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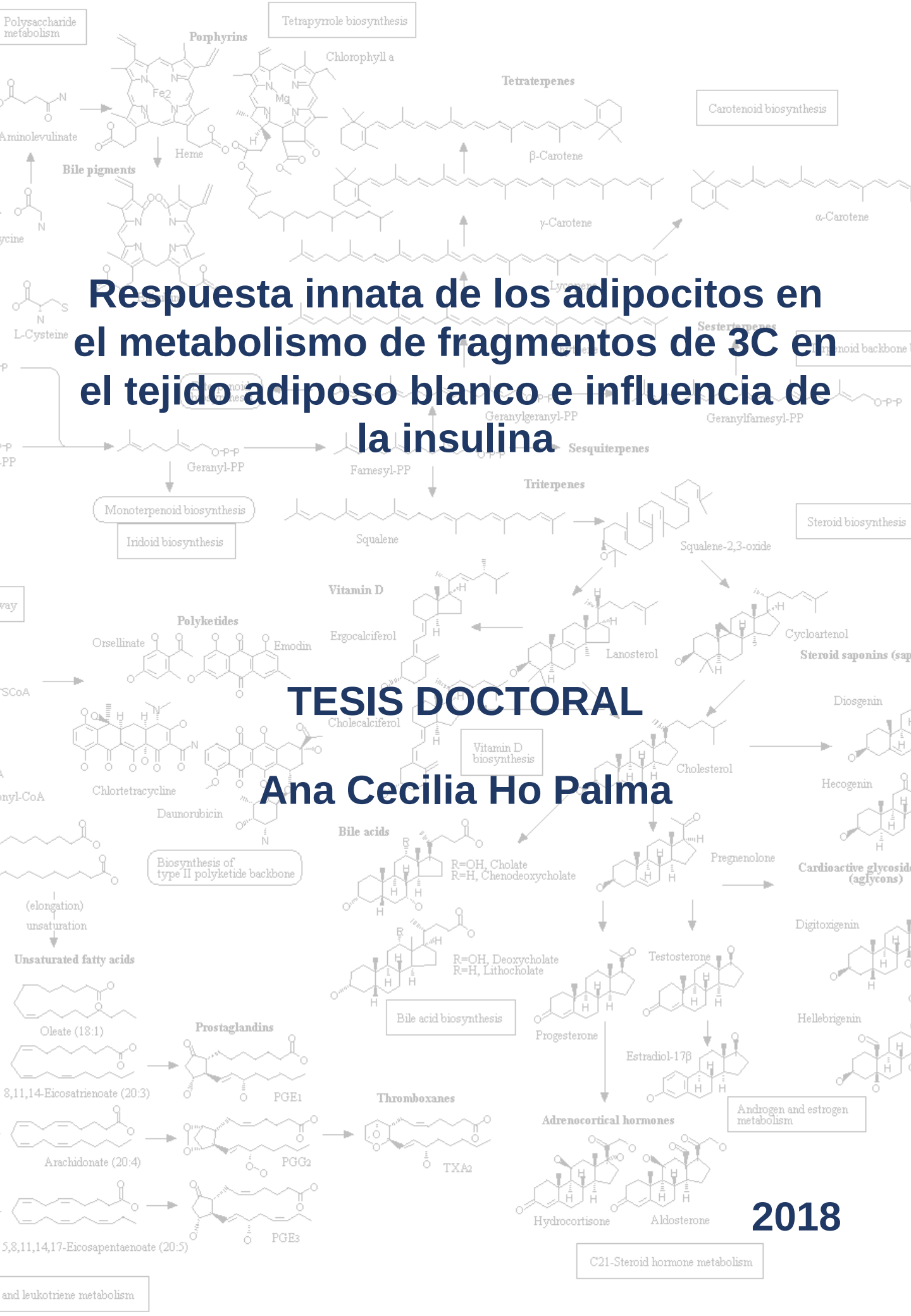
Respuesta innata de los adipocitos en el metabolismo de fragmentos de 3C en el tejido adiposo blanco e influencia de la insulina

Ana Cecilia Ho Palma



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TESIS DOCTORAL

Ana Cecilia Ho Palma

2018

UNIVERSITAT DE BARCELONA

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FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

Doctorado en Alimentación y Nutrición

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Memoria presentada por Ana Cecilia Ho Palma para optar el título de doctor por la Universitat de Barcelona

Codirector y Tutor	Codirector	Autor
		
Jose Antonio Fernandez-Lopez	Xavier Remesar Betlloch	Ana Cecilia Ho Palma

Trabajo realizado en el grupo de investigación Nitrógeno y Obesidad, del Departamento de Bioquímica y Biomedicina Molecular de la Facultad de Biología de la Universidad de Barcelona

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AGRADECIMENTOS

Estos cuatro años han sido toda una aventura cargada de nuevas experiencias, retos, crecimiento personal y autodescubrimiento, que han dejado recuerdos inolvidables, pero ha llegado la hora de agradecer a las personas que me han acompañado durante todo este tiempo.

Para empezar, quiero agradecer al Programa Nacional de Becas y Crédito Educativo del Gobierno Peruano (Pronabec) pues nada de esto hubiera sido posible sin su ayuda económica y a mi familia por su apoyo moral e incondicional, gracias por ayudarme a alcanzar mis objetivos profesionales.

A mis directores de tesis, José Antonio Fernández, por él llegué a este grupo de investigación y gracias a ello he aprendido y compartido nuevas experiencias. Gracias por confiar en mí y darme la oportunidad de trabajar bajo tu dirección, por tu paciencia, tiempo y por tranquilizarme en situaciones desesperantes. Y a Xavier Remesar, siempre preocupándose por nuestro bienestar y de que no nos faltara nada, siempre recordaré las pláticas de política en su despacho.

A Marià Alemany que, a pesar de no haber sido mi director de tesis, ha contribuido en gran medida en ella. Por las horas que ha invertido en mí, explicándome de todo un poco, desde bioquímica hasta etimología. Gracias por las interesantes y divertidas conversaciones.

A las profesoras Mar Grasa y Montse, por sus valiosos consejos y por compartir sus experiencias de vida conmigo.

A Eloy, mi compañero de vida, por su amor incondicional y por alentarme a ser quien quiero ser, gracias a él me embarque en este viaje. Ambos hemos aprendido del otro mientras compartíamos nuevas experiencias en esta aventura europea.

A Laia y Floriana, mis compañeras de doctorado, gracias por ayudarme cuando estaba perdida en el laboratorio y compartir sus experiencias conmigo, pero sobre todo gracias por su amistad incondicional. Por los cafés “delivery” al laboratorio, las cervezas y claras compartidas. Nunca olvidaré el viaje a Gotemburgo y sobre todo vuestra compañía y apoyo en los últimos meses de escritura.

A Jose, siempre dispuesto a ayudar, nunca olvidaré que fue el primero que me habló al llegar al laboratorio, dándome consejos y hablándome maravillas de una chica italiana que pronto conocería. Conocí la faceta algo paternal de Jose, pero también la bromista, pues a pesar de las canas, hay un niño en su interior. Gracias por alegrar el laboratorio con tus bromas y por los sustos.

A Lucia, que, aunque sólo estuvo pocos meses con nosotras, nuestra amistad creció rápidamente. Gracias por la sonrisa contagiosa que te animaba a pesar de tener un día horrible. Y a Edu, que luego de por fin conocerlo, pasamos momentos divertidos.

A Pau, que gracias a él pude terminar lo último que faltaba de la parte experimental y por tanto mi tesis. A pesar del estrés del tiempo, fue divertido compartir la poyata y música contigo.

Y por último a Mar Romero, por compartir sus experiencias profesionales conmigo. Y a mis compañeros de laboratorio, con los que coincidí en algún momento, Serena, Ricard, Zineb, Anna, Angelo y Sofi.

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ABSTRACT

Our research group has observed that both 3T3L1 cells and white adipose tissue *in vivo* are able to release, from glucose, large amounts of lactate under aerobic conditions and glycerol in greater quantity than which can be justified by lipolysis.

To study why adipocytes, act mainly in a glycolytic manner producing 3C fragments without being affected by hypoxia, we developed the necessary methodology applied to primary cultures. Since white adipose tissue is composed by other cell types, we also check whether hypoxia affects the stromal fraction cells in the same way. White adipose tissue responds to signals produced by the body as hormones, so we decided to study in adipocytes and the stromal fraction, how insulin can affect the adipocytes metabolism and their innate response, that is without any external stimulus.

The results indicate that white adipose tissue has a high metabolic activity in relative terms since, despite its small amount of "living" mass (only 1.3% of the total), adipose cells produce and release large amounts of 3C fragments. In adipocytes, lactate is produced from glucose, independently of the presence of oxygen and insulin, to be exported to other cells and used as an energy source. This idea is reinforced by the fact that at higher concentrations of glucose, more lactate is produced. However, although the stromal fraction cells also produce large amounts of lactate, this process is not influenced by glucose concentrations. On the other hand, glycerol is produced only by adipocytes, being regulated by insulin and constant over time. However, initially glycerol comes from the glycolytic pathway, when the glucose concentration is high, but when it is low it comes from lipolysis. At 48 h, its origin changes to glycolytic-lipolytic, except at 3.5 mM glucose. While free fatty acids are recycled to form triacyclglycerols, very little ^{14}C glucose is incorporated over time, indicating limited lipogenesis. But when insulin is added, this lipogenesis is promoted, even though at the same time the hormone limits, at the beginning, the incorporation of glycerol-3P for the synthesis of triacylglycerols.

The coexistence of these two processes, lipogenic and glycolytic simultaneously, is probably a consequence to prevent the accumulation of energy excess as a defense mechanism against "glycolipotoxicity", and a way to supply easily usable energy by other tissues in the form of 3C fragments.

RESUMEN

Se ha observado en nuestro grupo de investigación que tanto las células 3T3L1 como el tejido adiposo blanco *in vivo* son capaces de liberar, a partir de la glucosa, grandes cantidades de lactato en condiciones aeróbicas y glicerol en mayor cantidad que la que puede justificarse por lipólisis.

Para investigar por qué los adipocitos actúan principalmente de manera glucolítica produciendo fragmentos de 3C sin verse afectados por la hipoxia desarrollamos la metodología necesaria aplicada a cultivos primarios. Puesto que el tejido adiposo blanco está compuesto por otros tipos celulares, comprobamos también si la hipoxia afecta de la misma manera a las células de la fracción estromal del tejido. El tejido responde a señales que el organismo produce como hormonas, por ello nos propusimos estudiar en adipocitos y en la fracción estromal, la modulación del metabolismo provocada por la insulina y la respuesta innata del tejido, es decir sin ningún estimulo externo más que el de la glucosa.

Los resultados obtenidos señalan que el tejido adiposo blanco tiene una actividad metabólica elevada en términos relativos ya que a pesar de su escasa cantidad de masa "viva" (sólo el 1,3% del total), las células adiposas producen y liberan grandes cantidades de fragmentos de 3C. En los adipocitos, el lactato es producido a partir de la glucosa, independientemente de la presencia de oxígeno e insulina, para ser exportado a otras células y utilizado como fuente de energía. Esta idea se ve reforzada por el hecho que a concentraciones más elevadas de glucosa se produce más cantidad de lactato. Sin embargo, aunque las células de la fracción estromal también producen gran cantidad de lactato, este proceso no se ve influenciado por las concentraciones de glucosa. Por otra parte, el glicerol es producido solo por los adipocitos, siendo regulado por la insulina y constante en el tiempo. No obstante, al inicio el glicerol proviene de la vía glucolítica cuando la concentración de glucosa es elevada, pero cuando esta es baja proviene de la lipolisis. A las 48 h su origen cambia a glucolítico-lipolítico, excepto a 3,5mM de glucosa. Mientras que los ácidos grasos libres se reciclan para formar triacilgliceroles, muy poca glucosa ^{14}C es incorporada, lo que indica una lipogénesis limitada. Sin embargo, la insulina promueve esta lipogénesis, aunque al mismo tiempo limita, al inicio, la incorporación de glicerol-3P para la síntesis de triacilgliceroles.

La coexistencia de estos dos procesos, lipogénico y glucolítico a la vez, es probablemente una consecuencia para evitar la acumulación del exceso de energía, como un mecanismo de defensa contra la "glucolipotoxicidad" y una manera de suministrar energía en forma de fragmentos de 3C, fácilmente utilizable por otros tejidos.

INTRODUCCION

1. El Órgano adiposo

1.1 Tipos de células

El tejido adiposo estuvo relegado a un segundo plano durante muchos años ya que históricamente era considerado simplemente un reservorio de lípidos, ya sea en expansión en respuesta al exceso de nutrientes o liberando lípidos en respuesta al déficit de energía; sin embargo, desde la década de los 90 empieza a ser reconocido por su importante papel endocrino, por su activo rol en el metabolismo tanto de la glucosa como de los lípidos, y por ser capaz de modular la termogénesis en el proceso denominado "browning"[1].

Los adipocitos son las células principales del tejido adiposo y son los responsables de almacenar en sus gotas de lípidos el exceso de calorías en forma de triacilgliceroles sin sufrir daños por la lipotoxicidad, como es el caso de otros tejidos (principalmente músculo e hígado) bajo las mismas condiciones[2]. Se han identificado tres tipos distintos de células adiposas en mamíferos: adipocitos blancos, pardos y beige (brite), con orígenes, morfología, número de mitocondrias y genes de expresión termogénica diferentes[3].

Los adipocitos pardos, a diferencia de los blancos, tienen varios depósitos pequeños de grasa en los que también almacenan lípidos[2]; sin embargo, son células altamente especializadas en disipar la energía química almacenada en forma de calor[4]. Por ello, en la mayoría de los mamíferos, el tejido adiposo pardo desempeña un papel fundamental respondiendo a estímulos termogénicos (estrés provocado por el frio), manteniendo así la temperatura optima del organismo y previniendo la hipertermia, en un proceso denominado termogénesis[2]. De manera similar, se ha visto que el tejido adiposo pardo se activa (en roedores) en respuesta al consumo elevado de nutrientes, como una dieta rica en grasas, en un proceso conocido como termogénesis inducida por la dieta[5]. Prueba de ello es la elevada captación de lípidos y glucosa por gramo de tejido en los ratones modificados genéticamente para simular diabetes y dislipidemia, regularizando así la hiperglicemia e hiperlipidemia[6]. Los adipocitos pardos tienen esa coloración debido a los citocromos presentes en la gran cantidad de mitocondrias que estas células poseen[2]. Además, ubicada en la membrana interna de las mitocondrias se encuentra empaquetada densamente la proteína desacoplante 1 (UCP1, por sus siglas en inglés), que cataliza la fuga de protones a través de la membrana mitocondrial interna, "desacoplando" la oxidación de sustratos (combustible), a través de la cadena de transporte de electrones, de la producción de ATP, generando calor de esta

forma[4]. Este proceso consume gran cantidad de energía, por lo que además de sus efectos termogénicos, el tejido adiposo pardo (activado) posee también una gran capacidad de oxidación de sustratos. Por lo tanto, aunque el tejido adiposo pardo represente un porcentaje pequeño de la masa corporal, cuando está activado puede contribuir activamente en el fraccionamiento y utilización de nutrientes, así como en la regulación del peso corporal[6].

Aunque la UCP1 es una proteína particular de los adipocitos pardos, también se ha encontrado en el tejido adiposo blanco dentro de las mitocondrias de los llamados adipocitos beige o "brite". El número de adipocitos beige en el tejido adiposo blanco aumenta en roedores expuestos al frio prolongado o en respuesta a la señalización adrenérgica[4] ("browning"), al igual que los adipocitos pardos en el tejido adiposo pardo[7]; incrementándose así la capacidad termogénica de ambos tejidos. Sin embargo, aunque los adipocitos pardos y beige comparten los mismos genes requeridos para la termogénesis y expresan la misma proteína desacoplante 1, los adipocitos beige poseen una expresión génica propia, indicando que son un tipo distinto de célula termogénica[8]. Cuando no hay estimulación hormonal los adipocitos beige presentan una baja expresión basal de UCP1 y respiración desacoplada, equiparable al de las células blancas y menor que los adipocitos pardos. Sin embargo, la estimulación con un agonista β-adrenérgico eleva la expresión de UCP1, lo que sugiere que los adipocitos beige están programados para ser bifuncionales, adecuados para el almacenamiento de energía en ausencia de un estímulo termogénico pero totalmente capaces de proporcionar calor cuando se activan las señales apropiadas[4].

Finalmente, la clásica célula adiposa, la más estudiada por su expansión durante la obesidad y objeto de estudio en esta tesis, son los adipocitos blancos. Estos conforman la mayor parte del tejido adiposo blanco subcutáneo y visceral[2]; y aunque representan la mayor parte del volumen/masa del tejido adiposo blanco, ocupan sólo menos del 20% del total de las células que conforman este tejido[9], el resto lo componen preadipocitos, macrófagos, células endoteliales, fibroblastos y leucocitos. Estos adipocitos tienen un tamaño variable entre 25 a 200μm y poseen una sola vacuola grande de lípidos, unas pocas mitocondrias y baja capacidad oxidativa[10]. Además, cabe destacar que la vacuola de lípidos ocupa la mayor parte del adipocito, colocando el borde de la gota de grasa en las proximidades del retículo endoplasmático y las mitocondrias, donde los triacilgliceroles son esterificados e hidrolizados, respectivamente[2].

1.2 Participación del tejido adiposo blanco en el balance energético

El tejido adiposo blanco, además de ser un depósito de almacenamiento, es también el lugar de síntesis de distintos componentes con funciones endocrinas que regulan la energía almacenada de manera autónoma[11]. Las citoquinas secretadas por el tejido adiposo reciben el nombre de adipoquinas, siendo las dos más conocidas la leptina y la adiponectina, que desempeñan un papel importante en la regulación del equilibrio energético.

La leptina, es la encargada de iniciar un bucle de retroalimentación que, ante el exceso de tejido adiposo, genera una acción hipotalámica que provoca la disminución de la ingesta y activa el sistema nervioso simpático[12]. Esto último, promueve la secreción de catecolaminas a través de terminales nerviosas ubicadas en el tejido adiposo, activando los receptores β adrenérgicos en los adipocitos, lo que incrementa la lipólisis y activa los procesos termogénicos[12]. Además, la leptina ayuda a modular la homeostasis de la glucosa al actuar sobre las células β del páncreas propiciando la secreción y expresión de insulina[13]; también participa en la regulación de las respuestas inmunes (adaptativas e innatas) relacionadas a la adaptación metabólica[3]. Esta estrecha relación entre el eje cerebro-adipocito es un claro ejemplo de cómo los adipocitos controlan tanto el depósito de nutrientes como la movilización de la energía a través de señales entrecruzadas con otros tejidos[14].

Por otra parte, la adiponectina es capaz de inducir la sensibilidad a la insulina principalmente en el hígado, pero también ejerce acciones sobre otros tejidos[3]. Asimismo, se ha observado que la adiponectina activa la proteína quinasa dependiente de AMP (AMPK) y reduce la expresión de enzimas gluconeogénicas como la fosfoenolpiruvato carboxilasa y la glucosa-6-fosfatasa, inhibiendo así la gluconeogénesis[15] y activando de paso el catabolismo.

A nivel orgánico, la abundante adiposidad favorece la acumulación excesiva de lípidos en el tejido adiposo, que a la larga provoca una desregulación en la producción o secreción de las adipoquinas produciéndose tanto una suave inflamación crónica como una serie de respuestas inmunes derivadas, todo ello afectando profundamente el control y partición de la energía[16]. Finalmente, estos cambios alteran la función del tejido adiposo blanco, músculo, hígado e intestino (incluyendo a la microbiota) con la consiguiente resistencia a la acción de la insulina y secreción de citoquinas en general.

1.3 Localizaciones del tejido adiposo blanco

La distribución del tejido adiposo en determinadas zonas del organismo desempeña un papel fundamental en las complicaciones metabólicas relacionadas con la patogénesis de la obesidad. Aunque los adipocitos blancos nacen a partir de células residentes de origen mesenquimal en la grasa blanca, los adipocitos subcutáneos tienen un origen de desarrollo y propiedades metabólicas diferentes a los adipocitos viscerales, tanto en humanos como en roedores[3].

No obstante, aún existe cierta controversia sobre las funciones metabólicas de estas localizaciones. En general se acepta que la grasa visceral, que incluye la mesentérica, gonadal, epicardial, retroperitoneal, omental y las almohadillas perirrenales, tiende a ser más perjudicial[17]. El tejido adiposo visceral está compuesto por adipocitos blancos[11], mientras que en el subcutáneo probablemente se desarrollen también adipocitos beige[11] confiriéndole a esta zona efectos protectores ante el desarrollo de la obesidad y ante las enfermedades metabólicas asociadas[17] (al menos en roedores).

Es así como, el tejido adiposo visceral ubicado en las profundidades del abdomen se correlaciona con una elevada mortalidad y con el riesgo de sufrir enfermedades cardiovasculares y diabetes[17] en humanos, mientras que, la acumulación de tejido adiposo subcutáneo gluteofemoral, más frecuente en mujeres, se asocia con una mayor sensibilidad a la insulina y una disminución en el riesgo de padecer diabetes y enfermedades cardiovasculares. Por otra parte, en roedores, el tejido adiposo subcutáneo inguinal se asocia a la mejora de los parámetros metabólicos, de manera similar a lo observado en humanos en tejido gluteofemoral; sin embargo, el tejido perigonadal (visceral) se asocia a una disminución en la sensibilidad a la insulina[18].

Asimismo, la distribución del tejido adiposo está influenciado por las características inherentes a cada sexo. Por ejemplo, una dieta rica en grasas induce en ratones machos la hiperplasia de los adipocitos del tejido adiposo visceral, mientras que en las hembras esta adipogenesis ocurre tanto en tejido adiposo visceral como en el subcutáneo, siendo influenciada por las hormonas, en particular los estrógenos[19]. Adicionalmente, se ha observado que la acumulación visceral de tejido adiposo que se observa en machos es perjudicial para la salud metabólica, mientras que la acumulación subcutánea en hembras parece ser beneficiosa[19].

1.4 Hipertrofia e hiperplasia del adipocito blanco

Un atributo único del tejido adiposo es su increíble capacidad para cambiar sus dimensiones; ningún otro tejido (no neoplásico) es capaz de hacerlo al mismo nivel. Esto puede ser conseguido aumentando el tamaño individual de cada adipocito (hipertrofia) o reclutando en el tejido nuevos adipocitos a partir del acervo de progenitores residentes (adipogenesis o hiperplasia), aumentando así el número de células adiposas[4].

Al inicio de la obesidad, los depósitos de tejido adiposo se expanden por hipertrofia hasta un umbral crítico (~0.7 – 0.8 µg/célula) y con el progreso de la obesidad, a través de las señales liberadas que inducen la proliferación y diferenciación de los preadipocitos (hiperplasia)[4]. Además, se cree que el exceso de nutrientes induce la hipertrofia de la grasa subcutánea de la parte alta del cuerpo (en humanos) mientras que la hiperplasia se da en los depósitos ubicados por debajo de la cintura[20].

Aunque en general se considera que la hiperplasia del adipocito acontece tras un período de hipertrofia en respuesta al desequilibrio energético, y a partir de que los adipocitos alcancen su tamaño máximo, podría también suceder lo contrario, es decir que la adipogénesis ocurra mucho antes que los adipocitos lleguen a su máximo tamaño en respuesta a una dieta obesogénica[21] o que existan ciclos en los que se alterna la hiperplasia e hipertrofia a medida que la obesidad progresa[22].

Una vez iniciada la hiperplasia, es difícil perder los adipocitos ganados, incluso durante una perdida considerable de peso; los adipocitos sólo reducen su tamaño, pero no su número total[4]. Esto no significa que no exista muerte celular, de hecho, aproximadamente el 8% de los adipocitos subcutáneos (en humanos) son repuestos cada año, con tasas de nacimiento y muerte celular muy similares que resultan en un cambio casi imperceptible en la cantidad total de células[23].

La expansión hiperplásica es preferible a la expansión hipertrófica[17], puesto que existe una asociación directa entre la hipertrofia de los adipocitos y las enfermedades metabólicas[24]. Sin embargo, aún se desconocen los mecanismos intrínsecos que promueven la adipogenesis en la expansión de los depósitos de grasa.

La activación de células precursoras del adipocito puede ser regulada por el microambiente del depósito específico del adipocito y no por mecanismos intrínsecos de la propia célula[19]. Esto sugiere que las células precursoras del adipocito responden tanto a estímulos locales como

sistémicos, influyendo estos últimos en la diferenciación potencial de los adipocitos, independientemente de la localización del depósito; por lo que los nichos específicos de las células precursoras del adipocito coordinarían la distribución y crecimiento del depósito adiposo específico[19]. Es posible también, que los adipocitos hipertrofiados promuevan a través de señales el posterior llenado (con lípidos) de las células precursoras activadas del adipocito, ya que estas células demoran varias semanas en madurar[21].

Por otro lado, no solo se observa hipertrofia en la obesidad, sino también en personas no obesas, pero con diabetes tipo 2. Dicho crecimiento del adipocito puede que este mediado por una capacidad adipogénica deteriorada en las células progenitoras[25], lo que podría tener un impacto sobre la inflamación del tejido adiposo blanco, la liberación de ácidos grasos, la acumulación ectópica de lípidos y la sensibilización a la insulina.

2. Inflamación

2.1 Relación entre la respuesta metabólica e inmune

Entre los procesos más críticos para la supervivencia de las especies, se encuentran la capacidad para resistir el hambre y la capacidad de activar una respuesta inmune efectiva contra los patógenos. Esta última, la habilidad para combatir una infección, ha llevado a la selección de una fuerte respuesta inmune, particularmente después de la disminución masiva de una población durante períodos de epidemias y pandemias infecciosas[26]. La combinación de estos dos rasgos probablemente haya dado lugar a una organización biológica altamente capaz de procesar y almacenar energía, así como una poderosa respuesta inmune, quizá a veces demasiado sensible[27].

Además, existe una relación muy estrecha entre los sistemas de respuesta metabólica e inmune que han sido conservados evolutivamente. Por ejemplo, el cuerpo graso de la mosca *Drosophila*, integra lo que sería el equivalente mamífero al hígado y al sistema hematopoyético e inmune[28]. Igualmente, esta estructura es reconocida como análoga al tejido adiposo en mamíferos, compartiendo un desarrollo similar y rutas metabólicas funcionales[29]. En organismos más evolucionados, el tejido adiposo, hígado y sistemas hematopoyéticos se han especializado en unidades u órganos diferentes y funcionales que, a pesar de ello, aún conservan su origen común.

El hecho que la respuesta inmune y la regulación metabólica estén altamente integradas permite sugerir que el efecto producido por nutrientes y por los agentes infecciosos utiliza los mismos sistemas efectores y de señalización, por lo que esta interfaz puede considerarse un mecanismo homeostático central. La disfunción de alguno de estos sistemas puede originar trastornos metabólicos crónicos, lo que explica que los nutrientes puedan inducir inflamación[27], fenómeno que está presente particularmente en la obesidad, pero también en la diabetes tipo 2 y en las enfermedades cardiovasculares.

2.2 Inicio de la respuesta inflamatoria

La respuesta inflamatoria se inicia cuando se da algún tipo de estrés inicial. En el caso de la obesidad, deriva de un balance energético positivo que desencadena un estrés homeostático provocado por un estado anabólico, principalmente en los adipocitos[11]. Al inicio, las células del tejido adiposo responden liberando adipoquinas que generan una respuesta inflamatoria adaptativa que favorece la expansión saludable del tejido adiposo al mismo tiempo que se reduce el gasto de energía al inhibir la lipólisis y la termogénesis en el tejido, como consecuencia de una menor sensibilidad a las catecolaminas y la leptina[11], y con el fin de mantener el adecuado rango de glucosa en sangre y el balance energético. Sin embargo, con el tiempo el organismo se esfuerza por restablecer la homeostasis, que sólo puede ser alcanzada al fijar un nuevo punto de referencia para el peso[16,30], niveles circulantes de glucosa, lípidos y otras hormonas. Todos estos cambios van acompañados de la reducción en la flexibilidad metabólica; aumento de la resistencia a la acción de la insulina, la leptina y las catecolaminas, el remodelado anormal de tejidos y aparición de fibrosis en el tejido adiposo[11].

Aunque los mecanismos exactos que inician la respuesta inflamatoria asociada a la obesidad no se conocen del todo se han propuesto unos cuantos agentes causantes entre los que destacan sustancias derivadas del intestino y metabolitos procedentes de la dieta.

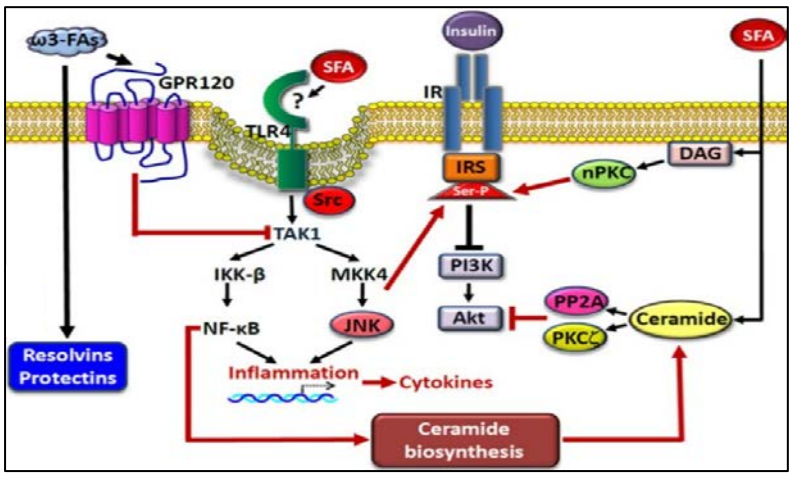
Durante la obesidad la permeabilidad intestinal está incrementada, lo que se traduce en el aumento de la concentración de sustancias derivadas del intestino en la circulación sanguínea como los lipopolisacáridos, que proceden de bacterias gram-negativas presentes en la microbiota intestinal[11], y que podrían ser un importante agente pro inflamatorio al iniciar una cascada inflamatoria, activando los receptores de patrón de reconocimiento, como por ejemplo el receptor TLR4 (Toll-like receptor 4) en los adipocitos[27]. El tejido adiposo mesentérico (visceral) podría ser el primero en sufrir los efectos inflamatorios de estas sustancias, ya que se encuentra

rodeando al intestino, pero también podría ser la primera línea de defensa debido a que su expansión podría prevenir la propagación de los lipopolisacáridos y por ende la inflamación[31]. Asimismo, como se ha comentado previamente, las señales que producen la inflamación son necesarias para el crecimiento y expansión del tejido adiposo en respuesta al exceso de nutrientes, lo que evita la acumulación ectópica de lípidos en otros tejidos y células (hígado, musculo y células β del páncreas) donde tendrían efectos tóxicos[11].

Otro de los responsables implicados en el inicio de la respuesta inflamatoria serían los niveles circulantes de lípidos (Figura 1), que se encuentran elevados en personas obesas o en estados diabéticos y que pueden influir negativamente en las acciones de la insulina. Por un lado, los ácidos grasos libres (en especial los saturados), circulan en el torrente sanguíneo activando rutas celulares proinflamatorias a través del receptor TLR4 (toll-like receptor 4), que induce la activación de las quinasas JNK e IKK, que fosforilan los residuos de serina del receptor del sustrato de insulina 1 (IRS-1) inactivando la señal de la insulina[32]. Adicionalmente la activación de estas quinasas por los ácidos grasos saturados promueve cambios en la expresión de genes que influencian negativamente la acción de la insulina a través de factores de transcripción como el factor nuclear NF-κB y el activador de proteína 1 (AP-1)[32]. Además, una vez activado, el factor NF-κB puede incrementar en el adipocito la síntesis y secreción de quimioquinas como la proteína quimiotáctica de monocitos 1 (MCP1, también conocida como CCL2) que incrementa la infiltración de macrófagos proinflamatorios en el tejido adiposo[11] (ver apartado 2.3). Otro efecto perjudicial de los ácidos grasos saturados se da cuando se metabolizan a ceramidas o diacilgliceroles en los tejidos, ya que estos compuestos pueden inactivar al sustrato del receptor de la insulina por fosforilación de los residuos de serina o al reducir la sensibilidad de esta señal a través de la proteína Akt[33]. También aquí se puede incluir a las proteínas chaperonas de los lípidos como la proteína de unión a ácidos grasos 4 (FABP4, por sus siglas en inglés) que se encuentran en los adipocitos y que podrían promover la señal proinflamatoria en macrófagos[34].

Finalmente, otro proceso que daría inicio a la respuesta inflamatoria, además de los mencionados anteriormente, sería la rápida expansión que se da en el adipocito como consecuencia de la obesidad. Ello produciría señales intrínsecas como la muerte del adipocito, la hipoxia del tejido adiposo y cambios en la matriz extracelular del tejido[11] que contribuyen al inicio de la inflamación. En particular, la flexibilidad de la matriz extracelular que sujeta a los adipocitos permite la expansión saludable del tejido[4]; sin embargo, la nociva expansión del adipocito da como resultado la rápida aparición de una densa matriz extracelular[35] y la expresión de un transcriptoma

profibrótico en el tejido[36]. Esta interacción mecánica entre el adipocito y su matriz fibrosa (fibrosis) restringe la flexibilidad metabólica de la célula y ha sido identificada como un proceso potencial que conduce a la disfunción del adipocito[2] causando la activación de rutas relacionadas con el estrés, acumulación ectópica de lípidos en otros tejidos[4] e inflamación a través de la vía de señalización del factor NF-κB[37].



Fuente: Glass CK/Cell Metabolism 2012

Figura 1: Efecto de los ácidos grasos saturados en la señalización de las rutas inflamatorias a través del receptor TLR4.

SFA: ácidos grasos saturados, DAG: diacilgliceroles, ω3-FAs: ácidos grasos omega 3

2.3 Respuesta inmune en el adipocito del obeso

Los tres diferentes tipos de tejido adiposo contienen células inmunes que velan por la integridad y sensibilidad hormonal de los adipocitos. En condiciones normales, los linfocitos T2 colaboradores, llamados "T helper 2" (T_H2) junto con otras células inmunes, secretan citoquinas con efectos antinflamatorios como la IL-13 y la IL-4[11]. La IL-13 activa a los eosinófilos que se encuentran en el tejido adiposo y al mismo tiempo estos secretan también IL-4, cuya función es mantener la polarización de los macrófagos hacia un estado antinflamatorio dando lugar a una población de macrófagos M2 o alternativamente activados[38]; también induce la diferenciación de los adipocitos beige[39]. Además, los macrófagos M2 secretan IL-10, la cual mantiene la flexibilidad metabólica del sistema al preservar la sensibilidad a la insulina en los adipocitos y suprimir a la vez las señales lipolíticas[40].

Por otra parte, las células inmunes en el tejido adiposo de animales obesos operan bajo un estado proinflamatorio gracias a la acción de los linfocitos T1 colaboradores, "T helper 1" (T_H1). Estas células secretan citoquinas proinflamatorias como el factor de necrosis tumoral α (TNF-α) y la IL-

1β [11]. El TNF-α, secretado también por los adipocitos, activa inhibidores claves de la acción de la insulina en el tejido adiposo de ratones obesos[41] lo que potencia la estrecha relación existente entre obesidad, diabetes e inflamación crónica.

Existen numerosos estudios que demuestran que los macrófagos presentan importantes cambios en la obesidad. Así, la obesidad se asocia con la presencia masiva de macrófagos (hasta un 40%) tanto en tejido adiposo de humanos como de ratones obesos[33]. Además, se cree que los adipocitos en expansión o los preadipocitos producen señales quimiotacticas que llevan al reclutamiento de macrófagos[42] polarizados hacia el tipo M1 (proinflamatorio). La combinación entre el aumento en la cantidad total de macrófagos y de la relación de macrófagos M1 a M2 es una señal distintiva de la inflamación del tejido adiposo. Una vez infiltrados, los macrófagos (mayormente M1, muy activos) secretan grandes cantidades de TNF-α, que activa quinasas intracelulares como la JNK y la IκB (IKK) que a su vez inhiben al receptor de sustrato de la insulina IRS-1 a través de la fosforilación de residuos específicos de serina (Figura 2)[33].

La concentración de citoquinas en el adipocito es mayor que los niveles circulantes de estas, por lo que es probable que, en la mayoría de los casos, los efectos más notorios de estos productos secretores sean locales en vez de sistémicos[43].

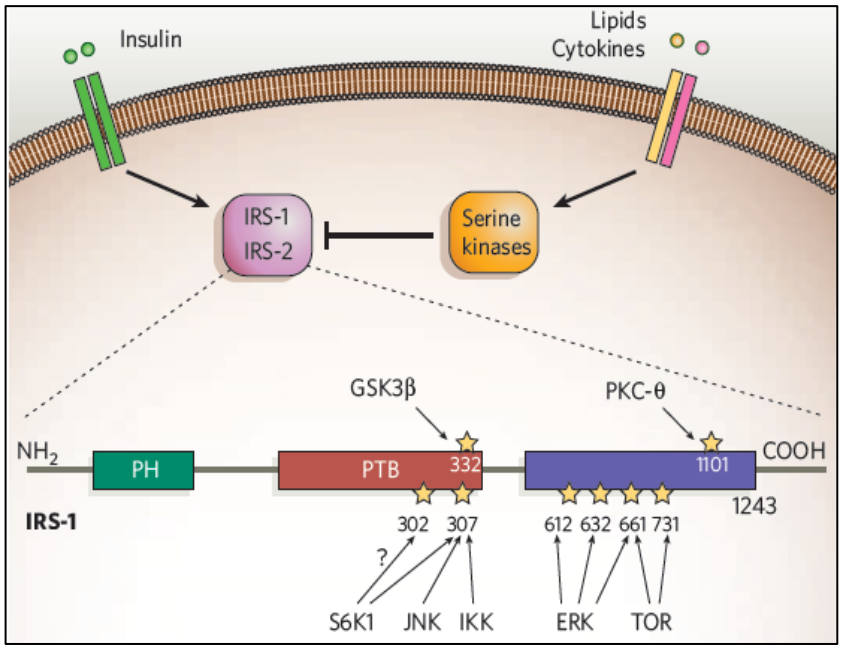


Figura 2: Ambos sustratos del receptor de la insulina, IRS-1 e IRS-2, contienen residuos de serina (estrellas amarillas) que son la diana de varias quinasas que cuando se fosforilan, inactivan las proteínas. La activación de estas quinasas se puede producir en respuesta a señales inflamatorias, como lípidos o citoquinas.

ERK, extracellular regulated mitogen-activated protein kinase; GSK3β, glycogen synthase kinase 3β; PH, pleckstrin homology; PTB, phosphotyrosine binding; S6K1, ribosomal protein S6 kinase polymerase 1; TOR, target of rapamycin.

Fuente: Gökhan S. Hotamisligil/Springer Nature 2006

Otros tejidos (incluyendo el hígado) también se inflaman con la obesidad, pero a diferencia del tejido adiposo, con la pérdida de peso desaparece la inflamación del hígado. Esto sugiere que el tejido adiposo podría tener una memoria obesogénica ya que retiene su estado inflamatorio a pesar de la pérdida de peso[44]. Este efecto no se da siempre, puesto que en algunos individuos la inflamación del tejido adiposo desaparece luego de una perdida extrema de peso, por ejemplo, tras una cirugía bariátrica, mientras que en otros no[44].

3. Resistencia a la acción de la insulina

3.1 Señalización de la insulina

A pesar de las fluctuaciones de la concentración de glucosa en plasma después de la ingesta de nutrientes o del ayuno, esta se mantiene en un rango estable entre 4 y 7 mM[45] gracias a un estrecho mecanismo de control orquestado por hormonas como la insulina, secretada por las células β del páncreas y cuya acción depende básicamente de la disponibilidad de la glucosa.

La insulina controla la captación de glucosa al regular la densidad del transportador de glucosa en la membrana plasmática de las células en vez de estimular directamente la actividad del transportador[46]. La estimulación en la captación de la glucosa es más pronunciada en las células musculares y adiposas, aunque la insulina también promueve una pequeña captación (dos veces menor) en muchos otros tipos celulares[46]. Los adipocitos y los miocitos expresan el transportador de glucosa tipo 4 (GLUT4) dependiente de insulina[46], un miembro de la familia de los trasportadores de glucosa GLUT. Por el contrario, el hígado expresa el transportador GLUT2 cuya estimulación no depende de la insulina[45]. Al parecer, GLUT4 es el transportador predominante de glucosa en el adipocito a pesar de la presencia del transportador GLUT1 (no de pendiente de insulina) en la membrana de este[2]. Cuando no hay insulina, GLUT4 se encuentra secuestrado en el interior de la célula; la señal de insulina induce su redistribución hacia la membrana plasmática (superficie celular), incrementando así la captación de glucosa. Cuando la señal se detiene, GLUT4 es recogido de la membrana plasmática, retornando a su sitio de almacenamiento intracelular, listo para ser reclutado nuevamente ante otra ronda de señalización de insulina[46].

En estado postprandial la insulina inhibe la producción hepática de glucosa y actúa favoreciendo la entrada de esta y su almacenamiento en forma de glucógeno en los miocitos[45] a través del GLUT4; y en mucha menor proporción al tejido adiposo, también a través del GLUT4. Además, la insulina promueve el almacenamiento de sustratos en el tejido adiposo, hígado y músculos a

través de la lipogénesis[47] y la síntesis de glucógeno y proteínas; al mismo tiempo inhibe la lipólisis, la glucogenólisis y el catabolismo proteico. En contraposición, durante el ayuno la insulina activa la glucogenólisis y la gluconeogénesis en el hígado[48], liberando luego la glucosa al torrente sanguíneo a través del GLUT2 (Figura 3). Por último, la insulina también suscita la estimulación del crecimiento y diferenciación celular[45].

La insulina ejerce sus acciones, activando la proteína quinasa Akt, a través de una serie de mecanismos que empiezan en la membrana celular (Figura 3). Como regla general, la fosforilación de la tirosina activa el receptor de insulina y las proteínas del sustrato del receptor de insulina, mientras que la fosforilación de la serina/treonina las inactiva[46].

Después de la unión de la insulina a su receptor, se activa la tirosina quinasa de dicho receptor de insulina; acción que fosforila a la tirosina del receptor y a la de las proteínas del sustrato del receptor de insulina (IRS). Los sitios de fosfotirosina que se encuentran en los IRS sirven como punto de anclaje para la PI3K (p85/p110 PI3 quinasa)[49,50], subunidad regulatoria que genera la formación de fosfatidil3,4,5-fosfato (PtdIns(3,4,5)P_3 o PIP3) en la membrana plasmática. Todo ello facilita el reclutamiento de la proteína quinasa dependiente de fosfoinositol 1 (PDK1), que directamente fosforila (activa) el residuo de Thr308 de la proteína Akt. Adicionalmente, se puede producir una segunda activación de la proteína Akt a través de la fosforilación de su residuo Ser473, en un proceso iniciado por un complejo proteico llamado mTOR2 (mTORC2)[46]. La Akt activada luego continúa fosforilando los residuos de serina/treonina de una serie de proteínas efectoras. Estas incluyen al factor de transcripción FOXO (Forkhead family box O) encargado de regular la producción y utilización de glucosa; la proteína tuberina, la cual permite la activación de mTORC1 que a su vez activa la proteína de unión a elementos reguladores de esteroles 1C (SREBP1c) responsable de regular la síntesis de lípidos y la proteína ribosómica quinasa S6 (S6K) encargada de regular la síntesis de proteínas; la glucógeno sintasa quinasa 3β (GSK3 β) involucrada en la síntesis de glucógeno; y finalmente de RabGAP TBC1, que interviene en la regulación de la captación de la glucosa[46]. Aunque se desconoce aún el mecanismo de activación de las proteínas mTOR, se cree que podrían funcionar como un sensor de nutrientes, ya que necesitan la presencia de aminoácidos en el medio para su completa activación por factores de crecimiento[51].

Todos los pasos de la cascada de señalización de la insulina mencionados son reacciones enzimáticas reversibles. Por lo tanto, para cada quinasa activada por la insulina, existen múltiples fosfatasas que terminan su acción. La desensibilización y desactivación de estos mecanismos regulatorios han sido estudiados ampliamente en relación con la obesidad[50,52], sugiriéndose como

el origen de la resistencia a la acción de la insulina en la obesidad. Estos mecanismos incluyen la internalización y degradación del receptor de insulina; el agotamiento de PtdIns(3,4,5)P_3 gracias a las fosfatasas PTEN y SHIP2; la inhibición por sustratos alternativos del receptor de la insulina tales como las proteínas GRB10, GRB14 y SOCS; y la eliminación de grupos de fosforilación activadores a través de proteínas fosfatasas como las PTP1B, PHLPP y la proteína fosfatasa 2A (PP2A), esta última en particular es otra fosfatasa de serina/treonina que desfosforila e inhibe a Akt[46]. Por último, otro mecanismo importante es la fosforilación inhibitoria del receptor de insulina o del sustrato del receptor de insulina (IRS), en el que intervienen diferentes quinasas. Por ejemplo, la activación de mTORC1 y posterior estimulación de S6K causa la fosforilación de la serina/treonina del IRS inactivándolo (letras azules de la figura 3). También existen factores nutricionales (como se menciona en el apartado inflamación) que influyen en la inhibición de esta respuesta y en la filtración de macrófagos de tipo proinflamatorio en el tejido adiposo blanco que secretan citoquinas como la IL-6, IL-1β y TNFα, que activan de manera paracrina a las quinasas IKKβ, JNK, S6K y mTOR en el adipocito, inhibiendo al IRS-1 y causando resistencia a la insulina en el adipocito; o la lipotoxicidad producida por el exceso de diacilgliceroles que activan a la PKCε (proteína quinasa Cε) que impide la autofosforilación del receptor de insulina al fosforilarlo primero durante el bucle de activación de dicho receptor e inhibiendo su activación de esta manera[46].

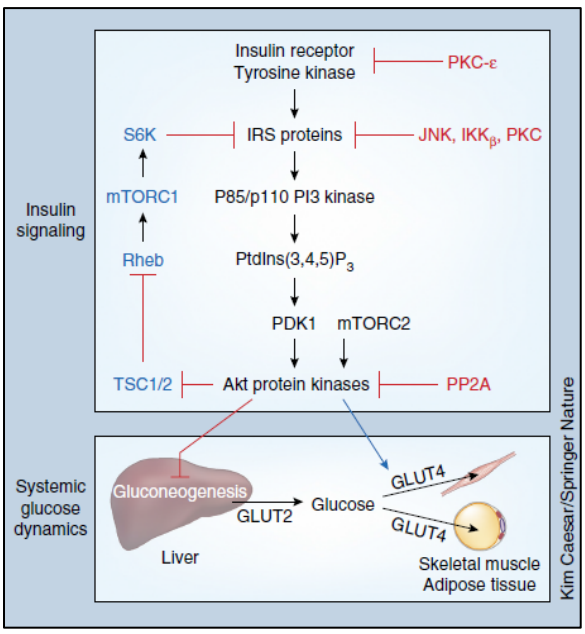


Figura 3: La insulina provoca una cascada de señalización que regula la homeostasis de la glucosa, mantenida por la coordinación de las rutas de señalización en el hígado (glicolisis y gluconeogénesis) durante el ayuno y a través de la captación de glucosa en el musculo y en menor proporción en el tejido adiposo, después de la ingesta de alimentos, para la síntesis de glucógeno y el metabolismo de la glucosa.

3.2 Captación de glucosa

Cuando la insulina es liberada en situación de euglucemia (estado basal), el músculo esquelético es responsable del 20% de la glucosa que se capta en el cuerpo[53]; de este porcentaje aproximadamente la mitad se da por procesos no mediados por insulina y la otra mitad por procesos mediados por insulina. Ello supone que el 10% de la captación de glucosa total del cuerpo se produce en el musculo esquelético mediante procesos dependientes de insulina; el resto es asumido por el tejido adiposo, músculo cardiaco y tejidos esplácnicos. Por el contrario, el sistema nervioso central es el responsable de la mayoría de la captación no mediada por insulina.

Sin embargo, el músculo esquelético tiene un rol protagónico cuando hay hiperinsulinemia llegando a representar entre el 75%-90% de toda la captación de glucosa mediada por insulina[54]. Además, la glucosa en músculo es rápidamente metabolizada ya que los niveles intracelulares de glucosa no cambian[55]. En contraposición, se ha demostrado que la captación de glucosa mediada por insulina en el tejido adiposo es mucho menor, tanto en condiciones de euglucemia como de hiperglicemia[56,57].

Cuando se bloquea la expresión del transportador dependiente de insulina en músculo, los ratones "knockout" aún siguen teniendo una tolerancia normal a la glucosa, así como niveles normales de glucosa sanguínea e insulina en suero[58]; sin embargo, presentan una elevación de ácidos grasos libres y de triacilgliceroles en suero, así como una elevada masa grasa, lo que apunta a que la resistencia a la acción de la insulina del músculo contribuye a la alteración del metabolismo lipídico asociado a la diabetes tipo 2.

En contraposición, los ratones "knockout" para el transportador dependiente de insulina GLUT4 en tejido adiposo presentan una grave alteración en la captación de glucosa por el adipocito[59], a pesar de tener un crecimiento y una cantidad de tejido adiposo normal. Además, estos ratones desarrollan resistencia a la acción de la insulina en músculo e hígado, a pesar de que la expresión de GLUT4 en músculo esta preservada[60]. Por tanto, la disminución de la función del transportador de glucosa GLUT4 en tejido adiposo puede causar resistencia a la acción de la insulina, incrementando el riesgo de padecer diabetes[45].

La diabetes lipoatrófica, una alteración que se caracteriza por la falta de tejido adiposo blanco, es uno de los rasgos presentes en pacientes infectados con el virus de inmunodeficiencia humana (VIH) en terapia antirretroviral, particularmente con inhibidores de la transcriptasa inversa[61] o en personas que nacen con una rara enfermedad congénita llamada lipodistrofia congénita generalizada (o síndrome de Berardinelli-Seip)[62]. Esta diabetes también se manifiesta con

alteraciones metabólicas como resistencia a la acción de la insulina, hiperglicemia, hiperlipidemia, hígado graso y organomegalia[63]. Alteraciones que pueden ser revertidas como se ha demostrado en ratones modificados genéticamente para presentar dicha diabetes, a los que se les trasplantó tejido adiposo de ratones normales[64], corrigiendo así la hiperglicemia y disminuyendo dramáticamente los niveles de insulina. Además, mejoró la captación de glucosa en el músculo demostrando también que el efecto positivo no sólo se debe a la entrada masiva de glucosa al tejido adiposo trasplantado[64].

Todo lo anterior sugiere que el tejido adiposo y músculo esquelético tienen roles complementarios y distintos en cuanto a la utilización de la glucosa con el fin de mantener sus niveles en equilibrio.

3.3 El origen de todo: ¿la resistencia a la acción de la insulina o la hiperinsulinemia?

Los factores que llevan al desarrollo de la desregulación metabólica y que conducen a la diabetes tipo 2 son muy complejos. No obstante, existen dos puntos de vista diferentes de cómo se origina.

El primero, sugiere que la obesidad causa inicialmente resistencia a la acción de la insulina, la cual origina la hiperinsulinemia, promoviendo la aparición de hígado graso e hipertrigliceridemia. El mecanismo que daría inicio a este proceso empezaría con la activación del factor de transcripción FOXO1 en el hígado[65], el cual aumenta la expresión de enzimas claves de la gluconeogénesis (Figura 4). Todavía es materia en investigación cómo la obesidad causa la alteración del factor de transcripción FOXO1. No obstante se cree que las dietas ricas en grasas y/o la obesidad provocan una señalización de la insulina activada por la proteína quinasa Akt ineficaz para inactivar a FOXO1 en el hígado[49]. Normalmente la insulina causa la supresión de su función al activar la proteína Akt. Sin embargo, hay suficiente cantidad de Akt activada para estimular mTORC1, que también estimula la expresión de enzimas lipogénicas en el hígado para la síntesis *de novo* de ácidos grasos, aumentado así la síntesis de triacilgliceroles y su incorporación a las lipoproteínas de muy baja densidad (VLDL)[49]. Con la activación de FOXO1 se incrementaría la gluconeogénesis, es decir la síntesis de glucosa a partir de otros sustratos (lactato, glicerol y aminoácidos) que llegan al hígado y aumentaría la salida de glucosa al torrente sanguíneo. A la vez, se produce la disrupción de la translocación del transportador de glucosa GLUT4 a la superficie de la membrana celular del músculo esquelético[66,67], lo que reduce la captación de glucosa en este. La resultante hiperglucemia junto con la hiperinsulinemia crónica disminuye el efecto supresor de la insulina en

el adipocito, activando la lipólisis[49] y cuyos productos liberados (glicerol y ácidos grasos) son captados en el hígado; el glicerol para la gluconeogénesis y los ácidos grasos para su oxidación.

Como se menciona anteriormente, los diacilgliceroles derivados de la oxidación de los ácidos grasos estimulan la actividad de la proteína quinasa C (PKC) que inhibe finalmente al receptor de insulina o al sustrato del receptor de insulina (figura 3)[68], suprimiendo así la actividad de la proteína Akt y con esto activando a FOXO1 y la gluconeogénesis, demostrando la contribución de la lipotoxicidad en la menor señalización de la insulina y dando origen a la resistencia a la acción de la insulina. Adicionalmente, como se menciona en el apartado 2.2, las grasas saturadas pueden convertirse en ceramidas, que también inactivan la acción de la proteína Akt[69].

Por el contrario, la obesidad genera la disminución de la capacidad de los adipocitos para almacenar y retener los triacilgliceroles, lo que genera la acumulación ectópica de grasa en el músculo y en el hígado (esteatosis) sin que exista necesariamente una disminución en la sensibilidad a la acción de la insulina, argumento en contra de que el origen de la desregulación metabólica sea la resistencia a la acción de la insulina[70].

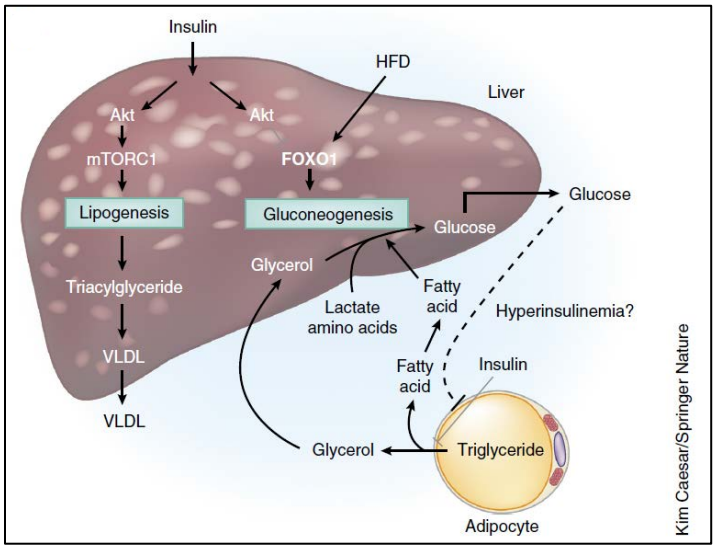


Figura 4: En condiciones de obesidad, FOXO1 se activa al no ser completamente suprimido por la señalización de la insulina a través de la proteína Akt. Lo que origina una mayor producción de glucosa.

HFD: Dieta rica en grasas, VLDL: lipoproteínas de muy baja densidad

El segundo punto de vista se basa en que las dietas ricas en grasas y el exceso de nutrientes generan hiperinsulinemia primaria[71] al aumentar la liberación de insulina ocasionada por la sobreestimulación de las células β del páncreas[72] y la supresión de la degradación de insulina[73]. Esto ocasiona un aumento de la glicolisis en el músculo con la consiguiente formación de lactato,

que será utilizado como sustrato en la gluconeogénesis hepática[74]. Además, la hiperinsulinemia activa la lipogénesis hepática e incrementa la secreción de VLDL, causando hiperlipidemia[75].

Por otro lado, la hiperinsulinemia activa en el tejido adiposo una respuesta inflamatoria que incrementa la lipólisis[76]. La activación de la ruta inflamatoria también puede vincular la acumulación en exceso de tejido adiposo con la resistencia a la acción de la insulina ya que la inflamación estimula la expresión de una serie de genes involucrados en la biosíntesis de ceramidas (apartado 2.2)[77].

El flujo constante hacia el hígado de ácidos grasos, provenientes del exceso de nutrientes de la dieta, además de la lipólisis en el adipocito y la disminución del almacenamiento de lípidos; estimula como ya vimos la gluconeogénesis[74]. Adicionalmente, este excesivo influjo de ácidos grasos puede sobrepasar la capacidad β oxidativa de la mitocondria dando como resultado la acumulación de intermediarios de acilcarnitina parcialmente oxidados, que podrían contribuir a la resistencia a la acción de la insulina.[78] Además, hoy en día existen una serie de aditivos que se agregan a los alimentos y que podrían estar contribuyendo al aumento de la secreción de insulina al ser también una fuente extra de ácidos grasos, como por ejemplo los monoacilgliceroles utilizados en los alimentos como conservantes[79].

A pesar de los extensos estudios y publicaciones sobre el tema, aun no existe un consenso claro respecto a cuál es la causa o el efecto. Los dos puntos de vista no son mutuamente excluyentes y es posible que la acumulación de tejido adiposo sea tanto un marcador biológico que resulta de la resistencia a la acción de la insulina como un factor que contribuye bajo ciertas circunstancias[33].

3.4 La resistencia a la acción de la insulina como mecanismo de defensa

Desde el punto de vista celular, el exceso de glucosa que entra y se almacena en las células musculares en ausencia de un gasto aumentado de energía puede ser perjudicial. Es entonces cuando la resistencia a la acción de la insulina podría ser una respuesta adaptativa beneficiosa.

Se necesita cierta versatilidad homeostática para afrontar las fluctuaciones en la ingesta de alimentos y gasto de energía, incluyendo la capacidad de modificar la sensibilidad a la insulina con el fin de optimizar la repartición y utilización de los sustratos entre los diferentes tejidos[80]. Dicha flexibilidad se puede observar en ciertas condiciones fisiológicas en las que se desarrolla una resistencia a la acción de la insulina no patológica.

Una de estas situaciones es el ayuno prolongado, en el que la relativa abundancia de ácidos grasos, en contraste con la escasa cantidad de glucosa, da prioridad en el músculo a la oxidación de estos sobre la glucosa (resistencia a la acción de la insulina). De esta forma la glucosa es reservada exclusivamente para ser usada en tejidos glucolíticos[16,81].

Similarmente, una sobrecarga de lípidos, que usualmente se utiliza como modelo de resistencia a la acción de la insulina, podría representar un modelo de la flexibilidad metabólica en vez de un mecanismo patológico de resistencia a la acción de la insulina. Un ejemplo de ello se da en atletas de larga distancia, en los que, en respuesta a una sobrecarga de lípidos, el músculo incrementa su capacidad oxidativa para utilizar los ácidos grasos, al mismo tiempo que se preserva el glucógeno del músculo a expensas de una reducción de la oxidación de glucosa[82].

Cuando hay un exceso de nutrientes a corto plazo, el músculo cardiaco y esquelético se vuelven temporalmente resistentes a la insulina, adaptación fisiológica que favorece la desviación del exceso de nutrientes hacia el tejido adiposo para ser almacenados[80]. Cuando este exceso se convierte en algo habitual, esta resistencia a la acción de la insulina podría transformarse en un factor protector contra el estrés metabólico y la disfunción inducida por estos nutrientes.

4. Síndrome metabólico

A través de la evolución, el ser humano ha desarrollado mecanismos para luchar contra el hambre priorizando la eficiencia energética y favoreciendo el almacenamiento de calorías en exceso cuando el acceso a los alimentos es intermitente[27]. Sin embargo, en un ambiente obesigénico como el de ahora, este mecanismo alguna vez ventajoso, podría sentar las bases para el exceso de adiposidad y de sus problemas asociados.

Hoy en día la incidencia de la obesidad a nivel mundial, originada por un consumo elevado de nutrientes y un comportamiento sedentario, ha aumentado dramáticamente. La organización mundial de la salud estima que más de 1900 millones de adultos mayores de 18 años tenían sobrepeso en el 2016 y de estos más de 650 millones tenía obesidad[83]. Estas alarmantes cifras suponen un serio problema de salud ya que la obesidad está asociada al aumento del riesgo de diversas enfermedades como diabetes tipo 2, aterosclerosis, resistencia a la acción de la insulina, hígado graso, desordenes degenerativos incluyendo la demencia, enfermedades respiratorias y algunos tipos de cáncer[27]. Debido a que la asociación entre estas y otras enfermedades no es casual, muchos autores se refieren al conjunto de ellas como síndrome metabólico.

El síndrome metabólico, por tanto, es definido como la confluencia de múltiples enfermedades en el cual se incluye un amplio rango de síntomas producidos por la combinación de una extensa variedad de rasgos metabólicos básicos que necesitan un periodo de tiempo considerable para desarrollarse y manifestarse. Además, son modulados por su desarrollo temprano, por diferentes genes que pueden actuar de modo distinto en función de la distribución especifica de alelos y los condicionantes epigenéticos, así como la exposición a ciertos factores ambientales a lo largo de la vida[84].

Así, aunque este síndrome afecta a todos los sistemas y órganos involucrados en el control del metabolismo energético, impactando directamente sobre todo el organismo a través de la alteración del suministro de energía[84], la genética, el ambiente y la dieta son los factores inmediatos que originan el síndrome metabólico. Sin embargo, cabe destacar el papel fundamental que desempeña la alimentación y en especial la ingesta excesiva de grasas[85]. Además, muchas de las complicaciones causadas por el síndrome metabólico comparten orígenes y rutas bioquímicas comunes a la inflamación, entendida no como la inflamación clásica, sino aquella que es leve pero duradera en el tiempo, es decir crónica (ver apartado inflamación).

El rasgo patogénico más importante del síndrome metabólico es la resistencia a la acción de la insulina, pero también se incluye dentro de estos rasgos, la disfunción del metabolismo de los aminoácidos y amoniaco, cambios en el sistema inmune, predominancia de glucocorticoides (principalmente cortisol), disminución del flujo sanguíneo del tejido adiposo y alteraciones en el sistema ponderostatico[30]. En consecuencia, el síndrome metabólico también está asociado a la diabetes tipo 2, hiperlipidemia, obesidad, hipertrofia e hiperplasia del tejido adiposo blanco, hipoxia, inflamación, hipogonadismo, estrés oxidativo y del retículo endoplasmático, alteración de la microbiota intestinal y de la excreción de urea, hipertensión arterial, e incremento en la producción de óxido nítrico[86].

En un ambiente como el actual, en el que existe una disponibilidad de nutrientes elevada (dietas hipercalóricas) y un gasto de energía reducido, el organismo es incapaz de deshacerse fácilmente del exceso de nitrógeno de los aminoácidos ya que evolutivamente estamos adaptados para conservar las reservas de proteínas y así preservar nuestra supervivencia cuando nos enfrentábamos a largos periodos de escasez[87]. Para la eliminación del N-amínico se necesita primero su conversión a amonio y su posterior incorporación a la urea para ser excretado. Sin embargo, la elevada disponibilidad de energía y lípidos restringe la utilización de glucosa, y la elevación de esta última, evita la producción de amonio a partir de los aminoácidos, limitando así

la síntesis de glutamina y su utilización en el intestino y riñones[88]. Por lo tanto, el normal funcionamiento del ciclo de la urea se ve afectado por la disminución de la formación de amoniaco y glutamina (y la excreción de urea). No obstante, el nitrógeno no se acumula en el organismo y debe ser excretado de alguna manera; estas otras formas de excreción se dan a través de la síntesis de óxido nítrico, sus metabolitos nitratos y nitritos, e incluso se ha postulado la posible eliminación en forma de gas nitrógeno [86].

Por otra parte, el principal vasodilatador del tejido adiposo blanco es el óxido nítrico, cuya síntesis usualmente es activada por la hipoxia[89]. A pesar de existir una elevada producción en estados inflamatorios[90], en el caso de la obesidad no se traslada directamente en vasodilatación. Esto debido, en primer lugar, a una biodisponibilidad reducida de óxido nítrico[91] y, en segundo lugar, a que este efecto es contrarrestado por las catecolaminas y otros vasoconstrictores que ayudan al desarrollo de la hipertensión[92], en respuesta a la resistencia a la acción de la insulina. La hipertensión también es una consecuencia del incremento de la resistencia periférica debido a una mayor masa corporal (obesidad)[93]; sin embargo, la principal causa de la hipertensión es el aumento en el tono vascular del musculo liso debido a la señalización de catecolaminas, endotelina o angiotensina II[94], que son excretadas en parte para contrarrestar la elevada producción de óxido nítrico. Asimismo, el aumento en la síntesis de óxido nítrico también deriva en el incremento de nitratos y nitritos, que son principalmente excretados a través de la saliva e introducidos por esta vía al tracto digestivo[86]. La disponibilidad en exceso de nutrientes, la presencia de estos nitritos y la activación evidente del sistema inmune en la obesidad induce cambios funcionales en la composición de la microbiota[95]. Al mismo tiempo, los lipopolisacáridos excretados por estas bacterias, que circulan libremente en la sangre, agravarían aún más la respuesta inflamatoria[96].

Adicionalmente, el aumento excesivo del tamaño de los adipocitos podría influir en la hipoxia del tejido adiposo blanco ya que se necesitarían más vasos sanguíneos para irrigar el tejido en expansión, así como también un flujo sanguíneo más potente y rápido[97]. Asimismo, el exceso de óxido nítrico contrarrestado por las catecolaminas induce vasoconstricción, lo que podría aumentar aún más la hipoxia e iniciar la inflamación endotelial debido al exceso de sustratos y lípidos junto con un relativo estado de inamovilidad[98]. Como consecuencia, los niveles de oxigeno decrecen, provocando una respuesta glucolítica para obtener ATP. La combinación de la disminución de oxígeno con el aumento de la glucolisis citoplasmática resulta en la masiva

producción de lactato en el tejido adiposo blanco, lo que contribuiría además al descenso de los niveles de pH[97].

Finalmente, en un estado constante de energía positiva, la combinación de niveles elevados de glucosa y de ácidos grasos libres que entran a la célula producen "glucolipotoxicidad", que originará una sobrecarga de equivalentes reductores en la cadena de transporte de electrones de la mitocondria, lo que generará la disfunción de esta y la producción de especies reactivas de oxígeno (ROS)[80]. Esta entrada masiva de nutrientes además se asocia con el cambio del metabolismo lipídico, pasando de oxidar ácidos grasos a esterificarlos, aumentando con ello la deposición de ceramidas. Al mismo tiempo que potencia la lipogénesis *de novo* al aumentar los niveles de malonil-CoA y disminuir la AMPK, favoreciendo también la producción de colesterol a partir del Acetil-CoA (también aumenta la acetil-CoA carboxilasa). Todo ello contribuye aún más al estrés del retículo endoplasmático y a la disfunción mitocondrial[99].

5. Integración de las rutas de señalización y regulación de sustratos

5.1 Estado postprandial y lipogénesis

El adipocito almacena el exceso de energía eficazmente gracias a la lipogénesis, la mayoría como triacilgliceroles, que representan aproximadamente un 90% de toda la energía almacenada[100], en un proceso anabólico mediado por la insulina. Aunque la mayoría de los ácidos grasos usados para la biosíntesis de triacilgliceroles en el adipocito provienen de la dieta[3], los adipocitos son perfectamente capaces de sintetizar nuevos lípidos a partir de los glúcidos en un proceso denominado lipogénesis *de novo*[4]. Incluso durante el ayuno, los adipocitos humanos son capaces de utilizar entre el 20-25% de la glucosa captada para su posterior uso en la síntesis de triacilgliceroles[101].

Las principales enzimas que intervienen en la síntesis de ácidos grasos son la ácido graso sintasa y la acetil-CoA carboxilasa. Durante el proceso de lipogénesis *de novo*, la glucosa aporta el acetil-CoA como sustrato, induciendo la expresión de la acetil-CoA carboxilasa, enzima limitante del paso de acetil-CoA a malonil-CoA y por tanto de la lipogénesis[3]. En el siguiente paso el malonil-CoA aporta el esqueleto de carbonos a los ácidos grasos en crecimiento, formándose ácido palmítico de 16C gracias a la ácido graso sintasa; en un proceso posterior una enzima elongasa se encarga de adicionar carbonos extras a la cadena en formación de ácidos grasos mientras que las

desaturasas se encargan de incorporar dobles enlaces. Tanto la expresión de la enzima ácido graso sintasa como la de la acetil-CoA carboxilasa son elevadas y controladas por la proteína de unión a elementos reguladores de esteroles 1C (SREBP1c) y por la proteína de unión a elementos sensibles a carbohidratos (ChREBP). Ambas actúan sinérgicamente en el hígado y tejido adiposo blanco, sin embargo ChREBP es la que predomina en el tejido adiposo blanco[4] y SREBP1c en el hígado[102]. Además, hay evidencia reciente señalando que ChREBP está implicada en la síntesis de FAHFAs (esteres de ácidos grasos de ácidos grasos hidroxilados), los cuales tienen la capacidad de mejorar la tolerancia a la glucosa[103].

En condiciones normales, esta síntesis de *novo* es relativamente baja en el tejido adiposo blanco, si se compara con el hígado y el tejido adiposo pardo de roedores, pero es aún más baja en el tejido adiposo blanco de humanos[104]. Extrañamente, la restricción calórica incrementa la síntesis *de novo* de ácidos grasos en el adipocito, aunque no está del todo claro si los efectos beneficiosos observados durante la disminución de la ingesta calórica se deban a dicha síntesis[105].

Por otro lado, como se menciona anteriormente, la mayoría de los ácidos grasos usados en la biosíntesis de los triacilgliceroles almacenados en el adipocito provienen de los quilomicra (que transportan los lípidos de la dieta) y de las lipoproteínas de muy baja densidad (VLDL) de la circulación sanguínea. La lipoproteína lipasa, secretada por los adipocitos en un proceso activado por la insulina, es la enzima clave que hidroliza los triacilgliceroles presentes en las lipoproteínas circulantes hasta ácidos grasos libres[3] para luego ser incorporados en el adipocito y una vez dentro, formar nuevamente triacilgliceroles. En el proceso de esterificación un ácido graso (Acil-CoA) se une al glicerol-3P para formar monoacilglicerol. Posteriormente se incorpora otro Acil-CoA al monoacilglicerol para formar diacilglicerol. Finalmente, la enzima diacilglicerol aciltransferasa desempeña un papel fundamental en la acumulación de lípidos en el adipocito ya que cataliza el último paso en la síntesis de triacilgliceroles a partir del diacilglicerol y Acil-CoA[3]. El glicerol, es comúnmente considerado un subproducto de la lipólisis de los triacilgliceroles, pero también puede derivar de la glucosa en el citoplasma del adipocito a través de la ruta glucolítica hasta producir triosas-P y a partir de estas, dihidroxiacetona fosfato. Esta última es reducida hasta sn-glicerol-3P que puede ser utilizado para la síntesis de acilgliceroles o puede producir glicerol libre gracias a la acción de una fosfatasa, la glicerol-3-fosfato fosfatasa[106]. El glicerol producido será transportado fuera de la célula gracias al transportador de glicerol libre, aquaporina 7.

Como se menciona anteriormente, la insulina es el principal estímulo que promueve la captación de la glucosa por el adipocito pero también la de los ácidos grasos y su esterificación a través de

múltiples mecanismos en los que se incluye la activación de enzimas lipogénicas y glucolíticas, la inducción en la translocación de las proteínas transportadoras de ácidos grasos y la sobreexpresión de los genes relacionados con la lipogénesis como la del gen SREBP1[3]. Además, actúa indirectamente al suprimir la lipólisis en el adipocito inhibiendo el recambio de glicerol y de ácidos grasos. Asimismo, cabe destacar que la secreción pancreática de insulina inducida por glucosa depende en parte de otros indicadores metabólicos y metabolitos en los que se incluye la proporción de NAD^+/NADH citosólico, el potencial de la membrana mitocondrial, ADP, Ca^{2+}, 1-monoacilglicerol, diacilglicerol, malonil-CoA y HMG-CoA. Así la liberación de insulina, además de la glucosa, depende de la capacidad pancreática para metabolizar los lípidos[107].

Por consiguiente, la capacidad del tejido adiposo blanco para almacenar lípidos es un factor fundamental ya que una acumulación excesiva de productos derivados de los lípidos dentro del adipocito favorece la aparición de resistencia a la acción de la insulina, y si se supera su capacidad de almacenamiento se produce la infiltración de lípidos en otros tejidos (hígado y músculo).

5.2 Ayuno y lipólisis

En contraposición, durante el ayuno, cuando los requerimientos de energía son estimulados por la necesidad de esta en otros órganos, las reservas de triacilgliceroles del adipocito son liberadas, una vez producida la lipólisis, como glicerol y ácidos grasos libres[3].

La lipólisis empieza con la ruptura sucesiva de los triacilgliceroles, pasando a di- y monoacilgliceroles hasta finalizar en ácidos grasos individuales. La maquinaria lipolítica al menos consiste de tres enzimas básicas (y algunos co-factores asociados) que juntas son responsables de cerca del 90% de la actividad lipolítica del adipocito[4]. Al inicio la ruptura del triacilglicerol hasta diacilglicerol es llevada a cabo por la enzima triacilglicerol lipasa del adipocito, luego la lipasa sensible a hormonas se encarga del paso de diacilglicerol a monoacilglicerol, por último, la monoacilglicerol lipasa completa el proceso al separar al glicerol de los ácidos grasos. Estas lipasas se movilizan hacia las gotas de lípidos para iniciar la lipólisis de los triacilgliceroles gracias a las proteínas asociadas a la superficie de las gotas de lípidos del adipocito como la perilipina (activa gracias a la proteína quinasa A, dependiente de AMPc, que la fosforila), que, una vez activada, se dobla o se aleja de la superficie de la gota de lípido permitiendo el acceso de la triacilglicerol lipasa y la lipasa sensible a hormonas para que inicien la lipólisis[108]. El glicerol liberado junto con los ácidos grasos puede luego ser transportado en el torrente sanguíneo y posteriormente entrar en el músculo, hígado y otros órganos[109]. Cabe destacar que mientras el glicerol suministra la energía necesaria al hígado para ser usado como sustrato energético o para la síntesis de glucosa a través

de la gluconeogénesis, los ácidos grasos libres son utilizados en la β-oxidación, localmente en el mismo tejido adiposo o en tejidos periféricos[3].

Además del glicerol, el tejido adiposo blanco también libera otros fragmentos de 3 carbonos durante el ayuno: alanina, piruvato y lactato. La alanina y principalmente el lactato proceden del piruvato. Este último se origina a partir de las triosas-P produciendo NADH en el proceso; este piruvato puede luego salir de la célula a través de su transportador MCT o puede ser reducido hasta lactato gracias a la enzima lactato deshidrogenasa con la utilización del NADH generado previamente. Luego el lactato puede salir del adipocito también por el transportador MCT. Adicionalmente, el piruvato puede producir alanina en tejidos periféricos como el músculo a través de reacciones de transaminación. En estas reacciones el α-cetoglutarato es convertido por la acción de la enzima glutamato deshidrogenasa a glutamato, cuyo grupo amonio es luego transferido al piruvato por la enzima alanina aminotransferasa, convirtiendo así el piruvato a alanina y regenerándose el α-cetoglutarato.

Cabe señalar que, si el ayuno se prolonga, se da una disminución de la disponibilidad de glúcidos y una elevación de ácidos grasos, lo que promueve la producción de cuerpos cetónicos. Estos se originan a partir de los ácidos grasos libres metabolizados en el hígado para ser utilizados como fuente de energía por el cerebro y en menor medida por el corazón y otros tejidos. De manera similar, estos cuerpos cetónicos son producidos en personas con diabetes tipo 1. En ellas, las células del organismo no disponen de glucosa ya que no es captada debido a la falta de insulina por lo que el hepatocito produce cuerpos cetónicos a partir de los ácidos grasos disponibles en la célula.

En esta fase del ayuno la insulina disminuye de la circulación sanguínea, lo que provoca la supresión de la lipogénesis mientras que se activan las rutas lipolíticas. Al mismo tiempo, los niveles circulantes de glucagón (elevados durante el ayuno) intervienen en la activación de la proteína quinasa A (PKA) y la lipólisis del adipocito[3]; mientras que el sistema nervioso simpático libera catecolaminas, las cuales se unen al receptor β adrenérgico del adipocito activando también la PKA y las rutas lipolíticas[3]. Clásicamente se cree que la lipólisis es dirigida por estas señales β adrenérgicas dentro del adipocito, pero también existen otros inductores que podrían tener relevancia fisiológica, como el factor de necrosis tumoral α (TNF- α)[4].

La insulina puede inhibir la lipólisis de maneras diferentes. En primer lugar, activa la fosfodiesterasa 3b (PDE3b) a través de la fosforilación mediada por Akt; ello reduce los niveles

celulares de AMPc y bloquea la activación de la proteína PKA[4]. Sin embargo, recientemente se ha descrito una ruta inusual en la que la insulina bloquea la activación de PKA selectivamente en la perilina 1 a través de la vía mediada por PI3K, independiente de Akt[110]. En segundo lugar, a largo plazo, la insulina suprime la lipólisis mediante el silenciamiento transcripcional de los genes de las lipasas a través de la represión de los factores de transcripción FOXO1 y IRF4[4].

Al mismo tiempo, la insulina regula el metabolismo hepático de la glucosa de manera directa en el hígado e indirecta a través de un complejo mecanismo molecular que vincula la acción de la insulina en el tejido adiposo blanco con la gluconeogénesis del hígado[52]. De manera directa, la insulina actúa activando los receptores de insulina en el hepatocito, lo cual a corto plazo estimula la síntesis de glucógeno por activación de la enzima glucógeno sintasa, lo que disminuye la producción hepática de glucosa; y a largo plazo, a través de la regulación transcripcional de las enzimas gluconeogénicas, principalmente gracias a la fosforilación de FOXO1[52]. Y de manera indirecta, al inhibir la lipólisis en el adipocito, lo que junto con la disminución del flujo de ácidos grasos hacia el hígado, reduce el contenido hepático de acetil-CoA, un activador alostérico de la enzima piruvato carboxilasa, lo que al final decrece la actividad de esta enzima[111]. Además, la supresión de la lipólisis en el adipocito también reduce el flujo de glicerol hacia el hígado y su conversión a glucosa (gluconeogénesis)[52].

Por el contrario, hay muchos factores que dificultan la capacidad de la insulina para suprimir la lipólisis del adipocito, incluyendo la inflamación. En la resistencia a la acción de la insulina y diabetes tipo 2, los macrófagos activados del tejido adiposo liberan citoquinas que promueven la lipólisis del adipocito, lo que incrementa la liberación de ácidos grasos y glicerol[52], al mismo tiempo que disminuye la esterificación de ácidos grasos y por tanto la capacidad de los adipocitos de almacenar el exceso de energía en forma de triacilgliceroles. El incremento de ácidos grasos libres aumentará la síntesis hepática de lípidos y activará la gluconeogénesis hepática mediante la activación de la piruvato carboxilasa gracias a su activador alostérico, el acetil-CoA; esto último junto con el flujo excesivo de glicerol hacia el hígado incrementa aún más la gluconeogénesis hepática[52]. Adicionalmente, este incremento de ácidos grasos libres impedirá la oxidación de la glucosa, particularmente en la mitocondria a nivel de la piruvato dehidrogenasa[80], de modo que se incrementará el flujo de glucosa hacia las rutas por encima de este paso, incluyendo la síntesis de glucógeno[112]. Por tanto, la desregulación de la acción de la insulina hepática y adiposa contribuye a la hiperglucemia.

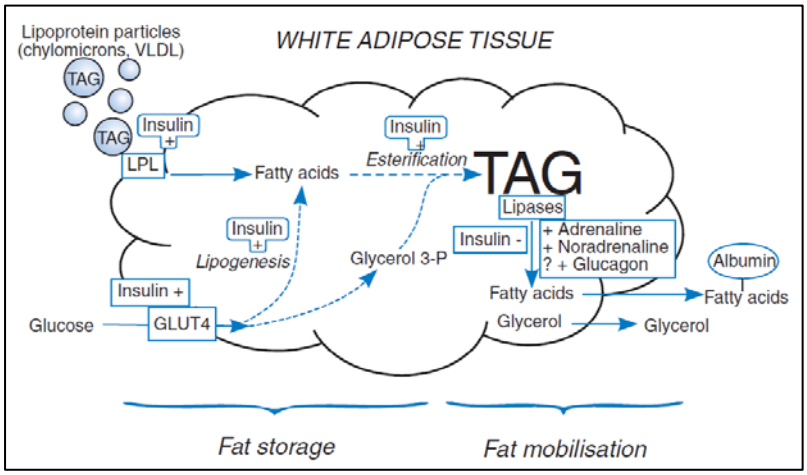


Figura 5: Metabolismo lipídico en el tejido adiposo blanco.

El principal depósito de almacenamiento de energía química en el organismo es el tejido adiposo blanco. Los triacilgliceroles son depositados en los adipocitos a través de la lipogénesis y son liberados a través de la lipólisis para su utilización en otros tejidos.

Fuente: Keith N Frayn/Metabolic Regulation: A Human Perspective 2010

OBJETIVOS

Los mecanismos de control actúan en situaciones de desbalance energético sin embargo nuestro organismo está genéticamente mejor preparado para afrontar periodos de escasez y cuando se produce un balance energético positivo y sostenido, los sistemas de control son incapaces de soportar dicha sobrecarga de nutrientes. Bajo estas condiciones el metabolismo energético cambia, no obstante, a pesar de la gran cantidad de información aún no se conocen muchos aspectos del metabolismo del tejido adiposo blanco. Por ejemplo, en el contexto del síndrome metabólico se asume que la inflamación endotelial producida en el tejido adiposo se debe a la hipoxia, sin embargo, en nuestro grupo de investigación se ha visto que tanto el tejido adiposo blanco *in vivo* como células murinas 3T3L1, incubados bajo condiciones normales sin hipoxia, consumen grandes cantidades de glucosa, mientras producen lactato[113,114] y glicerol (en cantidades no justificadas solo por la lipólisis)[115]. Además, aún continua el debate sobre qué tejido u órgano es el instigador inicial y principal de la resistencia a la acción de la insulina de todo el organismo.

En un esfuerzo por comprender mejor la naturaleza de los adipocitos y por qué dependen tanto del eflujo de los metabolitos de 3C procedentes de la glucolisis en detrimento de la lipogénesis, decidimos estudiar cuantitativamente la capacidad glucolítica (anaerobia a pesar de las condiciones de normoxia) de las células maduras del tejido adiposo blanco (adipocitos y estroma), identificando las diferencias en las funciones metabólicas, primero en estado basal, es decir en ausencia de cualquier estímulo externo como hormonas o fármacos; y luego con insulina para estudiar el efecto que tiene esta sobre dichas células, particularmente cómo afecta la utilización de la glucosa, la lipogénesis y la liberación de lactato y glicerol. Con este fin dividimos este trabajo de tesis en dos partes. Para empezar, nos centramos en el desarrollo de una adecuada metodología cuyo objetivo principal era adaptar, modificar y verificar los procesos necesarios para su correcta aplicación en los experimentos llevados a cabo durante la tesis, mientras que en la segunda parte nos concentramos en la aplicación de esta metodología para estudiar el destino de la glucosa y origen de los fragmentos de 3C.

Los objetivos específicos planteados serían:

- Desarrollar un método utilizando trazadores marcados con ^{14}C para investigar el destino de la glucosa en cultivos primarios (adipocitos de rata), y establecer las razones detrás de la producción masiva de lactato y de glicerol.

Objetivos

- Aislar y obtener células viables a partir del tejido adiposo blanco de rata para su posterior incubación, permitiendo un análisis cuantitativo de los distintos tipos celulares y su distribución en el tejido, así como su funcionalidad incluso a las 48 horas de incubación.

- Analizar el rumbo trazado por los carbonos de la glucosa en el metabolismo global de los adipocitos, con especial atención en la producción de fragmentos de 3C, particularmente, lactato y glicerol, en adipocitos obtenidos a partir de tejido adiposo blanco epididimal de rata. Así como las expresiones de los genes implicados en dichas rutas metabólicas para obtener un panorama más completo de lo que sucede dentro de los adipocitos en condiciones basales.

- Estudiar el metabolismo global de los adipocitos indicado en el apartado anterior, no sólo en condiciones basales, sino en una situación de estimulación hormonal con insulina, con el objeto de comprobar si se mantiene o no la elevada producción de fragmentos de 3C en estas condiciones y como se modifica el metabolismo.

- Evaluar la contribución cuantitativa de los adipocitos en comparación con las células estromales vasculares para generar fragmentos de 3C, a partir de la glucosa a través de la vía glucolítica bajo condiciones normóxicas.

INFORMES

1. Informe de participación

Esta tesis ha sido realizada en paralelo con la doctora Floriana Rotondo, antigua alumna de doctorado del grupo de investigación. La tesis de la Dra. Rotondo, así como la presente tesis comparten el mismo tema de investigación, sin embargo, ambas se enfocan en aspectos diferentes. La Dra. Rotondo desarrolló la parte metodológica del aislamiento e incubación de los adipocitos y Ana Cecilia Ho desarrolló el método para rastrear cuantitativamente el destino de la glucosa. Posteriormente, ambas participaron en la incubación de las células y en el análisis de los metabolitos; y mientras que la Dra. Rotondo analizó las expresiones y se centró en la investigación del glicerol; Ana Cecilia Ho estudió la distribución de la radiactividad y se enfocó en la investigación del lactato. Por tanto, ambas comparten la coautoría de los artículos publicados en Scientific Reports y en Adipocyte.

Consecutivamente, sin la participación de la Dra. Rotondo, Ana Cecilia Ho trabajó en otra línea de investigación, la determinación de la respuesta del tejido adiposo blanco a la insulina.

En cuanto a los artículos publicados, esta estrecha colaboración se podría repartir en porcentajes de participación de la siguiente manera:

1. A method for the measurement of lactate, glycerol and fatty acid production from 14C-glucose in primary cultures of rat epididymal adipocytes. Ana Cecilia Ho-Palma, Floriana Rotondo, María del Mar Romero, Serena Memmolo, Xavier Remesar, José Antonio Fernández-López and Marià Alemany. Analytical Methods 2016.

   80% Ana Cecilia Ho Palma — 20% Floriana Rotondo

2. Quantitative analysis of rat adipose tissue cell recovery, and non-fat cell volume, in primary cell cultures. Floriana Rotondo, María del Mar Romero, Ana Cecilia Ho-Palma, Xavier Remesar, José Antonio Fernández-López and Marià Alemany. PeerJ 2016.

   20% Ana Cecilia Ho Palma — 80% Floriana Rotondo

Informes

3. Glycerol is synthesized and secreted by adipocytes to dispose of excess glucose, via glycerogenesis and increased Acilglycerol turnover. Floriana Rotondo, Ana Cecilia Ho-Palma, Xavier Remesar, José Antonio Fernández-López, María del Mar Romero and Marià Alemany. Scientific Reports 2017.

   50% Ana Cecilia Ho Palma 50% Floriana Rotondo

4. Use of 14C-glucose by primary cultures of mature rat epididymal adipocytes. Marked release of lactate and glycerol, but limited lipogenesis in the absence of external stimuli. Ana Cecilia Ho-Palma, Floriana Rotondo, María del Mar Romero, José Antonio Fernández-López, Xavier Remesar and Marià Alemany. Adipocyte 2018.

   50% Ana Cecilia Ho Palma 50% Floriana Rotondo

5. Insulin only partially increases basal 14C-glucose utilization in isolated epididymal rat adipocytes, largely for glycerogenesis and lipogenesis. Ana Cecilia Ho-Palma, Pau Toro, Floriana Rotondo, María del Mar Romero, Marià Alemany, Xavier Remesar and José Antonio Fernández-López. Será sometido próximamente.

   100% Ana Cecilia Ho Palma

Codirector Codirector

Jose Antonio Fernandez-Lopez Xavier Remesar Betlloch

2. Informe sobre el factor de impacto de las publicaciones

Los artículos que forman parte de esta tesis doctoral presentada por Ana Cecilia Ho Palma han sido publicados o sometidos para su publicación en revistas internacionales indexadas tal como se detalla a continuación:

El artículo: "A method for the measurement of lactate, glycerol and fatty acid production from 14C-glucose in primary cultures of rat epididymal adipocytes" se publicó en la revista *Analytical Methods de* la Royal Society of Chemistry (U.K) el año 2016 en formato open access y con un índice de impacto de 2.073 (Q2).

El artículo: "Quantitative analysis of rat adipose tissue cell recovery, and non-fat cell volume, in primary cell cultures" se publicó en la revista *PeerJ* el año 2016 en formato open access y con un índice de impacto de 2,2 (Q2).

El artículo: "Glycerol is synthesized and secreted by adipocytes to dispose of excess glucose, via glycerogenesis and increased acilglycerol turnover" se publicó en la revista *Scientific Reports* del grupo Nature (USA) el año 2017 en formato open access y con un índice de impacto de 4,259 (Q1).

El artículo: "Use of 14C-glucose by primary cultures of mature rat epididymal adipocytes. Marked release of lactate and glycerol, but limited lipogenesis in the absence of external stimuli" se publicó en la revista *Adipocyte* del grupo Taylor & Francis (U.K) el año 2018. Actualmente no cuenta con índice de impacto.

El artículo: "Insulin only partially increases basal 14C-glucose utilization in isolated epididymal rat adipocytes, largely for glycerogenesis and lipogenesis" será sometido próximamente para su publicación.

Por lo que el trabajo de tesis presentado por Ana Cecilia Ho Palma corresponde a cuatro trabajos publicados del Q1-Q2 y uno pendiente de publicación.

Codirector

Jose Antonio Fernandez-Lopez

Codirector

Xavier Remesar Betlloch

PUBLICACIONES

1. Desarrollo de la metodología

La metodología desarrollada fue muy importante ya que al no existir un método apropiado para volúmenes pequeños como es el caso de los cultivos celulares, tuvimos que adaptar uno que permitiera el análisis desde el punto de vista cuantitativo. En el primer método se utilizó marcadores radiactivos permitiendo así seguir el destino de la glucosa y la producción de fragmentos de 3C a partir de esta, así como su participación en la lipogénesis e incorporación en los triacilgliceroles. Mientras que el segundo método surgió a consecuencia de la necesidad de aislar y obtener adipocitos viables, a partir del tejido adiposo de rata, para su posterior incubación. La combinación de ambos métodos aplicados a los experimentos posteriores nos permitió analizar los datos cuantitativamente a partir de la misma fuente (pozo de incubación) y conseguir así toda la información necesaria del sistema analizado.

1.1 A method for the measurement of lactate, glycerol and fatty acid production from 14C-glucose in primary cultures of rat epididymal adipocytes

Ana Cecilia Ho-Palma, Floriana Rotondo, María del Mar Romero, Serena Memmolo, Xavier Remesar, José Antonio Fernández-López and Marià Alemany.

Método que permite el análisis de la distribución de glucosa ^{14}C en los principales metabolitos utilizados por adipocitos aislados a partir de tejido epididimal de rata. Estos fueron incubados con glucosa ^{14}C a 7 mM durante 24 horas. La glucosa ^{14}C incorporada en las células fue examinada al extraer los lípidos (triacilgliceroles), saponificarlos y contar la radiactividad en las fracciones resultantes de glicerol de glicéridos, jabón de ácidos grasos y restos acuosos celulares. Mientras que el lactato, glicerol y glucosa presentes en el medio celular fueron separados a través de mini columnas de cromatografía de intercambio iónico recuperando los pequeños volúmenes utilizados por centrifugación. Sucesivos lavados de sólo 0,2mL y la posterior elución de las fracciones estudiadas, nos permitió la completa recuperación y un contaje preciso de la radiactividad. Primero se separó y contó el marcaje del lactato del medio celular. Luego se incubó el medio celular, libre de lactato, para separar la glucosa con glucosa oxidasa y catalasa; y el glicerol con gliceroquinasa y ATP, obteniendo ácido glucónico y glicerol-3P, respectivamente. Posteriormente se separó en simultaneo la glucosa y el glicerol con las mini columnas. Se midió la radiactividad de dichas fracciones y se analizaron los metabolitos fríos en medio. Finalmente, se

calcularon las actividades específicas por carbono de los principales metabolitos. Los resultados obtenidos de la aplicación completa del método confirman la elevada producción de lactato a partir de la glucosa, aún en condiciones fisiológicas normales (7mM de glucosa). Aproximadamente la mitad de la glucosa fue metabolizada después de 24 horas, 2/3 de la glucosa radiactiva incorporada fue hallada como lactato y, probablemente, otros fragmentos de 3C; y un 14% como glicerol libre. La radiactividad encontrada dentro de los ácidos grasos de los adipocitos, en el glicerol de glicéridos y en el dióxido de carbono estimado fue de aproximadamente 5% cada uno. El procedimiento experimental descrito es versátil bajo las condiciones probadas, con un alto grado de repetividad y permite la recuperación casi total de la radiactividad inicial (hasta un 97%).

1.2 Quantitative analysis of rat adipose tissue cell recovery, and non-fat cell volume, in primary cell cultures

Floriana Rotondo, María del Mar Romero, Ana Cecilia Ho-Palma, Xavier Remesar, José Antonio Fernández-López and Marià Alemany.

El tejido adiposo blanco, además de adipocitos, contiene otros tipos celulares con funciones heterogéneas. El estudio de los adipocitos implica su aislamiento con colagenasa a partir de tejido adiposo y su posterior incubación. Sin embargo, poco se conoce sobre la calidad y cantidad de los adipocitos utilizados en cultivos primarios y si se correlaciona con el tejido adiposo intacto. Para analizar la viabilidad y recuperación, aislamos con colagenasa adipocitos a partir de tejido adiposo blanco epididimal de 4 ratas jóvenes. La funcionalidad de los adipocitos, hasta las 48 horas de incubación, fue estudiada al medir la captación de la glucosa y los metabolitos producto de su metabolismo. La cantidad de contenido graso del tejido, así como de todos los volúmenes y pesos de las fracciones obtenidas durante el proceso fueron analizados minuciosamente permitiendo la estimación de la cantidad de grasa inicial de tejido adiposo blanco que queda en el cultivo primario. Se midió el tamaño y cantidad de los adipocitos y de las otras células que conforman el tejido (fracción vascular estromal). También se estimó la cantidad de eritrocitos, células no nucleadas, presentes en la fracción estromal. La recuperación final de los adipocitos viables en cultivo celular fue de 70 – 75% respecto al tejido intacto, siendo más activos en términos metabólicos durante el segundo día de incubación. Los adipocitos representaron un 7% del total de las células del tejido adiposo blanco mientras que los eritrocitos un 66% y las otras células nucleadas un 27%. No obstante, el volumen ocupado por los adipocitos en el tejido fue de un 90%, dejando un 0,05% para los eritrocitos y un 0,2% para otras células nucleadas. Finalmente, el

volumen que ocupa el citoplasma en el tejido adiposo blanco (la parte sin grasa del adipocito) fue de 1,3%. Por lo tanto, la cantidad de células “vivas” presentes en el tejido adiposo blanco, es relativamente pequeña: aproximadamente 13mL/g para los adipocitos y 2mL/g para el estroma; además de cerca de 1mL/g de eritrocitos a pesar de que las ratas fueron sacrificadas por desangrado. Sin embargo, según los resultados obtenidos, esta pequeña cantidad de células “vivas” del tejido, posee una actividad metabólica extremadamente elevada, lo que sugiere que el tejido adiposo blanco es sumamente importante para controlar el metabolismo energético. Además, la metodología desarrollada en los cultivos primarios permite la comparación directa y cuantitativa con el tejido adiposo intacto.

Analytical Methods

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c6ay01244b

A method for the measurement of lactate, glycerol and fatty acid production from ^{14}C-glucose in primary cultures of rat epididymal adipocytes

Ana Cecilia Ho-Palma,[a] Floriana Rotondo,[ab] María del Mar Romero,[abc] Serena Memmolo,[a] Xavier Remesar,[abc] José Antonio Fernández-López[abc] and Marià Alemany*[abc]

We have developed a method for the analysis of the main metabolic products of utilization of glucose by isolated adipocytes. They were incubated for 24 h with ^{14}C-glucose. The final label distribution and cold levels of medium glucose, lactate and glycerol were estimated. Medium lactate was extracted using ion-exchange resin minicolumns prepared with centrifugation-filtering tubes in which the filter was substituted by the resin. This allowed complete washing using only 0.2 mL. Repeated washings allowed for complete recovery of fractions with low volumes passing through or retained (and eluted), which permitted precise counting and a sufficient amount of sample for further analyses. Lactate was separated from glucose and glycerol; glucose was then separated by oxidizing it to gluconate with glucose oxidase, and glycerol was separated in parallel by phosphorylation with ATP and glycerol kinase. Cells' lipid was extracted with ether and saponified. Glycerides-glycerol and fatty acids (from the soaps) were counted separately. The complete analysis of cells incubated with labelled glucose resulted in about half of the glucose metabolized in 24 h, 2/3rds of the incorporated glucose label was found as lactate, and 14% as free glycerol. Their specific activities per carbon were the same as that of glucose. Production of fatty acids took about 5% of the label incorporated, an amount similar to that of glycerides-glycerol and estimated carbon dioxide. The procedure described is versatile enough to be used under experimental conditions, with a high degree or repeatability and with only about 3% of the label not accounted for.

Received 27th April 2016
Accepted 19th October 2016

DOI: 10.1039/c6ay01244b

www.rsc.org/methods

Introduction

The application of quantitative factors in the analysis of metabolic pathways allows a better understanding of main substrate partition, energy handling and inter-organ cooperation, under both physiological and pathological conditions.[1,2] Most of these studies imply the tracing of ^{14}C and ^{3}H-labelled substrates,[3,4] more recently substituted (in part) by stable isotopes.[5] The concurring availability of new powerful analytical techniques, largely based on mass spectrometry,[6] and the extended, albeit unjustified, assumption that our knowledge of the main metabolic pathways and their bulk regulation and operation are already well known, have certainly decreased the use of tracer techniques using radioactive labelled compounds. In any case, ^{14}C-labelled substrates remain to be the best option for studies of metabolic interconversion in which small amounts of material, and the absence of environmental control or major ethical constraints, allow their specific utilization.

White adipose tissue (WAT) is a disperse organ,[7] distributed in a number of locations in which its basic energy storage activity[8] is complemented by many other physiological functions.[9–11] In any case, its main role is to contribute to the defense of energy homoeostasis, helping to control glucose,[12] lipid,[13] and amino acid[14] metabolism overall, sharing a large part of the control of whole body energy availability,[15] and also acting as a platform for immune system protection and regeneration.[16,17] The complex (and varying) mixture of cell types in WAT sites largely determines and modulates these functions as part of its protean adaptability.[16,18]

Most of the WAT volume corresponds to a relatively small number of adipocytes. The rest of the cells (stromal) is made up of immune system, stem, blood, endothelial, and other types of cells,[19,20] which often play critical functions under conditions of inflammation and maintenance of energy homoeostasis.[21,22] Adipocytes, despite their small numbers but large volumes most of which are fat, have been intensely studied as the most "representative" cells of WAT.[23] To clarify their metabolic abilities, they are often isolated from WAT masses and studied in primary[24] or immortalized[25] cell cultures. The information

[a]Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, University of Barcelona, Av. Diagonal, 643, 08028 Barcelona, Spain. E-mail: malemany@ub.edu; Tel: +34 934034606
[b]Institute of Biomedicine, University of Barcelona, Barcelona, Spain
[c]CIBER-OBN Research Net, Barcelona, Spain

obtained is often taken as a direct representative of WAT *in vivo*, in spite of the large number of factors that are known to counter this simplistic approach.[26,27]

Many studies on adipocytes have been carried out using standard murine cell lines, as we recently did, finding that, under normoxic conditions, 3T3L1 cells were able to convert huge amounts of the medium glucose to lactate,[28] fulfilling most of their energy needs through anaerobic glycolysis. We postulated that this "wasting" of glucose might actually help in diminishing hyperglycemia because of the large combined mass of WAT; in *ex vivo* studies; we also observed the accumulation of lactate in WAT masses,[29] in line with the results previously observed in cultured cells. These results agreed with the low *in vivo* WAT oxygen consumption observed in humans and rats[30,31] parallel to its high production of lactate.[32,33] However, the origin of the circulating lactate could not be fully discerned (other than by bulk mass), leaving open its possible relationship with the utilization of glucose for other purposes, such as lipogenesis.[34] Fatty acid synthesis is the main metabolic pathway in the WAT of rodents which are fed with standard (largely carbohydrate-based) diets.[35] In order to help clarify the purpose of the massive production of lactate, we decided to analyze quantitatively the fate of glucose using primary cultures of rat adipocytes, but first we had to develop the necessary methodology to carry out the study applied to the small volumes used in standard cell culture.

Separation of glucose (uncharged molecules) from (ionized) lactate has been performed for a long time using ionic-interchange columns.[36,37] However, the problems posed by this approach limit the quantitative analyses, since high specific activity substrates are needed, and the large volumes required for effective ion-exchange column separation, washings and the inevitable dilution of label limit the possibility of using a multifaceted quantitative approach. In the present study, we adjusted this concept to the use of small volumes, thus limiting most of the unwanted effects of dilution by using a column centrifugation-based approach. These procedures allowed us to analyze, in the same culture well, both the fate of glucose to form lactate and its incorporation into lipids.

Experimental

Rats and housing conditions

All animal handling procedures and the experimental setup were in accordance with the animal handling guidelines of the corresponding European, Spanish and Catalan Authorities. The Committee on Animal Experimentation of the University of Barcelona specifically authorized the procedures used in the present study.

Male Wistar rats (Harlan Laboratory Models, Sant Feliu de Codines, Spain) were used after a 2 week acclimation period in a controlled environment. When used, the rats were 17 weeks old, and weighed 412 ± 35 g. The animals were kept in two-rat cages with wood shards as the bedding material, at 21.5–22.5 °C, and 50–60% relative humidity; lights were on from 08 : 00 to 20 : 00. They had unrestricted access to water and standard rat chow (Harlan-Teklad #2014).

Isolation and measurement of the number of adipocytes

The rats were killed under isoflurane anesthesia, at the beginning of a light cycle, by exsanguination, from the exposed aorta, using dry-heparinized syringes. The rats were rapidly dissected, taking samples of epididymal WAT. Tissue pieces were used immediately for adipocyte isolation. This procedure followed, essentially that described by Rodbell.[38] In short, tissue samples were weighed when already immersed in the digestion medium described below, and cut into small pieces with scissors. Samples were incubated, at 37 °C in a shaking bath for 60 min, with 2.5 volumes of Krebs-Henseleit medium pH 7.4, containing 5 mM glucose, 0.1 μM adenosine (Sigma-Aldrich, St Louis, MO USA),[39] and 10 g L^{-1} delipidated bovine serum albumin (Merck-Millipore, Billerica, MA USA). The medium was complemented with 3.5 μkat mL^{-1} collagenase (LS004196, type I, from Worthington Biomedical, Lakewood, NJ USA). At the end of the digestion process, the suspensions were gently "filtered" through a double layer of nylon mesh hose, which retained small vessels and undigested pieces (if any). The smooth crude suspension of isolated cells was left to stand for 5 minutes in capped syringes held vertically. The adipocytes floated to form a defined upper layer; then, the lower aqueous fraction was slowly drained off, capping again the syringe. The cells were washed three times with 2.5 volumes of the digestion buffer minus collagenase. The final supernatant layer contained intact adipocytes and a small amount of free fat from broken cells. In all cases, before re-suspending the cells, the buffer alone was subjected to 30 s of strong vortexing, to allow for equilibration with air oxygen. After the final washing, only the cells' fraction remained, from which aliquots were taken for incubation. The samples were extracted from the central part of this layer, trying not to disturb the thin floating fat layer. The whole procedure was carried out at room temperature; the cells were used immediately after the final washing.

A known volume of the adipocyte suspension was introduced into a Neubauer chamber (#717810 Neubauer improved bright line, Brand Gmbh, Wertheim Germany). Using an inverted microscope, four fields (following a pre-established selection pattern) were photographed at low power. Each adipocyte suspension sample was measured four times, obtaining about 16 photographs from each. The numbers and diameters of cells were counted (under the conditions used, all cells adopted a spheroid form) using the ImagingJ software (http://www.imagej.nih.gov/ij/)[40] for image analyses.

Conditions for the incubation of adipocytes

Incubations were carried out using 12-well plates (#CLS3513 Costar, Sigma-Aldrich) filled with 1.7 mL of DMEM (#11966-DMEM-no glucose; Gibco, Thermo-Fisher Scientific, Waltham MA USA), supplemented with 30 mL L^{-1} fetal bovine serum (FBS, Gibco). The medium also contained 25 mM hepes (Sigma-Aldrich), 2 mM glutamine (Lonza Biowhittaker, Radnor, PA USA), 1 mM pyruvate (Gibco), 30 g L^{-1} delipidated bovine serum albumin (Millipore Calbiochem, MA USA), 100 U mL^{-1} penicillin and 100 mg L^{-1} streptomycin (Gibco, Thermo-Fisher Scientific). Adenosine (Sigma-Aldrich) 100 nM was also added to

help maintain the integrity of the cells.[39] D-Glucose was added as a substrate, supplemented with ^{14}C-(U)-D-glucose, (#ARC0122B, American Radiolabeled Chemicals, St Louis MO USA, specific activity 11 GBq per mmol, *i.e.* 1.83 GBq per mmol-C). The final glucose concentration in the wells was, nominally, 7 mM. In all cases, the amount of label added per well was the same: 4.7 kBq (*i.e.* 430 pmol ^{14}C-glucose) resulting in specific activities in the range of 385 Bq per μmol. Labelled glucose was purified through a single pass of the product through an ion-exchange column as described below under "Estimation of medium label present in the "glucose" fraction". The removal of ionic contaminants contributed to the diminishment of overlapping of labels between the different fractions.

Each well received 400 μL of the adipocyte suspension, thus completing a final volume of 2.1 mL. A number of random counts of cells pipetted in wells gave a variation of about 2% in initial cell numbers, the standard amount of cells used ($702 \pm 46 \times 10^3$ cells per well) depended on the donor rat. Since the combined volume of the added cells was known, the net volume of the incubation medium (*i.e.* excluding the cell volume) was calculated for each single well from the initial volume and that of buffer added to the cells. This was estimated from the total volume of cell suspension added minus the volume of cells, obtained from the mean cell volume and the number of cells counted. Under these conditions, the cells floated freely and tended to accumulate on or near the surface.

The cells were incubated at 37 °C in an incubation chamber ventilated with air supplemented with 5% CO_2, which gave a theoretical pO_2 of 20 kPa (*i.e.* 0.2 mM of dissolved O_2). These values were in the range of those measured under the same conditions.[28] The calculated pCO_2 was in the range of 5 kPa, corresponding to 1.7 mM of dissolved CO_2.[41] The cells were incubated for 24 h without any further intervention.

The rate of evaporation of water from the medium was estimated, under the conditions of incubation, using medium-filled wells (*i.e.* no cells), and the loss of weight with time of incubation in the chamber was measured. Daily evaporation was in the range of 2.5% of the well water (*i.e.* about 48 μL per day). This value was included in the estimation of the volume of medium, used for all calculations of substrate utilization.

Cell harvesting and sample handling

The medium (carefully excluding the floating adipocytes) of the wells was transferred to 2 mL Eppendorf tubes. The cells (and the remaining medium bathing them) were transferred to another tube and left to stand. All media extractions were combined in a single tube and its weight was estimated by differential weighing; after thorough mixing, it was kept at −20 °C until processing. The cells were weighed likewise and re-suspended in 2 volumes of digestion buffer (without collagenase), pH 7.4, and containing 5 mM glucose; then, they were allowed to float again, removing the infranatant washing medium. The cells were then frozen in the same tube with liquid nitrogen and kept at −80 °C until being processed.

The incubation medium was used for the estimation of glucose, using a glucose oxidase-peroxidase kit (#11504, Biosystems, Barcelona Spain) to which we added 740 nkat mL^{-1} mutarotase (porcine kidney, 136A5000, Calzyme, St Louis, MO USA).[42] Lactate was measured with kit 1001330 (Spinreact, Sant Esteve d'en Bas, Spain), pyruvate was measured with NADH (Calbiochem EMD Millipore Merck, Darmstadt, Germany), and lactate dehydrogenase (rabbit muscle, 427217, Calbiochem EMD-Millipore Merck);[43] glycerol was estimated with kit #F6428 (Sigma-Aldrich). We decided to analyze the chemical concentrations of substrates in samples despite being tainted by radioactive tracers in order to be able to exactly match concentrations and label counts. We used that approach because of the small amounts of radioactivity involved, and the lower ionizing power of ^{14}C β radiation. These analyses were performed under conditions of high radioprotection safety. Debris and contaminated materials were safely disposed of as radioactive waste.

Estimation of label content

Counting of ^{14}C label in the samples was performed using typical 5 mL polyethylene vials. Samples of up to 400 μL were mixed with at least 10 volumes (minimum 3.5 mL) of water-miscible liquid scintillation cocktail (Ecoscint H; #LS-275, National Diagnostics, Atlanta, GA USA). Then, the tubes were shaken to obtain a single clear phase. They were left in the dark (at room temperature) for at least 12 h before being introduced in a scintillation counter (TriCarb 2100-TR, Perkin-Elmer Packard, Boston, MA USA). Automatic correction for counting efficiency was applied, and a number of blanks were introduced in each series to estimate the background, which was routinely discounted. When a sufficient amount of sample was available, counting was performed in duplicate. Specific radioactivity of the different fractions was expressed (and compared) on a molar (per carbon) basis. Thus total label (*i.e.* x Bq) in 1 μmol glucose was expressed in (x/6) Bq per μmol-C, because the glucose used was uniformly labelled in its 6 carbon atoms. In the case of lactate and glycerol, the factor used was 3, and for fatty acids, we used oleic acid (C18:1.9) as standard for comparisons because it is the most abundant fatty acid in the rat;[44] thus the molar specific activity of fatty acids was divided by 18 to obtain the molar-C (*i.e.* per carbon) value.

Preparation of micro ion-exchange columns

The basis for discrimination of the main substrates analyzed was the use of ion-exchange resins in microcolumns. The prepared columns were able to retain quantitatively ionized molecules, such as lactate at a pH in which its ionization will be sufficiently high, but non-ionic (acidic in the present setting) compounds such as glucose and glycerol would pass unaffected through the column. The acid retained in the column was later displaced with a low pH solution, releasing the lactate (and pyruvate) label.

Sieve-filter type centrifugation inserts (Ultrafree-MC, Millipore, Bedford, MA USA) were used as columns. The sieve filter was removed, leaving bare the insert plastic tube (Fig. 1). A small amount of analytical quality glass-fiber wool (Panreac, Castellar del Vallès, Spain) was used to cover the bottom of the

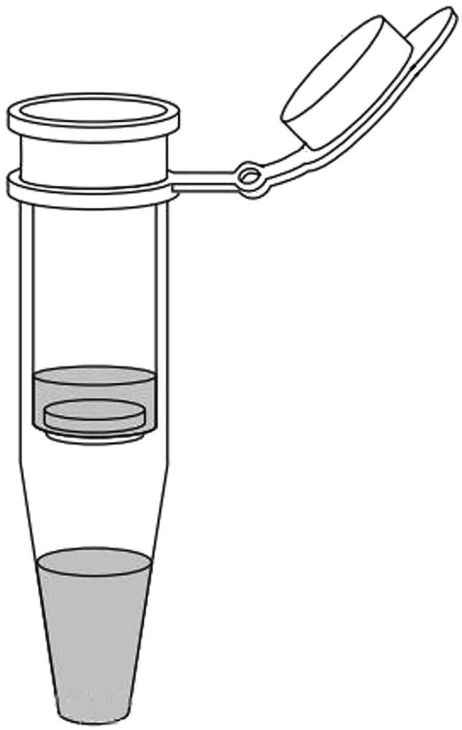


Fig. 1 Centrifuge-separation tube with plastic insert used in the preparation and use of ion-interchange columns.

insert tube, pressed with the help of a glass rod. The bottom of the insert was then filled with 250 mg of 24 h-hydrated and centrifuge spin-dried ion-exchange resin in cationic form (Dowex 1 × 2 [200–400 mesh], Serva Electrophoresis GmbH, Heidelberg, Germany). The insert was introduced into the holder tube and then 200 μL of pure water (18 MΩ resistivity) was added to the column. The tubes were centrifuged for 2 min at 200 × *g*. The recovered washing fluid was discarded. The column was already prepared for immediate use.

Overview of label distribution analysis after incubation

We used a sequential process for the separation and counting of fractions corresponding, essentially, to the main metabolites expected to be formed from glucose. The procedure used is presented in Fig. 2. At the end of the incubation of adipocytes in media containing labeled glucose, the cells were separated from the medium and processed as described below. The medium was passed though ion-exchange minicolumns (procedure M1) to retain all ionizable compounds (*i.e.* lactate), which were later recovered and counted (procedure M2). This process depleted the medium of ionizable (labelled) compounds; but containing all non-ionizable (*i.e.* glucose, glycerol) ones. These were separated through derivatization to a specific acidic compound (gluconate in the case of glucose, procedure M3; glycerol-3P in the case of glycerol, procedure M4) by means of parallel incubation of the depleted medium. The recovered fractions allowed a fair discrimination of the label present in the remaining medium glucose and that in lactate and glycerol.

Estimation of medium label present in the "lactate" fraction

The basic protocol for column retention of acidic compounds (procedure M1) consisted of the application of 300 μL of the incubation medium (its pH was already in the range 6.8–7.2) on top of the column. The tubes were capped and left to stand for 4 min to allow an even distribution of the liquid wetting the resin. No fluid left the column, since the mobile phase was retained by capillarity. The tubes were then centrifuged for 2 min at 200 × *g* (*i.e.* 20 MPa). The fluid recovered (about 300 μL) was reserved (washout 1, or W1). In order to remove any non-ionic labeled remnants, the column was washed with 200 μL of pure water, quantitatively recovering the washout (W2). The column was then eluted (procedure M2) with the addition (and separation by centrifugation) of four successive 200 μL aliquots of 250 mM HCl, recovering the combined acidic eluates (W3). Finally, the column was washed again with 200 μL of pure water (W4). This procedure extracted all ionic labelled compounds of the medium previously retained by the column; the combined volume of W3 + W4 was about 1 mL. Depending on the volume to process and sample dilution, higher volumes of medium were used, or pairs of columns could be used in tandem. The radioactivity of the samples was estimated after neutralization with solid sodium bicarbonate, in order to limit the acid-generated quenching.

In order to check the effectiveness of the columns, an analysis of lactate label distribution was carried out under otherwise standard conditions (but no cells). We used cold (5 mM) and labeled L-lactate [317 Bq per well] (^{14}C-(U)-L-lactate; #NEC-599, Perkin-Elmer, Boston, MA USA; specific activity 4.80 MBq per μmol, *i.e.* 1.60 MBq per μmol-C). Table 1 shows the distribution of total label between the different fractions recovered.

The column completely retained the lactate label; its elution was practically complete with the first 400–600 μL of HCl; the successive washings did not provide further recoveries of lactate label. The final process was established leaving only one additional water washing, which was used, in all the cases, to check for the completeness of column elution. When label was detected in the second washout (it was always less than 5% of that in the first washing), the net Bq were simply added to the corresponding column washout for final calculations.

Estimation of medium label present in the "glucose" fraction

After retention of lactate in the ion-exchange column, the medium (plus the water washing) passing through (W1 + W2) contained the entire non-retained glucose label. We modified enzymatically the glucose to gluconic acid by means of the same glucose-oxidase method used to measure its levels (procedure M3).

Aspergillum glucose oxidase (EC 1.1.3.4) specifically oxidizes C1 of glucose with oxygen to yield gluconic acid and hydrogen peroxide:[45]

β-D-glucose + O_2 → D-glucono-β-lactone + H_2O
D-glucono-β-lactone + H_2O → D-gluconic acid
(spontaneous non-enzymatic hydrolysis)

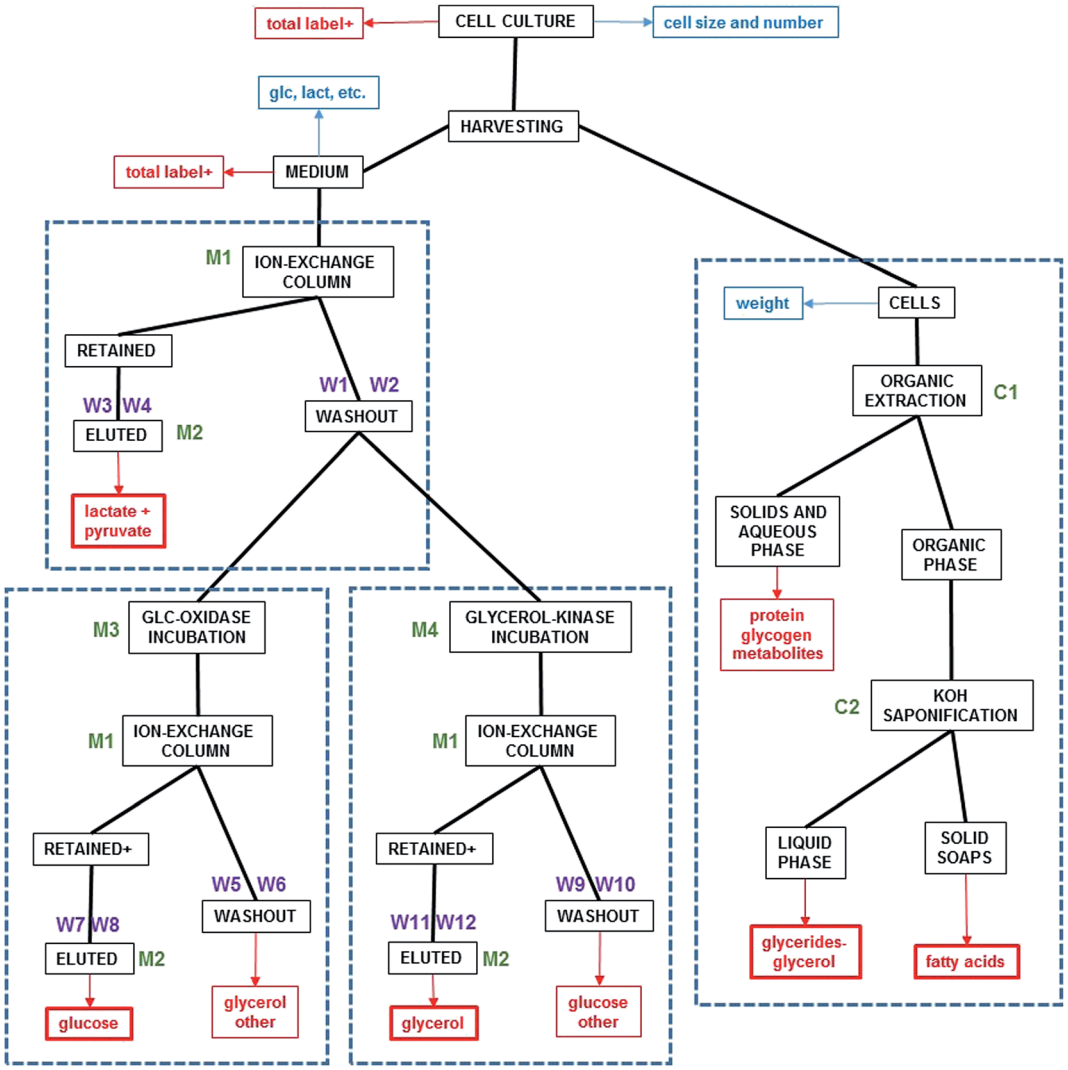


Fig. 2 Flow diagram of the process of analysis of the fate of ^{14}C-glucose label in the medium and cells of rat epididymal WAT incubated for 24 hours. The process is marked in black, red fractions refer to the counting of the fraction radioactivity, and blue is reserved for non-radioactive measurements (metabolites, cell parameters). The specific procedures explained under "Experimental" are marked in green, and the fractions eluted in purple. The four rectangles marked with dashed lines show the specific procedures for lactate, glucose and glycerol label separation, and for the processing of cell's fractions. glc = glucose; lact = lactate.

This same reaction has been used previously to discriminate the presence of radioactive label in glucose[46] using classical minicolumns filled with an ion-exchange resin (cationic form) adapted to the retention of acidic compounds (as in the case described above for lactate).[37] In order to diminish possible sources of interference, we used purified enzymes, glucose oxidase (type VII from *Aspergillus niger*, Sigma-Aldrich); as well as catalase (from bovine liver, Sigma-Aldrich). Catalase (EC 1.11.1.6) was added in excess to drive the reaction to the right, to limit unwanted oxidative damage and to recycle part of the medium oxygen to sustain the glucose oxidase activity, since oxygen availability may be the limiting step of the reaction under the conditions tested.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The addition of mutarotase (EC 5.1.3.3) to favor the conversion of α to β glucose[47] was considered unnecessary because of the length of the incubation period (allowing for a complete drainage of α-glucose to the β form, substrate of glucose oxidase). A test including mutarotase showed no effects in glucose oxidation under the experimental conditions described below. The test results, obtained using labelled glucose (initially most of it as the α-isomer), which resulted in its complete oxidation, were a definitive argument for omitting mutarotase in all the ensuing experiments.

An aliquot 100 μL of combined fractions W1 and W2 (eluted after lactate retention) was used for the measurement of total label. Another 100 μL aliquot was mixed with 1 mL of 50 mM acetate buffer pH 5.2, containing 1 g L^{-1} dithiothreitol, 1 g L^{-1} defatted bovine serum albumin (all from Sigma-Aldrich), about 400 nkat of glucose oxidase and an excess (350 μkat) of catalase

Table 1 Test distribution of glucose, lactate and glycerol label along the M1 procedure of separation using micro ion-exchange columns and elution by centrifugation[a]

Fraction	% *vs.* initial
Labelled lactate	
Initial 5 mM	100.0
First pass of medium W1	0.29 ± 0.08
Water washing W2	0.51 ± 0.03
HCl elution W3	99.8 ± 0.7
Water washing W4	0.15 ± 0.09
Total recovery	100.8 ± 0.6
Labelled glucose	
Initial 7 mM	100.0
First pass of medium after enzyme incubation W5	0.45 ± 0.03
Water washing W6	0.53 ± 0.09
HCl elution W7	100.4 ± 3.2
Water washing W8	0.26 ± 0.14
Total recovery	101.6 ± 3.4
Labelled glycerol	
Initial 1.3 mM	100.0
First pass of medium after enzyme incubation W9	1.4 ± 0.8
Water washing W10	2.7 ± 0.3
HCl elution W11	99.5 ± 1.2
Water washing W12	1.7 ± 0.4
Total recovery	105.6 ± 2.0

[a] The data correspond to the mean ± sem values of four separate analyses for each label, as explained in the text.

in open Eppendorf tubes. They were incubated overnight at room temperature. The long incubation was not justified by the high enzyme load added, but by the need to maintain an adequate interchange with atmospheric oxygen to allow a prompt oxidation of glucose by glucose-oxidase. The periodic ventilation of samples with small volumes (10 mL) of air using syringes and a capillary tube showed no significant effect on the effectiveness of the reaction, and was cumbersome. Thus additional ventilation was not considered further, using time and passive gas diffusion instead. The incubation was stopped by the addition of 1 mL of 20 mM glycine buffer pH 9.8 containing 0.5 mM $MgCl_2$, changing the medium pH to 8.5. Then, the sample was divided into two aliquots of the same volume, which were passed through two parallel Dowex 1 × 2 columns as described previously for the M1 procedure. The washouts W5 + W6 from both columns were combined, and were used to determine the label content of the fraction. The label in W5 and W6 did not correspond to glucose but to glycerol, and (possibly) other unknown nonionic compounds, which were not substrates for glucose oxidase.

The label retained in the column was eluted following the same procedure described above for lactate, which yielded washouts W7 and W8 (total volume of 1 mL), which were counted using several scintillation vials. They contained the eluted gluconic acid whose label was that of the original glucose present in the medium.

In order to check the effectiveness of the derivatization procedure, an analysis of glucose label distribution was carried out under otherwise standard conditions. As shown in Table 1, the conversion of glucose to acidic-labeled compounds (essentially gluconate) was quantitative, being retained in the mini-column. Elution with HCl released, again, quantitatively the glucose label.

Estimation of medium label present in the "glycerol" fraction

As outlined above, after retention of lactate in the ion-exchange column, washouts W1 + W2 contained the entire non-retained nonionic label (*i.e.* glucose and glycerol, essentially). However, to find out whether the label corresponded to glycerol, we used a specific enzymatic reaction to convert all free (nonionic) glycerol to an ionic form retaining the label (procedure M4).

Glycerol kinase (EC 2.7.1.30) specifically esterifies glycerol with ATP to yield glycerol-3P and ADP:[48]

$$\text{Glycerol} + \text{ATP} \rightarrow \text{glycerol-3P} + \text{ADP}$$

This same reaction has been used previously to discriminate the presence of radioactive label in glycerol,[49] using an ion-exchange resin to retain all acidic compounds (*i.e.* remaining ATP, ADP and glycerol-3P). In order to diminish possible sources of interference, we used a purified enzyme, glycerol kinase from *Escherichia coli* (#G6278, Sigma-Aldrich).

A 100 μL aliquot of combined fractions W1 and W2 had been already used for the measurement of total label. Another 100 μL aliquot was mixed in an Eppendorf tube with 0.3 mL of 50 mM tris–HCl buffer pH 9.8. The buffer also contained about 100 pkat of the enzyme, as well as disodium-ATP, streptomycin + penicillin, magnesium acetate, lipid-free bovine serum albumin and dithiothreitol (all from Sigma-Aldrich) at final concentrations, respectively, of 8 mM, 10 mg L^{-1} + 10 U mL^{-1}, 8 mM, 1 g L^{-1} and 1 g L^{-1}. The tubes were closed and incubated at room temperature. The incubation was stopped by adding 18 μL of 50 mM HCl to each sample; their pH decreased to about 7. Aliquots of the sample were passed through Dowex 1 × 2 columns as described previously for the M1 procedure. The washouts W9 + W10 were combined, and used to determine their label content, that belonged essentially to glucose and other remaining compounds not susceptible to phosphorylation by glycerol kinase.

The label retained in the column was eluted following the same procedure described above for lactate, which yielded washouts W11 and W12 (total volume of 800 μL), which were counted. They contained the eluted glycerol-P, whose label was that of the original glycerol present in the medium.

This procedure showed a number of additional problems, not encountered in the case of glucose oxidation to gluconate. The amount of enzyme added was sufficient to convert the substrate in a few minutes, but initially we left the tubes under incubation overnight to make sure that all glycerol was phosphorylated. However, we observed that the long incubation resulted in yields of label higher than expected in the W9 + W10 effluxes. In fact, the label obtained after the retention of glycerol-3P in the column was higher than that found in the W5 + W6 obtained as washouts of glucose retention. We traced the

Table 2 Effect of the presence of antibiotics in the yield of labelled "glycerol" in the acid-eluted fractions of the fraction of glycerol kinase-treated medium after its retention in the ion-interchange columns[a,b]

	Glucose label: "glycerol" fraction		
Units	Without added antibiotics	With added antibiotics	Glucose label: "other nonionic components fraction"[c]
Glycerol label in Bq per well	724 ± 190	524 ± 93	428 ± 104
Bq per μmol of glycerol	591 ± 123	387 ± 64	363 ± 84
Bq per μmol-C of glycerol	197 ± 41	129 ± 21	121 ± 28

[a] Data are the mean ± sem of 4 different analyses using glucose as the initial source of label. [b] There were no statistically significant differences between groups for any of the parameters shown (unpaired Student's *t* test). [c] These data were obtained from the analysis of glucose oxidation.

discordance to the presence of glucose in the glycerol incubation with glycerol-kinase, and to the fact that the M1 procedure removed the antibiotics present in the medium, which prevented the growth of microorganisms. The differences between the fractions indicated above were due to such growths yielding lactate or other acids. The problem was solved by reintroducing the antibiotics in the glycerol-kinase incubation medium, as indicated above in the general procedure. The results of this test can be seen in Table 2. This was only part of the problem, since by using labelled glucose and no glycerol standards, part of the glucose was retained in the columns. The long incubation, thus, resulted, in a partial ionization (phosphorylation?) of glucose, yielding erroneous label values for glycerol. The problem was finally solved by cutting down the incubation time to only 2 hours and increasing the amount of glycerol kinase to the 100 pkat indicated above.

In order to check the effectiveness of the specific derivatization procedure used for glycerol, an analysis of its label distribution was carried out under otherwise standard conditions. We used cold (1.3 mM) and labelled glycerol [242 Bq per well] (^{14}C-(U)-glycerol, #CFB174 Amersham Pharmacia Biotech, Amersham UK; specific activity 5.25 MBq per μmol, *i.e.* 1.75 MBq per μmol-C). Table 1 shows the distribution of total label in each case between the different fractions recovered. This experiment was performed using an overnight incubation time, which can explain the relative variability of the results obtained when compared with lactate and glucose. The retention of label in the column was not quantitative, but almost, and the eluted fractions of the label obtained with HCl allowed us to recover the initial label.

Cell fraction handling

Frozen (*i.e.* assumedly broken) adipocytes were used for the extraction of lipids (procedure C1, Fig. 2), using diethyl-ether (peroxide-free, containing 7 ppm BHT, Sigma-Aldrich), in the proportion of about 1.5 mL of chilled diethyl ether for each sample (*i.e.* 179 ± 22 mg) of frozen cells. The tubes containing the already freeze-fractured cells were mixed by vortexing several times and cells thawed when subjected to extraction in the cold. Then 500 μL chilled pure water were added and the tubes were briefly centrifuged (5 min at 600 × *g*) and two phases appeared. The lower (aqueous) phase was suctioned off, including the delicate inter-phase film of protein (and membrane fragment) debris. This fraction was used for counting; it contained cell protein, (mainly) glycogen, metabolites, membrane microsomes and non-lipophilic debris. The (upper) organic phase was recovered, left to dry at room temperature and weighed. Then, the lipid residue was dissolved/mixed with 2 mL of diethyl-ether and 1.5 mL of hydroalcoholic KOH. The latter was prepared immediately before use by mixing 1 volume of 8.4 M potassium hydroxide (in water) with 3 volumes of ethanol. The alkali broke down the acylglycerols through saponification, forming potassium soaps and freeing glycerol. The tubes in which the reaction took place were vortexed and allowed to react at room temperature for 10 min (procedure C2). The tubes were then left to stand and two layers were separated, the bottom one contained the glycerides' glycerol. The upper phase promptly solidified and contained, essentially, the potassium soaps, not soluble in ether–ethanol. The phases' weights were estimated, and the whole samples were counted.

The cells' fractioning was not checked using standards, as was the case of lactate and glucose, since its total retention of label was small, the procedure could not be repeated from scratch with known labelled compounds in the cells, and, especially, because all the manipulations described are common and have been previously used. Instead, the overall evaluation of the effectiveness of the whole process described was based on a precise accounting of the distribution of all label added to the incubation well.

Additional methodologic considerations

A critical factor in the development of this procedure was to keep track of volumes and incorporate into the calculations all aliquots used for testing (*i.e.* cold glucose or lactate levels, amount of label in a given fraction). Unless the whole fraction was used for counting, a sizeable part of it was used to determine its label content. All data were introduced in a spreadsheet in which the volumes were justified with an (pipetting) error of ±3%. A similar error level was estimated for counts, which were routinely repeated and the mean value was used. When possible, or when no other avenue was available, volumes were estimated from differential weights and the application of densities, estimated, as described, using the same samples.

Statistical calculations and analyses were performed using the Prism 5 package (Graph-Pad Software, La Jolla, CA USA).

Results

Experimental application of the method described

Analysis of label and substrates/metabolites in fractions of incubated adipocytes. Cells from four different rats were used. The results obtained are presented in Tables 3–5. Table 3 shows the composition of the media at the beginning of the experiment and after 24 h of incubation. The data were given both in molar units and in well content of each metabolite analyzed.

Table 4 presents the weights/volumes of samples, their label content and the extrapolation of the results to the whole incubation well, in spite of the size of the sample analyzed. The presence of label in the different fractions is presented as mean values of the four rats used.

Table 5 presents the specific radioactivity of the different fractions analyzed: both in the initial medium and after incubation and cell harvesting. We did not find sufficiently high levels of non-esterified fatty acids in the media to include them in the calculations. Lactate (plus pyruvate) and the remaining glucose showed values for specific radioactivity very close to those of the initial glucose used, which helps supporting the adequacy of the methodology used.

As expected, we found much lower specific radioactivity, in lipids, with roughly half of their label incorporated into fatty acids and the rest in the glycerol moiety of triacylglycerols. As expected, the specific radioactivity, expressed in Bq per μmol of C, of the remaining glucose, lactate (plus pyruvate), and medium free glycerol were the same as that of the glucose initially added to the cell cultures, which proved that the origin of the free 3C units was the glucose added to the medium. The specific activity of glycerides-glycerol was more than one order of magnitude higher than that of fatty acids, but much lower than that of the glycerol found in the medium.

Final distribution of the glucose label. Fig. 3 presents the results of the separation of medium and cell label in fractions. The data are a translation of the experimental figures shown in Table 4, and are expressed as percent values of the distribution of the label initially introduced in the incubation medium. Most of the label remained in the medium, about half as unused glucose. Thus, sufficient glucose remained after 24 h to sustain

Table 3 Results obtained in the application of the described analytical process to epididymal adipocytes from adult male Wistar rats. Cells and medium parameters before and after 24 h of incubation[a]

	mM			Cells or μmol per well (metabolites)	
	Initial	After 24 h	% of change	Initial	After 24 h
Adipocytes (cells × 10^3)			−2	702 ± 47	688 ± 33
Glucose	6.78 ± 0.29	3.73 ± 0.87	−45	12.23 ± 0.41	6.57 ± 1.54
Lactate	0.14 ± 0.04	2.81 ± 0.38	+2 × 10^3	0.26 ± 0.08	4.93 ± 0.65
Glycerol	0.014 ± 0.006	0.70 ± 0.07	+5 × 10^3	0.023 ± 0.011	1.22 ± 0.12

[a] Data are the mean ± sem of 4 different complete runs (as described in the text) using glucose as the initial source of label.

Table 4 Results obtained in the application of the described analytical process to epididymal adipocytes from adult male Wistar rats. Fraction size and label content[a]

	Major compound	Weight mg	Volume μL	Bq (whole well)
Medium initial			1806 ± 24	4730
Medium aliquot used			275 ± 25	4186 ± 294
Medium pass column [W1 + W2]	Glucose + glycerol		503 ± 7	2893 ± 280
Retained and eluted [W3 + W4]	Lactate + pyruvate		1125 ± 153	1293 ± 228
Glucose-oxidase incubation [W1 + W2 aliquot]			100 ± 2	
Incubation pass column [W5 + W6]	Glycerol + other		1911 ± 18	429 ± 104
Retained and eluted [W7 + W8]	Glucose		1985 ± 16	2465 ± 326
Glycerol-kinase incubation [W1 + W2 aliquot]			100 ± 3	
Incubation pass column [W9 + W10]	Glucose + other		455 ± 44	2491 ± 342
Retained and eluted [W11 + W12]	Glycerol		842 ± 41	269 ± 58
Cells total (density: 0.9075 g mL^{-1})		179 ± 22	197 ± 24	297 ± 116
Solids and aqueous phases	Glycogen + protein + metabolites	497 ± 83		72 ± 9
Lipid extract	Triacyl-glycerols	106 ± 12		214 ± 97
Potassium soaps	Fatty acids	159 ± 23		106 ± 56
Liquid phase	Glycerol + other	205 ± 49		109 ± 43
Losses in cells' fractions		22 ± 25		16 ± 16
Glucose oxidation, losses of label, and experimental, measuring and accounting errors				148 ± 210

[a] Data are the mean ± sem of 4 different complete runs (as described in the text) using glucose as the initial source of label. For calculations, we used glyceroyl-trioleate [MW 887] for triacylglycerol molar equivalences.

Table 5 Results obtained in the application of the described analytical process to epididymal adipocytes from adult male Wistar rats. Specific activities[a]

	μmol per well	Bq per well	Bq μmol^{-1}	Bq per μmol-C
Initial medium glucose	12.2 ± 0.4	4730	388 ± 13	65 ± 3[b]
Medium "glucose" fraction	6.57 ± 1.50	2465 ± 326	444 ± 98	74 ± 16[b]
Medium "lactate" fraction	4.93 ± 0.65	1290 ± 228	196 ± 26	65 ± 9[b]
Medium "glycerol" fraction	1.22 ± 0.12	269 ± 58	202 ± 36	67 ± 12[b]
Cell fatty acids' (soaps) fraction	357 ± 41	106 ± 56	0.26 ± 0.11	0.014 ± 0.006
Cell glycerides-glycerol fraction	119 ± 14	109 ± 43	0.84 ± 0.26	0.28 ± 0.09

[a] Data are the mean ± sem of 4 different complete runs (as described in the text) using glucose as the initial source of label. [b] These values were not statistically different.

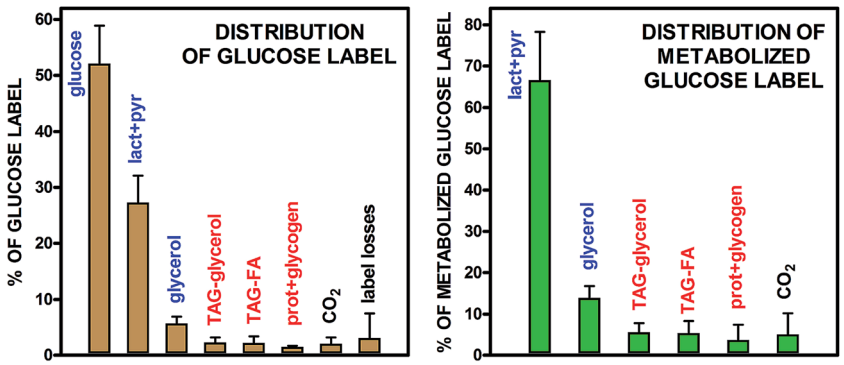


Fig. 3 Percent distribution of labeled glucose (total label and metabolized glucose) after 24 h of incubation by epididymal adipocytes. The results are the mean ± sem of four different rats. TAG = triacylglycerols; FA = fatty acids; prot = protein; lact + pyr = lactate + pyruvate.

the cells' metabolism. The other major label containing fractions in the medium were lactate (+pyruvate) and glycerol. Cells incorporated only about 6% of the label, with a similar distribution of total label between glycerides-glycerol and fatty acids, but containing also a significant proportion of label in hydrophilic compounds (probably glycogen). In all, only *ca.* 0.3% of the cell label was not accounted for. This discrepancy was uncharacteristically small for this type of studies, and helps in understanding the limited number of molecule types that were marked from the glucose label in the relatively short time of incubation. The global loss of label was, similarly, low (in the range of 3% overall) given the usual imprecision of separation of labelled compounds, probably because of reasons similar to those of the cells' label.

Fig. 3 also shows the fate of label from the glucose actually metabolized. About two thirds corresponded to the lactate fraction, but the sum of free and triacylglycerol-incorporated glycerol accounted for 1/5th of the total, which contrasts with the (low) 5% of fatty acids. Since there was a definite, albeit small, net synthesis of fatty acids (they incorporated glucose label), we necessarily have to take into account the coexistence, in a mainly glycolytic environment, of oxidative processes (at least the pentose-phosphate pathway —or malic enzyme— and pyruvate dehydrogenase). The oxidative metabolism was necessary to provide NADPH and 2C units to build acyl-CoA. The production of CO_2 from glucose in the pentose-phosphate pathway yields 6 carbon-units as CO_2 (*i.e.* equivalent to a whole glucose) for each 12 NADPH produced; and pyruvate dehydrogenase produces another CO_2 for each acetyl-CoA formed. To synthesize an 18C fatty acid we need 9 acetyl-CoA units and $2 \times 8 = 16$ NADPH; this results, necessarily, in the production of $9 + 8 = 17$ CO_2, which is liberated for each fatty acid produced, *i.e.* roughly 1 CO_2 per carbon in the fatty acid. The minimum oxidative needs for the metabolic picture depicted are the equivalent, in glucose units of the label in fatty acids of about 1/3rd of the label found in fatty acids. This is a minimum value, which does not preclude other oxidative pathways. It may be also lower than this figure depending on the activity of the malic enzyme as the provider of cytoplasmic reducing power for lipogenesis. Thus, it is only an approximation to the actual oxidative capabilities of the adipocytes. In any case, the proportion of oxidative activities of adipocytes on the medium glucose could not go beyond a 4% of metabolized glucose, which represents a maximum figure for measuring errors not corrected, including the variability of ^{14}C counting, and the probable loss of cells and medium during the harvesting process.

Discussion

We have developed a complex, but doable, procedure for analysis of the fate of a substrate, as is glucose, using a primary cell culture of adipocytes just extracted from WAT. There are a considerable number of procedures similar to this one, which

have been used to follow up the uptake of labelled substrates,[4,50] or their (usually one-step) transformation, or oxidation.[51] The problem, shared by all similar procedures, being largely the small volumes of media and cells used in cell culture, which strongly limits the actual amount of label that can be incorporated and, especially, the later analysis of the metabolites, intact substrate and distribution along different pathways. Studies using tissue pieces, sections or isolated cells have been used in the past for experiments such as those described here, but the critical step of quantitative separation of substrates requires a careful balance between the amount of tissue, medium (and labelled compounds) used, and the effects of dilution on label distribution. The measurement of labelled CO_2,[52] and up to a degree, its incorporation in cells[53] or removal from the medium[54] have been used often with success. However, the studies using perfused[55] or perifused[56] organs and incubations with pieces of tissue,[57] now seldom used, have been the main methods available for analyses of the utilization of glucose and release of 3C units by live tissues.

The objective of this study was to design a flexible system for the study of the metabolism of WAT cells (or tissue pieces) under well-controlled conditions, which can provide coherent results from a single incubation plate well. We have found, recently, that adipocytes glycolyze huge amounts of glucose to lactate and glycerol,[28] acting, essentially, as an anaerobic tissue under normoxic conditions, such as those used in the present study, and this mechanism extends to the *in vivo* conditions.[29] We have also found that WAT contains a full urea cycle[58] and a potentially active amino acid metabolism.[14] The results obtained, and a growing list of unique capabilities of WAT[59–64] has decided us to focus on the quantitative analysis of WAT metabolism. Thus, we checked these parameters against the known functional and morphologic differences of WAT sites,[65–67] and their modulation by sex and diet,[14,68] as well as, especially, the effects of metabolic syndrome.[69,70] In our opinion, there is yet much to learn from WAT from the "basic" biochemical-metabolic point of view. We know much about its metabolic modulation by cytokines[71] and hormones,[72–75] and even its responses to oxidative stress[76] and hypoxia,[77,78] however, we know very little on the quantitative importance of Randall's glucose-fatty acid cycle, the classical Cori cycle and the interchange of glutamine and ornithine–arginine with the splanchnic bed. Consequently, we developed a tool, which can be used for that purpose, adapted, modified, and, we hope, improved to fulfill its function. In the present study, we did a full experiment of label distribution on rat adipocytes, but our focus was not on the spectacular results (we plan to extend further these studies, in adequate depth), but on obtaining a proven tool to carry out these types of investigations. A test *in toto* seems the most adequate way to check the defects (and correct them) of such a complex approach. The results may need further development for specific experiments, but the backbone of quantitative approaches and reduced column efflux may allow these developments further than presented here.

The large volumes involved in the use of ion-exchange columns, and the difficulties posed by the dilution of samples linked to their use make it difficult to discriminate a sufficient number of main metabolites from the same cell culture. This approach is of limited usefulness in modern cell culture (immortalized or primary); consequently, in the last few decades, the use of these methods has been largely abandoned, in part because of the limited advantages of using radioactive tracers for the small volumes and live material mass involved in cell culture-based studies. The availability of other analytical tools and the focus set on regulation, assuming that the main metabolic pathways are already known, have compounded the situation.

For decreasing the handling of volumes needed for ion-exchange column separation, we have developed a methodology that may be directly applicable to the analysis of metabolic flow of substrates in (at least primarily) adipose tissue cells. The critical points of adaptation of the existing methodology to the problem described are:

(a) The use of micro-columns, which require microliter samples to process, by using centrifugation instead of simple gravity for eluent recovery, eliminating most of the contamination induced by capillary retention of effluents in the columns.

(b) The control of volumes and of presence of label in different fractions, including a critical analysis of losses (and possible sources of error) and incorporation of all data in the final computation of the results.

(c) To combine these label analyses with those of the main metabolites studied using standard micro-analytical methods in the same well, and thus being able to compare the specific radioactivity of these fractions. This requires special training and care for non-label dependent analyses of labelled compounds, but the homology of the results achieved justifies the additional application of safety measures.

The main problems posed by this type of methodology are already well-known: it is fairly difficult to match the "cold" measurements of concentrations and the analysis of the labelled fractions which, essentially, correspond to the same molecules. In the case of lactate, we were aware that this fraction contained a variable amount of pyruvate, in part because it is a standard component of the media for adipocytes (as is our case), but also for its known secretion by adipose tissue.[33] Its formation is also a consequence of the production of glycerol since the synthesis of glycerol-3P redirects NADH from the glycolytic pathway (that generated by glycerol-3P dehydrogenase). This relative deficit of NADH in the main glycolytic pathway results in a relative excess of pyruvate (that cannot be reduced to lactate for lack of enough NADH to maintain the stoichiometry of the process). This "extra" pyruvate, however, was not fully directed to produce the acetyl-CoA later used to synthesize the fatty acids found in cells' triacylglycerols, lipogenesis was limited, as observed in the difference between the label presence in the fatty acids and glycerol moieties of triacylglycerols, even not taking into account the free glycerol in the medium. However, the specific activity (per carbon unit) of lactate was in the same range as those of glucose and glycerol. The explanation is not simple, since pyruvate was retained, as was lactate in the columns in the first pass, its "cold" levels were not measured as was the case with the major component,

lactate. The specific activity of the "lactate + pyruvate" fraction should be, consequently, somewhat lower than that of glucose, unless we take into account the preferential utilization of pyruvate for fatty acid synthesis. This possibility is supported by the specific activity of this fraction (per C) being similar to that of the other medium metabolites.

It has been found that under hypoxic conditions WAT decreases the synthesis of acyl-CoA, storing less fat.[77] WAT consumed very little oxygen *in vivo*,[78] suggesting that its anaerobic glycolysis may sustain a sizeable amount of its needs[28] for an unexpectedly active metabolism.[14] In fact, about 98.5% of the adult large adipocytes such as those used here is made up of fat, the small active cytosol, however, has enzyme activities (*i.e.* lactate dehydrogenase, amino acid metabolism enzymes) in the range of most other tissues.[14]

The results obtained from the application of the combined study of label fate are in agreement with the known high production of lactate even under normoglycemic conditions. The specific activity of lactate is the same as that of the initial (and remaining) medium glucose. However, in spite of a largely glycolytic environment, there was a small but significant synthesis of fatty acids from glucose, stored in the enormous triacylglycerol stores. The animals used were normal adult rats, thus, the glycolysis could not be attributed to hypoxia (the availability of oxygen was high), or metabolic stress/inflammation.

The data presented support the assumption that glucose is practically the only origin of the medium glycerol. The high specific activities found were much higher than what could be expected if the medium glycerol were produced *via* acyl-glycerol lipolysis, even if this was only a minor fraction of the total.

In the adipocyte, the flow of label to glycerol (*i.e. via* glycerol-3P) was considerable, even when corrected by the number of carbons. We can only assume that a significant part of the glycerol-3P pathway resulted in the patent presence of high specific activity-glycerol in the medium.[28] The results observed in the test of the method are, thus, coincident with those observed using other systems and conditions. The high label content and the absence of labelled fatty acids seem to confirm that glycerol was not formed by lipolysis as previously postulated,[79,80] but it was formed directly from the direct conversion of glucose.

In sum, the results we obtained in the samples of epididymal WAT analyzed agree with what we know of WAT, established using other means and techniques, but the results presented here contribute further to show the proportions of glucose carbon going through different pathways to form a combination of glycolytic and oxidative metabolism. The methodology we present may be especially useful to analyze the effects of substrate availability/concentration, hypoxia, inflammation, and regulatory factors on the modulation of these effects.

Conclusions

We have developed a systematic analysis of the fate of ^{14}C-label from glucose in primary cultures of adipocytes. Its application to epididymal rat adipocytes has allowed us to analyze in detail the proportion of label incorporated into medium lactate and glycerol. Cells glycerydes' glycerol and fatty acid synthesis were also measured to complete the picture. The coherent and intertwined methodological approach establishes the basis for further detailed confirmatory studies of the findings presented here. In particular the full glucose (*i.e.* not lipolytic) origin of the glycerol secreted by adipocytes under conditions of abundant glucose in the medium, and the predominance of an active glycolysis in adipocytes (*i.e.* high production of lactate) parallel to the (oxidative) synthesis of fatty acids, albeit the latter occurring in smaller proportions under the conditions tested. The main advantage of the method described, which warrants its further utilization, is the ability to carry out all determinations quantitatively and using a single cell incubation well.

Acknowledgements

This study did not obtain any specific financing. A. C. Ho-Palma was the recipient of a fellowship of the Peruvian Government. F. Rotondo obtained also a predoctoral fellowship from the Government of Catalonia. We would like to acknowledge the generous gift of labelled compounds by Dr Marçal Pastor and the late Dr Anna Maria Gómez-Foix, which was critical for the development of this paper. We had no opportunity in the end to thank Dr Gómez Foix for her kindness, friendship, scientific soundness and quality as a person. We miss her.

References

1 R. G. P. Denis, A. Joy-Amado, C. Cansell, J. Castel, S. Martinez, A. S. Delbes and S. Luquet, *Diabetes Metab.*, 2014, **40**, 191–197.
2 M. Alemany, *RSC Adv.*, 2013, **3**, 1636–1651.
3 B. Kalderon, A. Gopher and A. Lepidot, *FEBS Lett.*, 1987, **213**, 209–214.
4 L. Hue and H. G. Hers, *Biochem. Biophys. Res. Commun.*, 1974, **58**, 532–539.
5 E. J. Parks and M. K. Hellerstein, *J. Lipid Res.*, 2006, **47**, 1651–1660.
6 C. M. Hasenour, M. L. Wall, D. E. Ridley, C. C. Hughey, F. D. James, D. H. Wasserman and J. D. Young, *Am. J. Physiol.*, 2015, **309**, E191–E203.
7 S. Cinti, *Prostaglandins, Leukotrienes Essent. Fatty Acids*, 2005, **73**, 9–15.
8 M. D. M. Romero, S. Roy, K. Pouillot, M. Feito, M. Esteve, M. D. M. Grasa, J.-A. Fernández-López, M. Alemany and X. Remesar, *PLoS One*, 2014, **9**, e90995.
9 P. Trayhurn, *Acta Physiol. Scand.*, 2005, **184**, 285–293.
10 H. Hauner, *Physiol. Behav.*, 2004, **83**, 653–658.
11 M. Alemany and J. A. Fernández-López, *Curr. Nutr. Food Sci.*, 2006, **2**, 141–150.
12 E. D. Rosen and B. M. Spiegelman, *Nature*, 2006, **444**, 847–853.
13 P. Arner, S. Bernard, M. Salehpour, G. Possnert, J. Liebl, P. Steier, B. A. Buchholz, M. Eriksson, E. Arner, H. Hauner, T. Skurk, M. Ryden, K. N. Frayn and K. L. Spalding, *Nature*, 2011, **478**, 110–113.

14 S. Arriarán, S. Agnelli, X. Remesar, J. A. Fernández-López and M. Alemany, *PeerJ*, 2015, **3**, e1399.
15 S. I. Anghel, E. Bedu, C. D. Vivier, P. Descombes, B. Desvergne and W. Wahli, *J. Biol. Chem.*, 2007, **282**, 29946–29957.
16 S. Winer and D. A. Winer, *Immunol. Cell Biol.*, 2012, **90**, 755–762.
17 H. Mizuno, Y. Itoi, S. Kawahara, R. Ogawa, S. Akaishi and H. Hyakusoku, *Cells Tissues Organs*, 2008, **187**, 177–185.
18 S. Cinti, *Am. J. Physiol.*, 2009, **297**, E977–E986.
19 I. Kheterpal, G. Ku, L. Coleman, G. Yu, A. A. Ptitsyn, Z. E. Floyd and J. M. Gimble, *J. Proteome Res.*, 2011, **10**, 1519–1527.
20 S. N. O'Brien, K. A. Mantzke, M. W. Kilgore and T. M. Price, *Anal. Quant. Cytol. Histol.*, 1996, **18**, 137–143.
21 A. Sorisky, A. S. D. Molgat and A. Gagnon, *Adv. Nutr.*, 2013, **4**, 67–75.
22 J. H. Kim, S. H. Kim, S. Y. Song, W. S. Kim, S. U. Song, T. Yi, M. S. Jeon, H. M. Chung, Y. Xia and J. H. Sung, *Cell Biol. Int.*, 2014, **38**, 32–40.
23 W. Leonhardt, M. Hanefeld and H. Haller, *Int. J. Obes.*, 1978, **2**, 33–45.
24 M. E. Fernyhough, J. L. Vierck, G. J. Hausman, P. S. Mir, E. K. Okine and M. V. Dodson, *Cytotechnology*, 2004, **46**, 163–172.
25 V. P. Knutson and Y. Balba, *In Vitro Cell. Dev. Biol.: Anim.*, 1997, **33**, 77–81.
26 S. Gesta, K. Lolmède, D. Daviaud, M. Berlan, A. Bouloumié, M. Lafontan, P. Valet and J. S. Saulnier-Blanche, *Horm. Metab. Res.*, 2003, **35**, 158–163.
27 Y. H. Yu and H. N. Ginsberg, *Circ. Res.*, 2005, **96**, 1042–1052.
28 D. Sabater, S. Arriarán, M. M. Romero, S. Agnelli, X. Remesar, J. A. Fernández-López and M. Alemany, *Sci. Rep.*, 2014, **4**, 3663.
29 S. Arriarán, S. Agnelli, D. Sabater, X. Remesar, J. A. Fernández-López and M. Alemany, *PLoS One*, 2015, **10**, e0119572.
30 N. Hosogai, A. Fukuhara, K. Oshima, Y. Miyata, S. Tanaka, K. Segawa, S. Furukawa, Y. Tochino, R. Komuro, M. Matsuda and I. Shimomura, *Diabetes*, 2007, **56**, 901–911.
31 L. Hodson, M. Neville, M. F. F. Chong, I. Rogers, S. S. Huda, D. J. Freeman, K. N. Frayn and B. A. Fielding, *Nutr., Metab. Cardiovasc. Dis.*, 2013, **23**, 1128–1133.
32 E. Hagström, P. Arner, U. Ungerstedt and J. Bolinder, *Am. J. Physiol.*, 1990, **258**, E888–E893.
33 M. DiGirolamo, F. D. Newby and J. Lovejoy, *FASEB J.*, 1992, **6**, 2405–2412.
34 S. I. Taylor and R. L. Jungas, *Arch. Biochem. Biophys.*, 1974, **164**, 12–19.
35 J. Flatt, *J. Lipid Res.*, 1970, **11**, 131–143.
36 R. H. Hammerstedt, *Anal. Biochem.*, 1980, **109**, 443–448.
37 B. Yu, S. Pugazhenthi and R. L. Khandelwal, *Biochem. Pharmacol.*, 1994, **48**, 949–954.
38 M. Rodbell, *J. Biol. Chem.*, 1964, **239**, 375–380.
39 R. C. Honnor, G. S. Dhillon and C. Londos, *J. Biol. Chem.*, 1985, **260**, 15122–15129.
40 S. N. Baviskar, *Am. Biol. Teach.*, 2011, **73**, 554–556.
41 J. J. Carroll, J. D. Sluosky and A. E. Mather, *J. Phys. Chem. Ref. Data*, 1991, **20**, 1201–1209.
42 L. Oliva, C. Baron, J.-A. Fernández-López, X. Remesar and M. Alemany, *PeerJ*, 2015, **3**, e1101.
43 F. Noll, *Methods Enzym. Anal.*, 1983, **6**, 682–688.
44 I. Rafecas, M. Esteve, J. A. Fernández-López, X. Remesar and M. Alemany, *Int. J. Obes.*, 1992, **16**, 775–787.
45 A. S. G. Huggett and D. A. Nixon, *Lancet*, 1957, **270**, 368–370.
46 M. Azain, T. Kasser and C. Baile, *J. Nutr. Biochem.*, 1999, **10**, 205–209.
47 I. Miwa, K. Maeda, J. Okuda and G. Okuda, *Clin. Chim. Acta*, 1972, **37**, 538–540.
48 C. Bublitz and O. Wieland, *Methods Enzymol.*, 1962, **5**, 354–361.
49 C. Crespi, G. Gastalver, A. Palou and P. Roca, *J. Biochem. Biophys. Methods*, 1995, **30**, 179–183.
50 P. R. Bally, G. F. Cahill, B. Leboeuf and A. E. Renold, *J. Biol. Chem.*, 1960, **235**, 333–336.
51 R. H. Hammerstedt, *Anal. Biochem.*, 1980, **109**, 443–448.
52 N. C. Juhr, J. Hardieck and J. Franke, *J. Exp. Anim. Sci.*, 1991, **34**, 195–197.
53 P. Schadewaldt, M. Rossig and W. Staib, *Biol. Chem. Hoppe-Seyler*, 1988, **369**, 181–192.
54 F. J. Casado, J. A. Fernández-López, J. M. Argilés and M. Alemany, *Anal. Biochem.*, 1988, **172**, 509–513.
55 D. Lincová, D. Miseková, E. Kmonícková, N. Canová and H. Farghali, *Physiol. Res.*, 2002, **51**, 387–394.
56 J. L. King and M. DiGirolamo, *Obes. Res.*, 1998, **6**, 69–75.
57 J. Christophe, J. Dehaye and J. Winand, *Biochem. Soc. Trans.*, 1974, **2**, 1214–1215.
58 S. Arriarán, S. Agnelli, X. Remesar, J.-A. Fernández-López and M. Alemany, *RSC Adv.*, 2015, **5**, 93403–93414.
59 A. Trzeciak-Ryczek, B. Tokarz-Deptula, P. Niedwiedzka-Rystwej and W. Deptula, *Cent. Eur. J. Immunol.*, 2011, **36**, 95–99.
60 J. Corre, C. Barreau, B. Cousin, J. P. Chavoin, D. Caton, G. Fournial, L. Penicaud, L. Casteilla and P. Laharrague, *J. Cell. Physiol.*, 2006, **208**, 282–288.
61 Y. W. Jiang, D. C. Berry, W. Tang and J. M. Graff, *Cell Rep.*, 2014, **9**, 1007–1022.
62 L. E. Kokai, K. Marra and J. P. Rubin, *Transl. Res.*, 2014, **163**, 399–408.
63 Y. Shingyochi, H. Orbay and H. Mizunot, *Expert Opin. Biol. Ther.*, 2015, **15**, 1285–1292.
64 C. Lu, L. Y. Su, R. M. K. W. Lee and Y. J. Gao, *Eur. J. Pharmacol.*, 2010, **634**, 107–112.
65 A. Galinier, A. Carrière, Y. Fernandez, S. Caspar-Bauguil, B. Periquet, A. Periquet, L. Pénicaud and L. Casteilla, *FEBS Lett.*, 2006, **580**, 6391–6398.
66 M. M. Romero, J. A. Fernández-López, E. Esteve and M. Alemany, *Cardiovasc. Diabetol.*, 2009, **8**, 42.
67 A. Gil, J. Olza, M. Gil-Campos, C. Gomez-Llorente and C. M. Aguilera, *Int. J. Pediatr. Obes.*, 2011, **6**, 13–20.
68 D. Lemonnier, *J. Clin. Invest.*, 1972, **51**, 2907–2915.
69 C. M. Elks and J. Francis, *Curr. Hypertens. Rep.*, 2010, **12**, 99–104.

70 C. P. Wang, H. L. Hsu, W. C. Hung, T. H. Yu, Y. H. Chen, C. A. Chiu, L. F. Lu, F. M. Chung, S. J. Shin and Y. J. Lee, *Clin. Endocrinol.*, 2009, **70**, 876–882.
71 A. Rodríguez, S. Ezquerro, L. Méndez-Giménez, S. Becerril and G. Frühbeck, *Am. J. Physiol.*, 2015, **309**, E691–E714.
72 M. E. E. Jones, K. J. McInnes, W. C. Boon and E. R. Simpson, *J. Steroid Biochem. Mol. Biol.*, 2007, **106**, 3–7.
73 J. Galitzky and A. Bouloumie, *Cell Metab.*, 2013, **18**, 3–5.
74 A. Hirata, N. Maeda, H. Nakatsuji, A. Hiuge-Shimizu, T. Okada, T. Funahashi and I. Shimomura, *Biochem. Biophys. Res. Commun.*, 2012, **419**, 182–187.
75 D. Feldman, *Am. J. Physiol.*, 1977, **2**, E147–E151.
76 F. Boyer, J. B. Vidot, A. G. Dubourg, P. Rondeau, M. F. Essop and E. Bourdon, *Oxid. Med. Cell. Longevity*, 2015, **2015**, 534873.
77 E. García Fuentes, C. Santiago-Fernández, C. Gutiérrez-Repiso, M. D. Mayas, W. Oliva-Olivera, L. Coin-Aragüez, J. Alcaide, L. Ocaña-Wilhelmi, J. Vendrell, F. J. Tinahones and L. Garrido-Sánchez, *J. Transl. Med.*, 2015, **13**, 373.
78 L. Hodson, S. M. Humphreys, F. Karpe and K. N. Frayn, *Diabetes*, 2013, **62**, 1417–1425.
79 M. Vaughan, *J. Biol. Chem.*, 1962, **237**, 3354–3358.
80 P. Jansson, A. Larsson, U. Smith and P. Lönnroth, *J. Clin. Invest.*, 1992, **89**, 1610–1617.

Quantitative analysis of rat adipose tissue cell recovery, and non-fat cell volume, in primary cell cultures

Floriana Rotondo[1,2], María del Mar Romero[1,2,3], Ana Cecilia Ho-Palma[1], Xavier Remesar[1,2,3], José Antonio Fernández-López[1,2,3] and Marià Alemany[1,2,3]

[1] Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, University of Barcelona, Barcelona, Spain
[2] Institute of Biomedicine, University of Barcelona, Barcelona, Spain
[3] CIBER OBN, Barcelona, Spain

ABSTRACT

Background. White adipose tissue (WAT) is a complex, diffuse, multifunctional organ which contains adipocytes, and a large proportion of fat, but also other cell types, active in defense, regeneration and signalling functions. Studies with adipocytes often require their isolation from WAT by breaking up the matrix of collagen fibres; however, it is unclear to what extent adipocyte number in primary cultures correlates with their number in intact WAT, since recovery and viability are often unknown.
Experimental Design. Epididymal WAT of four young adult rats was used to isolate adipocytes with collagenase. Careful recording of lipid content of tissue, and all fraction volumes and weights, allowed us to trace the amount of initial WAT fat remaining in the cell preparation. Functionality was estimated by incubation with glucose and measurement of glucose uptake and lactate, glycerol and NEFA excretion rates up to 48 h. Non-adipocyte cells were also recovered and their sizes (and those of adipocytes) were measured. The presence of non-nucleated cells (erythrocytes) was also estimated.
Results. Cell numbers and sizes were correlated from all fractions to intact WAT. Tracing the lipid content, the recovery of adipocytes in the final, metabolically active, preparation was in the range of 70–75%. Cells showed even higher metabolic activity in the second than in the first day of incubation. Adipocytes were 7%, erythrocytes 66% and other stromal (nucleated cells) 27% of total WAT cells. However, their overall volumes were 90%, 0.05%, and 0.2% of WAT. Non-fat volume of adipocytes was 1.3% of WAT.
Conclusions. The methodology presented here allows for a direct quantitative reference to the original tissue of studies using isolated cells. We have also found that the "live cell mass" of adipose tissue is very small: about 13 μL/g for adipocytes and 2 μL/g stromal, plus about 1 μL/g blood (the rats were killed by exsanguination). These data translate (with respect to the actual "live cytoplasm" size) into an extremely high metabolic activity, which make WAT an even more significant agent in the control of energy metabolism.

Subjects Cell Biology, Metabolic Sciences
Keywords Adipocyte, Adipose tissue, Stromal cells, Cell culture, Cell volume, Cell viability, Primary cell cultures

Submitted 17 August 2016
Accepted 26 October 2016
Published 29 November 2016

Corresponding author
Marià Alemany, malemany@ub.edu

Academic editor
Eric Schirmer

Additional Information and Declarations can be found on page 17

DOI **10.7717/peerj.2725**

INTRODUCTION

White adipose tissue (WAT), which has been defined as the adipose organ (*Cinti, 2001*), is dispersed in a large number of locations, in which its basic energy storage activity is complemented by many other physiological functions (*Alemany & Fernández-López, 2006*). In any case, its main acknowledged role is to contribute to the defense of energy homoeostasis, helping to control glucose (*Sabater et al., 2014*), lipid (*Deschênes et al., 2003*; *Wang et al., 2016*), and amino acid (*Arriarán et al., 2015a*) metabolism overall. It is responsible for an important share of the control of whole body energy availability (*Hall, Roberts & Vora, 2009*; *Choe et al., 2016*), and acts as a platform for the immune system, being actively implicated in processes of protection and repair (*Parker & Katz, 2006*; *Dixit, 2008*). The complex (and varying) mixture of cell types in WAT depots largely determines and modulates these functions as part of its adaptability (*Vielma et al., 2013*; *Oishi & Manabe, 2016*).

Most of WAT volume is taken up by a relatively small number of large cells, the mature adipocytes, which are generally considered the genuine cells of this tissue and thus the main target for the fight against obesity (*Nawrocki & Scherer, 2005*). However, most of the adipocyte volume is filled by (triacylglycerol) energy reserves (*Kotronen et al., 2010*). This can be extended, obviously in similar proportions, (often higher than 80%) to the WAT/adipose organ taken as a whole. This is a variable but significant share of total body weight (5–50%) in humans and most animal phyla. The rest of WAT cells are loosely called stromal, despite most of them not being actually connective tissue cells (*Da Silva Meirelles et al., 2015*). In this text, we will use the general term "stromal cell" to refer to all WAT cells different from fat-laden adipocytes.

The stromal fraction of WAT is made up of immune system, stem, blood, endothelial, true stromal and other types of cells, with relevant functions in the maintenance of adipocyte energy homoeostasis (*Sadie van Gijsen et al., 2012*), defense (*Hill, Bolus & Hasty, 2014*), regeneration (*Domergue et al., 2016*), differentiation (*Gimble et al., 2011*; *Mitterberger et al., 2014*) and others (*Sumi et al., 2007*; *Takahara et al., 2014*). Many of these functions become critical under conditions of inflammation (*Lee, 2013*), changing the cell composition and overall WAT metabolism (*Lolmède et al., 2011*; *Cignarelli et al., 2012*). Adipocytes, despite their small numbers (but huge volume due to their fat stores), have been intensely studied as "representative" of WAT (*Leonhardt, Hanefeld & Haller, 1978*). To study their metabolic or regulatory capabilities, the cells are isolated from WAT masses and studied using primary (*Garvey et al., 1987*) or immortalized (*Tordjman, Leingang & Mueckler, 1990*) cell cultures. The information obtained is often taken as directly representative of WAT *in vivo,* in spite of the large number of factors that are known to rebut this excessively simplistic approach (*O'Brien et al., 1996*), including the ordeal of cell isolation (*Thompson et al., 2012*).

When dealing with WAT, the data obtained from most experiments is deeply conditioned by the methodology used, i.e., isolated cells, tissue pieces or slices, or *in vivo* functional analyses. Seldom can we obtain quantitative data which could be referred to the live tissue. Comparison of different locations, individuals, metabolic or pathologic conditions is severely hampered by the size of fat depots (*Cinti, 2001*; *Wronska & Kmiec, 2012*), the varying proportion of adipocyte/stromal cells (in fact, only when the latter are actually taken

into account (*Pasarica et al., 2009*) and the blood flow/oxygen and substrates' availability (*Mjös & Akre, 1971*). Quantification of adipocyte recovery from whole tissue samples, and the analysis of the proportion of "live" cell space in the tissue are necessary steps for direct comparison of data from different sources. Unfortunately, cell number is dependent on the method of quantification used, and is logically affected by cell volume. The proportion of fat in the tissue and cells also proportionally "reduces" the live-cell mass. This is further confounded by the direct estimation of cell numbers via DNA analysis which (at least in mammals) would not detect the number of erythrocytes, but would detect numbers of small hematopoietic cell (*Luche et al., 2015*) macrophages and lymphocytes (*Sell & Eckel, 2010*). The latter non-adipocyte populations would then be counted as "adipocytes," despite having a volume about 10^4-fold smaller.

Referring cell or tissue experimental data to protein content may be a fair index for comparison, but the large presence (also deeply varying depending on location (*Alkhouli et al., 2013*)) of extracellular fibrous proteins, such as collagen (*Liu et al., 2016*) also modifies the quantitative evaluation of the metabolically active fraction of the tissue; this fraction is also deeply affected by obesity and inflammation (*Li et al., 2010*).

In the present study, we have devised a method for the estimation of actual recovery of viable adipocytes with respect to WAT mass based on the unique presence of large amounts of fat in them. We have also intended to present an estimation of the size of the metabolically active WAT cell mass with respect to the mass/volume of the tissue. We used, as reference, the epididymal WAT fat pads of non-obese healthy adult rats (to limit the known effects of inflammation on WAT cell profile). This location is considered to be one of the less metabolically active (*Arriarán et al., 2015b*), and is widely used for "representative" WAT adipocyte function for its size, easy dissection and absence of contamination by neighboring tissues.

MATERIALS AND METHODS

Rats and housing conditions

All animal handling procedures and the experimental setup were in accordance with the animal handling guidelines of the corresponding European, Spanish and Catalan Authorities. The Committee on Animal Experimentation of the University of Barcelona specifically authorized the procedures used in the present study.

Male Wistar rats (Harlan Laboratory Models, Sant Feliu de Codines, Spain), 18-week old, weighing 435 ± 84 g (mean, SD), were used after a 2-week acclimation period in a controlled environment. The animals were kept in two-rat cages with wood shards as bedding material, at 21–22 °C, and 50–60% relative humidity; lights were on from 08:00 to 20:00. They had unrestricted access to water and standard maintenance rat chow (Harlan #2014).

Isolation of adipocytes

The rats were killed, under isoflurane anesthesia, at the beginning of a light cycle, by exsanguination from the exposed aorta, using dry-heparinized syringes. The rats were rapidly dissected, taking samples of epididymal WAT, used immediately for adipocyte isolation. This procedure followed, essentially that described by *Rodbell (1964)*. In short, tissue

samples were weighed, immersed in the digestion medium described below, and cut in small pieces with scissors. Samples were incubated, at 37 °C in a shaking bath for 60 min, with 2.5 volumes of Krebs-Henseleit buffer pH 7.4, containing 5 mM glucose, 0.1 μM adenosine (Sigma-Aldrich, St Louis, MO, USA) (*Honnor, Dhillon & Londos, 1985*), and 10 g/L lipid-free bovine serum albumin (Merck-Millipore, Billerica, MA USA). This was complemented with 3.5 mkat/L collagenase (LS004196, type I; Worthington Biomedical, Lakewood, NJ, USA). The collagenase-containing digestion buffer was prepared in the cold room (4 °C), and was used within 1 h.

At the end of the digestion process (carried at 37 °C), the suspensions were gently sieved using a double layer of nylon mesh hose (plain commercial sheer tight stocking; 90% polyamide, 10% elastomer, parallel woven with 15 den cylindrical single-filament threads; with approximate mean—flexible—pores in the range of 300 μm), which retained vessel fragments and (eventually) undigested tissue pieces. The smooth crude suspension of isolated cells was left standing for 5 min in stoppered polypropylene syringes (#SS+10ES1, Terumo, Tokyo, Japan), held vertically, at room temperature (22–24 °C). The adipocytes floated to form a defined upper layer. Then, the lower aqueous fraction was slowly drained off, capping again the syringe to retain the adipocytes. The cells were washed this way three times, using 2.5 volumes of the buffer each time. Before re-suspending the cells in it, the buffer was subjected to 30 s vortexing, to allow for equilibration with air oxygen. The final supernatant fraction contained intact adipocytes and a thin layer of free fat from broken cells. After the final washing, 400 μL aliquots of the cells' fraction were taken for incubation. The samples were slowly extracted from the central part of the adipocytes' layer, trying not to disturb the thin-floating lipid layer. The cells were manipulated and maintained at room temperature for a time as short as possible, and used immediately after the final washing.

Stromal cell space in the isolated cell suspension, used to relate their numbers and volumes to initial tissue weight, was considered the sum of the volume of the lower phase of adipocyte separation in the syringes, plus the volume of the adipocyte phase, to which the volume of adipocytes (calculated from cell numbers and volumes) was subtracted. Obviously, the first separation of adipocytes and stromal cells left a high number of the latter mixed with adipocytes. The three successive washings resulted in the presence (calculated) of, at most, 0.1% of the initial stromal cells in the final washed adipocyte fraction (down from an initial 7.3%). This assumption does not take into account stromal cells bound, retained or attached to the larger adipocytes.

Estimation of the efficiency of adipocyte extraction

Practically all fat in WAT is limited to adipocytes. All types of cells contain lipids, mainly as membrane components; the small size of the combined mass and their density do not alter the cells' density and, consequently their buoyancy. A few types of cells, i.e., macrophages, foam cells and differentiating preadipocytes may contain sizeable amounts of fat, but they only appear under precise physiological conditions (foam cells, differentiating preadipocytes) and their numbers and size (and thus their combined content of fat) make their contribution small (negligible in the present case). All other cells do not have sufficient

lipid to generate enough floatability to allow their separation from the rest of cells by just standing—i.e., at 1 × *g*—for five minutes. We used this differential fat content to establish an approximate estimation of the efficiency of the digestion-extraction procedure for adipocyte isolation described above, simply by estimating the recovery of fat from the intact tissue to a preparation containing only viable functional cells.

A sample of just dissected WAT was divided in two parts, one was processed to obtain washed adipocytes as described above, and the other was divided in several aliquots, used to measure the water (dry weight after 24 h at 90 °C) and lipid content. To measure lipids, fragments of about 300 mg of intact tissue were weighed and extracted with trichloromethane: methanol (2:1 v/v) (*Folch, Lees & Sloane-Stanley, 1957*). The resulting values were used to establish the proportion of lipids in the intact tissue. Using this method as originally described, most of membrane lipids were not extracted (*Rose & Oklander, 1965*; *Eder, Reichlmayr-Lais & Kirchgener, 1993*), but the recovery of WAT-vacuole lipids (i.e., fat, essentially triacylglycerols) was quantitative. The weights of the lipids present in the fat layer on top of the cells' suspension (washed and essentially free of stromal cells, as explained above), and those of stromal cells' fraction and extraction debris were measured. The weight of the recovered adipocyte fraction and their water and lipid content were also estimated, thus obtaining the total weight of lipid present in the isolated adipocytes.

The density of WAT was estimated using tightly capped tubes, which were weighed both dry and completely full of deionized water at 20 °C. The net weight of water was used to calculate the volume of the tube. The process was repeated including weighed 300–500 mg pieces of intact WAT in the tubes and completely filling them with water (nevertheless, no different values were obtained using pieces of 200–1,000 mg). The difference in weight of the tubes with and without WAT samples allowed us to calculate the volume of the samples; their density was estimated from the volume and weight. Other samples of WAT were used to extract its lipid as described above. The density of the extracted lipid was estimated using the same procedure using cold-solidified fat samples.

The weight of lipid extracted from the adipocyte preparation was compared with the initial weight and the actual proportion of lipid present in the intact tissue, after discounting the weight of debris eliminated during the process of extraction. Lipid in the stromal cell fraction was negligible, statistically not different from zero.

Measurement of isolated cell parameters

A known volume of the suspension of adipocytes was introduced in a Neubauer chamber (#717810 Neubauer improved bright line; Brand Gmbh, Wertheim, Germany). Using an inverted microscope, four fields (following a pre-established selection pattern) were photographed at low power (Fig. 1). Four samples of each adipocyte suspension were inspected, taking 16 photographs from each. Cells were identified, counted, and their diameters analyzed (under the conditions used, all cells adopted a spheroid form), using the *FIJI ImageJ* software (http://imagej.nih.gov/ij/), following a simple procedure (*Baviskar, 2011*). The data were computed (range, mean and SD for diameter, cell volume and number, including their combined volume). In this experiment, the final range of counted cells (mean, SD) was 96 ± 10 μm in diameter (when assuming the form of a sphere), i.e., 475

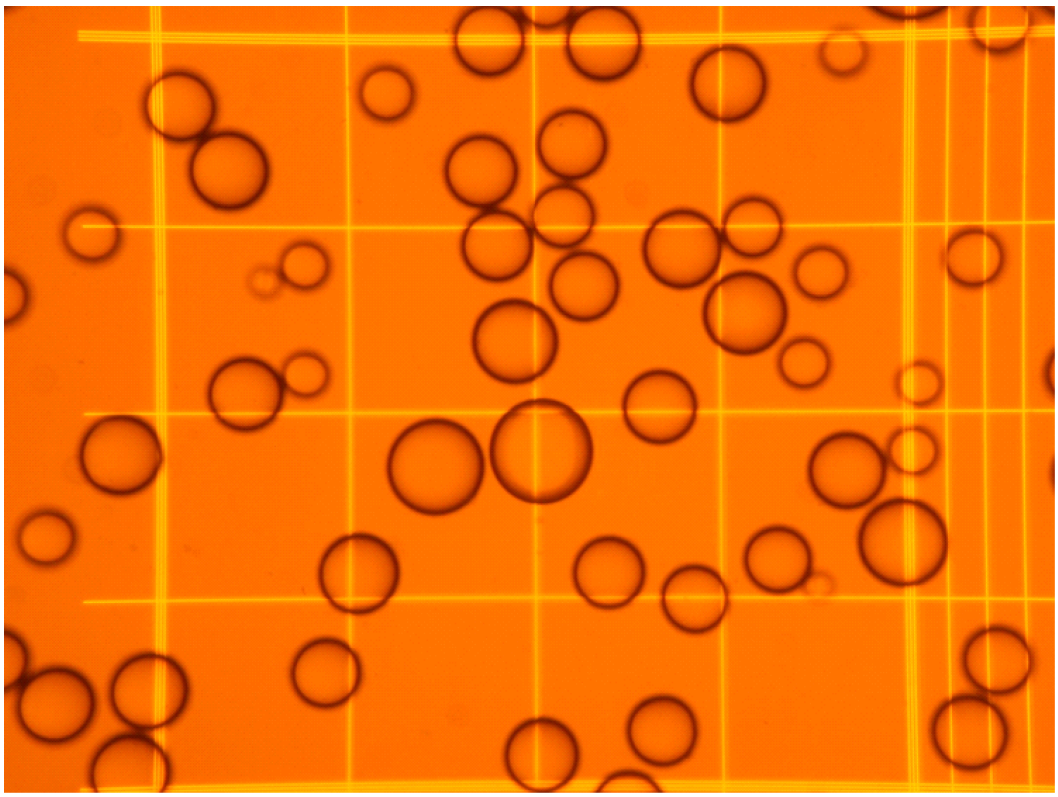


Figure 1 Representative microphotography of an adipocyte preparation observed at the microscope using a Neubauer chamber. The squares in the grid have a width of 250 μm, and correspond to a volume of 6.25 nL.

± 147 pL in volume. Figure 2 shows a representative example of the range of cell sizes obtained using this procedure on epididymal WAT.

Non-nucleated cells (essentially red blood cells: RBCs) were identified by their smaller size (in the fL range) using the Scepter 2.0 cell counter (EDM Millipore Corp, Billerica, MA USA) hand-held cell sizer. Total stromal cells, (i.e., including RBCs) were analyzed for each sample using two different cell-range tips for the Scepter: Sensor 40, for 3–18 μm particles' size (PHCC40050; Merck Millipore, Darmstadt, Germany) and Sensor 60, for 6–36 μm particles' size (PHCC60050; Merck Millipore). The data for both ends of the superimposed size graphs were taken as final values, and those in the overlapping zone were used taking in both series of data against diameter. After the data were arranged, the measured volumes were plotted and the data were statistically analyzed.

Using stromal cell fraction samples from all rats tested, a cytometric flow analysis (Fig. 3) was performed to distinguish the proportion of small non-nucleated cells (i.e., red blood cells) from those nucleated and either dead or viable. The analyses were done using a FacsAria I SORP sorter (Beckton-Dickinson, San Jose, CA, USA). The cells were stained with propidium iodide (Sigma-Aldrich) and Syto-13 (Life Technology, Thermo-Fisher Scientific, Waltham, MA USA) used to estimate the proportion of non-nucleated red blood cells in the samples as a percentage of total stromal cells. We used this value to estimate

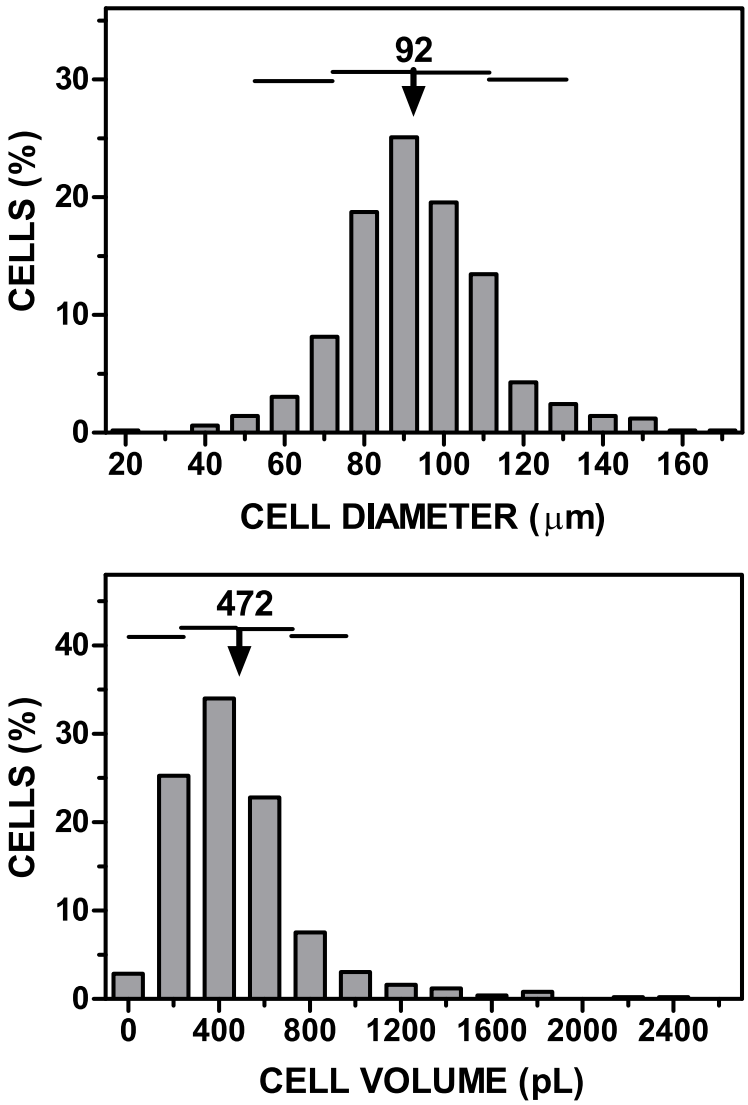


Figure 2 Representative graph of cell size (diameter, volume) *vs.* cell numbers representation obtained applying the cell extraction procedure described in the text to a sample of epididymal adipose tissue. The data have been grouped to facilitate the presentation. The arrow (and the number above) represent the mean cell diameter and volume. The horizontal lines represent each one the extent of one SD.

the presence of blood cells in the whole tissue and stromal cell counts, incorporating these data in the calculations.

Cell viability

We analyzed the functionality of the cells checking their metabolic integrity along a 2-day incubation study. We used 12-well plates (#CLS3513 Costar; Sigma-Aldrich) filled with 1.7 ml of DMEM (#11966-DMEM-no glucose; Gibco, Thermo-Fisher Scientific, Waltham, MA, USA), supplemented with 30 mL/L fetal bovine serum (FBS, Gibco). The medium (*Romero et al., 2015*) also contained 25 mM hepes (Sigma-Aldrich), 2 mM glutamine (Lonza Biowhittaker, Radnor, PA, USA), 1 mM pyruvate (Gibco), 30 mg/mL delipidated

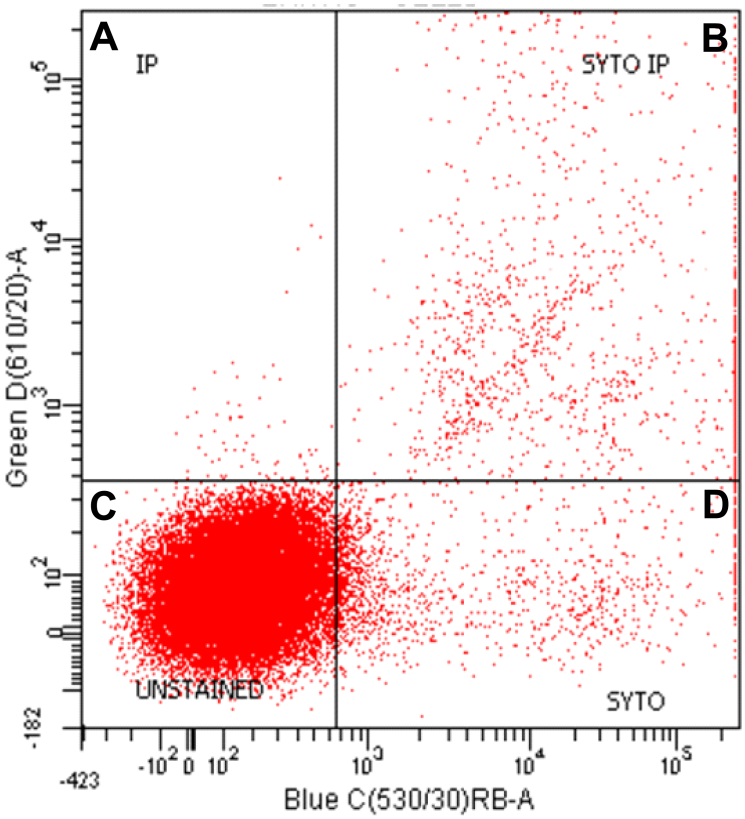


Figure 3 Representative graph of flow cell analysis of stromal fraction of epididymal rat WAT to discriminate nucleated from non-nucleated cells. Both propidium Iodide (IP) and Syto-13 (SYTO) bind DNA-positive and double positive particles (i.e., cells). The dots in the (A), (B) and (D) correspond to nucleated stromal cells; dots in (C) show the unstained cells, largely corresponding to the high proportion of erythrocytes.

bovine serum albumin (Millipore Calbiochem, MA, USA), 100 U/mL penicillin and 100 mg/L streptomycin (Gibco). Adenosine (Sigma-Aldrich) 100 nM was also added to help maintain the integrity of the cells. D-glucose (7 mM) was added as substrate. Each well received 400 µL of the adipocyte suspension (a second 100 µL aliquot was taken simultaneously to determine the adipocyte content in the well), thus completing a final volume of 2.1 mL. Under these conditions, the cells floated freely (as spheres) and tended to accumulate on the surface of the well. The cells were incubated at 37 °C in an incubation chamber ventilated with air supplemented with 5% CO_2, which gave a theoretical pO_2 of 20 kPa (i.e., 0.2 mM of dissolved O_2) (*Romero et al., 2015*). The calculated pCO_2 was in the range of 5 kPa, corresponding to 1.7 mM of dissolved CO_2. The cells were incubated for 24 h or 48 h without any further intervention. At the end of the experiment, a sample of the well contents was used to determine the number of cells. Then, the cells were harvested and the medium was extracted and frozen.

The incubation medium was used for the estimation of glucose, using a glucose oxidase-peroxidase kit (#11504; Biosystems, Barcelona, Spain) to which we added 740 nkat/mL mutarrotase (porcine kidney, 136A5000; Calzyme, St Louis, MO, USA) (*Oliva et al., 2015*).

Lactate was measured with kit 1001330 (Spinreact, Sant Esteve d'en Bas, Spain), glycerol was estimated with kit #F6428 (Sigma-Aldrich); NEFA were measured using kit NEFA-HR(2) (Wako Life Sciences, Mountain View, CA, USA).

Calculations

A critical factor in the development of this procedure was to keep track of all weights/volumes and incorporate into the calculations all aliquots extracted for testing (i.e., glucose or lactate levels). All data were introduced in a spreadsheet in which the volumes were justified with a (pipetting) error of ±3%. When possible, or when no other avenue was available, volumes were estimated from differential weights and the application of the densities calculated as described above.

The calculations used to determine the cell parameters, adipocyte recovery and WAT cell distribution are described in the Tables, presenting the original experimental data along with the derived or calculated data, as well as the formulas used for their estimation.

Statistical analyses were carried out using the Prism 5 Program (Graphpad Software Inc., La Jolla, CA, USA). Statistical differences between groups of data were determined with the unpaired Student's *t* test.

RESULTS

Analysis of the recovery of adipocytes from intact epididymal WAT

Table 1 shows the main experimental data for the quantitative analysis of free isolated adipocyte yield from just-dissected epididymal WAT. Both weight, water and fat content, as expected, showed little variation. The suspension obtained after collagenase digestion was estimated by weight, as were the floating fat layer and the debris retained in the nylon mesh. The number, and mean volume of intact adipocytes was also fairly uniform. The number of free (i.e., unattached to adipocytes) stromal cells was 17-fold higher than that of adipocytes, but almost 3/4ths of them were just red blood cells. All stromal cells had cell volumes in the range of 10^{-4} of those of adipocytes. The volumes of all stromal cells, including erythrocytes were measured after separation via high-speed centrifugation, which may have altered their original shape and volume, a treatment that the large adipocytes could not endure.

All tables contain a first column, labeled #, in which a letter and number are given to each row (or datum). These references are later used, in Tables 2–6 to present the origin of the data and the calculations done using the experimental data.

Table 2 presents the calculations (largely based on the data in Table 1) used to determine the recovery of viable isolated adipocytes from the intact tissue sample. Since all experimental data referred to weight (its measurement was several-fold more precise than volumetric measurements, especially those implying solids—such as cells—in suspension and mixed-phase systems) the main column of data is that indicated by weights, and have been referred to mg in 1 g of initial tissue. These values were converted to volumes using the densities experimentally measured for fat and tissue shown in Table 1. The third column shows the origin of the data and the calculations used to obtain the corresponding values.

Table 1 Results obtained from the collagenase digestion of rat epididymal WAT and the analysis of the tissue and fractions of tissue obtained in the process of separation of viable isolated adipocytes. The data presented as mean ± SD are direct experimental results obtained from four different rats.

#	Parameter	Units	Values
A1	Epididymal WAT weight	g	4.32 ± 0.44
A2	WAT fat content	mg/g	869 ± 15
A3	WAT water content	mg/g	45 ± 6
A4	Adipocyte suspension (digested tissue)	g	4.78 ± 0.86
A5	Floating fat derived from broken adipocytes	mg	105 ± 96
A6	Intact adipocytes suspension (A4–A5)	g	4.67 ± 0.85
A7	Fat in the intact adipocytes suspension	mg/g	537 ± 199
A8	Total fat in the intact adipocytes suspension	g	2.51 ± 1.06
A9	Water in the intact adipocytes suspension	mg/g	287 ± 68
A10	Recovery of intact adipocytes	cells × 10^6	5.82 ± 3.06
A11	Adipocyte mean volume	pL	475 ± 147
A12	Extraction debris mass (dry weight)	mg	356 ± 13
A13	Number of total stromal cells freed	cells × 10^6	103 ± 45
A14	Stromal cells' mean volume	fL	96.6 ± 43.0
A15	Red blood cells (proportion of A13, total stromal cells)	%	71.4 ± 8.5
A16	Red blood cells' mean volume	fL	25.9 ± 1.1
dt	Intact WAT density	g/mL	0.940 ± 0.013
dl	WAT fat density	g/mL	0.922 ± 0.022

Table 2 Analysis of the effectivity of the adipocyte isolation procedure used based on the analysis of lipid distribution, from intact tissue to the final preparation of adipocytes. The data are mean values calculated from the experimental data in Table 1. The column "calculations" explains the data used in each case. Volumes were calculated with *dt* or *dl* (Table 1) when applied to tissue ($V = W/dt$) or lipid ($V = W/dl$), where W is weight (in g) and V volume (in mL). In the calculations marked (W and V), the values were calculated directly from weights and volumes, i.e., not applying the density factors.

#	Parameter	Weight mg/g intact WAT	Volume μL/g intact WAT	Calculations
B1	Intact epididymal WAT	1,000	1,064	
B2	Extraction debris (dry weight)	83	88	(A12 × B1)/A1
B3	WAT fat content	869 ± 15	943	A2
B4	WAT mass minus debris	917	976	B1 − B2 (W and V)
B5	WAT fat content corrected by debris	797	865	(B3 × B4)/B1 (W and V)
B6	Lipid, from broken adipocytes, in the fat layer	24	26	(A5 × B1)/A1
B7	Total WAT fat in the extracted adipocytes	773	838	B5 − B6 (W and V)
B8	Total fat in the intact adipocytes recovered	581	630	(A8 × B1)/A1
B9	Total fat in the adipocytes recovered (intact or broken)	605	657	B6 + B8 (W and V)
B10	Fat loss during adipocyte isolation	192	208	B5 − B9 (W and V)
B11	Percentage of adipocyte fat recovery	75.9	–	(B9/B5) × 100
B12	Percentage of adipocytes (fat) lost in the fat layer	3.1	–	(B6/B5) × 100
B13	Percentage of intact adipocytes (expressed as fat) in the final preparation	72.8	–	(B8/B5) × 100

The detailed calculations of the efficiency of adipocyte recovery can be seen on Tables 1 and 2. We assumed that practically all WAT fat was present in the adipocyte fraction, essentially in adipocytes, since membrane lipids were not extracted with the procedure used (*Rose & Oklander, 1965*; *Remesar et al., 2015*), the eventual presence of fat in stromal cells went undetected and, in any case, could not represent a significant amount of material given the combined volume of these cells and their density. Consequently, all the fat present in the final intact adipocyte preparation should correspond to that of adipocytes, since free fat was measured and removed, and there were no other fat-carrying cells in the system in mass and/or numbers sufficient to alter the results, and neither membrane lipids could interfere in a significant way. Our previous work provides additional calculations that further support this conclusion (*Remesar et al., 2015*). Thus, we could equate the losses of fat (with respect to intact tissue) with losses of adipocytes. These losses were found to be significant, and the manipulation of the cells resulted in additional cells breakup. Under the conditions described, the collagenase incubation and extrusion through the nylon mesh resulted in a loss of about 24% of the cells (in fact, losses of fat), and the washings of the isolated cells added an additional loss in the range of 3%, which resulted in a recovery of about 73% of intact functional cells in the final adipocyte preparation, used for incubations, and referred to intact WAT (Table 2).

Isolated adipocyte viability

The viability of the cells obtained was high in the final preparation, with a negligible number of cells broken. The incubation of cells (about 700,000 per well) resulted in a loss of cells of approximately 4% in the first 24 h and an additional 9% in the second 24 h period. Consequently, the cells were viable and remained functional for 2 additional days in primary culture. The rate of glucose uptake (and metabolic utilization) per cell increased significantly in the second day of incubation (Fig. 4). However, the lactate efflux rates were maintained. Glycerol efflux rate also rose several fold in the 24–48 h period, maintaining, in the end, a much higher efflux rate than that of NEFA, which attests to its mainly glycolytic origin (parallel to the increase in glucose uptake and the maintenance of lactate production). However, the sole presence of NEFA proves that lipolysis was clearly present in the second day, probably as a consequence of the loss of about half of the glucose initially present in the medium (i.e., decreasing its availability to support cell metabolism). The higher rates of glycerol efflux in comparison with those of NEFA also support the finding that most of glycerol was not of lipolytic origin (*Smith, 1972*; *Romero et al., 2015*), since then the reverse would be true. In any case, the data prove that metabolic activity (at least glucose uptake, glycolysis to lactate, glycerogenesis and lipolysis) were fully functional in the 48 h period studied, in fact increasing during the second day of incubation.

Analysis of WAT cell type distribution and proportions, cumulative volumes

Table 3 shows the calculations derived from the data of Table 1 to obtain an approximate estimation of the combined proportions of tissue volume filled by the three main types of cells we were able to discriminate: adipocytes, nucleated stromal cells and red blood cells.

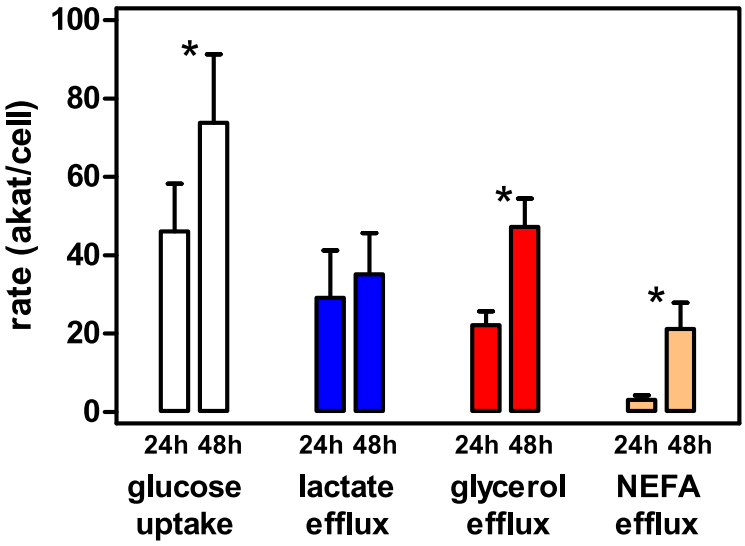


Figure 4 Metabolic activity of epididymal WAT adipocytes in primary culture at 24 h and 48 h of incubation. The data represent the mean ± SD of four different rats (triplicate wells). The data are presented as rates of uptake (glucose, white bars), or efflux to the medium (lactate, blue bars, glycerol red bars and NEFA beige bars), in concordant units (akat/cell) to facilitate comparisons. Statistical significance of the differences between 24 h and 48 h data: an asterisk * represents a $P < 0.05$ difference (Student's t test).

Table 3 Calculation of the volumes of cells from rat epididymal WAT. Data calculated using the experimental results presented in Tables 1 and 2.

#	Parameter	Units	Values	Calculations
C1	Adipocytes in WAT	cells × 10^6/g WAT	1.85	(B5/B1) × (A10/A8)
C2	Combined volume of WAT adipocytes	µL/g WAT	878	(A11 × C1)/10^6
C3	Stromal cells in WAT	cells × 10^6/g WAT	23.9	A13/A1
C4	Red blood cells in WAT	cells × 10^6/g WAT	17.0	(C3 × A15)/100
C5	Nucleated stromal cells in WAT	cells × 10^6/g WAT	6.8	C3 − C4
C6	Total volume of stromal cells in WAT	µL/g WAT	2.3	(C3 × A14)/10^9
C7	Total volume of red blood cells in WAT	µL/g WAT	0.44	(C4 × A16)/10^9
C8	Total volume of nucleated stromal cells in WAT	µL/g WAT	1.87	C6 − C7
C9	Mean nucleated stromal cell volume	fL	273	(C8/C5) × 10^9

The total mass of adipocytes was scaled up to the tissue volume from the measured data of mean adipocyte volume and its numbers (estimated from tissue and isolated cells' fat content). Adipocytes constituted almost 0.88 mL/g WAT volume. Total stromal cells and erythrocytes' volumes were, likewise, calculated from their mean cell size and numbers, scaled up to the volume of 1 g of intact WAT. Despite their larger numbers, the combined total volume of all stromal cells accounted for a little more than 2 µL/g WAT.

Using the adipocyte fat content and its volume (both referred to 1 g of tissue weight minus debris), as shown in Table 4, we obtained an approximate estimation of the "live cell mas" of adipocytes in epididymal WAT. This volume included all the cell organelles, systems and cytoplasm, since the fat vacuole volume corresponds to the fat content, estimated from tissue mass and its direct measurement of fat content. The total cell volume, only slightly

Table 4 Calculation of the non-fat cell volume of adipocytes in rat epididymal WAT. Data calculated using the experimental data presented in Tables 1–3.

#	Parameter	Volume (μL/g WAT)	% of total cells volume	Calculations
D1	Total volume of adipocytes in 1 g of WAT	878	100	C2
D2	Total fat volume in 1 g of WAT	865	98.5	B5
D3	Non-fat adipocyte cell volume in 1 g of WAT	13	1.5	D1 − D2

Table 5 Distribution of cell types by volume and number in rat epididymal fat. Data calculated using the results presented in Tables 1–4.

#	Parameter	Volume μL/g WAT	% of WAT volume	Cells (10^6/g WAT)	% of WAT cells	Calculations
E1	Initial WAT weight (minus debris)	976	100.0			B4
E2	Adipocytes	878	90.0	1.85	7.2	D1
E3	Red blood cells	0.44	0.05	17.0	66.2	C7
E4	Nucleated stromal cells	1.87	0.19	6.84	26.6	C8
E5	Total cells	880	90.2	25.7	100.0	E2 + E3 + E4
E6	Extracellular space	96	9.8			E1 − E5
E7	Fat	865	88.6			D2
E8	Total stromal cell volume	2.3	0.24			C6
E9	Total nucleated cell volume	880	90.2			E2 + E4
E10	Adipocyte non-fat cell volume	13.0	1.3			D3

larger, was calculated from another set of data: cell counting and mean volumes, tracing the cell losses from those of fat. The small difference between both entities was in the range of 1.5% of the cell volume, and taken as such, despite the wide margin of error and the small number of animals used to calculate this mean value, it represents a very small proportion of the whole tissue, which magnifies its active metabolic performance.

Table 5 shows the global distribution of epididymal WAT volume and the space taken up by the three types of cells analyzed. Adipocytes took up 90% of the tissue volume (excluding the "debris," largely vessels and other structures or undigested tissue), but their number was only 7% of the total number of cells. Nucleated stromal cells hardly took 0.2% of the volume but accounted for 27% of the cells. Red blood cells were the most abundant, 66% of numbers, but their space was only 0.05%, a value that roughly corresponds to 1 μL of whole blood per g of WAT (the rats were exsanguinated, thus this is a residual tissue blood volume). Cells did not occupy all the tissue space, since about 10% of the tissue volume was extracellular space (interstitial and vascular). Fat alone filled 89% of the tissue space.

Table 6 summarizes the mean characteristics of the adipocytes extracted from rat epididymal WAT. Their estimated non-fat cell volume was in the range of 13 pL, much larger than the 273 fL of nucleated stromal cells and the 26 fL of red blood cells (Table 1). Adipocytes' "live cell volume" was 48× higher than nucleated stromal or 500× higher than red blood cells. But their complete volume (i.e., including the single fat vacuole) was, respectively, 1,700× and 18,000× larger. The combined non-fat adipocyte volume was (Table 5) about one order of magnitude higher than that of nucleated stromal cells. Thus,

Table 6 Characteristics of the adipocytes isolated from rat epididymal adipose tissue. Data calculated using the results shown in Tables 1–3.

#	Parameter	Units	Values	Calculations
F1	Lipid content	mg/g	797	B5
F2	Cell lipid weight	ng/cell	431	B5/(C1 × 10^6)
F3	Cell lipid volume	pL/cell	468	(B5/C1) × 10^6/dl
F4	Cell mean volume	pL/cell	475	A11
F5	Non-fat cell volume	pL/cell	13	F4 − F3

despite their lower numbers, the mass of "live-cell material" of adipocytes remains the main active component of WAT at least using these gross comparison tools.

DISCUSSION

Probably, the most striking conclusion of the present study is the very small proportion of "live cell matter" found in epididymal WAT of normal young adult rats. Fat stores take up an inordinate amount of the tissue space, the interstitial space found is close to that described in previous reports and is in the range of other tissues (*Robert & Alemany, 1981*). However, after excluding the inert fat deposits, the remaining "cell material" accounts for about 1.5% of the total tissue mass, which seems very little even in relation to the assumedly limited metabolic activity of the tissue.

The data and viability of cells obtained with our customized version of the *Rodbell (1964)* method for isolation of adipocytes reflect a specific experimental condition, and their absolute values are obviously subjected to a number of possible modifying conditions, such as small changes in the conditions of extraction, the length of incubation, the inflammatory condition of WAT, the location of WAT depots, and the age, mass of WAT and sex of the animals used. Primary adipocytes may be incubated for long periods without loss of response to hormonal or paracrine stimuli (*Marshall, Garvey & Miriam, 1984*; *Fain & Madan, 2005*; *Giovambattista et al., 2006*). The lineal response to excess medium glucose producing lactate for up to 48 h is comparable to that described previously by us in 3T3L1 cells (*Sabater et al., 2014*). The increased secretion of glycerol and NEFA during the second day of incubation attest not to a loss of metabolic response and viability but to a change in the mechanisms of control of substrate efflux; these results agree with the known glycerogenesis and limited lipolysis of adipocytes when exposed to glucose (*Romero et al., 2015*).

It is well known that adipose tissue presents considerable difficulties to work with, the main problem being the dilution of cell proteins, RNA and DNA, as well as its wide variation in almost any parameter, largely attributed to the space occupied by huge fat stores. Evidently, this is not new, but the actual quantification, albeit approximate, of this entity is. The results may seem perhaps extreme, but the combined volume of fat (we often measure the weight, not the volume of fat depots) and extracellular space (i.e., plasma, and interstitial space) markedly limit the possible volume of the sum of blood cells, nucleated stromal cells and adipocyte non-fat cell volume. These considerations support, at least the range of "live cell" volume we have presented here for WAT. It is obvious

that the data calculated from the actual experimental results is only an approximation to the real values of this "live-cell" volume of adipose tissue cells. However, the data involved: percentage of fat in the intact tissue, and the combination of mean cell volume and number of adipocytes yield very close figures, with a small difference in cell size over vacuolar fat size. The different origin of the data, plus the use of different animals to get the means (the individual variability gave too much dispersion), decided us to work with experimentally-derived mean values to diminish the noise or clutter of individual data on the calculated/derived parameters. In previous works, we have proven the remarkable metabolic activity of the sum of WAT depots (i.e., taken as adipose organ) (*Arriarán et al., 2015b*; *Arriarán et al., 2015c*), especially its considerable glycolytic capability (under normoxic conditions) (*Arriarán et al., 2015c*; *Romero et al., 2015*), which adds to its known ability to store fatty acids taken from plasma lipoproteins (*Garfinkel, Baker & Schotz, 1967*; *Wang et al., 2016*) or synthesized from glucose (*Guerre-Millo, 2003*). Its important contribution to amino acid metabolism (*Arriarán et al., 2015a*), second only to liver (*Agnelli et al., 2016*; *Arriarán et al., 2016*), supports the long-proposed active WAT implication in energy and intermediate/substrate metabolism (*Cahill, 1962*). The data presented here only compound the puzzle, since the actual mass of cells doing the work is only a small fraction of the tissue, much lower than usually assumed. This small number of cells (including the stromal nucleated cells) is able to produce a large number of signaling cytokines (*Gerner et al., 2013*; *Wisse, 2004*), hormones (*Killinger et al., 1995*; *Stimson et al., 2009*) and maintain an active capacity to defend (immune system) (*Chmelar, Chung & Chavakis, 2013*), and repair or regenerate (i.e., stem cells) (*Ogura et al., 2014*) tissues. Compared to liver, which cell volume is upwards of 75% of its volume, the 50-fold lower proportion of WAT "live cell" volume has to show a much higher metabolic activity to be able to carry out the large number of functions and active metabolism that we keep discovering in recent times in WAT. The actual quantitation of the mass of adipocyte cytosol and its correlation with metabolic activity is a study worth carrying out, to definitively establish that WAT cells metabolism is extremely active, and not a dump for excess energy.

Surprisingly, the most abundant cells found in WAT were red blood cells, which accounted for roughly two thirds of the total. The volume of red blood cells was the approximate equivalent to about 1 μL of blood per g WAT, lower than previously published data using ^{65}Fe-labelled red blood cells (*Robert & Alemany, 1981*). Probably, the blood figure will be higher *in vivo*, since the rats were killed by exsanguination, so that most of the blood was drained. Consequently, we can assume that *in vivo,* WAT blood content may justify a hefty proportion of the tissue cells.

For operative methodological simplicity, we have analyzed all non-adipocyte cells ("stromal") as a single entity, but we have considered apart, independently, red blood cells, first for their relatively large proportion, and second because of their limited metabolic activity (and absence of nuclei). Nevertheless, the combined volume of the nucleated stromal cells was, again, smaller than expected. We are reasonably certain that the methodology used accounted for all free tissue cells in this fraction, since only low-density cells (i.e., adipocytes, and—probably—differentiating preadipocytes) (*Grégoire et al., 1990*) were separated by the low centrifugation force used. Our stromal cell data are difficult to compare with

the large number of studies available that analyze WAT cell populations under different metabolic conditions, since in practically all cases, the studies are not quantitative, neither referred to initial tissue mass, and are usually centered on preadipocytes (*Grégoire et al., 1990*), macrophages (*Makkonen et al., 2007*), vascular (*Kajimoto et al., 2010*) or other specific cell types (*Villaret et al., 2010*). In addition, most data on WAT adipocyte counts were done in fixed and stained WAT histologic cuts, where, usually, only section areas (of adipocytes) are taken into account, irrespective of the level of the cell at which they have been sliced and then estimated.

The ever-present problem of lipid droplets in cell suspensions has been partially solved in this case by letting them coalesce in a lipid layer before counting adipocytes in microphotographs. Nuclear staining may open new possibilities for counting, but the probable presence of other cells attached to adipocytes (i.e., not removed by the washings) and the need to maintain the integrity of the cells for sizing has prevented the use of this approach in the present study.

We expected to find larger numbers of stromal cells, obviously more than blood cells, because this relatively small part of the tissue is responsible for a large number of its metabolic functions and control responses as explained above, and is subjected to considerable variability related to its location and to inflammation (*Cildir, Akincilar & Tergaonkar, 2013*; *Villaret et al., 2010*). In any case, adipocytes remain by large (percentage of WAT volume either counting the fat vacuoles or not) the main component of WAT cell populations, but this primacy was lost when considering the numbers of cells.

One of the critical points this study tried to address was the efficiency of viable cell isolation from freshly dissected WAT and the maintenance of their functions for up to two days of incubation. The cell separation method we used is standard, and so widely used that seldom the source is cited, ensuring a fair recovery of the delicate adipocytes with minimal losses. We quantified these losses, and found that the recovery was initially close to 76% of the cells initially present in the tissue; but incubation resulted in the additional loss of significant (albeit relatively small) numbers of cells. In any case, we presented a method that allows the establishment of a quantitative relationship between the numbers of functional cells obtained with respect to the initial tissue mass, in the range of 73%. The data refer to viable cells, able to take up glucose from the medium, glycolyse it to produce lactate, and synthesize glycerol, part of which (as attested by the production of NEFA) was the product of lipolysis. In fact, the data presented show a marked increase in the efflux of glycerol and NEFA during the second day of incubation, at the expense of higher glucose uptake, proof that the cells were not losing functionality during the 24–48 h incubation, but increasing their utilization of glucose, which was comparable to that of 3T3L1 adipocytes (*Sabater et al., 2014*). The maintenance of function of adipocytes obtained with the *Rodbell (1964)* method, as is our case, has been repeatedly tested for periods of two days (or longer) in a wide variety of metabolic pathways and response to hormonal or chemical stimuli (*Marshall, Garvey & Miriam, 1984*; *Fain & Madan, 2005*; *Giovambattista et al., 2006*). However, the analysis of recovery was based essentially on the analysis of lipid in all fractions, so that the measurement of volumes (or weights) was critical and introduced a number of factors to be considered for success. First, all cells

floating in the buffer after treatment with collagenase and separation of debris (i.e., low density, and preferentially of large volume), were considered adipocytes. Just leaving the cells standing (i.e., centrifugation at $1\times g$) 5 min prevented pressure-caking of adipocytes and their breakage, but allowed a uniform distribution of smaller stromal cells between both phases. This was no problem for their estimation (numbers and volumes), but introduced a possible source of error when using isolated adipocytes for metabolic analysis, since the nucleated stromal cells remained a significant fraction of the crude adipocyte suspension. Three washings resulted in the loss of about 3% of adipocytes, but theoretically removed almost all non-attached stromal cells, down to a negligible proportion of the initial stromal cells content in the adipocyte fraction. The numbers and volumes of adipocytes found were in the range of those described in the literature (*DiGirolamo & Owens, 1976*; *Francendese & Digirolamo, 1981*). In addition, the cell volumes estimated, combined with the numbers of cells measured accounted for almost all the tissue space available, which is, in itself, an internal check that our calculations and estimations were essentially correct.

CONCLUSIONS

The methodology presented here for the estimation of adipocyte recovery allows for a direct quantitative reference to the original intact tissue of studies with isolated cells. This way, the cultured cell data can be used as an approximation to metabolic activity and function related to whole organism.

We have presented proof that the "live cell mass" of adipose tissue is very small. This fact, translates into an extremely high (with respect to the actual "live cytoplasm" size) metabolic activity to justify the overall activity of WAT in glucose-fatty acid relationships, but also in amino acid metabolism. These data justify that comparison of epididymal WAT, often considered the less metabolically active part of the adipose organ, with more metabolically relevant tissues such as liver should take into account these quantitative data, which make WAT an even more significant agent in the control of energy metabolism.

ACKNOWLEDGEMENTS

Thanks are given to Dr. Marta Camps for her help with practical aspects cell isolation methodology. We also thank the staff of the Scientific and Technological Services of the University of Barcelona for their help in the use of cell technologies.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

The authors received no funding for this work.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Floriana Rotondo performed the experiments, analyzed the data, prepared figures and/or tables, reviewed drafts of the paper.
- María del Mar Romero conceived and designed the experiments, performed the experiments, reviewed drafts of the paper.
- Ana Cecilia Ho-Palma performed the experiments, reviewed drafts of the paper.
- Xavier Remesar performed the experiments, analyzed the data, reviewed drafts of the paper.
- José Antonio Fernández-López analyzed the data, prepared figures and/or tables, reviewed drafts of the paper.
- Marià Alemany conceived and designed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

The Committee on Animal Experimentation of the University of Barcelona authorized the procedures used in the present study.

The animals were only subjected to euthanasia under isoflurane anesthesia, this is not a procedure requiring special permission but simple verbal communication of the execution of the procedure to the Acting Director of the Animal House. The sacrifice of the animals was carried out within a periodic culling procedure to reduce the population of the animal room. The animals were simply used for tissue sampling after anaesthesia instead of leaving them to die because of overdose of anaesthesia. No other manipulation was done on the animals, in accordance with the rules established by the Committee.

Data Availability

The following information was supplied regarding data availability:

University of Barcelona Repository http://hdl.handle.net/2445/102243.

REFERENCES

Agnelli S, Arriarán S, Oliva L, Remesar X, Fernández-Lápez J-A, Alemany M. 2016. Modulation of rat liver urea cycle and related ammonium metabolism by sex and cafeteria diet. *RSC Advances* **6**:11278–11288 DOI 10.1039/C5RA25174E.

Alemany M, Fernández-López JA. 2006. Adipose tissue: something more than just adipocytes. *Current Nutrition and Food Science* **2**:141–150 DOI 10.2174/157340106776818817.

Alkhouli N, Mansfield J, Green E, Bell J, Knight B, Liversedge N, Tham JC, Welbourn R, Shore AC, Kos K, Winlove CP. 2013. The mechanical properties of human adipose tissues and their relationships to the structure and composition of the extracellular matrix. *American Journal of Physiology* **305**:E1427–E1435 DOI 10.1152/ajpendo.00111.2013.

Arriarán S, Agnelli S, Remesar X, Alemany M, Fernández-López JA. 2016. White adipose tissue urea cycle activity is not affected by one-month treatment with a hyperlipidic diet in female rats. *Food and Function* **7**:1554–1563 DOI 10.1039/C5FO01503K.

Arriarán S, Agnelli S, Remesar X, Fernández-López J-A, Alemany M. 2015a. The urea cycle of rat white adipose tissue. *RSC Advances* **5**:93403–93414 DOI 10.1039/C5RA16398F.

Arriarán S, Agnelli S, Remesar X, Fernández-López JA, Alemany M. 2015b. Effects of sex and site on amino acid metabolism enzyme gene expression and activity in rat white adipose tissue. *PeerJ* **3**:e1399 DOI 10.7717/peerj.1399.

Arriarán S, Agnelli S, Sabater D, Remesar X, Fernández-López JA, Alemany M. 2015c. Evidences of basal lactate production in the main white adipose tissue sites of rats. Effects of sex and a cafeteria diet. *PLoS ONE* **10**:e0119572 DOI 10.1371/journal.pone.0119572.

Baviskar SN. 2011. A quick & automated method for measuring cell area using ImageJ. *American Biology Teacher* **73**:554–556 DOI 10.1525/abt.2011.73.9.9.

Cahill GF. 1962. Metabolic role of adipose tissue. *Transactions of the American Clinical and Climatologic Association* **73**:22–29.

Chmelar J, Chung KJ, Chavakis T. 2013. The role of innate immune cells in obese adipose tissue inflammation and development of insulin resistance. *Thrombosis and Haemostasis* **109**:399–406 DOI 10.1160/TH12-09-0703.

Choe SS, Huh JY, Hwang IJ, Kim JI, Kim JB. 2016. Adipose tissue remodeling: its role in energy metabolism and metabolic disorders. *Frontiers in Endocrinology* **7**:Article 30 DOI 10.3389/fendo.2016.00030.

Cignarelli A, Perrini S, Ficarella R, Peschechera A, Nigro P, Giorgino F. 2012. Human adipose tissue stem cells: relevance in the pathophysiology of obesity and metabolic diseases and therapeutic applications. *Expert Reviews in Molecular Medicine* **14**:e19 DOI 10.1017/erm.2012.13.

Cildir G, Akincilar SC, Tergaonkar V. 2013. Chronic adipose tissue inflammation: all immune cells on the stage. *Trends in Molecular Medicine* **19**:487–500 DOI 10.1016/j.molmed.2013.05.001.

Cinti S. 2001. The adipose organ: morphological perspectives of adipose tissues. *Proceedings of the Nutrition Society* **60**:319–328 DOI 10.1079/PNS200192.

Da Silva Meirelles L, Maistro Malta T, Wagatsuma VMD, Viana Bonini Palma P, Goes Araújo A, Ribeiro Malmegrim KC, Morato de Oliveira F, Alexandre Panepucci R, Araújo Silva W, Kashima Haddad S, Tadeu Covas D. 2015. Cultured human adipose tissue pericytes and mesenchymal stromal cells display a very similar gene expression profile. *Stem Cells and Development* **24**:2822–2840 DOI 10.1089/scd.2015.0153.

Deschênes D, Couture P, Dupont P, Tchernof A. 2003. Subdivision of the subcutaneous adipose tissue compartment and lipid-lipoprotein levels in women. *Obesity Research* **11**:469–476 DOI 10.1038/oby.2003.64.

DiGirolamo M, Owens JL. 1976. Water content of rat adipose tissue and isolated adipocytes in relation to cell size. *American Journal of Physiology* **231**:1568–1572.

Dixit VD. 2008. Adipose-immune interactions during obesity and caloric restriction: reciprocal mechanisms regulating immunity and health span. *Journal of Leukocyte Biology* **84**:882–892 DOI 10.1189/jlb.0108028.

Domergue S, Bony C, Maumus M, Toupet K, Frouin E, Rigau V, Vozenin MC, Magalon G, Jorgensen C, Noel D. 2016. Comparison between stromal vascular fraction and adipose mesenchymal stem cells in remodeling hypertrophic scars. *PLoS ONE* **11**:e0156161 DOI 10.1371/journal.pone.0156161.

Eder K, Reichlmayr-Lais AM, Kirchgeßner M. 1993. Studies on the extraction of phospholipids from erythrocyte membranes in the rat. *Clinica Chimica Acta* **219**:93–104 DOI 10.1016/0009-8981(93)90200-N.

Fain JN, Madan AK. 2005. Insulin enhances vascular endothelial growth factor, interleukin-8, and plasminogen activator inhibitor 1 but not interleukin-6 release by human adipocytes. *Metabolism Clinical and Experimental* **54**:220–226 DOI 10.1016/j.metabol.2004.08.016.

Folch J, Lees M, Sloane-Stanley GH. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry* **226**:497–509.

Francendese AA, Digirolamo M. 1981. Alternative substrates for triacylglycerol synthesis in isolated adipocytes of different size from the rat. *Biochemical Journal* **194**:377–384 DOI 10.1042/bj1940377.

Garfinkel A, Baker N, Schotz MC. 1967. Relationship of lipoprotein lipase activity to triglyceride uptake in adipose tissue. *Journal of Lipid Research* **8**:274–280.

Garvey WT, Olefsky JM, Matthaei S, Marshall S. 1987. Glucose and insulin co-regulate the glucose transport system in primary cultured adipocytes. A new mechanism of insulin resistance. *Journal of Biological Chemistry* **262**:189–197.

Gerner RR, Wieser V, Moschen AR, Tilg H. 2013. Metabolic inflammation: role of cytokines in the crosstalk between adipose tissue and liver. *Canadian Journal of Physiology and Pharmacology* **91**:867–872 DOI 10.1139/cjpp-2013-0050.

Gimble JM, Bunnell BA, Chiu ES, Guilak F. 2011. Concise review: adipose-derived stromal vascular fraction cells and stem cells: let's not get lost in translation. *Stem Cells* **29**:749–754 DOI 10.1002/stem.629.

Giovambattista A, Piermaría J, Suescun MO, Calandra RS, Gaillard RC, Spinedi E. 2006. Direct effect of ghrelin on leptin production by cultured rat white adipocytes. *Obesity* **14**:19–27 DOI 10.1038/oby.2006.4.

Grégoire F, Todoroff G, Hauser N, Remacle C. 1990. The stroma-vascular fraction of rat inguinal and epididymal adipose tissue and the adipoconversion of fat cell precursors in primary culture. *Biology of the Cell* **69**:215–222 DOI 10.1016/0248-4900(90)90348-7.

Guerre-Millo M. 2003. Extending the glucose/fatty acid cycle: a glucose/adipose tissue cycle. *Biochemical Society Transactions* **31**:1161–1164 DOI 10.1042/bst0311161.

Hall J, Roberts R, Vora N. 2009. Energy homeostasis: the roles of adipose tissue-derived hormones, peptide YY and ghrelin. *Obesity Facts* **2**:117–125 DOI 10.1159/000208517.

Hill AA, Bolus WR, Hasty AH. 2014. A decade of progress in adipose tissue macrophage biology. *Immunological Reviews* **262**:134–152 DOI 10.1111/imr.12216.

Honnor RC, Dhillon GS, Londos C. 1985. cAMP-dependent protein-kinase and lipolysis in rat adipocytes. 1. Cell preparation, manipulation, and predictability in behavior. *Journal of Biological Chemistry* **260**:15122–15129.

Kajimoto K, Hossen MN, Hida K, Ohga N, Akita H, Hyodo M, Hida Y, Harashima H. 2010. Isolation and culture of microvascular endothelial cells from murine inguinal and epididymal adipose tissues. *Journal of Immunological Methods* **357**:43–50 DOI 10.1016/j.jim.2010.03.011.

Killinger DW, Strutt BJ, Roncari DA, Khalil MW. 1995. Estrone formation from dehydroepiandrosterone in cultured human breast adipose stromal cells. *Journal of Steroid Biochemistry and Molecular Biology* **52**:195–201 DOI 10.1016/0960-0760(94)00164-H.

Kotronen A, Seppänen-Laakso T, Westerbacka J, Kiviluoto T, Arola J, Ruskeepää AL, Yki-Jarvinen H, Oresic M. 2010. Comparison of lipid and fatty acid composition of the liver, subcutaneous and intra-abdominal adipose tissue, and serum. *Obesity* **18**:937–944 DOI 10.1038/oby.2009.326.

Lee J. 2013. Adipose tissue macrophages in the development of obesity-induced inflammation, insulin resistance and type 2 diabetes. *Archives of Pharmacal Research* **36**:208–222 DOI 10.1007/s12272-013-0023-8.

Leonhardt W, Hanefeld M, Haller H. 1978. The adipocyte volume in human adipose tissue: 1. Lipid space, normal and maximum values, and the relation to body weight index. *International Journal of Obesity* **2**:33–45.

Li QK, Hata A, Kosugi C, Kataoka N, Funaki M. 2010. The density of extracellular matrix proteins regulates inflammation and insulin signaling in adipocytes. *FEBS Letters* **584**:4145–4150 DOI 10.1016/j.febslet.2010.08.033.

Liu YJ, Aron-Wisnewsky J, Marcelin G, Genser L, Le Naour G, Torcivia A, Bauvois B, Bouchet S, Pelloux V, Sasso M, Miette V, Tordjman J, Clement K. 2016. Accumulation and changes in composition of collagens in subcutaneous adipose tissue after bariatric surgery. *Journal of Clinical Endocrinology and Metabolism* **101**:293–304 DOI 10.1210/jc.2015-3348.

Lolmède K, Duffaut C, Zakaroff-Girard A, Bouloumie A. 2011. Immune cells in adipose tissue: key players in metabolic disorders. *Diabetes and Metabolism* **37**:283–290 DOI 10.1016/j.diabet.2011.03.002.

Luche E, Sengenès C, Arnaud E, Laharrague P, Casteilla L, Cousin B. 2015. Differential hematopoietic activity in white adipose tissue depending on its localization. *Journal of Cellular Physiology* **230**:3076–3083 DOI 10.1002/jcp.25045.

Makkonen J, Westerbacka J, Kolak M, Sutinen J, Corner A, Hamsten A, Fisher RM, Yki-Jarvinen H. 2007. Increased expression of the macrophage markers and of 11β-HSD-1 in subcutaneous adipose tissue, but not in cultured monocyte-derived macrophages, is associated with liver fat in human obesity. *International Journal of Obesity* **31**:1617–1625 DOI 10.1038/sj.ijo.0803635.

Marshall S, Garvey WT, Miriam G. 1984. Primary culture of isolated adipocytes. A new model to study insulin receptor regulation and insulin action. *Journal of Biological Chemistry* **259**:6375–6384.

Mitterberger MC, Lechner S, Mattesich M, Zwerschke W. 2014. Adipogenic differentiation is impaired in replicative senescent human subcutaneous adipose-derived stromal/progenitor cells. *Journals of Gerontology* **69A**:13–24 DOI 10.1093/gerona/glt043.

Mjös OD, Akre S. 1971. Effect of chatecolamines on blood flow, oxygen consumption, and release/uptake of free fatty acids in adipose tissue. *Scandinavian Journal of Clinical and Laboratory Investigation* **27**:221–225 DOI 10.3109/00365517109080211.

Nawrocki AR, Scherer PE. 2005. The adipocyte as a drug discovery target. *Drug Discovery Today* **10**:1219–1230 DOI 10.1016/S1359-6446(05)03569-5.

O'Brien SN, Mantzke KA, Kilgore MW, Price TM. 1996. Relationship between adipose stromal-vascular cells and adipocytes in human adipose tissue. *Analytical and Quantitative Cytology and Histology* **18**:137–143.

Ogura F, Wakao S, Kuroda Y, Tsuchiyama K, Bagheri M, Heneidi S, Chazenbalk G, Aiba S, Dezawa M. 2014. Human adipose tissue possesses a unique population of pluripotent stem cells with nontumorigenic and low telomerase activities: potential implications in regenerative medicine. *Stem Cells and Development* **23**:717–728 DOI 10.1089/scd.2013.0473.

Oishi Y, Manabe I. 2016. Integrated regulation of the cellular metabolism and function of immune cells in adipose tissue. *Clinical and Experimental Pharmacology and Physiology* **43**:294–303 DOI 10.1111/1440-1681.12539.

Oliva L, Baron C, Fernández-López J-A, Remesar X, Alemany M. 2015. Marked increase in rat red blood cell membrane protein glycosylation by one-month treatment with a cafeteria diet. *PeerJ* **3**:e1101 DOI 10.7717/peerj.1101.

Parker AM, Katz AJ. 2006. Adipose-derived stem cells for the regeneration of damaged tissues. *Expert Opinion on Biological Therapy* **6**:567–578 DOI 10.1517/14712598.6.6.567.

Pasarica M, Sereda OR, Redman LM, Albarado DC, Hymel DT, Roan LE, Rood JC, Burk DH, Smith SR. 2009. Reduced adipose tissue oxygenation in human obesity-evidence for rarefaction, macrophage chemotaxis, and inflammation without and angiogenic response. *Diabetes* **58**:718–725 DOI 10.2337/db08-1098.

Remesar X, Antelo A, Llivina C, Albà E, Berdié L, Agnelli S, Arriarán S, Fernández-López JA, Alemany M. 2015. Influence of a hyperlipidic diet on the composition of the non-membrane lipid pool of red blood cells of male female rats. *PeerJ* **3**:e1083 DOI 10.7717/peerj.1083.

Robert M, Alemany M. 1981. Water compartments in the tissues of pentobarbital anesthetized rats. *IRCS Medical Science* **9**:236–237.

Rodbell M. 1964. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *Journal of Biological Chemistry* **239**:375–380.

Romero MM, Sabater D, Fernández-López JA, Remesar X, Alemany M. 2015. Glycerol production from glucose and fructose by 3T3-L1 cells: a mechanism of adipocyte

defense from excess substrate. *PLoS ONE* **10**:e0139502 DOI 10.1371/journal.pone.0139502.

Rose H, Oklander M. 1965. Improved procedure for the extraction of lipids from human erythrocytes. *Journal of Lipid Research* **6**:428–431.

Sabater D, Arriarán S, Romero MM, Agnelli S, Remesar X, Fernández-López JA, Alemany M. 2014. Cultured 3T3L1 adipocytes dispose of excess medium glucose as lactate under abundant oxygen availability. *Scientific Reports* **4**:3663 DOI 10.1038/srep03663.

Sadie van Gijsen H, Smith W, Du Toit EF, Michie J, Hough FS, Ferris WF. 2012. Depot-specific and hypercaloric diet-induced effects on the osteoblast and adipocyte differentiation potential of adipose-derived stromal cells. *Molecular and Cellular Endocrinology* **348**:55–66 DOI 10.1016/j.mce.2011.07.030.

Sell H, Eckel J. 2010. Adipose tissue inflammation: novel insight into the role of macrophages and lymphocytes. *Current Opinion in Clinical Nutrition and Metabolic Care* **13**:366–370 DOI 10.1097/MCO.0b013e32833aab7f.

Smith U. 1972. Studies of human adipose tissue in culture. I. Incorporation of glucose and release of glycerol. *Anatomical Record* **172**:597–602 DOI 10.1002/ar.1091720401.

Stimson RH, Andersson J, Andrew R, Redhead DN, Karpe F, Hayes PC, Olsson T, Walker BR. 2009. Cortisol release from adipose tissue by 11β-Hydroxysteroid Dehydrogenase type 1 in humans. *Diabetes* **58**:46–53 DOI 10.2337/db08-0969.

Sumi M, Sata M, Toya N, Yanaga K, Ohki T, Nagai R. 2007. Transplantation of adipose stromal cells, but not mature adipocytes, augments ischemia-induced angiogenesis. *Life Sciences* **80**:559–565 DOI 10.1016/j.lfs.2006.10.020.

Takahara K, Ii M, Inamoto T, Komura K, Ibuki N, Minami K, Uehara H, Hirano H, Nomi H, Kiyama S, Asahi M, Azuma H. 2014. Adipose-derived stromal cells inhibit prostate cancer cell proliferation inducing apoptosis. *Biochemical and Biophysical Research Communications* **446**:1102–1107 DOI 10.1016/j.bbrc.2014.03.080.

Thompson ACS, Nuñez M, Davidson R, Horm T, Schnittker K, Hart MV, Suarez AM, Tsao TS. 2012. Mitigation of isolation-associated adipocyte interleukin-6 secretion following rapid dissociation of adipose tissue. *Journal of Lipid Research* **53**:2797–2805 DOI 10.1194/jlr.D031286.

Tordjman K, Leingang K, Mueckler M. 1990. Differential regulation of the HepG2 and adipocyte/muscle glucose transporters in 3T3L1 adipocytes. Effect of chronic glucose deprivation. *Biochemical Journal* **271**:201–207 DOI 10.1042/bj2710201.

Vielma SA, Klein RL, Levingston CA, Young MRI. 2013. Adipocytes as immune regulatory cells. *International Immunopharmacology* **16**:224–231 DOI 10.1016/j.intimp.2013.04.002.

Villaret A, Galitzky J, Decaunes P, Esteve D, Marques MA, Sengenes C, Chiotasso P, Tchkonia T, Lafontan M, Kirkland JL, Bouloumie A. 2010. Adipose tissue endothelial cells from obese human subjects: differences among depots in angiogenic, metabolic, and inflammatory gene expression and cellular senescence. *Diabetes* **59**:2755–2763 DOI 10.2337/db10-0398.

Wang MY, Gao MM, Liao JW, Qi YF, Du XM, Wang YH, Li L, Liu G, Yang HY. 2016. Adipose tissue deficiency results in severe hyperlipidemia and atherosclerosis in the low-density lipoprotein receptor knockout mice. *Biochimica et Biophysica Acta* **1861**:410–418 DOI 10.1016/j.bbalip.2016.02.018.

Wisse BE. 2004. The inflammatory syndrome: the role of adipose tissue cytokines in metabolic disorders linked to obesity. *Journal of the American Society of Nephrology* **15**:2792–2800 DOI 10.1097/01.ASN.0000141966.69934.21.

Wronska A, Kmiec Z. 2012. Structural and biochemical characteristics of various white adipose tissue depots. *Acta Physiologica* **205**:194–208 DOI 10.1111/j.1748-1716.2012.02409.x.

2. Origen y liberación de fragmentos de 3C

Los adipocitos maduros provenientes de tejido adiposo blanco producen grandes cantidades de lactato, glicerol y otros fragmentos de 3C a partir de la glucosa. Por lo tanto, en estos estudios se utilizó la metodología previamente descrita para investigar la composición del medio tras la incubación con glucosa, la distribución de los carbonos provenientes de la glucosa en los metabolitos estudiados y la expresión de los genes involucrados en las rutas metabólicas estudiadas. Además, se combinaron los datos de la concentración de metabolitos con los datos radiactivos del destino de esta glucosa para obtener la radiactividad específica por carbono de los metabolitos resultantes. Todo ello, nos permitió elaborar un mapa metabólico relativamente completo de la utilización de la glucosa, en el cual los datos tienen el mismo origen, así como una metodología coherente que utiliza diversas fuentes, métodos y estrategias; y que por lo tanto refuerza las conclusiones del estudio.

2.1 Glycerol is synthesized and secreted by adipocytes to dispose of excess glucose, via glycerogenesis and increased acilglycerol turnover

Floriana Rotondo, Ana Cecilia Ho-Palma, Xavier Remesar, José Antonio Fernández-López, María del Mar Romero and Marià Alemany.

Se utilizaron adipocitos de tejido epididimal con el objetivo de establecer la importancia cuantitativa de la producción de glicerol a través de la vía glucolítica respecto al proveniente de la lipogénesis. El efecto de la concentración de glucosa (7 y 14 mM) durante la incubación fue limitado, sin embargo, con el tiempo (24 y 48h) de incubación disminuyó la lipogénesis, aumentando la liberación de glicerol, al mismo tiempo que el eflujo del lactato se mantenía estable (sin verse afectado por la concentración de glucosa). La incorporación de marca radiactiva en el glicerol de los triacilgliceroles aumentó en paralelo con la expresión de las enzimas glicerogénicas y de las lipasas, coincidiendo con la disminución de la radiactividad especifica por carbono del glicerol liberado con el tiempo (mientras que la de acil-glicerol aumento a expensas de la glucosa). La expresión de la glicerofosfatasa no se vio afectada con la incubación, pero la importancia relativa de su ruta metabólica para liberar glicerol disminuyó con el tiempo. El resultado fue un cambio del origen del glicerol, pasando de un origen principalmente glucolítico a lipolítico, liberado a consecuencia del incremento del recambio de triacilgliceroles, de manera que la mayoría de los ácidos grasos, pero no el glicerol, fueron reciclados. Por lo tanto, la producción

de glicerol, parece ser una función primaria importante de los adipocitos, mantenida tanto por el proceso de glicerogénesis como por el recambio de acil-gliceroles. El glicerol y el lactato incorporaron aproximadamente 2/3 de la glucosa radiactiva, mientras que los ácidos grasos sólo el 10%. Todo ello apunta a que la producción de fragmentos de 3C ayuda a eliminar el exceso de glucosa y, por consiguiente, disminuye de esta forma la glucemia. Además, de proporcionar sustratos más pequeños, listos para ser utilizados por casi todas las células del organismo y que no están sujetos a limitaciones regulatorias como es el caso de la glucosa.

2.2 Use of 14C-glucose by primary cultures of mature rat epididymal adipocytes. Marked release of lactate and glycerol, but limited lipogenesis in the absence of external stimuli

Ana Cecilia Ho-Palma, Floriana Rotondo, María del Mar Romero, José Antonio Fernández-López, Xavier Remesar and Marià Alemany.

Como se menciona en el artículo anterior, la concentración de glucosa no afecta al eflujo del lactato ni a la mayoría de los parámetros estudiados. La incorporación de los carbonos de la glucosa ^{14}C en los adipocitos fue mínima mientras que el eflujo del glicerol aumentó debido al incremento en el recambio de triacilgliceroles, coincidiendo con la inhibición de la lipogénesis. Sin embargo, la producción de lactato se mantuvo estable y en paralelo a la glicerogénesis. Todos los cambios en el metabolismo de los adipocitos se reflejaron en la expresión de los genes. El eflujo constante del lactato demuestra que el proceso de glucolisis anaerobia es la principal fuente de energía en el adipocito y que el tejido adiposo blanco regula la glucemia al producir fragmentos de 3C, reduciendo de este modo la lipogénesis.

2.3 Insulin only partially increases basal 14C-glucose utilization in isolated epididymal rat adipocytes, largely for glycerogenesis and lipogenesis

Ana Cecilia Ho-Palma, Pau Toro, Floriana Rotondo, María del Mar Romero, Marià Alemany, Xavier Remesar and José Antonio Fernández-López.

Para este estudio se aislaron adipocitos a partir de tejido adiposo blanco epididimal de rata y se incubaron con glucosa ^{14}C (3,5mM, 7mM y 14mM) durante 24 horas sin insulina o con 175 nM de insulina, en condiciones normóxicas. Se analizó la contribución cuantitativa de los principales

metabolitos descritos anteriormente con la metodología desarrollada para la presente tesis. La captación de glucosa por parte de los adipocitos permaneció prácticamente constante, aumentando sólo ligeramente a una concentración de glucosa inicial de 7mM; no obstante, la presencia de insulina incrementó la captación de glucosa en todas las concentraciones utilizadas. Se produjo una mayor producción de lactato en los adipocitos incubados a 7 y 14mM, respecto a los incubados a 3,5mM. La insulina incrementó la liberación de lactato y disminuyó la de glicerol. Aunque la liberación de glicerol fue independiente de la concentración de glucosa, el origen vario según la situación, de forma que, con altas concentraciones de glucosa, el glicerol provenía casi exclusivamente de la glucosa ^{14}C (excepto a 7 mM con insulina), mientras que, a bajas concentraciones iniciales de glucosa, se produjo un cambio, pasando a ser obtenido mayoritariamente a partir de la lipólisis. Al igual que en los experimentos previos el glicerol de glicéridos y en mucha menor proporción los ácidos grasos del adipocito incorporaron pequeñas cantidades de glucosa marcada a través de la lipogénesis, afectando la presencia de insulina de manera evidente sólo a los ácidos grasos de los triacilgliceroles. Todas las expresiones de los genes acompañaron los cambios metabólicos producidos. Por tanto, la insulina produce una serie de respuestas anabólicas en los adipocitos, aumentando la captación de glucosa, así como el flujo de carbono a través de la glucólisis y la lipogénesis. Sin embargo, aunque la insulina promueve la lipogénesis, limita la incorporación de glicerol-3P para la síntesis de triacilgliceroles, lo que puede representar un mecanismo de autodefensa del tejido adiposo para evitar la acumulación excesiva de grasa.

SCIENTIFIC REPORTS

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Glycerol is synthesized and secreted by adipocytes to dispose of excess glucose, via glycerogenesis and increased acyl-glycerol turnover

Received: 16 March 2017
Accepted: 26 July 2017
Published online: 21 August 2017

Floriana Rotondo[1,2], Ana Cecilia Ho-Palma[1], Xavier Remesar[1,2,3], José Antonio Fernández-López[1,2,3], María del Mar Romero[1,2,3] & Marià Alemany[1,2,3]

White adipose tissue (WAT) produces large amounts of lactate and glycerol from glucose. We used mature epididymal adipocytes to analyse the relative importance of glycolytic versus lipogenic glycerol in adipocytes devoid of external stimuli. Cells were incubated (24/48 h) with 7/14 mM glucose; half of the wells contained ^{14}C-glucose. We analysed glucose label fate, medium metabolites, and the expression of key genes coding for proteins controlling glycerol metabolism. The effects of initial glucose levels were small, but time of incubation increased cell activity and modified its metabolic focus. The massive efflux of lactate was uniform with time and unrelated to glucose concentration; however, glycerol-3P synthesis was higher in the second day of incubation, being largely incorporated into the glycerides-glycerol fraction. Glycerophosphatase expression was not affected by incubation. The stimulation of glycerogenic enzymes' expression was mirrored in lipases. The result was a shift from medium glycolytic to lipolytic glycerol released as a consequence of increased triacylglycerol turnover, in which most fatty acids were recycled. Production of glycerol seems to be an important primary function of adipocytes, maintained both by glycerogenesis and acyl-glycerol turnover. Production of 3C fragments may also contribute to convert excess glucose into smaller, more readily usable, 3C metabolites.

Intact white adipose tissue (WAT) (and isolated adipocytes) secrete significant amounts of glycerol[1]. It has been long assumed that this glycerol is a by-product of lipolysis, released by cell lipases acting on triacylglycerol (TAG) stores[2], and/or lipoprotein-carried TAGs (i.e. by lipoprotein lipase)[3]. WAT capacity to recycle free glycerol is limited[4], but glycerol is a main substrate for hepatic gluconeogenesis[5], and a viable substrate for energy or TAG synthesis in many tissues[6,7].

Glycerol is synthesized from glucose via the glycolytic pathway through reduction of dihydroxyacetone-P by glycerol dehydrogenase, yielding *sn*-glycerol-3P[8]. Under conditions of high glucose availability, there is a steady supply of glycerol-3P for the synthesis of acylglycerols; this is achieved by condensation with the acyl-CoA; produced by the lipogenic pathway, from glucose[9] or other substrates[10]. In most tissues, including WAT, acyl-CoA can be alternatively synthesized from extracellular fatty acids[11], such as those released by lipoprotein lipase. The rates of TAG deposition, in addition depend on the excess energy and type of substrate available, but also on the size of cells. Small young adipocytes showed higher lipogenic flows[12], whereas mature large adipocytes preferentially incorporate preformed fatty acids[13].

Despite pyruvate and lactate, both 3C fragments, being good lipogenic substrates[10,14], free glycerol does not seem to be used by WAT in significant amounts, neither for lipogenesis nor as energy substrate[15], and is not recycled in significant proportions to glycerol-3P[16,17]. Glycerol kinase is present, with low activity, in WAT[4,17]; but tends to increase in the obese[18], and under high-fat diets[17]. Adrenal glycerol kinase K_M is in the range of 10^{-4} M[19] which may, theoretically, allow for a significant recycling. However, the main WAT glycerol transporter,

[1]Department of Biochemistry and Molecular Biomedicine, Faculty of Biology University of Barcelona, Barcelona, Spain. [2]Institute of Biomedicine, University of Barcelona, Barcelona, Spain. [3]CIBER-OBN Research Web, Barcelona, Spain. Correspondence and requests for materials should be addressed to M.A. (email: malemany@ub.edu)

aquaporin 7[20], markedly limits the process by facilitating its rapid excretion[21], thus effectively preventing significant intracellular recycling.

Glycerol is an excellent gluconeogenic substrate[5], which has the advantage, over lactate, pyruvate and alanine (the four being the main inter-organ 3C glucose precursors), that it has no charge nor N burden to dispose of. In addition to its hepatic utilization for gluconeogenesis, in most tissues glycerol can be easily converted again to glycerol-3-P by glycerol kinases, to be used in the synthesis of acyl-glycerols. Glycerol is also a good substrate for energy, since it can be rapidly incorporated into the glycolytic pathway[22].

Alternative catabolic pathways have been described in which glycerol is oxidized by alcohol and aldehyde dehydrogenases[23]. The quantitative transcendence of this mechanism is probably small because of the high K_M values for glycerol of these unspecific enzymes, low cell concentrations and the competence of the specific and thermodynamically-favoured processes described above. Direct acylation of glycerol has been also described in most mammal tissues[24], but the information available on this pathway is scant.

In birds' eggs, glycerol is the main low molecular weight carbohydrate present, fully substituting glucose in the first stages of embryonic development[25]. In a number of plants and yeasts, glycerol production from glycerol-3P allows its accumulation in cells as part of an extended mechanism for protection against environment-induced metabolic stress[26]. It is produced through a free glycerol shunt[27] not found in mammals; however, an enzyme structurally related to the yeast glycerol cycle, showing a marked glycerol-3P phosphatase activity[28], has been recently described in mammals. This phosphatase is also present in WAT and is modulated by diet[28]. Probably, this enzyme may, finally, complete the identification of the gene coding for the high glycerophosphatase activity described in earlier studies on WAT[29], but which has not been, so far, related to the known mammalian cell phosphatases[30].

The control of glycerol-3P availability has been considered a critical node in the control of TAG synthesis in mammals. However, the availability of dihydroxyacetone-P is not subjected to specific control other than that of the whole glycolytic/gluconeogenic flow of substrate, since both triose-P isomerase and the fructose-1,6-*bis*P aldolase are enzymes catalysing physiologically reversible reactions. A similar situation may affect glycerol-3P dehydrogenase, which is NADH dependent, and which reduces reversibly the C2 of dihydroxyacetone-P. This means that glycerol-3P may be synthesized in sufficient amounts only when there is enough glucose available (i.e. yielding both dihydroxyacetone-P and cytoplasmic NADH). Its production, thus, depends essentially on the bulk flow of substrates through the glycolytic pathway. Consequently an excess of glucose availability should favour a production in excess of glycerol-3P.

It has been postulated that, under conditions of insufficient glucose (but not energy and 3C substrate) availability, glycerol-3P can be synthesized from phosphoenolpyruvate[31]. This process, however, requires a high availability of oxaloacetate, plus ATP and NADH in the cytosol to synthesize glycerol. These conditions are incompatible with a robust glycolytic flow because of the need of NADH to produce lactate using the pyruvate formed from phosphoenolpyruvate.

The margins for a fine control of glycerol-3P availability should be necessarily narrow. Sufficient acyl-CoA may drive the synthesis of TAG by bulk effect, drawing 3C from the glycolytic path as needed. However, this picture does not correspond to physiological conditions, since the synthesis of TAG is highly regulated[32] by mechanisms other than substrate mass action.

We have recently observed the massive efflux of 3C units (lactate, glycerol) in normoxic 3T3L1 cells incubated with glucose[33], and of lactate *in vivo* from rat WAT[34]. Glycerol efflux was not accompanied by the expected efflux of NEFA (non-esterified fatty acids) to justify a lipolytic origin[30]. We assumed that with ample glucose available, a high sustained release of glycerol could not be solely supported by lipolysis, because: a) it was not paralleled by the canonical molar proportion of glycerol to NEFA; b) the mass of lipid present (at least in 3T3L1 cells) could no account for the large mass of glycerol liberated to the medium; and c) glycerol and lactate efflux were proportional to glucose[30]. Thus, bulk glycerol release could be sustained only by newly formed glucose-derived glycerol[30]. This process may help decrease the glycolytic pressure, both supplying glycerol-3P for the eventual synthesis of TAG (if the conditions favour this avenue) or to release glycerol as a 3C fragment for gluconeogenesis or use as energy substrate elsewhere.

In the present study we intended to widen the scope of our previous work with 3T3L1 cells[30] using, instead, primary cultures of rat adipocytes, and analysing the problem from three points of view: (a) The proportions of release of free glycerol (and lactate), plus NEFA, for up to 2 days; using glucose as substrate in the absence of external stimuli; (b) the quantification of ^{14}C-labelled glucose flow in adipocytes to glycerol, using the specific radioactivity of the metabolites and glucose to determine the lipolytic or glycerogenic (glycolytic) origin of the glycerol efflux; (c) the analysis, under the same experimental conditions, of the expression of the genes coding for the enzymes directly involved in glycerol metabolism in WAT.

Specific methodology has been developed to enable this line of work, both establishing the conditions of incubation, cell counting and viability[35], and the analysis of different label fractions[36].

Results

Isolated adipocytes glycerol release to the incubation medium. Table 1 shows the initial (glucose) and final concentrations of glucose, lactate, glycerol and NEFA in the medium after 24 or 48 h of incubation. Glucose levels steadily decreased and both lactate and glycerol increased during the incubation. The presence of NEFA in the medium also increased dramatically from 24 to 48 h. However, in all cases, NEFA levels were only a fraction (when compared in molar units) of that of glycerol.

Figure 1 shows the effect of initial glucose concentration on its uptake by the adipocytes and the efflux of NEFA and glycerol per cell over time. Glucose uptake was lineally dependent on the time of incubation, but independent of medium glucose. NEFA efflux was low during the first 24 h of incubation, markedly increasing when the whole 48 h period was analysed, showing high efflux rates, and no significant effect of glucose concentration.

fraction	units	7 mM glucose			14 mM glucose			P_T	P_G
		initial	24 h	48 h	initial	24 h	48 h		
medium glucose	µmol/well	12.6 ± 0.2	10.0 ± 0.2	5.03 ± 0.49	26.5 ± 0.5	23.5 ± 0.4	17.6 ± 0.5	< 0.0001	< 0.0001
medium lactate	µmol/well	< 0.05	1.78 ± 0.20	4.45 ± 0.34	< 0.05	2.15 ± 0.20	6.15 ± 0.75	< 0.0001	0.0171
medium glycerol	µmol/well	< 0.1	1.16 ± 0.12	5.12 ± 0.24	< 0.1	1.33 ± 0.13	5.28 ± 0.47	< 0.0001	NS
medium NEFA	µmol/well	< 0.1	0.11 ± 0.03	1.82 ± 0.18	< 0.1	0.09 ± 0.02	1.43 ± 0.25	< 0.0001	NS
adipocyte number*	10^3cells/well	591 ± 57	568 ± 55	515 ± 50	591 ± 57	568 ± 55	515 ± 50		
adipocyte volume	pL (SD)	449 ± 165			449 ± 165				
adipocyte TAG	µmol/well		107 ± 11	111 ± 4		130 ± 21	134 ± 11	NS	NS

Table 1. Medium levels of glucose, metabolites and cell counts. The data are presented as mean ± sem of eight different two-rat pools (i.e. labelled + parallel). *Estimated values (cell counts were obtained from combined "parallel" well samples). The adipocyte % of lipid (990 g/L) was measured using tissue pooled samples as previously described[35]. The levels of cell TAG were calculated from their lipid content; a standard molecular weight of 884 (i.e. trioleoyl-glycerol) has been used for the calculations. Statistical significance of the differences between groups (2-way-ANOVA). P_T represents the effect of time of incubation and P_G the effects of initial glucose in the medium.

Medium glycerol was also dependent on the time of incubation, but not on the initial glucose levels, the efflux rates practically doubling glycerol appearance in the medium. The molar ratio of NEFA to glycerol in the medium after incubation was far from the canonical value of 3 (the ratio in TAG) corresponding to pure lipolysis, being in the range of 0.07 to 0.36, the lowest values corresponding to the initial 24 h of incubation. The ratios for the efflux rates showed the same values.

Fate of glucose label. Table 2 shows the distribution of the label, initially present only in glucose, distributed after incubation in the different metabolite fractions. A large proportion of the initial glucose was recovered intact after 24 h or 48 h. These data agree with the fairly uniform rate of glucose uptake by adipocytes, essentially independent of medium glucose concentration. The largest individual label fraction was recovered as lactate. The results obtained with "cold" glucose presented in Table 1 and Fig. 1 are paralleled by the labelled data. The 14 mM glucose groups showed a significantly higher accumulation of label. At 24 h, free glycerol share was highest than that of glycerides-glycerol (especially in the 7 mM glucose group); the differences disappearing at 48 h. The possible complete oxidation of glucose for energy, may be considered improbable, since the label recovered was in the range of 95%.

A significant proportion of label, corresponding to an unidentified fraction (up to 20% of that of used glucose) was found in the medium, especially after 48 h of incubation; the values at 24 h were much lower. We could not identify the nature of this important fraction, not previously detected[36]. We are certain that it is not an acid (i.e. pyruvic, which is retained into the "lactate" fraction), and were neither glycerol (already measured) nor CO_2, since the data were not related to the estimated production of CO_2. The results were, then, incompatible with mitochondrial oxidation of Acetyl-CoA. NEFA also were an improbable option, because they would be retained by the columns, more because of lipophilic binding than because of its limited acidity at the pH of extraction. In addition, label in fatty acids, despite its considerable increase in concentration in the medium had a very low specific activity that could not justify not even a small fraction of the label in this important new fraction. Alanine could be a fair candidate, but the source of N was limited.

The values for CO_2 were calculated from the minimal amount needed to incorporate the radioactivity found in the labelled fatty acids fraction. For that reason, we counted this label together with that found in fatty acids and considered the sum as the fraction of label that went through the lipogenic pathway (i.e. 18–36% of total label), values comparatively lower than those retained as 3C units, most of which was returned to the medium (46–66%), probably in the range of 70% if the unknown medium factor is definitively confirmed to be alanine.

Specific radioactivity of the products of incubation. Figure 2 shows the changes in specific radioactivity experienced by the label fractions isolated and identified after 24 h or 48 h of adipocyte incubation in the presence of glucose. To facilitate the comparisons the data have been plotted on a logarithmic scale, with a value of 1 given to the specific radioactivity of the labelled glucose added to the medium. The specific radioactivity of glucose showed no changes with time, remaining all the time at values not different from 1 (i.e. 10^0). The values for lactate tended to show a limited decline with the time of incubation (only for glucose 14 mM), but the change was not statistically significant. Neither were the changes experienced by the medium glycerol, despite a clear trend to decrease with time and higher glucose concentration. The variability of the measurements was considerable, especially for the data obtained with 14 mM glucose. The effect of incubation time was, however, statistically significant for the 7 mM glucose group ($P = 0.0479$, Student's *t* test). The decrease in free glycerol specific radioactivity contrasted with the marked, significant increase in glyceride-glycerol data (in any case more than one order of magnitude lower than glycerol). The increase in TAG-incorporated glycerol attests to a marked flow of newly synthesized glycerol into the cell lipid stores, whereas, the decrease in free glycerol shows that only part of this free glycerol can be a direct product of glycerogenesis, the rest being produced via lipolysis of the TAG, which glycerol had a much lower specific radioactivity: it was free of label when the incubation started.

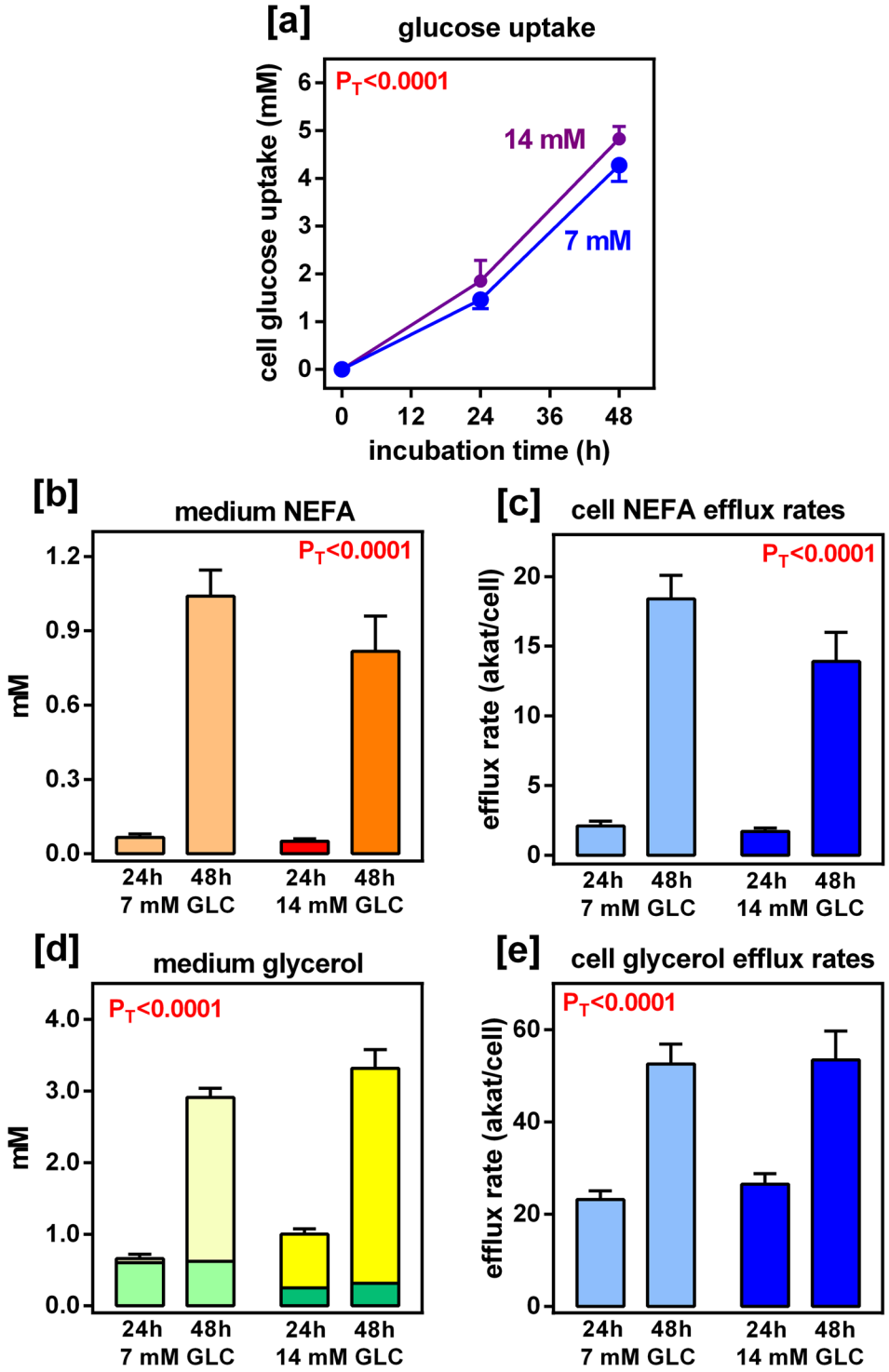

Figure 1. Effects of medium glucose concentration and incubation time on glucose uptake, and on the efflux of NEFA and glycerol, by primary cultures of rat epididymal adipocytes. The data are the mean ± sem of four different pairs (pooled) of rats; [**a**]: Glucose uptake vs. time. Blue circles: nominal initial glucose concentration 7 mM; purple circles: glucose 14 mM. In the histograms, pale shades of color correspond to 7 mM glucose in the medium, and the darker ones to 14 mM; [**b**] Effect of incubation with glucose on medium NEFA levels; [**c**] Cell NEFA efflux rates expressed in amol·s-1 per cell; [**d**]: Effect of incubation time and glucose on medium glycerol; the stacked parts of the columns show the approximate contribution of lipolytic (yellow) or phosphatase-released glycerol (green): [**e**] Effect of glucose and incubation time on cell glycerol efflux rates, also expressed as amol·s^{-1} per cell. Statistical significance of the differences between groups (2-way ANOVA): PT correspond to the differences with respect to time of incubation; PG correspond to the differences with respect to initial glucose, and Pi to their interaction. Not significant values ($P > 0.05$) were not represented.

fraction	label(total ^{14}C)	7 mM glucose		14 mM glucose	
		24 h	48 h	24 h	48 h
glucose metabolized	% of initial	*22.2 ± 1.4*	*51.9 ± 1.9*	*12.5 ± 1.1*	*25.6 ± 3.3*
	% of metabolized glucose	100	100	100	100
medium lactate		28.2 ± 7.3	24.1 ± 1.6	39.6 ± 3.1	35.2 ± 9.6
medium glycerol		23.4 ± 7.9	13.4 ± 4.9	19.5 ± 16.8	6.9 ± 3.8
glyceride-glycerol		10.4 ± 1.5	11.7 ± 1.9	6.8 ± 0.5	13.9 ± 3.1
total glycerol		*34 ± 7*	*25 ± 3*	*26 ± 18*	*19 ± 3*
total 3C		62 ± 14	49 ± 5	66 ± 16	46 ± 13
TAG fatty acids		12.7 ± 2.0	9.3 ± 0.8	18.5 ± 5.9	9.5 ± 2.8
estimated CO_2		12.0 ± 1.9	8.8 ± 0.8	17.4 ± 5.5	9.0 ± 2.7
total lipogenic		*25 ± 4*	*18 ± 2*	*36 ± 11*	*19 ± 5*
glycogen, metabolites		2.8 ± 0.2	1.4 ± 0.3	5.2 ± 1.3	2.2 ± 0.5
other medium labelled compounds		1.2 ± 0.7	20.7 ± 1.9	3.9 ± 2.4	18.7 ± 4.5

Table 2. Percent distribution of label from metabolized initial glucose in the main metabolite fractions of rat epididymal adipocytes in primary culture. Values calculated using only the "labelled" well data. Total glycerol corresponds to the sum of the label in medium free glycerol plus acyl-glycerides-glycerol. Statistical significance of the differences between groups (2-way ANOVA). Total "lipogenic" label includes that of cells esterified fatty acids and the calculated minimum CO_2 needed for their synthesis as explained in the text. The effect of "incubation time" was significant for glucose metabolized ($P_T < 0.0001$), glycogen ($P_T = 0.0087$) and for other medium labelled compounds ($P_T < 0.0001$), whereas the significance of "initial glucose concentration" affected only the glucose metabolized ($P_G < 0.0001$). No significant interactions were observed except for metabolized glucose ($P_i = 0.0021$).

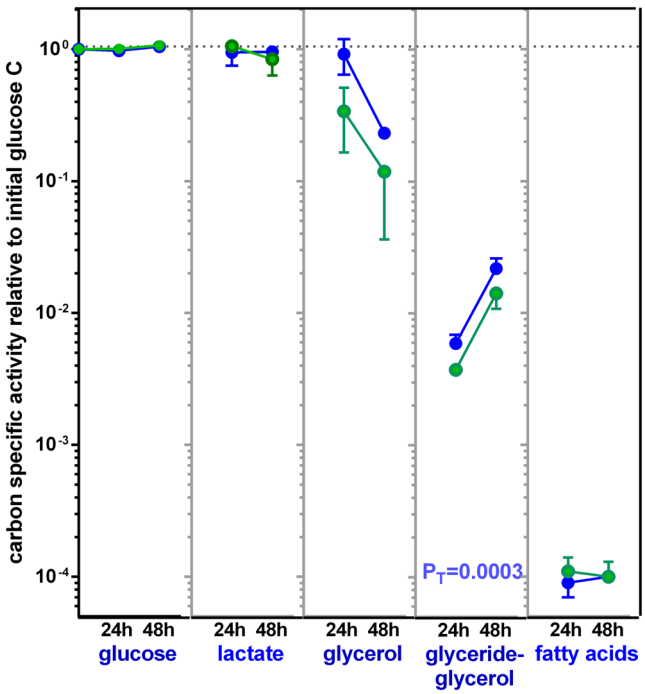

Figure 2. Carbon specific radioactivity of the main label fractions obtained after incubation of epididymal adipocytes in a primary culture in the presence of ^{14}C-glucose. The data are presented as mean ± sem of four different rats, and are presented in a log scale to show the wide differences between fractions. C-specific radioactivity correspond to the quotient of label found in the fraction divided by the molar concentration and the number of carbons the compound contains. In this case, all data have been referred to initial glucose C-specific radioactivity, to which a value of 1 (i.e. 10^0) was given. Blue dots and lines: incubation in 7 mM glucose; green dots and lines: 14 mM glucose. The statistical significance data and conventions are the same as in Figure 1.

Figure 1d shows an approximation to the glycerogenic and lipolytic origin of the free glycerol in the medium calculated from the mean values of Fig. 2. In the 7 mM glucose group, at 24 h, practically all free glycerol had been synthesized from glucose, but at 48 h, practically no additional glycolytic glycerol was produced, and the surge in medium glycerol was fuelled by lipolysis. At 14 mM glucose, the pattern was the same, but at 24 h of incubation, lipolytic glycerol was about twice that of direct glycerogenesis. It is remarkable that the pattern of glycerol efflux, shown in Fig. 1e, was the same, irrespective of the availability of glucose in the medium. The specific radioactivity of glycerides-glycerol was 1–2 orders of magnitude higher than that of fatty acids. In addition, the changes described for glycerides-glycerol with incubation time were not observed in the esterified fatty acids fraction. The data agree with lipogenesis being arrested after 24 h in contrast with massive incorporation of labelled glycerol into TAG.

In any case, lipolysis diluted the specific radioactivity of glucose-derived glycerol, but increased its efflux. In spite of lipolysis being the source of part of glycerol, this was not translated into the secretion of NEFA in the high proportions expected. Pure lipolysis produces 3 moles fatty acids per mole of glycerol, but the results were just the reverse, about 3 moles of glycerol per mole of NEFA. Since only part of free glycerol was of lipolytic origin, this ratio may be lower (2–2.5 times more glycerol than NEFA), but in any case was far from that expected for a complete lipolysis. Since comparisons of specific radioactivity were done in terms of C content, not moles, the relationship was drawn even further away. A mean fatty acid has 6-fold more C than glycerol: i.e. 18 to 3. Consequently, the label per C in TAG could not correspond to lipogenic activity matched to the large amounts of newly incorporated glycerol to glycerides, which prompts us to speculate that glycerol turnover in the cell TAG droplet should be much faster than expected. The incorporation of fatty acids newly synthesized from glucose would represent only a fraction of those used to re-synthesize TAG, since most of them were simply recycled, in contrast with the one-way-out of the lipolysis-generated glycerol.

Analysis of gene expression of glycerol metabolism-related proteins. Figure 3 shows the levels of expression of transporters, enzymes and other proteins related to the metabolism of glycerol/glycerol-3P in adipocytes, already presented in Table 3, and depicted in the metabolic map of Fig. 4. The data are expressed as the approximate number of copies of the corresponding mRNA per cell, and are presented in a logarithmic scale to allow for comparison of the levels of expression in addition to the trends of change elicited by glucose concentration and incubation time.

The gene for glycerol-3P dehydrogenase, *Gpd1*, presents a sizeable number of copies per cell, which increased by one order of magnitude in one day (48 h *vs*. 24 h) of incubation; no effect of glucose concentration was observed. The glycerol phosphatase gene (*Pgp*), also showed a high basal number of copies, and a moderate (albeit significant) increase with incubation time. Again glucose availability did not affect the expression of the enzyme. Glycerol kinase gene (*Gk*) presented a low number of copies compared with *Gpd1* and *Pgp*, but also increased its expression with incubation time and was not affected by glucose. The incorporation of glycerol-3P to form acylglycerols by glycerol-P acyl-transferase (*Gpam*) showed a similar pattern to *Gpd1* and *Gk*, with a number of copies per cell similar to the latter. Again no effect of glucose concentration in the medium was observed, but incubation time increased the expression of the gene, theoretically facilitating the synthesis of acyl-glycerols if sufficient substrate was available.

The main glycerol transporter, aquaporin 7 (*Aqp7*), presented a high number of copies per cell, especially when the cells were exposed to 14 mM glucose, and was affected differently by incubation (decreasing under high glucose and increasing when it was low). The 3P-glycerate dehydrogenase gene (*Phgdh*) is not considered a control node in the glycolytic pathway, but its function is critical to allow the flow of C from trioses-P to pyruvate. In this case, there was a clear interaction between incubation time and glucose availability, observable only at 48 h, in which higher glucose resulted in less marked increases in gene expression. The malic enzyme gene (*Me1*) related to lipogenesis as NADPH provider, repeated the pattern of increase in expression with time of incubation with nil effect of glucose concentration; its pattern and level of expression being similar to that of *Pgp*.

The three main WAT lipase genes showed the same pattern than *Gpd1*, despite acting in the opposite direction of glycerogenesis and glycerol incorporation into TAG. Hormone sensitive lipase (*Hsl*), and adipose TAG-lipase (*Atgl*), but also lipoprotein lipase (*Lpl*) showed a large (highest for *Lpl*) initial number of copies that increased considerably in the second day of incubation, with nil effects of glucose levels. The gene (*Cpt1*), coding for carnitine-palmitoyl transferase, which allows the entry of acyl-CoA into the mitochondria, usually for its oxidation, also showed this increasing pattern with time, but glucose also increased its expression (at 7 mM *vs*. 14 mM), with significant interaction between time and glucose. In any case, the number of copies was very low, which hints at both a complex regulation and limited activity under the metabolic circumstances studied. *CD36*, one of the main fatty acid membrane transporters, repeated the same pattern of lipases, with similar high number of copies of its mRNA per cell, which may favour uptake rather than release of NEFA.

The expression of pyruvate dehydrogenase kinase 4 gene (*Pdk4*) was especially interesting. Its small number of copies may be explained by its regulative function on an enzyme, pyruvate dehydrogenase. The increase of almost one order of magnitude from 24 h to 48 h suggests a similarly powerful effect on the synthesis of acetyl-CoA from pyruvate, effectively blocking mitochondrial lipogenesis, and thus the complete oxidative utilization of glucose. This effect was also dependent on glucose concentration. Probably, the changes in *Pdk4* were not related to mitochondrial alterations, since the expression of cytochrome C (*Cycs*), a key mitochondrial marker, remained unaltered by glucose and/or time of incubation.

Discussion

Using primary cultures of mature epididymal adipocytes, we have found that under conditions of maintained glucose availability (even markedly hyperglycaemic), the cells convert a sizeable part of glucose to 3C metabolites such as lactate and glycerol. The use of ^{14}C-labelled glucose as precursor has proven the mostly glycolytic origin

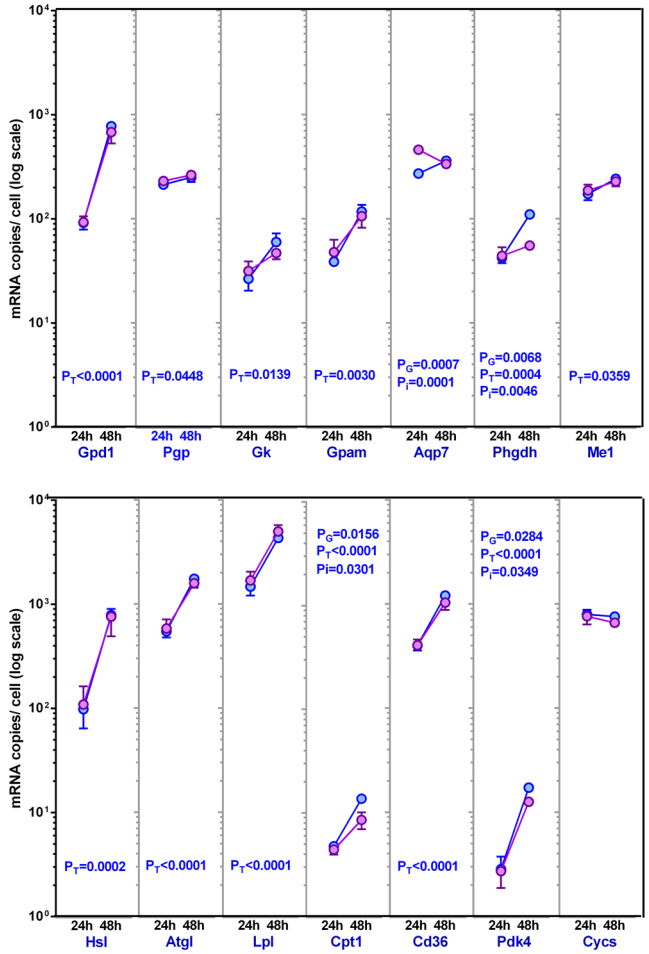

Figure 3. Gene expression of proteins related to glycerol metabolism in adipocytes incubated under varying glucose concentration for up to 48 h. The data are presented as number of copies of the corresponding mRNA per cell, and are mean ± sem of data from four rats. The data were obtained from the "parallel" incubations (i.e. no label). The results are shown in a log scale to facilitate comparisons of copies per cell between genes and groups. Blue dots and lines: initial 7 mM glucose, mauve dots and lines: 14 mM glucose. The statistical significance data and conventions are the same as in Figure 1. The correspondence between gene names and those of the proteins they code are given in the text and can be seen in Table 3.

of the free glycerol released to the medium. The rate of glycerogenesis was largely in excess of the cell needs of *sn*-glycerol-3P for the synthesis of acyl-glycerols, since the rate of lipogenesis from glucose did not match the larger flow of labelled glucose-C towards the synthesis of glycerol.

A key question for the credibility of this investigation is the validity of the methodology used, thus we invested considerable time and resources to establish its effectiveness and limits. A classical method[37] for adipocyte isolation was adapted, checked and complemented to obtain a basic system of incubation with relevant inclusion of quantitative factors and control of viability[35]. The surge in selective expression of enzymes and transporters, and increased metabolite handling was, in itself, an additional (albeit indirect) proof of the metabolic viability of the cells during a two-day incubation. The use of labelled glucose, a critical point to discern the origin of glycerol and the fate of glucose, was the subject of another previous specific methodology paper[36].

The main novelty of the present study lies on the combined use of the methodology primarily developed for this investigation, and the combination of different quantitative data obtained from the same source (levels of metabolites, cell counting and size estimation, label distribution and gene expression analyses). The methodological complexity and the large number of data obtained from the same sources, at the same time and conditions, facilitates comparisons, but do not preclude the existence of problems. We believe that the main weaknesses of the present study are:

(1) Constrictions affecting the number of samples studied, pooled in pairs. (2) The finding of a large fraction of unidentified labelled compound(s) released to the medium in parallel to the lipolytic surge; we have indications, that this fraction contains alanine (unpublished results). (3) Absence of data on NEFA specific radioactivity (too small samples, and low expected fatty acid label). (4) The non-viability of measuring the small amounts of evolved $^{14}CO_2$ in an atmosphere containing already 5% CO_2, allowing us only to calculate the minimum cost in CO_2 of lipogenesis; in any case, this figure should be low, given the small proportion of label not accounted for.

gene	protein (and EC code)	direction	sequences	bp
Gpd1	glycerol-3P dehydrogenase (NAD^+) [EC 1.1.1.8]	5′>	CTGGAGAAAGAGATGCTGAACG	113
		>3′	GCGGTGAACAAGGGAAACTT	
Pgp	phosphoglycolate phosphatase[glycerophosphatase] [EC 3.1.3.18]	5′>	CCTGGACACAGACATCCTCCT	100
		>3′	TTCCTGATTGCTCTTCACATCC	
Gk	glycerol kinase [EC 2.7.1.30]	5′>	ACTTGGCAGAGACAAACCTGTG	74
		>3′	ACCAGCGGATTACAGCACCA	
Gpam	glycerol-3P acyl-transferase [EC 2.3.1.15]	5′>	GGTGAGGAGCAGCGTGATT	129
		>3′	GTGGACAAAGATGGCAGCAG	
Aqp7	aquaporin 7	5′>	ACAGGTCCCAAATCCACTGC	127
		>3′	CCGTGATGGCGAAGATACAC	
Hsl	hormone-sensitive lipase [EC 3.1.1.79]	5′>	TCCTCTGCTTCTCCCTCTCG	108
		>3′	ATGGTCCTCCGTCTCTGTCC	
Atgl	triacylglycerol lipase (adipose tissue) [EC 3.1.1.3]	5′>	CACCAACACCAGCATCCAAT	120
		>3′	CGAAGTCCATCTCGGTAGCC	
Lpl	lipoprotein lipase [EC 3.1.1.34]	5′>	TGGCGTGGCAGGAAGTCT	116
		>3′	CCGCATCATCAGGAGAAAGG	
Cpt1b	carnitine-O-palmityl transferase (type1) [EC 2.3.1.21]	5′>	TGCTTGACGGATGTGGTTCC	152
		>3′	GTGCTGGAGGTGGCTTTGGT	
Cd36	platelet glycoprotein 4[fatty acid transporter]	5′>	TGGTCCCAGTCTCATTTAGCC	154
		>3′	TTGGATGTGGAACCCATAACT	
Me1	$NADP^+$-dependent malic enzyme [EC 1.1.1.39]	5′>	GGAGTTGCTCTTGGGGTAGTGG	143
		>3′	CGGATGGTGTTCAAAGGAGGA	
Phgdh	3-phosphoglycerate dehydrogenase [EC 1.1.1.95]	5′>	CTGAACGGGAAGACACTGGGAA	138
		>3′	AACACCAAAGGAGGCAGCGA	
Pdk4	pyruvate dehydrogenase kinase 4 [EC 2.7.11.2]	5′>	CTGCTCCAACGCCTGTGAT	142
		>3′	GCATCTGTCCCATAGCCTGA	
Cycs	cytochrome c, somatic	5′>	GGTCTGTTTGGGCGGAAG	70
		>3′	TACCTTTGTTCTTGTTGGCATCTG	
Arbp	0 S acidic ribosomal phospho-protein PO [housekeeping gene]	5′>	CCTTCTCCTTCGGGCTGAT	122
		>3′	CACATTGCGGACACCCTCTA	

Table 3. Primers used for the analysis of gene expression. E.C. = Enzyme Code Number.

(5) The often large variability of some of the label fraction measurements, resulting in statistical uncertainty. (6) The need to use "parallel" wells with no label for the measurement of protein expressions. (7) Too many interdependent results showing complex interactions, which forced us to limit the data presented and discussed here.

The results of our study support an active role of WAT in the handling of glucose, probably helping maintain glycaemia. The main findings were:

(A) Glucose uptake was higher than the actual cell energy needs, since even in the absence of insulin, the glycolytic production of lactate apparently provided sufficient ATP to sustain the adipocyte under practically anaerobic conditions[33]. This process is characterized by an increased expression of *Glut1* (independent of insulin[38]) and the production of lactate[33,34], being the main cell energy-sustaining pathway. The regular, and quantitatively significant conversion of glucose to lactate has been linked to the synthesis of ATP, ADP availability being postulated as the main regulatory factor[30]. Lactate efflux proceeds at a steady pace within a wide range of medium glucose levels (7–14 mM in this study), which agrees with the automatism of the anaerobic metabolism of the thin cytoplasm layer stretched between cell membrane and the lipid droplet surface. Thus, lactate production helps sustain the basic energy needs of most of the cell through a fully anaerobic process[33]. It seems that this source of ATP may suffice to sustain the activity of the small amount of "live cytoplasm" of mature adipocytes[35]. Lactate secretion by WAT may be considered, thus, primarily a normal consequence of the need for ATP generation, and not a specific indicator of hypoxia, despite the generalized association of lactate to hypoxia[39]. In this sense, this mechanism to obtain energy may be more comparable to the Warburg effect of cancer cells[40,41] than to hypoxia.

A marked glycerogenic flow provided glycerol as a final 3C export substrate together with lactate. A direct extrapolation to the *in vivo* situation in which lactate is released in large amounts from WAT[42] may hint at this tissue helping lower glycaemia, in fact breaking up 6C to 3C molecules. Glucose was substituted in large proportions by less-regulated 3C substrates, used elsewhere for energy or, eventually, for splanchnic lipogenesis or gluconeogenesis. The existence of a phosphatase directly hydrolysing glycerol-3P was previously postulated by us[30]; recently, a new glycerol-3P phosphatase has been described in liver, which is also present in WAT[28]. We have found that the corresponding gene was robustly expressed in isolated adipocytes, which agrees with the high glycerophosphatase activity of WAT[29]. The expression of the enzyme (*Pgp*) increased with time but not with the concentration of glucose; and was neither correlated with the rates of synthesis and efflux of glycerol.

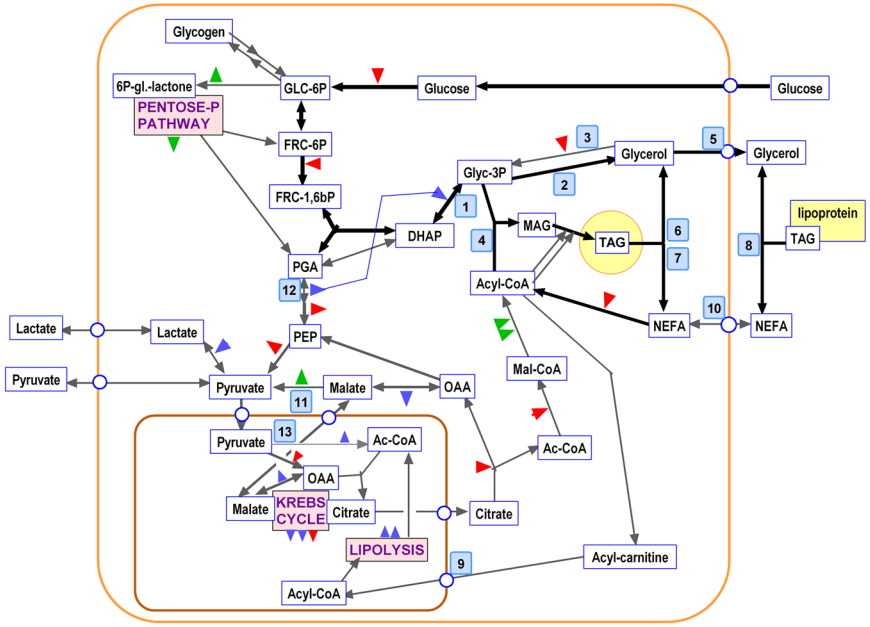

Figure 4. Main metabolic pathways affecting glycerol in the context of glucose-fatty acid metabolism in the adipocyte. The graph presents the main intermediate metabolites and substrates. Wide lines correspond to main pathways activated by incubation with glucose, whilst thin lines represent inhibited pathways. The figure represents the situation of the adipocyte during incubation with glucose, and have incorporated the data from label fate, metabolite concentrations, specific radioactivity and gene expression. Red triangles represent ATP, blue triangles represent NADH, and green triangles NADPH; in all cases, utilization by the path is represented by the tip pointing to the line, and synthesis or production by the tip pointing away from the line. The blue line represents the alternative use of phosphoglycerate dehydrogenase-generated NADH by glycerol-P dehydrogenase instead of lactate dehydrogenase as described in the text. The blue squares with numbers represent the proteins/genes controlling the corresponding path: 1- *Gpd1* (glycerol-3P dehydrogenase); 2- *Pgp* (glycerophosphatase); 3- *Gk* (glycerokinase); 4- *Gpam* (glycerol-3P acyl-transferase); 5- *Aqp7* (aquaporin 7); 6 *Hsl* (hormone-sensitive lipase); 7- *Atgl* (TAG lipase, adipose tissue); 8- *Lpl* (lipoprotein lipase); 9- *Cpt1b*(carnitine-palmitoleyl transferase); 10- *Cd36* (CD36 fatty acid transporter); 11- *Me1* (NADP-dependent malic enzyme); 12- *Phgdh* (3-phosphoglycerate dehydrogenase); 13- *Pdk4* (pyruvate dehydrogenase kinase 4).

The expression of *Pgp* seems to be affected by diet and by the lipolytic/lipogenic orientation of the specific adipose tissue analysed[28]. The small, but significant, rise in expression observed here hints at a modulated response. Perhaps the phosphatase activity is more dependent on hormonal control than on bulk substrate. Glycerol phosphatase provides the most direct (and specific) known mechanism to control the availability of glycerol-3P for synthesis of acyl-glycerols, via modulation of the direct hydrolysis of the phosphate ester cosubstrate. The production of free glycerol through this process has been demonstrated in yeasts and plants[43], where it is catalysed by an enzyme which structure is closely related to that encoded by *Pgp*[28]. Further study of modifying factors (i.e. exposure to hormones or marked inflow of fatty acids) other than simple glucose availability is needed to check/understand the role of glycerol phosphatase in the control of the glycerol-3P node. The relatively high number of copies found in comparison with those of glycerol kinase and the also high numbers for aquaporin all point to a clear predominance of the phosphatase over the kinase[4] and the effective removal of glycerol from the cell by aquaporin 7[44].

(B) The synthesis of acyl-glycerols is a highly regulated process[32] which increased with time of incubation, incorporating large amounts of newly formed glycerol (from glucose) into TAG. Using label distribution data, we found that glycerogenesis was more active than lipogenesis in isolated mature adipocytes, at least when cultured with sufficient glucose. Fatty acids synthesis used only about 1/4th of metabolized glucose (half of its carbon being lost as CO_2). The massive efflux of glycerol in cultured adipocytes has been attributed to a non-lipolytic origin, in part because it was not accompanied by a parallel secretion of NEFA[30,45]. Glucose was postulated as the source of glycerol released into the medium by adipocytes[46], and our results with labelled glucose confirm this origin. The sheer size of adipocytes, and the stretched layer configuration of most of its cytoplasm around the huge lipid vacuole, physically hampers the timely intracellular circulation of substrates. The long (peripheral) distances, the limitations of cytoplasmic currents in adipocytes due to simple geometry, and the rates of diffusion limit most metabolic activities. The resulting layered microenvironments are the consequence of almost unsurmountable difficulties for fast and continuous cytoplasm/mitochondrion interactions, such as pyruvate oxidation and lipogenesis. In most of the cell, glucose or fatty acids can be taken up easily from the interstitial space, and the glucose converted anaerobically to lactate, pyruvate or glycerol, with minimal needs of ATP. But the production of acetyl-CoA requires access to mitochondria, sparsely distributed on large adipocytes[47]. This is not the case with small or growing cells, such as the 3T3L1 converted fibroblasts[48], where mitochondria and multiple fat vacuoles

are interspersed in the surrounding cytoplasm. The physical constrictions may help explain why, in mature adipocytes, glycerogenesis and incorporation of exogenous fatty acids prevail over lipogenesis[45].

(C) The adipocytes are able to redirect the glycolytic flow towards lipogenesis, glycerogenesis or oxidative metabolism according to their size/geometry limitations and exposure to glucose, irrespective of the concentration of the sugar. These changes were spontaneously activated by adipocytes in the absence of external stimuli other than glucose, and/or the products of its catabolism. We postulate that the coordinated changes (and their direction) observed may be part of a fail-safe automatic mechanism established in the adipocyte to maintain metabolic control against an excess of substrates even in the absence of external regulatory signals. In our study, the absence of insulin did not affect the maintained incorporation of glucose by the cell, and neither lipogenesis, which is known to depend on insulin[49].

In the present experimental setup, lipolysis was activated by exposure to glucose, without other external stimuli. Glycerol-3-P fate shifted, in part, from being essentially hydrolysed yielding glycerol to being incorporated into acyl-glycerols. This process, however, decreased the availability of glycolytic NADH, needed to convert pyruvate to lactate, thus increasing the availability of pyruvate for oxidative decarboxylation to acetyl-CoA. This process was markedly hindered not only by cell geometry, but also by the marked rise of the expression of *Pdk4*, an inhibitor of pyruvate dehydrogenase. The consequence was a decrease in lipogenesis in spite of the excess pyruvate available. The absence of an increase in TAG-fatty acids label of adipocytes (in comparison to their glycerides-glycerol) is further proof that lipogenesis practically ceased after the first 24 h coinciding with *Pdk4* activation.

In the cell TAG stores, the amount of label incorporated as glycerol was of the same order of magnitude than that of fatty acids (similar number of labelled carbons, not molecules). The stoichiometry of production of one glycerol molecule for each pyruvate, and the utilization of the latter for the synthesis of acyl-CoA via acetyl-CoA is suggestive of lipogenesis as some sort of automatic process for disposal of pyruvate. The synthesis of additional acyl-CoA could be more a consequence than a key objective for disposal of glucose carbon. The glycerogenesis process, we postulate, would modify the glycolytic pathway to produce net pyruvate (not lactate) and excess glycerol-3P. This situation may facilitate both lipogenesis and the synthesis of TAG, provided that glucose supply is maintained. This combination of mechanisms has not been described before, but is supported by the results: in mature adipocytes, the existence of (aerobic) lipogenesis, fuelled by (anaerobic) glycolysis (in the absence of insulin), results in active TAG turnover, sustained by glycerogenesis.

(D) The outflow of glycerol does not follow the steady glycolytic rhythm shown by lactate efflux (unpublished results). Over time (in the second day of incubation), lipolytic-origin glycerol largely substituted direct glycerol-3P hydrolysis as main source of medium glycerol. This was the consequence of a marked rise in lipolysis, which was not paralleled by a matching efflux of fatty acids. Medium NEFA levels increased considerably, but in a proportion much lower than that of glycerol, even when only lipolytic glycerol (and not that coming directly from glycerol-3P) was taken into account. We assumed that most fatty acids freed by intracellular lipolysis were recycled. And those eventually produced by lipoprotein lipase from droplets or exosomes were largely incorporated into cell TAG with freshly formed glycerol-3P; this extracellular lipolytic glycerol adding to that released from the cell via aquaporin 7[50]. In sum, glycerogenesis from glucose shifted from massively freeing glycerol (necessarily via phosphatase) to increase its incorporation into TAG which turnover freed even more glycerol.

The contradictory coexistence of increased lipolysis (proven by the decreasing specific radioactivity of glycerol efflux) and increased synthesis of acyl-glycerols (enhanced glycerol label incorporation), plus higher lipogenesis (ultimately from glucose, as shown by the label found in the fatty acids of TAG) can only be explained by an increase in TAG turnover. This may be considered an example of "futile cycles" spendthrift mechanisms postulated to provide ways to eliminate excess energy, such as thermogenesis. Another postulated futile cycle, based on glycerol kinase was found to be activated by thiazolidinediones[51], but is actual operation, i.e. free glycerol recycling, has been refuted[16]. However, the steady production of glycerol, and the sequentially compensatory action of the phosphatase and TAG turnover paths, suggest that glycerol synthesis from glucose and its release from adipocytes may be an objective in itself, irrespective of the mechanism used. The main and primary consequence of this process was the net release of free glycerol. That is, glucose-derived glycerides-glycerol was freed by lipolysis, but most of the fatty acids were recycled. Probably, the justification of lipogenesis may be, at least under these conditions, only a consequence of enhanced glycerogenesis and the equilibrium of NADH usage in the cytosol of the adipocyte (unpublished results). Perhaps this glycerol plays an important role elsewhere, as has been suggested for heart normal operation[22]. This hypothesis is also supported by the effort/energy expense devoted to its massive production and release by the adipocyte through two different complementary (sequential?) pathways (phosphatase and TAG turnover). This is a critical open point that deserves further detailed experimental investigation.

(E) We had postulated previously that adipocytes (or WAT) take up more glucose than needed when confronted with high glucose levels, converting a large proportion of it into 3C fragments, such as lactate[52], pyruvate[53], alanine[54] and glycerol[1]. These 3C units may be used as energy substrate elsewhere; largely, by the liver in the gluconeogenic[5] and/or lipogenic pathways[55]. But with this action, WAT also disposes of (or defends from) an excess of glucose that may damage its function by dramatically inducing an inordinate enlargement its TAG stores[33]. This is part of a defence process that includes the limitation of blood flow as part of its protection against excess energy substrates[56]. Since WAT accounts for a sizeable part of body mass, and produces large amounts of lactate, pyruvate, glycerol an (probably) alanine, blood glucose levels should decrease, thus helping lower the inflammation and other damaging (i.e. glycosylation) effects caused by hyperglycaemia. The entry of 3C fragments in most tissues goes unhindered by insulin resistance and the tight control of glucose uptake[57]. This approach provides ready to use energy substrates, which are already partially metabolized in a way comparable to that of 3-hydroxybutyrate *vs*. NEFA or TAG. These fragments are massively used by liver[5,55], muscle[58], heart[59], brain[60] and other tissues, including the adipose tissues (WAT, BAT) themselves[61].

The main purpose of all these processes may be summarized in the contribution of WAT to decrease the glycaemic load of the body[62]; of all the glucose consumed by the adipocytes, about 70% found its way into glycerol, lactate and other metabolites. We included here the portion we suspect corresponds to alanine and that of cell metabolites, largely glycogen, fairly abundant in WAT in relation to live cell volume[63]. In contrast, only about 10% was found as fatty acids. Despite the probable errors and variability in accounting, after discounting the losses and estimated CO_2 production, most of the glucose was simply converted to 3C units. This is indeed a remarkable feat that goes against the general assumption that most of the glucose arriving at the adipocyte is inexorably converted to fat by the cells' lipogenesis-oriented metabolism.

The amount of glucose managed by the adipocytes is considerable, in spite of its small active cytoplasm proportion (in the range of 1% of tissue mass)[35]. The large mass of body WAT reinforces the postulated importance of this tissue in the control of glycaemia.

The uniform proportion of glucose taken up and converted into 3C, irrespective of glucose concentration, points towards an intrinsic automatic mechanism of compensation (and, perhaps, protection). The process could be modulated by the mass of substrate available rather than by external regulatory factors. This may be part of a fail-safe mechanism that takes place under conditions of generalized deregulation. If this hypothesis is finally proven, then WAT would be more of a protagonist of energy triage than the obliged recipient (depot) of excess energy[64]. The signalling role of glycerol has been analysed[65], and WAT is the choice organ source for its release[66]. However, this line of thought needs a more complex experimental scheme to discuss, or even to allow us to speculate further. In any case, it remains a troubling idea, which may in the end move us to reconsider the unanimous assumption of the pathologic nature of WAT accumulation, as, simply, a partly derailed element of a defence system unable to cope with a disordered availability of substrates. The alternative interpretation of an actual effective defence function is in concordance with the beneficial effect of insulin resistance in starvation becoming the basis of type 2 diabetes under conditions of excess.

In sum, we have found that mature adipocytes in primary cultures synthesize and release lactate and a large proportion of glycerol. The latter is a mechanism that needs some time of exposure to glucose to elicit a massive glycerogenic response, parallel to the synthesis and release of fatty acids, albeit in markedly lower proportions. This is paralleled by matched changes in gene expression. The pattern of change was different from the uniform rates of lactate production, unrelated to the concentration of glucose. The changes in glycerol production were paralleled by deep modifications of the enzymes of glycerogenesis and utilization of glycerol-3P. However, the expression of a robust glycerophosphatase was not modified by glucose availability. The stimulation of glycerogenic enzymes was mirrored by similar increases, with time, in WAT main lipases, and largely substituted glycolytic glycerol by the lipolytic product of TAG turnover. This turnover contributed to a higher efflux of glycerol (and, partially, of NEFA), while recycling most of the fatty acids, in a process far from being energetically efficient when compared with lactate production. Consequently, it is postulated that production of glycerol is an important primary function of adipocytes, supported by glycolysis and TAG turnover. Both lactate and glycerol production are assumed to contribute significantly to convert glucose to 3C units, thus lowering the negative effects of excess glucose.

Methods

Rats, housing, handling and sampling. All animal handling procedures and the experimental setup were in accordance with the animal treatment guidelines set forth by the corresponding European, Spanish and Catalan Authorities. The Committee on Animal Experimentation of the University of Barcelona specifically authorized the procedures used in the present study.

Male Wistar rats (Janvier, Le Genest-Saint Isle, France), 14-week old (N = 16), were used after at least 1-week acclimation period. The rats had free access to food (standard rat chow: #2014, Teklad Diets, Madison WI USA) and water at any time, and were kept in two-rat cages with wood shards as bedding material, at 21.5–22.5 °C, and 50–60% relative humidity; lights were on from 08:00 to 20:00.

The rats were killed, under isoflurane anaesthesia, at the beginning of a light cycle, by exsanguination from the exposed aorta. They were rapidly dissected, excising samples of epididymal WAT. Tissue samples of each pair of rats were coarsely minced and pooled. Thus, eight 2-rat samples were used.

Isolation, measurement and incubation of adipocytes. Adipocytes were isolated by incubation with collagenase as described in a previous paper[35], essentially following the Rodbell procedure[37]. Cells were counted, and their spherical (when free) diameters measured using the ImageJ software (http://imagej.nih.gov/ij/)[67]. The cells yield (with respect to WAT sample mass) was estimated in a number of randomly selected samples as previously described[35]. Incubations were carried out using 12-well plates (#734-2324VWR International BVBA/Sprl., Leuven Belgium) filled with 1.7 ml of DMEM (#11966-DMEM-no glucose; Gibco, Thermo-Fisher Scientific, Waltham MA USA), supplemented with, 30 mL/L foetal bovine serum (FBS, Gibco). The medium also contained 25 mM hepes (Sigma-Aldrich), 2 mM glutamine (Lonza Biowhittaker, Radnor, PA USA), 1 mM pyruvate (Gibco), 30 mg/mL delipidated bovine serum albumin (Millipore Calbiochem, MA USA), 100 U/mL penicillin and 100 mg/L streptomycin (Sigma-Aldrich). Adenosine (Sigma-Aldrich) 100 nM was also added to help maintain the integrity of the cells.

For each experiment, two series of incubations were carried out: (a) Adipocytes incubated in the presence of labelled glucose used to determine the glucose fate and specific radioactivity of metabolites; and a parallel group, (b) incubated in the same conditions except for the label, used for cell counting, to analyse gene expressions, and to obtain additional data on media metabolites.

The incubation medium was supplemented with ^{14}C-(U)-D glucose, (#ARC0122B, American Radiolabeled Chemicals, St Louis MO USA; specific radioactivity 11 GBq/mmol). Final glucose concentrations in the wells were, nominally, 7 or 14 mM. In the labelled samples the amount of label added per well was the same: about

1.8 kBq of ^{14}C-glucose. Specific radioactivity was expressed in Bq/μmol-C i.e. per micromole of the substrate divided by the number of C in the molecule, thus allowing a direct comparison of specific radioactivity between molecules of different size[36].

Each well received 400 μL of the cell suspension. Since 0.1 mL of medium was used for initial measurements, the final incubation volume was 2.0 mL. The cell plates were kept at 37 °C in an incubation chamber, ventilated with air supplemented with 5% CO_2, which gave a theoretical pO_2 of 20 kPa (i.e. 0.2 mM of dissolved O_2). These values were in the range of previous experimental measurements done under the same conditions[33]. The cells were incubated for 24 or 48 h without any further intervention, as previously described[36].

Cell recovery, measurements and processing of labelled cell components. The incubation of adipocytes was stopped by harvesting the cells. The medium was pipetted out, mixed, aliquoted and frozen. The procedure for measuring label distribution in the different fractions of cells and media was developed, tested and quantified previously[36]. Briefly, the cells of wells incubated with labelled glucose were weighed, frozen with liquid nitrogen, transferred to glass tubes and immediately extracted with chilled peroxide-free diethyl ether. The aqueous fraction contained small remnants of medium, but essentially cell metabolites and glycogen. The interphase contained most of the cell proteins. The aqueous (and interface) fraction was used whole to estimate its radioactivity. The organic phase, essentially containing TAG, was dried, weighed, re-dissolved in ethyl ether and saponified using KOH in ethanol. The potassium soaps were extracted and counted. The aqueous phase essentially contained only glycerides-glycerol label; it was also removed and counted[36]. Soap label was that of TAG fatty acids. Total cell label was estimated from the cells suspension, TAG label was the sum of total glyceride-glycerol and fatty-acid soaps counts.

The cells of the "parallel" wells were used to extract their RNA for analysis of gene expression. Total cell volume was also calculated from cell numbers and mean cell size. Since cell lipid proportion was known (as indicated in Table 1), we were able to estimate their TAG content[35], as a way to check (or correct the values in small size samples) the weight of adipocyte ethyl ether-extracted lipid from labelled cells.

Processing of the incubation media. We used the media of both "parallel" and label-containing wells to estimate the levels of glucose, lactate, glycerol and non-esterified fatty acids (NEFA). We also applied the protocol for labelled metabolite fractioning previously described[36].

Glucose concentration was measured using a glucose oxidase-peroxidase kit (#11504, Biosystems, Barcelona Spain) to which we added 740 nkat/mL mutarrotase (porcine kidney, 136A5000, Calzyme, St Louis, MO USA)[68]. Lactate was measured with kit 1001330 (Spinreact, Sant Esteve d'en Bas, Spain); glycerol was estimated with kit #F6428 (Sigma-Aldrich); NEFA were measured using kit NEFA-HR (Wako Life Sciences, Mountain View, CA USA).

Lactate (including pyruvate) label was determined using centrifuge microcolumns made up with sieve-filter type centrifugation inserts (Ultrafree-MC, Millipore, Bedford, MA USA) containing 250 mg of just hydrated, spin dried cationic-form Dowex 1 × 2 ion exchange resin (Serva Electrophoresis GmbH, Heidelberg, Germany) as previously described[36]. The retained lactate fraction was eluted with acid and counted.

The medium free of lactate was used in part to convert all glucose to gluconate by incubation with glucose oxidase (type VII from *Aspergillus niger*, Sigma-Aldrich). Catalase (from bovine liver, Sigma-Aldrich) was added to break up H_2O_2 and help maintain O_2 availability. The change of non-ionic glucose to gluconate allowed its retention (and acidic elution) using microcolumns as those described for lactate. The label retained was that of the unaltered glucose remaining in the medium after incubation[36,69].

A second aliquot, of the label-containing medium (already free of lactate) was treated with glycerol kinase (from *Escherichia coli*, #G6278, Sigma-Aldrich) with ATP in a medium adequate for the complete conversion of glycerol to glycerol-3P. The change in ionization was used to remove the glycerol (as glycerol-3P) from the medium using a microcolumn, eluting it with acid and thus counting the label retained in the glycerol moiety[36,70].

Combination of "cold" metabolite measurements and their radioactivity allowed us to calculate the fate of the initial glucose label under all conditions tested and to estimate the specific-C radioactivity for all of them.

Carbon dioxide production along the lipogenic process was estimated by the calculation of NADPH needed to synthesize one (~C18) acyl-CoA molecule (equivalent to one fatty acid residue in TAG) and assuming that 1 mole of CO_2 was produced in the pentose-P pathway for each 2 moles of NADPH generated (explained in more detail in Ho-Palma *et al.*[36]). The label present in TAG fatty acids allowed us to calculate the amount of glucose needed to be oxidized to CO_2 to provide C and reducing power for that synthesis. Since the ratio was constant, (minimum) label in CO_2 was calculated as a correlate of that found in the cell (soaps fraction) fatty acids.

Gene expression analyses. Total cell RNA was extracted from the harvested cells (from "parallel" wells) using the Tripure reagent (Roche Applied Science, Indianapolis IN USA), and were quantified in a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington DE USA). RNA samples were reverse transcribed using the MMLV reverse transcriptase (Promega, Madison, WI USA) system and oligo-dT primers.

Real-time PCR (RT-PCR) amplification was carried out using 10 μL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), 4 ng of reverse-transcribed RNA and 150 nM primers. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) using a fluorescent threshold manually set to 0.5 for all runs.

A semi-quantitative approach for the estimation of the concentration of specific gene mRNAs per unit of tissue weight was used[71]. *Arbp* was used as the charge control gene[72]. We expressed the data as the number of transcript copies per cell, in order to obtain comparable data between the groups, given the uniformity of the samples in that aspect. The genes analysed and a list of primers used are presented in Table 3. Their relationships to the metabolic glycerol node are shown in Fig. 4.

All final processed data for this study have been already incorporated into the text, Tables and Figures.

Statistical analyses and comparisons between groups (two-way ANOVAs) were done with the Prism 5 program (GraphPad Software, San Diego CA USA).

References

1. Jansson, P. A., Larsson, A., Smith, U. & Lönnroth, P. Glycerol production in subcutaneous adipose tissue of lean and obese humans. *J. Clin. Invest.* **89**, 1610–1617 (1992).
2. Langin, D. Control of fatty acid and glycerol release in adipose tissue lipolysis. *C. R. Biol.* **329**, 598–607 (2006).
3. Miles, J. M. *et al*. Systemic and forearm triglyceride metabolism. Fate of lipoprotein lipase-generated glycerol and free fatty acids. *Diabetes* **53**, 521–527 (2004).
4. Chakrabarty, K., Bombeck, C. T., Sigel, B., Tauber, J. W. & Jeffay, H. Glycerokinase activity in human adipose-tissue as related to obesity. *Int. J. Obesity* **8**, 609–622 (1984).
5. Ross, B. D., Hems, R. & Krebs, H. A. The rate of gluconeogenesis from various precursors in the perfused rat liver. *Biochem. J.* **102**, 942–951 (1967).
6. Robinson, J. & Newsholme, E. A. Glycerol kinase activities in rat heart and adipose tissue. *Biochem. J.* **104**, 2C–4C (1967).
7. Bortz, W. M., Pauil, P., Haff, A. C. & Holmes, W. L. Glycerol turnover and oxidation in man. *J. Clin. Invest.* **51**, 1537–1546 (1972).
8. Fondy, T. P., Herwig, K. J., Sollohub, S. J. & Rutherford, D. B. Isolation and structural properties of cytoplasmic glycerol-3-phosphate dehydrogenase from rat liver. *Arch. Biochem. Biophys.* **145**, 583–590 (1971).
9. Prior, R. L. & Jacobson, J. J. Effects of fasting and refeeding and intravenous glucose infusion on *in vitro* lipogenesis in bovine adipose tissue. *J. Nutr.* **109**, 1279–1283 (1979).
10. Katz, J. & Wals, P. A. Lipogenesis from lactate in rat adipose tissue. *Biochim. Biophys. Acta* **348**, 344–356 (1974).
11. Ookhtens, M., Montisano, D., Lyon, I. & Baker, N. Transport and metabolism of extracellular free fatty acids in adipose tissue of fed and fasted mice. *J. Lipid Res.* **28**, 528–539 (1987).
12. Roberts, R. *et al*. Markers of de novo lipogenesis in adipose tissue: associations with small adipocytes and insulin sensitivity in humans. *Diabetologia* **52**, 882–890 (2009).
13. Hood, R. L., Beitz, D. C., Best, D. J. & Trankina, M. L. Relationship between adipocyte size and lipid-synthesis in the Zucker rat. *Nutr. Rep. Int* **27**, 791–797 (1983).
14. Jungas, R. L. Effect of insulin on fatty acid synthesis from pyruvate, lactate, or endogenous sources in adipose tissue: Evidence for the hormonal regulation of pyruvate dehydrogenase. *Endocrinology* **86**, 1368–1375 (1970).
15. Vaughan, M. The production and release of glycerol by adipose tissue incubated *in vitro*. *J. Biol. Chem.* **237**, 3354–3358 (1962).
16. Tan, G. D. *et al*. A "futile cycle" induced by thiazolidinediones in human adipose tissue? *Nat. Med* **9**, 811–812 (2003).
17. Stern, J. S. *et al*. Glycerol kinase-activity in adipose-tissue of obese rats and mice. Effects of diet composition. *J. Nutr.* **113**, 714–720 (1983).
18. Kaplan, M. L. & Leveille, G. A. Development of lipogenesis and insulin sensitivity in tissues of the ob-ob mouse. *Am. J. Physiol.* **240**, E101–E107 (1981).
19. Seltzer, W. K. & McCabe, E. R. Human and rat adrenal glycerol kinase: subcellular distribution and bisubstrate kinetics. *Mol. Cell. Biochem.* **62**, 43–50 (1984).
20. Kishida, K. *et al*. Aquaporin adipose, a putative glycerol channel in adipocytes. *J. Biol. Chem.* **275**, 20896–20902 (2000).
21. Hara-Chikuma, M. *et al*. Progressive adipocyte hypertrophy in aquaporin-7-deficient mice. Adipocyte glycerol permeability as a novel regulator of fat accumulation. *J. Biol. Chem* **280**, 15493–15496 (2005).
22. Hibuse, T. *et al*. The heart requires glycerol as an energy substrate through aquaporin 7, a glycerol facilitator. *Cardiovasc. Res.* **83**, 34–41 (2009).
23. Sillero, M. A. G., Sillero, A. & Sols, A. Enzymes involved in fructose metabolism in liver and the glyceraldehyde metabolic crossroads. *Eur. J. Biochem.* **10**, 345–350 (1969).
24. Lee, D. P. *et al*. A novel pathway for lipid biosynthesis: the direct acylation of glycerol. *J. Lipid Res.* **32**, 1979–1986 (2001).
25. Roca, P., Sáinz, F., González, M. & Alemany, M. Energetic components in the unincubated egg fractions of several avian species. *Comp. Biochem. Physiol. B* **72**, 439–443 (1982).
26. Saito, H. & Posas, F. Response to hyperosmotic stress. *Genetics* **192**, 289–318 (2012).
27. Chen, H., Lu, Y. & Jiang, J.-G. Comparative analysis on the key enzymes of the glycerol cycle metabolic pathway in *Dunaliella salina* under osmotic stresses. *PLoS One* **7**, e37578 (2012).
28. Mugabo, Y. *et al*. Identification of a mammalian glycerol-3-phosphate phosphatase: Role in metabolism and signaling in pancreatic beta-cells and hepatocytes. *Proc. Natl. Acad. Sci. USA* **113**, E430–E439 (2016).
29. Margolis, S. & Vaughan, M. α-Glycerophosphate synthesis and breakdown in homogenates of adipose tissue. *J. Biol. Chem.* **237**, 44–48 (1962).
30. Romero, M. M., Sabater, D., Fernández-López, J. A., Remesar, X. & Alemany, M. Glycerol production from glucose and fructose by 3T3-L1 cells: A mechanism of adipocyte defense from excess substrate. *PLoS One* **10** (2015).
31. Ballard, F., Hanson, R. W. & Leveille, G. Phosphoenolpyruvate carboxykinase and the synthesis of glyceride-glycerol from pyruvate in adipose tissue. *J. Biol. Chem.* **242**, 2746–2750 (1967).
32. Coleman, R. A. & Lee, D. P. Enzymes of triacylglycerol synthesis and their regulation. *Prog. Lipid Res.* **43**, 134–176 (2004).
33. Sabater, D. *et al*. Cultured 3T3L1 adipocytes dispose of excess medium glucose as lactate under abundant oxygen availability. *Sci. Rep* **4**, 3663 (2014).
34. Arriarán, S. *et al*. Evidences of basal lactate production in the main white adipose tissue sites of rats. Effects of sex and a cafeteria diet. *PLoS One* **10**, e0119572 (2015).
35. Rotondo, F. *et al*. Quantitative analysis of rat adipose tissue cell recovery, and non-fat cell volume, in primary cell cultures. *PeerJ* **4**, 2725 (2016).
36. Ho-Palma, A. C. *et al*. A method for the measurement of lactate, glycerol and fatty acids production from ^{14}C-glucose in primary cultures of rat epididymal adipocytes. *Anal. Meth* **8**, 7873–7885 (2016).
37. Rodbell, M. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239**, 375–380 (1964).
38. Pedersen, O. *et al*. Evidence against altered expression of GLUT1 or GLUT4 in skeletal-muscle of patients with obesity or NIDDM. *Diabetes* **39**, 865–870 (1990).
39. Sahlin, K. Lactate formation and tissue hypoxia. *J. Appl. Physiol.* **67**, 2640 (1989).
40. Warburg, O. On the origin of cancer cells. *Science* **132**, 309–314 (1956).
41. Gatenby, R. A. & Gillies, R. J. Why do cancers have high aerobic glycolysis? *Nat. Rev. Cancer* **4**, 891–899 (2004).
42. DiGirolamo, M., Newby, F. D. & Lovejoy, J. Lactate production in adipose tissue: a regulated function with extra-adipose implications. *FASEB J.* **6**, 2405–2412 (1992).
43. Påhlman, A. K., Granath, K., Ansell, R., Hohman, S. & Adler, L. The yeast glycerol 3-phosphatases Gpp1 and Gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic, and oxidative stress. *J. Biol. Chem.* **376**, 3555–3563 (2001).

44. Maeda, N., Funahashi, T. & Shimomura, F. Metabolic impact of adipose and hepatic glycerol channels aquaporin 7 and aquaporin 9. *Nat. Clin. Pract. Endocrinol. Metab.* **4**, 627–634 (2008).
45. Nye, C. K., Hanson, R. W. & Kalhan, S. C. Glyceroneogenesis is the dominant pathway for triglyceride glycerol synthesis *in vivo* in the rat. *J. Biol. Chem.* **283**, 27565–27574 (2008).
46. Nurjhan, N. *et al*. Quantification of the glycolytic origin of plasma glycerol: implications for the use of the rate of appearance of plasma glycerol as an index of lipolysis *in vivo*. *Metabolism* **37**, 386–389 (1988).
47. Zhang, Y., Zeng, X. G. & Jin, S. K. Autophagy in adipose tissue biology. *Pharmacol. Res.* **66**, 505–512 (2012).
48. Newton, B. W. *et al*. Proteomic analysis of 3T3-L1 adipocyte mitochondria during differentiation and enlargement. *J. Proteome Res.* **10**, 4692–4702 (2011).
49. Baquer, N. Z., Sinclair, M., Kunjara, S., Yadav, U. C. S. & Mclean, P. Regulation of glucose utilization and lipogenesis in adipose tissue of diabetic and fat fed animals: Effects of insulin and manganese. *J. Biosci. (Bangalore)* **28**, 215–221 (2003).
50. Hibuse, T., Maeda, N., Nagasawa, A. & Funahashi, T. Aquaporins and glycerol metabolism. *Biochim. Biophys. Acta* **1758**, 1004–1011 (2006).
51. Guan, H. P. *et al*. A futile metabolic cycle activated in adipocytes by antidiabetic agents. *Nat. Med* **8**, 1122–1128 (2002).
52. Crandall, D. L., Fried, S. K., Francendese, A. A., Nickel, M. & DiGirolamo, M. Lactate release from isolated rat adipocytes: Influence of cell size, glucose concentration; insulin and epinephrine. *Horm. Metab. Res.* **15**, 326–329 (1983).
53. Saggerson, E. & Greenbaum, A. The regulation of triglyceride synthesis and fatty acid synthesis in rat epididymal adipose tissue. *Effects of altered dietary and hormonal conditions. Biochem. J* **119**, 221–242 (1970).
54. Snell, K. & Duff, D. A. Alanine release by rat adipose tissue *in vitro*. *Biochem. Biophys. Res. Commun.* **77**, 925–931 (1977).
55. Geelen, M. J. H. & Hindriks, G. A. Studies on the substrate for hepatic lipogenesis in the rat. *FEBS Lett* **175**, 8–12 (1984).
56. Alemany, M. Regulation of adipose tissue energy availability through blood flow control in the metabolic syndrome. *Free Radical Biol. Med.* **52**, 2108–2119 (2012).
57. Williamson, J. Effects of insulin and diet on the metabolism of L (+)-lactate and glucose by the perfused rat heart. *Biochem. J.* **83**, 377 (1962).
58. van Hall, G. Lactate kinetics in human tissues at rest and during exercise. *Acta Physiol* **199**, 499–508 (2010).
59. Taegtmeyer, H., Hems, R. & Krebs, H. A. Utilization of energy-providing substrates in the isolated working rat-heart. *Biochem. J.* **186**, 701–711 (1980).
60. Dringen, R., Schmoll, D., Cesar, M. & Hamprecht, B. Incorporation of radioactivity form [^{14}C] lactate into the glycogen of cultured mouse astroglial cells- Evidence for gluconeogenesis in brain cells. *Biol. Chem. Hoppe Seyler* **374**, 343–347 (1993).
61. Saggerson, E. D., Mcallister, T. W. J. & Baht, H. S. Lipogenesis in rat brown adipocytes - effects of insulin and noradrenaline, contributions from glucose and lactate as precursors and comparisons with white adipocytes. *Biochem. J.* **251**, 701–709 (1988).
62. Alemany, M. The defense of adipose tissue against excess substrate-induced hyperthrophia: Immune system cell infiltration and arrested metabolic activity. *J. Clin. Endocrinol. Metab.* **96**, 66–68 (2011).
63. Rigden, D. J., Jellyman, A. E., Frayn, K. N. & Coppack, S. W. Human adipose tissue glycogen levels and responses to carbohydrate feeding. *Eur. J. Clin. Nutr.* **44**, 689–692 (1990).
64. Rosen, E. D. & Spiegelman, B. M. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* **444**, 847–853 (2006).
65. Wirtshafter, D. & Davis, J. D. Body weight: Reduction by long-term glycerol treatment. *Science* **198**, 1271–1274 (1977).
66. van der Merwe, M. T. *et al*. Lactate and glycerol release from subcutaneous adipose tissue in black and white lean men. *J. Clin. Endocrinol. Metab.* **84**, 2888–2895 (1999).
67. Baviskar, S. N. A quick & automated method for measuring cell area using ImageJ. *Am. Biol. Teach* **73**, 554–556 (2011).
68. Oliva, L., Baron, C., Fernández-López, J.-A., Remesar, X. & Alemany, M. Marked increase in rat red blood cell membrane protein glycosylation by one-month treatment with a cafeteria diet. *PeerJ* **3**, 1101 (2015).
69. Azain, M., Kasser, T. & Baile, C. A method for the determination of glucose synthesis in isolated bovine hepatocytes. *J. Nutr. Biochem.* **10**, 205–209 (1999).
70. Crespí, C., Gastalver, G., Palou, A. & Roca, P. Enzymatic determination of carbon (^{14}C)-labeled glycerol in biological samples. *J. Biochem. Biophys. Methods* **30**, 179–183 (1995).
71. Romero, M. M., Grasa, M. M., Esteve, M., Fernández-López, J. A. & Alemany, M. Semiquantitative RT-PCR measurement of gene expression in rat tissues including a correction for varying cell size and number. *Nutr. Metab* **4**, 26 (2007).
72. Bamias, G. *et al*. Comparative study of candidate housekeeping genes for quantification of target gene messenger RNA expression by real-time PCR in patients with inflammatory bowel disease. *Inflamm. Bowel Dis.* **19**, 2840–2847 (2013).

Acknowledgements

This study has not been specifically funded, and part of the expenses have been endorsed by the researchers themselves. F. Rotondo and A.C. Ho-Palma were recipients of pre-doctoral fellowships of the Governments of Catalonia and Peru, respectively. The CIBER-OBN Research Web paid the salary of Dr. Romero.

Author Contributions

Development and checking of methodology: F.R., A.C.H.P and M.A. Cell incubations, media and gene expression analyses: F.R. and M.M.R. Work with labelled glucose: A.C.H.P. Statistical analyses, calculations and data organization: F.R., A.C.H.P., J.A.F.L., X.R. Design and draft writing: M.A. All Authors reviewed the manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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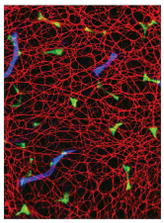
ADIPOCYTE
Volume 4 • Issue 4 • October/November/December 2015



Adipocyte

ISSN: 2162-3945 (Print) 2162-397X (Online) Journal homepage: http://www.tandfonline.com/loi/kadi20

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To cite this article: Ana Cecilia Ho-Palma, Floriana Rotondo, María del Mar Romero, José Antonio Fernández-López, Xavier Remesar & Marià Alemany (2018): Use of ^{14}C-glucose by primary cultures of mature rat epididymal adipocytes. Marked release of lactate and glycerol, but limited lipogenesis in the absence of external stimuli, Adipocyte, DOI: 10.1080/21623945.2018.1460020

To link to this article: https://doi.org/10.1080/21623945.2018.1460020

Accepted author version posted online: 05 Apr 2018.
Published online: 30 Apr 2018.

ADIPOCYTE, 2018
VOL. 0, NO. 0, 1–14
https://doi.org/10.1080/21623945.2018.1460020

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

Use of ^{14}C-glucose by primary cultures of mature rat epididymal adipocytes. Marked release of lactate and glycerol, but limited lipogenesis in the absence of external stimuli

Ana Cecilia Ho-Palma[a], Floriana Rotondo[a], María del Mar Romero[a,b,c], José Antonio Fernández-López[a,b,c], Xavier Remesar[a,b,c], and Marià Alemany[a,b,c]

[a]Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, University of Barcelona, Barcelona, Spain; [b]Institute of Biomedicine, University of Barcelona, Barcelona, Spain; [c]CIBER-OBN Research Web, Barcelona, Spain

ABSTRACT

White adipose tissue can metabolize large amounts of glucose to glycerol and lactate. We quantitatively traced glucose label to lactate, glycerol and fats in primary cultures of mature rat epididymal adipocytes. Cells were incubated with 7/14 mM ^{14}C-glucose for 24/48 h. Medium metabolites and the label in them and in cells' components were measured. Gene expression analysis was done using parallel incubations. Glucose concentration did not affect lactate efflux and most parameters. Glycerol efflux increased after 24 h, coinciding with arrested lipogenesis. Steady production of lactate was maintained in parallel to glycerogenesis. Changes in adipocyte metabolism were paralleled by gene expression. Glucose use for lipogenesis was minimal, and stopped (24 h-onwards) when glycerol efflux increased because of triacylglycerol turnover. Lactate steady efflux showed that anaerobic glycolysis was the main adipocyte source of energy. We can assume that adipose tissue may play a quantitatively significant effect on glycaemia, returning 3C fragments thus minimizing lipogenesis.

ARTICLE HISTORY
Received 2 February 2018
Revised 19 March 2018
Accepted 26 March 2018

KEYWORDS
Adipose tissue; adipocyte; lactate; glycerol; lipogenesis; anaerobic glycolysis

Introduction

WAT is a disperse organ, often considered a metabolically inert dump for unwanted energy, causing obesity due in part to its threshold for insulin resistance [1]. However, WAT is also a main site for defense against surplus substrate availability [2], where inflammation spreads through adipocytokine diffusion [3], and where steroid hormones interact and modulate the response to an excess energy challenge [4].

The physical discontinuity of the adipose organ requires their effective communication in order to coordinate physiological responses, hence the qualitative and quantitative importance of cytokine signaling [5]. There is an interrelationship between the nervous system and WAT [6], but specific site signaling may be hampered by extreme dispersion. These considerations portrait a unique, complex and often misunderstood organ made up from several different cell types, compromised in the defense against excess energy availability. This problem, never encountered before along evolution, turns part of our systems of protection against scarcity, such as insulin resistance [7], into deadly components of metabolic syndrome molecular inflammation [8]. We have not had yet sufficient evolution time to develop methods to cope with the derangement caused by affluence [8]. However, there are biological responses to the challenge, albeit limited and often ineffective: higher energy consumption (enhanced protein turnover, exercise) and wasting (thermogenesis), accompanied by (temporal) storage of fats, as well as secular trends to diminish energy intake. Thus, a trend to reduce the global incidence of obesity and co-morbidities is beginning to be observed at the population level [9].

WAT, as main fat storage space (but not exclusive [10]) also defends itself from this 'excess energy aggression', first limiting blood flow, but also through hypertrophia and inflammation [11], making lipogenesis difficult because of the sheer size of the cells [12]. Under basal conditions, isolated adipocytes and intact white adipose tissue (WAT) secrete significant amounts of 3C metabolites derived from glucose: lactate, glycerol, alanine, etc [13]. These 3C compounds may be used for hepatic gluconeogenesis [14], for lipogenesis [15] or directly used for energy elsewhere.

CONTACT Dr. Marià Alemany malemany@ub.edu Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, University of Barcelona, Av. Diagonal, 643, Barcelona 08028, Spain.

The 3C substrates released by WAT present two distinct biochemical origins: (a) those derived from pyruvate (i.e. pyruvate, alanine and, principally, lactate), and (b) those coming from the triose-P level of glycolysis, i.e. glycerol.

Pyruvate is the primary product of cytoplasmic glycolysis, reduced to lactate as a way to eliminate excess cytoplasmic NADH. Alanine is also a common 3C export product from peripheral tissues [16] and is formed by transamination of pyruvate with alanine transaminases. Glycerol is synthesized from glucose via the glycolytic pathway down to triose-P. Dihydroxyacetone-P is reduced to *sn*-glycerol-3P , which can produce free glycerol by the action of a phosphatase [17] or used in the synthesis of acyl-glycerols. WAT-released glycerol is commonly assumed to be a byproduct of lipolysis.

Despite pyruvate and lactate being potentially good lipogenic substrates [15], neither alanine [18] nor free glycerol seem to be used in significant amounts by WAT for energy or as lipogenic substrates [19] under basal conditions or under energy deprivation [20].

In WAT, the steady supply of glycerol-3P sustains the synthesis of acylglycerols using acyl-CoA provided by the lipogenic pathway, which depends on glucose availability [21]. There are other sources of acetyl-CoA [15], such as free fatty acids, from lipolysis or taken up from the extracellular space.

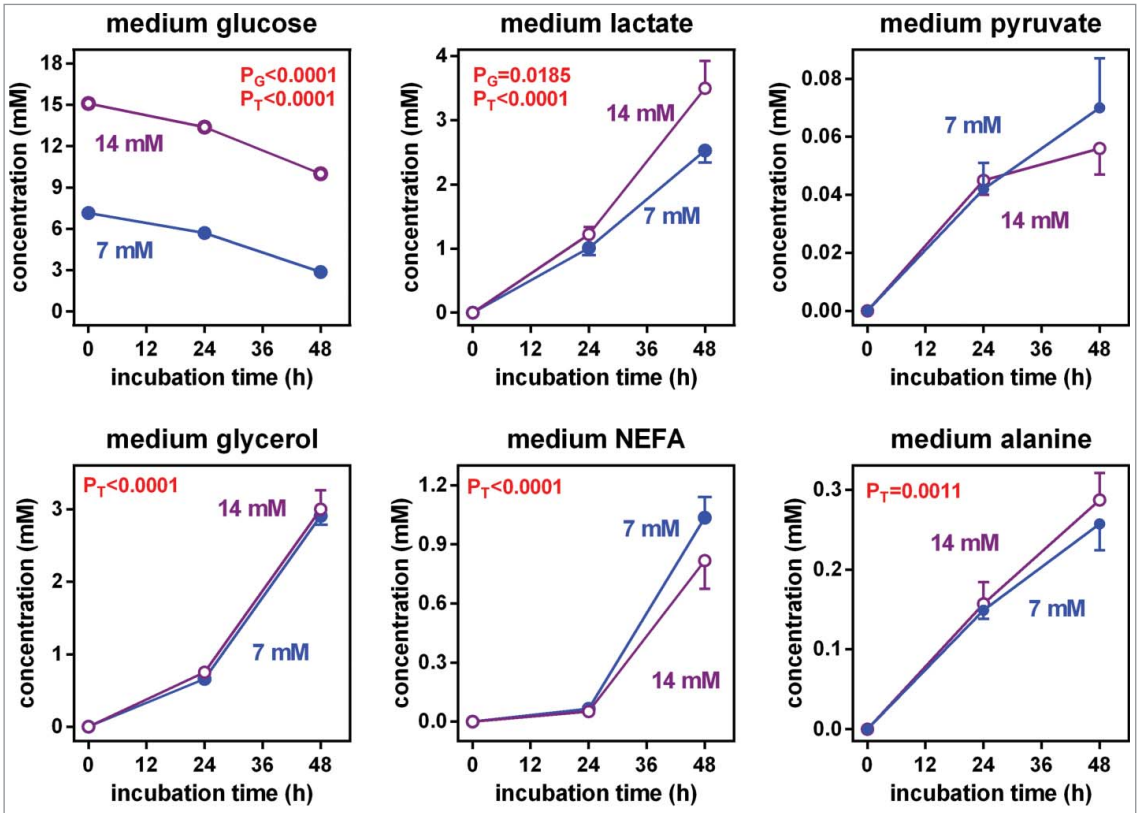
3T3L1 cells can convert large amounts of glucose into lactate through (anaerobic) glycolysis in the presence of abundant oxygen [22]. Similarly, when studied *in vivo,* rat WAT [23], also produces large amounts of lactate in normoxic conditions. Despite sufficient oxygen availability, in WAT, 6C-glucose was also converted to other 3C-metabolites [12,24]. In normal cells, with sufficient proximity to mitochondria, pyruvate is oxidized to acetyl-CoA in a fully aerobic process. The conversion of glucose into 3C fragments, together with the low lipogenic activity from glucose and high recycling rate of lipolytic-freed fatty acids shown by incubated adipocytes [12,24] has been attributed, largely, to the geometry of mature (large) adipocytes. In them, most cell content is essentially restricted to a thin layer of 'live' cytoplasm between the cell membrane and the border of the huge lipid vacuole [25], far from oxidative mitochondria. Thus, in addition to hamper (oxidative) energy production from glucose, lipogenesis is also severely reduced [12,26]. Cell size, thus, has a deep influence on adipocyte function. Smaller, younger, adipocytes, show a comparatively remarkable lipogenic capacity [27] in contrast with the mature cells [28]. Plurivacuolar small cells, such as 3T3L1 fibroblast-derived adipocytes, show both active glycolysis [22] and lipogenesis [29]. Glycerol is released by adipocytes under basal conditions without a parallel efflux of NEFA (i.e. the products of lipolysis) to justify its appearance [24]. In a recent paper, we have analyzed how adipocytes can produce such high amounts of glycerol without destabilizing the cytoplasm NADH homoeostasis and the flow of C through the glycolytic pathway [12]. The glycerol secreted by incubated adipocytes is generated from glucose via glycerol-3P [24]. However, the direct, phosphatase-mediated path, main responsible of the initial production of free glycerol was main substituted, with longer incubations, by glycerol from increased cell TAG turnover [12,24]. Most glycerol was released to the medium via aquaporin 7 [30], but fatty acids were recycled, minimizing the actual NEFA (non-esterified fatty acids) efflux [12].

The present study is part of a wider effort to understand better why mature adipocytes rely so much on 3C metabolite efflux via glycolysis in detriment of lipogenesis. We have already published two papers describing the methodology used here [25,26] and distributed the final study in two papers, the first centered on how glycerol is produced and released [12] and this one, focused on the quantitative utilization of glucose to yield essentially lactate and glycerol, as well as fueling lipogenesis. Our objective was to establish a basis for the quantification of the products of adipocyte lipogenesis, and the relative importance of 3C metabolite production under basal normoxic conditions, i.e. whether the abundance of oxygen may revert the glycolytic habit of largely hypoxic adipocytes. On the present study, we analyzed quantitatively the use of labelled glucose and the metabolic adaptations (gene expressions, essentially) that justify the changes observed. We intended to differentiate the purposeful synthesis of glycerol, even at the expense of accelerated TAG turnover [12,24] from the maintenance, even in the presence of sufficient oxygen of anaerobic glycolysis. We investigated whether this option was a consequence of the need for rapid cytoplasmic ATP availability, as in the Warburg effect of neoplastic cells [31], or if it was a mechanism of WAT to defend itself from excess glucose [2,24] to help lower glycaemia and limit TAG accumulation.

Results

Metabolite efflux

Figure 1 shows the concentrations of metabolites in the medium after the incubation of adipocytes for 24 or 48 h. Glucose levels decreased steadily during incubation, in a way similar for 7 mM and 14 mM. When the data were expressed as percentages of the initial values (data not shown), no differences were found, either, between the two glucose concentrations. Medium lactate



[glucose] mM	incubation time	METABOLITE RATIOS lactate/ pyruvate	alanine/ pyruvate	glycerol/ NEFA
7	24	28±6	4.4±1.5	20±3
	48	49±11	4.1±0.9	3.6±0.4
14	24	31±7	3.6±0.7	30±10
	48	64±16	5.7±1.2	5.6±0.6
"glucose" P		NS	NS	NS
"time" P		0.0233	NS	0.0015

Figure 1. Concentrations of glucose, lactate, pyruvate, glycerol, NEFA and alanine in the incubation medium of adipocytes isolated from rat epididymal WAT.
The data are the mean ± sem of 8 different 2-rat pools (4 for alanine and pyruvate). Each well contained about 4.7 × 10^5 adipocytes, equivalent to those present in 0.25 g of WAT. The cells were incubated 24 or 28 h. Blue dots and lines correspond to initial 7 mM glucose, and mauve represents the 14 mM data. Statistical significance of the differences between groups (two-way ANOVA); only significant values are shown. The P values corresponding to the effect of initial glucose concentration are shown under P_G; the effect of time of incubation by P_T and their interaction by P_i. The inserted Table shows the metabolite concentration ratios for lactate/pyruvate, alanine/pyruvate and glycerol/NEFA, as well as its statistical analysis. These ratios were calculated from the data shown in the graphs; NS = not statistically significant.

increased steadily and almost linearly with time, showing small (albeit statistically significant) differences between the glucose groups. Pyruvate levels were much lower than those of lactate, and no statistically significant differences were observed between the groups, in fact, the presence of pyruvate was practically unchanged between 24 and 48 h. The lactate/pyruvate concentrations ratio was not affected by medium glucose concentration, but increased with incubation time from 30:1 at 24 h to 50–65:1 at 48 h.

Medium glycerol showed a two-phase increase depending on the time of incubation, from 0 to 24 h and a much steeper increase in the 24–48 h period. The glycerol changes were unrelated to glucose concentration. The NEFA efflux showed a similar pattern, but the difference between the first and second day was even more pronounced. The glycerol/NEFA ratio sharply changed from 20–30:1 in the first 24 h to values around 5:1 in the second. Again, time of incubation marked the differences and glucose did not influence the results.

The pattern for alanine levels in the medium followed the general trend of lactate and pyruvate, but with a steady, linear rise up to 48 h, again with no effect of initial glucose concentration. The alanine/pyruvate concentration ratio was fairly uniform for all groups, in the range of 4:1, suggesting a direct

relationship between the concentrations of both compounds.

The rates of glucose uptake and metabolite efflux during incubation are shown in Table 1. The data have been presented in uniform, comparable, units: amol/s and cell. Rates for glucose uptake tended to increase with time (at the limit of statistical significance) but were –again— unaffected by glucose concentration itself. Lactate efflux was high, and closely related to glucose uptake, with a ratio between both parameters maintained at a steady 0.7. Since one glucose molecule may yield two of lactate, we can infer that about 35 % of all glucose input was returned to the medium as lactate, irrespective of glucose concentration or incubation time. The rates of efflux for pyruvate and alanine were lower than those of lactate but also remained fairly uniform with time and glucose concentration.

As expected from the data shown in Fig. 1, both glycerol and NEFA showed marked changes in efflux rates with time, albeit unrelated to initial glucose levels.

Label distribution in the cells and medium

We used the incubation wells in a way similar to a closed system, in which the glucose label added was distributed in the fractions later analyzed and compared. Fig. 2 shows the label found in the cell and medium fractions after 24 h or 48 h of incubation. The data are presented as raw values (Bq) and do not include the label not accounted for. Since the label remaining in glucose was much higher than the small fraction going into 'other' cell fractions (protein, metabolites and, especially glycogen), we presented the data on a log scale to include all fractions in a single graph.

Glucose label decreased during the second day of incubation, and showed differences related to glucose concentration and incubation time. This seems logical, since the amount of label per well was the same irrespective of the glucose present. The label in lactate increased from 24 h to 48 h. That in glycerol tended to decrease, but the differences were not significant because of the wide variability of the data. The same can be said of fatty acids (with smaller errors) which maintained a similar amount of label. The rise in glycerides-glycerol label was considerable, significant for both time and glucose. Changes in the 'others' (glycogen) fraction did not show significant effects of time or glucose; the label present in this fraction was extremely low.

When the data in Fig. 2 were tabulated and adjusted to the actual amount of glucose label we obtained the stacked histograms of Fig. 3, in which the fate of glucose label used is shown. Since the label in 7 mM and 14 mM groups was the same (but there was twice as much glucose in the 14 mM group); to facilitate comparison, the scale for 14 mM has been halved with respect to that of 7 mM. Each group contains two columns, for 24 and 48 h. The considerable similitude in height and distribution of both 7 mM and 14 mM glucose groups attests to the practically nil effect of doubling glucose levels in the medium. Its consumption and utilization showed little effect on its utilization and fate. The only fractions with significant differences between 24 h and 48 h were lactate, glycerides-glycerol and 'unaccounted for' label for both glucose groups. At 48 h, the 7 mM glucose group used slightly more than 46 % of all glucose available in the well, whereas that of 14 mM consumed about 24 % of the glucose available, that is 5.8 to 6.4 μmol glucose, respectively. If the 'unaccounted for' label were discounted, the final amount of glucose consumed would remain practically the same in both groups.

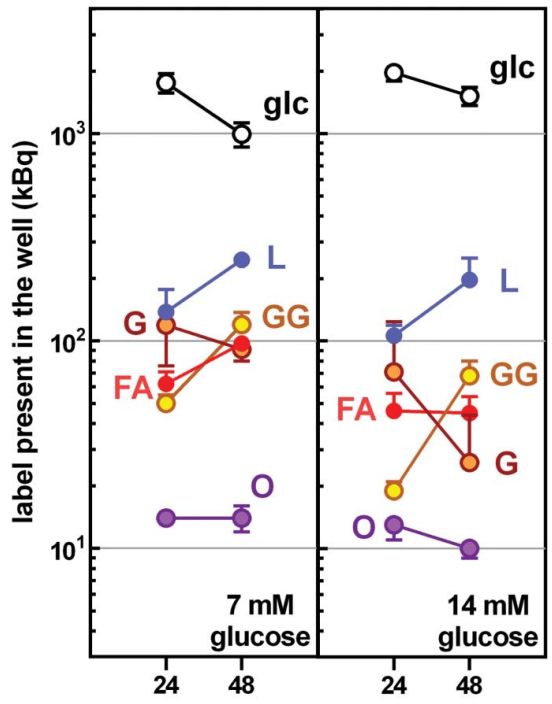
The sum of 3C and glycogen justified about half to 60 % of all glucose metabolized, and that of fatty acids represented only about 10–12% in most groups. The proportion of preserved 3C metabolites (plus glycogen) *vs.* lipogenic products was (at 48 h) in a range close to 6-fold.

Figure 4 shows the specific carbon radioactivities of the five fractions in which direct measurement of cold and labelled compounds were done. The data are shown as percentages of the initial glucose carbon specific activity. In spite of the considerable variability of individual data, the simple differences in scale of the specific

Table 1. Rates of glucose uptake and efflux of, lactate, pyruvate, alanine, glycerol and NEFA from isolated epididymal WAT adipocytes in primary culture.

process and units (attomol/cell·s)	7 mM glucose		14 mM glucose		
	24 h	48 h	24 h	48 h	P values
glucose uptake	52.5 ± 8.1	77.1 ± 7.4	68.5 ± 15.7	89.6 ± 10.8	$P_T = 0.0471$
lactate efflux	38.0 ± 6.2	48.0 ± 7.2	46.7 ± 8.3	69.1 ± 13.1	NS
pyruvate efflux	1.81 ± 0.44	1.64 ± 0.51	1.97 ± 0.17	1.24 ± 0.26	NS
alanine efflux	6.63 ± 0.86	5.81 ± 1.14	7.16 ± 1.64	6.48 ± 1.26	NS
glycerol efflux	23.2 ± 1.86	52.6 ± 4.3	26.5 ± 2.3	53.5 ± 6.2	$P_T<0.0001$
NEFA efflux	2.1 ± 0.4	18.4 ± 1.7	1.7 ± 0.2	13.9 ± 2.1	$P_T<0.0001$

Conventions and conditions of incubation are the same as in Fig. 2 and/or described in the text. The rates have been presented in uniformed units: attomoles per second and cell (comparable to akat/cell).



2-way ANOVA		"time" P	"glucose" P
glc	glucose	<0.0001	<0.0001
L	lactate	0.0003	NS
G	glycerol	NS	NS
GG	glycerides-glycerol	0.0010	0.0171
FA	glycerides-fatty acids	NS	NS
O	other (glycogen)	NS	NS

Figure 2. Well label distribution in absolute values for the analyte compartments of mature epididymal rat adipocytes incubated 24 h or 48 h in a medium with 7 mM or 14 mM glucose. glc = glucose; L = lactate; G = free (medium) glycerol; GG = glycerides-glycerol; FA = glycerides-fatty acids; O = other cell fractions (protein, metabolites and, mainly, glycogen).
The data correspond to the mean ± sem of 4 different 2-rat pools, and are presented in a log scale. The statistical analysis (2-way-ANOVA) results are shown in the embedded Table. NS = not statistically significant.

radioactivity of the fractions, all derived from glucose (the only initial source of label) shows that, as expected, glucose specific activity was maintained. That of lactate, was also maintained (no statistically significant effects of glucose or time were observed) on the same range than glucose.

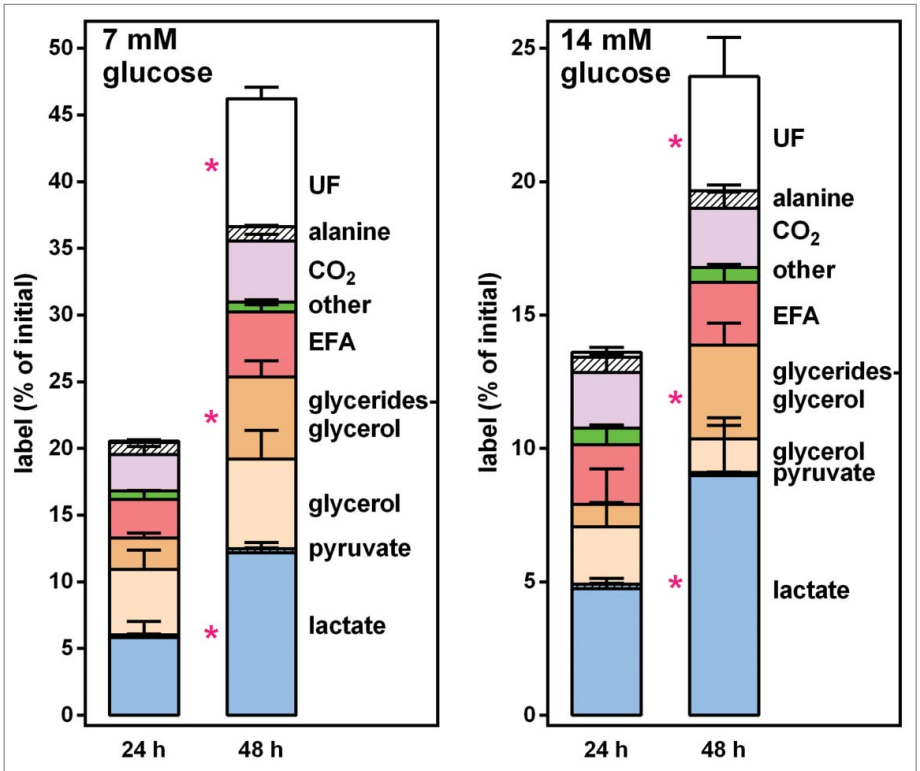
The specific radioactivities of free glycerol in the medium decreased with time. At 48 h, they were only a fraction of the initial glucose values. The data for glycerides-glycerol showed an opposite pattern, from practically zero at 24 h the values increased steadily to about 2 % of the initial glucose specific radioactivity at 48 h, also showing a significant effect of incubation time. The effect of glucose concentration in both glycerol groups was not statistically significant (but was in the limit of significance). The glycerides-glycerol values, however, were much lower at 48 h, at least by one order of magnitude, than those of medium free glycerol. The specific radioactivity of the glycerides-fatty acids was very low, close to four orders of magnitude lower than initial glucose, and did not change either with time or glucose concentration. Despite forming part of the same TAG molecules than glycerides-glycerol, their C specific radioactivity was more than two orders of magnitude lower.

Protein gene expression

The high adipocyte production of lactate may be related to the high number of copies of the gene for main isozyme of lactate dehydrogenase (*Ldha*), which repeated the same pattern of the other glycolytic enzymes analyzed again without any observable effect of medium glucose levels. On the contrary, the other isoenzyme (*Ldhb*) showed no significant effects neither for glucose nor for time of incubation. *Ldhb* showed a lower number of copies (about one order of magnitude) than *Ldha*, but it was, nevertheless, relatively high, in the same range of *Hk* and *Pfkl*. The monocarboxylate transporter gene (*Mct1*), responsible of lactate (and pyruvate) efflux showed the same pattern of change already described for glycolytic enzymes and lactate dehydrogenase.

Figure 5 presents the changes in gene expression of key enzymes and transporters implicated in the glycolytic and lipogenic utilization of glucose by adipocytes. The glucose transporter gene *Glut1*, showed similar number of copies of mRNA per cell for both glucose concentration groups, and increased (practically doubled) its expression from 24 h to 48 h. The pattern for hexokinase *Hk* expression was similar, but the total number of copies was higher. The glycolytic control enzyme P-fructokinase (isozyme genes *Pfkj* and *Pfkm*) showed the same pattern (i.e. no effects of glucose concentration and increased expression with longer incubations), but the *Pfkl* isozyme showed a more powerful increase response and higher levels of gene expression than *Pfkm*. The glyceraldehyde-P dehydrogenase gene (*Phgdh*), despite catalyzing a fully reversible path showed a marked difference in expression induced by glucose availability, with a higher increase at the lower glucose levels.

The enzyme P-enol-pyruvate carboxy-kinase gene (*Pck1*) was, at 24 h of incubation, poorly expressed in adipocytes, but in the next 24 h its expression increased steeply. Again, no effects of glucose were observed. This dramatic increase in expression was paralleled by *Pdk4*, the gene controlling pyruvate dehydrogenase kinase 4, main inhibitor of pyruvate dehydrogenase activity. The effect of glucose was in the limit of significance. This



	p (time)	p [glucose]		p (time)	p [glucose]
lactate	<0.0001	NS	other cell	NS	NS
pyruvate	NS	0.0457	CO_2 minimum	NS	0.0244
glycerol	NS	0.0352	alanine	NS	NS
AG-glycerol	<0.0001	0.0200	unaccounted for	<0.0001	0.0099
AG-fatty acids	NS	0.0244			

Figure 3. Final distribution of the medium glucose label, calculated from the radioactivity incorporated into the different label-containing fractions studied.
The data have been stacked up to show the total glucose label not recovered in the intact glucose fraction at the end of the study, i.e. the glucose taken up by the cells. The values presented correspond to the mean ± sem of 4 different 2-rat pools. The initial values were considered to be 100 % in each well, the degree of variation of this parameter was 1.98 ± 0.19 mBq/cell. The shadowed areas, representing pyruvate and alanine label, were not measured directly, but calculated in relation to the specific radioactivity of the lactate fraction and the concentrations of pyruvate and alanine in these same wells. UF = unaccounted for; CO_2 represents an estimation of (only) the carbon oxidized during the process of lipogenesis. EFA = esterified fatty acids (in the cell lipid droplet). The 2-way-ANOVA statistical analysis data of the results is presented in the embedded Table. Red asterisks represent statistically significant ($P<0.05$) differences between the 24 h and 48 h data; NS = not statistically significant.

strong activation during the second day of incubation can be interpreted as a blockage of the oxidation of pyruvate to acetyl-CoA, thus preventing its incorporation to lipogenesis or the Krebs cycle.

One of the main providers of NADPH in the cytoplasm to sustain lipogenesis is the reductive part of the pentose-phosphate cycle. The expression of its key enzyme, glucose-6P dehydrogenase gene (*G6pdx*) reflected the same pattern described for glycolytic enzymes, with an increase in expression induced by time and no effects of glucose levels. When we analyzed the expression of three key points of control of lipogenesis: ATP: citrate lyase (*Acly*), acetyl-CoA carboxylase (*Acaca*) and fatty acid synthase (*Fas*) genes, no statistically significant effects of time of incubation or glucose initial concentration were observed. The number of copies of *Acaca* was lower than the other lipogenic enzyme genes studied. Nevertheless, the expression of a gene (*Gpam*) coding a critical enzyme for TAG synthesis, glycerol-3P acyl-transferase, was considerably activated by time (albeit not by glucose).

Discussion

Adipocytes (or WAT) take up excess glucose, when confronted with high levels, converting a large proportion of it into 3C metabolites, such as lactate [32], pyruvate,

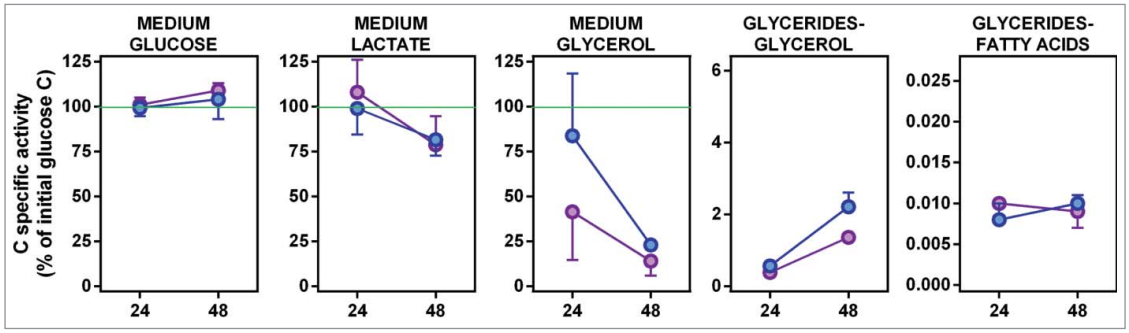


Figure 4. Carbon-specific radioactivity of medium glucose, lactate, glycerol and the cell glycerides components: glycerol and fatty acids.

The data correspond to the mean ± sem of 4 different 2-rat pools. The data are shown as percentages of the initial labelled glucose added to the medium. The medium components (glucose, lactate and glycerol) are drawn at the same scale; cell components scales are grossly extended. The conventions are the same as in Fig. 1. Statistical analysis of the differences between groups (2-way ANOVA): The only significant data correspond to time: P = 0.0003 (glycerides-glycerol). Glycerol fractions showed no significant differences to glucose.

alanine [33] and glycerol [12,13], which may be used as energy substrate elsewhere, or, largely, by the liver in the gluconeogenic [14] and/or lipogenic pathways [34]. But with this action, WAT also disposes of (or defends from) an excess of glucose that may damage its function by dramatically enlarging its TAG stores [22], a process that enhances the limitation of blood flow as defense system against excess energy substrates [11]. By releasing lactate, glycerol, alanine or pyruvate in large proportions (when factoring in the large body WAT mass), blood glucose levels are reduced, thus helping lower inflammation and eventual toxic effects of excess glucose. The incorporation of 3C metabolites goes unhindered by insulin resistance and/or hexose uptake control in most tissues [35], and provides, instead, directly usable energy substrates, which are already partially metabolized if compared with glucose. These small molecules are used for energy (or source of C) by liver , muscle, heart, brain and other organs [36], including the adipose tissues (WAT, BAT) [37].

The results presented here agree with this interpretation, showing, in quantitative terms, that most of the glucose taken up by adipocytes is just returned to the medium as 3C metabolites, essentially lactate and glycerol, thus lowering the circulating glucose availability. However, this process was practically not influenced by glucose in a range going from normal plasma levels, 7 mM to twice this figure (akin to postprandial state or sustained hyperglycemia). It must be noted that the

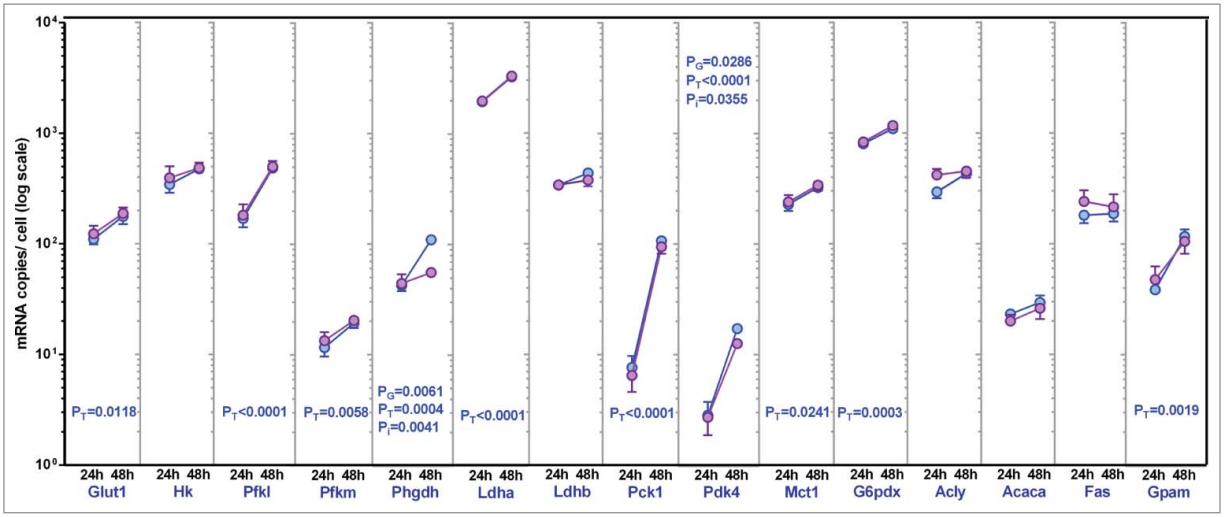


Figure 5. Level of expression, in adipocytes, of the main enzymes related to glucose uptake and glycolysis shown in Fig. 1, and are expressed as the number of copies of the corresponding mRNAs per cell.

The data correspond to the mean ± sem of 4 different 2-rat pools, and are presented in a log scale. Blue: 7 mM glucose, mauve 14 mM glucose. Statistical analysis of the differences between groups was done using a 2-way-ANOVA; only P values for significant differences are shown. The conventions used are the same as in Fig. 1.

hacking of glucose to 3C metabolites proceeded during a 2-day incubation of the cells, in which no external hormones or signals (including those of other WAT cells not firmly attached to adipocytes) were able to affect the processes described and quantified. Thus, we can conclude that isolated adipocytes' conversion of glucose to 3C metabolites (and, to a minor extent, fatty acids) was not elicited by external signals and neither by glucose concentration itself. Consequently, we can assume that this active conversion of 6C glucose to 3C metabolites may be a pre-established innate process, which potentiates glucose break up by defect. One of the most relevant consequences of this response, which our label tracing data proved, is the limited importance of lipogenesis in the disposal of glucose by mature adipocytes.

A critical finding of this study is the unwavering reliance of adipocytes on glucose (probably when and if available) to provide energy (and release lactate) via glycolysis, independently of the medium glucose concentration over a wide range. This may be a consequence of the mass of the cells, which limit access of most of the cytoplasm to more efficient oxidative processes in mitochondria [38], another consequence of the adipocyte geometry. However, this factor deeply affects the tissue function: WAT (at least adipocytes) are practically anaerobic, and can subsist under this condition for a long time; fully in line with the successful utilization of incoming blood flow limitation to decrease excess energy unloading [2]. This way, lipogenesis (an aerobic process) may be reduced by controlling oxygen availability, whilst the cell maintains its glycolytic energy supply. Oxygen levels are already low in WAT under *in vivo* conditions [39], probably because oxygen is needed only for oxidative processes such as lipogenesis. Low oxygen, and limited access to mitochondria may become essential factors for the control of lipogenesis. There is no hypoxia as pathologic sign because adipose tissue oxygen consumption is already low [40]. The widely assumed relationship between supposed WAT hypoxia, often justified by lactate production [41], and inflammation needs to be revised using quantitative terms [23]. Perhaps the low WAT blood flow, which we link to a defense system preventing substrate loading, may help, also limit the conversion of glucose to lipid favoring, instead its return as 3C metabolites.

The data presented showed that the influence of glucose concentration was indeed minimal. The alterations in substrate handling were clearly correlated with gene expression data, and represented two different and well defined successive time groups, as previously observed [22]. The only effect largely unchanged affecting equally both periods was the production of lactate (i.e. the glycolytic pathway and its production of the ATP needed for the cell maintenance) [12,22,24].

Since, during the two consecutive days of incubation, there were no external hormonal or pharmacological stimuli, or environmental changes differently affecting the cells, the changes observed could be elicited only by (internal) factors developed during incubation. In fact, glucose consumption and gene expression increased in the second day with respect to the first.

Glycerogenesis was highly active on the first day, with most of the glycerol-3P generated from the glycolytic pathway finding its way into medium glycerol: here glucose concentration affected the process; 7 mM glucose converted most of glycerol-3P into glycerol, but 14 mM glucose included part of TAG-turnover-derived glycerol [12], lowering its specific radioactivity. However, on day 2, most of the medium glycerol came from TAG turnover [12], with an even lower specific radioactivity. Inversely, glycerides-glycerol specific radioactivity increased because of the huge influx of new glucose-derived glycerol into TAG. Similarly, on day 1, lipogenesis was sufficiently active to produce a measurable proportion of labelled fatty acids, incorporated into the cell TAG vacuole. This was possible because of the sufficient expression of lipogenic enzyme genes and *G6pdx*, providing NADPH. However, on day 2, lipogenesis was stopped. There were no changes in lipogenic marker genes *Acly*, *Acaca*, *Fas*, but the high increase in *Pdk4* expression necessarily blocked the function of pyruvate dehydrogenase [42], preventing the conversion of pyruvate into acetyl-CoA. The lack of substrate resulted in the maintenance (not increase) of label and specific radioactivity (already very low) of fatty acids. We can also deduce, that lipogenesis is not an 'automatic' process to dispose of glucose, since it ceased to be effective after one day, and the pO_2 in the medium was higher than under *in vivo* conditions. Lipogenesis must be activated via external signals for the adipocyte to proceed even under excess glucose available.

The smooth uniformity of lactate production (despite increased expression of *Ldha*) contrasts with the biphasic production of glycerol by adipocytes. First releasing glycerol essentially derived from glycerol-3P and hydrolysis of the phosphate ester [24]; and, largely on the second day, when glycolytic gene expression also increased, by redirecting glycerol-3P to the synthesis of TAG, through the increased expression of *Gpam*. The data on glycerides-glycerol label accumulation and increasing specific radioactivity prove that TAG synthesis was highly increased in the second day over the first. This process was countered by a considerable increase in lipase expression [12] that resulted in accelerated TAG turnover, in which most of fatty acids were recycled and glycerol excreted [12].

From the point of view of metabolic efficiency, the glycolytic use of one molecule of glucose to produce two of

lactate results in a net gain of about 2 ATP/glucose, since the 2 NADH produced at the triose-P dehydrogenase level are used by lactate dehydrogenase to render 2 lactate molecules, maintaining the stoichiometry of cytoplasm reducing power. However, when part of the glucose is used to produce glycerol-3P, only one triose can be oxidized by triose-P dehydrogenase, and the ATP net gain is lost; leaving a deficit of NADH, needed to convert the excess pyruvate into lactate [12].

Apparently, an internal signal, or reaction to the products of glycolysis resulted in deep changes in gene expression that altered the fate of both glycerol-3P and pyruvate families of 3C substrates. The first was used to produce (and release) free glycerol in large proportions via incorporation into TAG and activated TAG turnover. On the other side, pyruvate was prevented to produce acetyl-CoA, its C being returned to the cytosol (assumedly as malate) [12]. This C, probably in the form of oxaloacetate was reincorporated to the glycolytic pathway thanks to a marked rise in P-enol-pyruvate carboxykinase (*Pck1*) expression.

The results presented confirm the metabolic effort of mature adipocytes, in the absence of other external regulatory signals, to continue using glucose as main energy source, using glycolysis, an energy-wasting (but 3C metabolite-preserving) mechanism for maintenance, irrespective of the possible excess of substrate, but converting part of this glucose into fatty acids, stored in their TAG vacuole.

The nature and origin of the process in which the adipocyte metabolic focus shifts from lipogenesis to TAG turnover and glycerol efflux, without affecting lactate production, is unknown, but its effects are extensive, marked and coordinated. The data presented suggest that in spite of the common nature of the 3C substrates produced by the adipocyte, and its role in the possible preservation of glucose recovery, the efflux of lactate and that of glycerol show different patterns and seem to respond to different causes. The uniform rate of lactate production *vs*. deep changes in the glycerol-3P fate, and paths to free glycerol efflux, agree with a different physiological role and regulation for them. The also different timing of gene expression and metabolite production rates or label flow give support to this differential 3C substrate handling by the adipocyte. These processes also share a considerable wasting of energy, and the ultimate reduction of glucose levels. Lactate is cheaper and easier to produce, but it is an acid, whereas glycerol is a non-reactive polyol, easily incorporated into metabolism via widely distributed glycerokinases [43] . Lactate may trigger the rapid release of oxygen by red blood cells (Bohr effect) , and can easily substitute glucose as main energy staple for developing nervous system [44]. Glycerol is essentially the only carbohydrate in the avian egg, and sustains the early life and development of birds [45]. Both 3C compounds can fully substitute glucose for most biochemical functions.

The quantitative estimation of glucose conversion into 3C fragments or fatty acids (x2C), established that adipocytes (and by extension WAT) actively participate in the control of glycemia [12], lowering glucose levels and contributing to limit its pro-inflammatory effect via insulin resistance.This glucose is largely recycled to 3C metabolites, i.e. usable as energy substrate by almost any tissue, as indicated above. The 3C substrates are not subjected to the same strict controls as glucose (insulin), and can be easily reconverted again (if needed) to glucose via hepatic gluconeogenesis.

The glucose arriving at the adipocyte is not converted to fatty acids in significant proportions in the absence of pathological conditions or signaling, at least not by adipocytes themselves, which soon modulate their pro-lipogenic proteome to block this process, as shown here. This does not prevent, however, that the 3C substrates would be used by other organs or tissues, such as the liver, for lipogenesis [46], being then carried to WAT via lipoproteins, and their fatty acids incorporated into the adipocyte TAG via lipoprotein lipase and fatty acid uptake and re-esterification [47]. But, as presented here, this widely accepted irreversible conversion of 3C to 2C units (linked to lipogenesis) is not carried out as main fate of glucose by mature (i.e. not growing) adipocytes.

The metabolic prowess of WAT, a tissue with so small proportion of 'live' cytoplasm [25], does not cease to surprise us with a widespread and powerful participation in the overall control of body energy . Also by the growing number of functional metabolic pathways it contains and 'hides' in between so much fat. Perhaps we should look more beyond this fat and its assumed perils, to discover (probably) that WAT may be a main actor in the fight against the ravages of excess energy intake, using inadequate tools but achieving, nevertheless, remarkable effectiveness.

Methods

Rats, housing, handling and sampling

All animal handling procedures and the experimental setup were in accordance with the animal treatment guidelines set forth by the corresponding European, Spanish and Catalan Authorities. The Committee on Animal Experimentation of the University of Barcelona specifically authorized the procedures used in the present study (procedure DAAM6911).

Male Wistar rats (Harlan Laboratory Models, Sant Feliu de Codines, Spain) were used after a 1-week acclimation period. The rats had free access to food (standard rat chow: Teklad #2014, Harlan) and water at any time, and were kept in two-rat cages with wood shards as bedding material, at 21.5–22.5°C, and 50–60% relative humidity; lights were on from 08:00 to 20:00. When used, the rats were 14-weeks old.

The rats were killed under isoflurane anesthesia, at the beginning of a light cycle, by exsanguination from the exposed aorta. They were rapidly dissected, taking samples of epididymal WAT, used immediately for adipocyte isolation.

Isolation, measurement and incubation of adipocytes

Adipocytes were isolated by incubation with collagenase as described in a previous paper [25], essentially following the Rodbell procedure [48]. Cells were counted, and their (spherical) diameters measured using the ImageJ software (http://imagej.nih.gov/ij/) [49]. The yield with respect to WAT sample mass was estimated in a number of randomly selected samples as previously described [25]. The adipocytes recovered were in the range of 73–75 % of those present in the tissue. Cell incubations were carried out using 12-well plates (#734-2324VWR International BVBA/Sprl., Leuven Belgium) filled with 1.7 ml of DMEM (#11966-DMEM-no glucose; Gibco, ThermoFisher Scientific, Waltham MA USA), supplemented with, 30 mL/L fetal bovine serum (FBS, Gibco). The medium also contained 25 mM hepes (Sigma-Aldrich), 2 mM glutamine (Lonza Biowhittaker, Radnor, PA USA), 1 mM pyruvate (Gibco), 30 mg/mL delipidated bovine serum albumin (Millipore Calbiochem, MA USA), 100 U/mL penicillin and 100 mg/L streptomycin (Sigma-Aldrich). Adenosine (Sigma-Aldrich) 100 nM was also added to help maintain the integrity of the cells.

The incubation medium was supplemented with ^{14}C-(U)-D glucose, (#ARC0122B, American Radiolabeled Chemicals, St Louis MO USA), specific radioactivity 11 GBq/mmol. Final glucose concentrations in the wells were, nominally, 7 or 14 mM. In the labelled samples the amount of label added per well was about 1.8 kBq of ^{14}C-glucose. Specific activities were expressed in Bq/μmol-C i.e. per micromole of the substrate divided by the number of C in the molecule, thus allowing a direct comparison of specific activities between molecules with different number of C atoms [26]. The initial incubation medium containing 7 mM glucose had a specific radioactivity of 141 Bq/μmol glucose (23.5 Bq/μmol-C) that for 14 mM was 71 Bq/μmol glucose (11.8 Bq/μmol-C).

Each well received 400 μL of the cell suspension to a final volume of 2.0 mL, since 0.1 mL was used for the initial measurements. The cells were incubated at 37°C in an incubation chamber, ventilated with air supplemented with CO_2 (5%), which gave a theoretical pO_2 of 20 kPa, in the range of those previously measured under the same conditions [22]. The cells were incubated for 24 or 48 h without any further intervention, as previously described [25]. A 'parallel' series of wells was developed, containing the same adipocytes' suspension and identical medium composition and other conditions than those described above, but in which no label was added. These wells were used for cell gene transcription and medium metabolite analyses.

Cell harvesting and processing of labelled cell components

The incubation of adipocytes was stopped by harvesting the cells after the medium was extracted, mixed, aliquoted and frozen. The procedure for measuring label distribution in the different fractions of cells and media have been previously developed, tested and quantified [26]. Briefly, the cells of wells incubated with labelled glucose were weighed, frozen with liquid nitrogen, transferred to glass tubes and immediately extracted with chilled peroxide-free diethyl ether, since it is non-reactive, and is highly effective for TAG [50]. The aqueous fraction contained small remnants of medium, most cell metabolites and glycogen. The interphase contained most of the cell proteins. This aqueous (and interface) fraction was wholly used to estimate the radioactivity. The organic phase, containing essentially TAG, was dried, weighed, re-dissolved in ethyl ether and saponified with KOH in ethanol in the cold [51]. The ether-insoluble potassium soaps were extracted and counted. The aqueous phase contained all glycerides-glycerol; it was also removed and counted [26]. Soap label was that of TAG fatty acids. Total cell label was estimated from the harvested cells suspension. TAG label was taken as the sum of fatty acids (soaps) and glycerides-glycerol. The cells of the 'parallel' wells were used to extract their RNA for analysis of gene expression.

Sample radioactivity was measured by liquid scintillation (EcoScint #LS275, National Diagnostics, Atlanta, GA USA)), in 6 mL plastic vials (#90010 mini vial. Delta Lab, Rubí, Barcelona, Spain). Using a counter (2100TR TriCarb, Perkin-Elmer, Billarica, MA USA), which partly corrected for quenching, providing the results as dpm (i.e. Bq/60).

Processing of the incubation media: metabolites.

We used both labelled and parallel well media to estimate the levels of glucose, lactate, glycerol and non-esterified fatty

acids (NEFA). Glucose was measured using a glucose oxidase-peroxidase kit (#11504, Biosystems, Barcelona Spain) to which we added 740 nkat/mL mutarrotase (porcine kidney, 136A5000, Calzyme, St Louis, MO USA) [52]. Lactate was measured with kit 1001330 (Spinreact, Sant Esteve d'en Bas, Spain); glycerol was estimated with kit #F6428 (Sigma-Aldrich); NEFA were measured using kit NEFA-HR (Wako Life Sciences, Mountain View, CA USA).

Pyruvate and alanine were measured sequentially [53] in 1.5 mL of tris-HCl buffer 92 mM pH 7.2, containing 100 nM mM NADH (Calbiochem San Diego CA USA) and 1 mM 2-ketoglutarate (Sigma-Aldrich), to which 25 μL of incubation medium (adequately diluted with Krebs-Ringer bicarbonate buffer) were added. In standards, samples were substituted by different concentrations of alanine and pyruvate. The decrease in 5 min of the absorbance at 340 nm was measured after the addition of 20 μL (6 μkat) of lactate dehydrogenase (rabbit muscle #427217 Calbiochem). Pyruvate was estimated from the fall in absorbance (i.e. consumption of NADH) [26]. When the lecture was stabilized, alanine was measured [53] with the addition to the cuvettes of 20 μL (170 nkat) of alanine transaminase (porcine heart #G8255 Sigma-Aldrich), and comparing the rates of absorbance decrease *vs.* time in the samples against alanine standards.

Processing of the incubation media: label distribution

The label-containing samples were used to fraction the label distribution applying a protocol previously described by us [26]. Lactate (including pyruvate) label was determined using centrifuge microcolumns made up with sieve-filter type centrifugation inserts (Ultrafree-MC, Millipore, Bedford, MA USA) containing 250 mg of hydrated, spin dried cationic-form Dowex 1 × 2 ion exchange resin (Serva Electrophoresis GmbH, Heidelberg, Germany) as previously described [26]. The retained lactate was eluted with acid and counted.

The medium free of lactate was used in part to convert all glucose to gluconate by incubation with glucose oxidase (type VII from *Aspergillus niger*, Sigma-Aldrich); as well as catalase (from bovine liver, Sigma-Aldrich). Catalase was added to destroy H_2O_2 and to help maintain O_2 availability. The change of nonionic glucose to gluconate allowed its retention (and acidic elution) using microcolumns as described above for lactate. The label retained was that of the unaltered glucose remaining in the medium after incubation [26,54].

A second aliquot, of the label-containing medium free of lactate, was treated with glycerol kinase (from *Escherichia coli*, #G6278, Sigma-Aldrich) and ATP in a medium adequate for the complete conversion of glycerol to glycerol-3P. The change in ionization was used to remove the glycerol (as glycerol-3P) from the medium, eluting it with acid and thus counting the label retained in the glycerol moiety [26,55].

Combination of 'cold' metabolite measurements and their radioactivity allowed us to calculate the fate of the initial glucose label under all conditions tested and to estimate the specific-C radioactivity for all of them.

Carbon dioxide production along the lipogenic process was estimated by the calculation of NADPH needed to synthesize one (~C18) acyl-CoA molecule (equivalent to one fatty acid residue in TAG) and assuming that 1 mole of CO_2 was produced in the pentose-P pathway for each 2 moles of NADPH generated (explained in more detail in Ho-Palma et al. [26]). The label present in TAG fatty acids allowed us to calculate the amount of glucose oxidized to CO_2 to allow for that synthesis; since the ratio was constant, label in CO_2 was calculated from that found in the cell (soaps fraction) fatty acids.

Gene expression analyses

Total cell RNA was extracted from the harvested cells ('parallel' wells) using the Tripure reagent (Roche Applied Science, Indianapolis IN USA), and were quantified in a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington DE USA). RNA samples were reverse transcribed using the MMLV reverse transcriptase (Promega, Madison, WI USA) system and oligo-dT primers (Gene Link, Westchester, NY USA).

Real-time PCR amplification was carried out using 10 μL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), 4 ng of reverse-transcribed RNA and 150 nmol of primers. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) using a fluorescent threshold manually set to 0.5 for all runs. Table 2 presents a list of the primers used.

A semi-quantitative approach for the estimation of the concentration of specific gene mRNAs per unit of tissue weight was used [56]. *Arbp* was used as the charge control gene. We expressed the data as the number of transcript copies per cell, in order to obtain comparable data between the groups, given the uniformity of the samples in that aspect. The genes analyzed and a list of primers used are presented in Table 2.

It was not feasible to use a meaningful 'zero time' for gene expression data because the cells has been just subjected to the process of extraction, facing different medium and physical conditions. Thus, we had to rely only on the 24 and 48 h data. The remarkable uniformity in behavior of metabolite, label and expression data support the credibility to this approach. The loss of cells was

Table 2. List of primers used in the present study.

gene	protein	direction	sequences	bp
Glut-1	glucose transporter type 1, erythrocyte/ brain	5′ >	GCTCGGGTATCGTCAACACG	97
		> 3′	ATGCCAGCCAGACCAATGAG	
Hk1	hexokinase type 1	5′ >	TGGATGGGACGCTCTACAAA	100
		> 3′	GACAGGAGGAAGGACACGGTA	
Pfkl	phospho-fructokinase, liver, b-type	5′ >	CAGCCACCATCAGCAACAAT	90
		> 3′	TGCGGTCACAACTCTCCATT	
Pfkm	phospho-fructokinase, muscle, a-type	5′ >	CATCCCATTTGTGGTCATTCC	149
		> 3′	TAAACACTCGCCGCTTGGT	
Phgdh	phospho-glycerate dehydrogenase	5′ >	CTGAACGGGAAGACACTGGGAA	138
		> 3′	AACACCAAAGGAGGCAGCGA	
Ldha	L-lactate dehydrogenase a	5′ >	AAAGGCTGGGAGTTCATCCA	96
		> 3′	CGGCGACATTCACACCACT	
Ldhb	L-lactate dehydrogenase b	5′ >	GCGAGAACTGGAAGGAGGTG	145
		> 3′	GGGTGAATCCGAGAGAGGTTT	
Pck1	phospho-enol-pyruvate carboxykinase, cytosolic	5′ >	CGGGTGGAAAGTTGAATGTG	142
		> 3′	AATGGCGTTCGGATTTGTCT	
Pdk4	pyruvate dehydrogenase kinase, isoenzyme 4	5′ >	CTGCTCCAACGCCTGTGAT	142
		> 3′	GCATCTGTCCCATAGCCTGA	
Mct	monocarboxylate transporter	5′ >	CCCAGAGGTTCTCCAGTGCT	133
		> 3′	ACGCCACAAGCCCAGTATGT	
G6pdx	glucose-6-phosphate dehydrogenase X-linked	5′ >	GACTGTGGGCAAGCTCCTCAA	77
		> 3′	GCTAGTGTGGCTATGGGCAGGT	
Acly	ATP: citrate lyase	5′ >	TGTGCTGGGAAGGAGTATGG	137
		> 3′	GCTGCTGGCTCGGTTACAT	
Acaca	acetyl-CoA carboxylase 1	5′ >	AGGAAGATGGTGTCCGCTCTG	145
		> 3′	GGGGAGATGTGCTGGGTCAT	
Fas	fatty acid synthase	5′ >	CCCGTTGGAGGTGTCTTCA	117
		> 3′	AAGGTTCAGGGTGCCATTGT	
Gpam	glycerol-3-phosphate acyl-transferase, mitochondrial	5′ >	GGTGAGGAGCAGCGTGATT	129
		> 3′	GTGGACAAAGATGGCAGCAG	
Arbp	0S acidic ribosomal phospho-protein PO [housekeeping gene]	5′ >	CCTTCTCCTTCGGGCTGAT	122
		> 3′	CACATTGCGGACACCCTCTA	

minimal [25], and there were no changes in the levels of oxygen during the 2-day incubation [22].

Statistical analyses and comparisons between groups (two-way ANOVAs) were done with the Prism 5 program (GraphPad Software, San Diego CA USA).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Part of the expenses have been covered by the University of Barcelona and the researchers themselves. A.C. Ho-Palma and F. Rotondo had pre-doctoral fellowships of the Governments of Peru and Catalonia, respectively. Dr. M.M. Romero contract was supported by the CIBER-OBN Research Web of the Government of Spain.

ORCID

José Antonio Fernández-López http://orcid.org/0000-0002-2856-7223
Xavier Remesar http://orcid.org/0000-0002-9707-7287
Marià Alemany http://orcid.org/0000-0002-9783-8293

References

[1] Pénicaud L, Doré E, Ferré P, et al. Development of obesity in Zucker rats – early insulin resistance in muscles but normal sensitivity in white adipose-tissue. Diabetes. 1987; 1987;36(5):626–631.
[2] Alemany M. The defense of adipose tissue against excess substrate-induced hyperthrophia: Immune system cell infiltration and arrested metabolic activity. J Clin Endocrinol Metab. 2011;96(1):66–68.
[3] Wisse BE. The inflammatory syndrome: The role of adipose tissue cytokines in metabolic disorders linked to obesity. J Am Soc Nephrol. 2004;15(5):2792–2800.
[4] Maneschi E, Morelli A, Filippi S, et al. Testosterone treatment improves metabolic syndrome-induced adipose tissue derangements. J Endocrinol. 2012;215 (3):347–362.
[5] Shao L, Feng BY, Zhang YY, et al. The role of adipose-derived inflammatory cytokines in type 1 diabetes. Adipocyte. 2016;5(3):270–274. doi:10.1080/21623945.2016.1162358
[6] Pénicaud L, Cousin B, Leloup C, et al. The autonomic nervous system, adipose tissue plasticity, and energy balance. Nutrition. 2000;16:903–908.
[7] Soeters MR, Soeters PB. The evolutionary benefit of insulin resistance. Clin Nutr. 2012;31(6):1002–1007.
[8] Alemany M. Metabolic syndrome: A multifaceted disease of affluence. J Endocrinol Metab. 2012;2(4-5):155–165.
[9] di Cesare M, Bentham J, Stevens GA, et al. Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based

measurement studies with 19.2 million participants. Lancet. 2016;387(10026):1377–1396.
[10] Romero MdM, Roy S, Pouillot K, et al. Treatment of rats with a self-selected hyperlipidic diet, increases the lipid content of the main adipose tissue sites in a proportion similar to that of the lipids in the rest of organs and tissues. PLoS One. 2014;9(3):e0090995. doi:10.1371/journal.pone.0090995
[11] Alemany M. Regulation of adipose tissue energy availability through blood flow control in the metabolic syndrome. Free Radical Biol Med. 2012;52(10):2108–2119.
[12] Rotondo F, Ho-Palma AC, Remesar X, et al. Glycerol is synthesized and secreted by adipocytes to dispose of excess glucose, via glucerogenesis and increased acylglycerol turnover. Sci Rep. 2017;7:8983. doi:10.1038/s41598-017-09450-14
[13] Jansson PA, Larsson A, Smith U, et al. Glycerol production in subcutaneous adipose tissue of lean and obese humans. J Clin Invest. 1992;89(5):1610–1617.
[14] Ross BD, Hems R, Krebs HA. The rate of gluconeogenesis from various precursors in the perfused rat liver. Biochem J. 1967;102:942–951.
[15] Katz J, Wals PA. Lipogenesis from lactate in rat adipose tissue. Biochim Biophys Acta. 1974;348:344–356.
[16] Felig P. The glucose-alanine cycle. Metabolism. 1973;22(2):179–207.
[17] Margolis S, Vaughan M. a-Glycerophosphate synthesis and breakdown in homogenates of adipose tissue. J Biol Chem. 1962;237(1):44–48.
[18] Christophe J, Dehaye J, Winand J. Utilization *in vitro* of [U-^{14}C] glucose, [U-^{14}C] fructose, L-[U-^{14}C] Alanine and [^{3}H] water in adipose-tissue fragments from bar harbor obese-hyperglycaemic mice. Biochem Soc Trans. 1974;2:1214–1215.
[19] Guan HP, Li Y, Jensen MV, et al. A futile metabolic cycle activated in adipocytes by antidiabetic agents. Nat Med.200:1122–1128.
[20] Palacín M, Lasunción MA, Herrera E. Utilization of glucose, alanine, lactate, and glycerol as lipogenic substrates by periuterine adipose tissue *in situ* in fed and starved rats. J Lipid Res. 1988;29(1):26–32.
[21] Prior RL, Jacobson JJ. Effects of fasting and refeeding and intravenous glucose infusion on in vitro lipogenesis in bovine adipose tissue. J Nutr. 1979; 1979;109(7):1279–1283.
[22] Sabater D, Arriarán S, Romero MM, et al. Cultured 3T3L1 adipocytes dispose of excess medium glucose as lactate under abundant oxygen availability. Sci Rep. 2014;4:92573. doi:10.1038/srep03663
[23] Arriarán S, Agnelli S, Sabater D, et al. Evidences of basal lactate production in the main white adipose tissue sites of rats. Effects of sex and a cafeteria diet. PLoS One. 2015;10(3):e0119572. doi:10.1371/journal.pone.0119572
[24] Romero MdM, Sabater D, Fernández-López JA, et al. Glycerol production from glucose and fructose by 3T3-L1 cells: A mechanism of adipocyte defense from excess substrate. PLoS One. 2015;10(10):e0139502. doi:10.1371/journal.pone.0139502
[25] Rotondo F, Romero MM, Ho-Palma AC, et al. Quantitative analysis of rat adipose tissue cell recovery, and non-fat cell volume, in primary cell cultures. PeerJ. 2016;4:e2725. doi:10.7717/peerj.2725
[26] Ho-Palma AC, Rotondo F, Romero MdM, et al. A method for the measurement of lactate, glycerol and fatty acids production from ^{14}C-glucose in primary cultures of rat epididymal adipocytes. Anal Methods. 2016;8(44):7873–7885. doi:10.1039/c6ay01244b
[27] Roberts R, Hodson L, Dennis AL, et al. Markers of *de novo* lipogenesis in adipose tissue: associations with small adipocytes and insulin sensitivity in humans. Diabetologia. 2009;52(5):882–890.
[28] Hood RL, Beitz DC, Best DJ, et al. Relationship between adipocyte size and lipid-synthesis in the Zucker rat. Nutr Rep Int. 1983;27(4):791–797.
[29] Student AK, Hsu R, Lane M. Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. J Biol Chem. 1980;255(10):4745–4750.
[30] Maeda N, Funahashi T, Hibuse T, et al. Adaptation to fasting by glycerol transport through aquaporin 7 in adipose tissue. Proc Natl Acad Sci U S A. 2004;101(51):17801–17806.
[31] López-Lázaro M. The Warburg effect: Why and how do cancer cells activate glycolysis in the presence of oxygen? Anticancer Agents Med Chem. 2008;8:305–312.
[32] Crandall DL, Fried SK, Francendese AA, et al. Lactate release from isolated rat adipocytes: Influence of cell size, glucose concentration; insulin and epinephrine. Horm Metab Res. 1983;15:326–329.
[33] Snell K, Duff DA. Alanine release by rat adipose tissue *in vitro*. Biochem Biophys Res Commun. 1977;77(3):925–931.
[34] Geelen MJH, Hindriks GA. Studies on the substrate for hepatic lipogenesis in the rat. FEBS Lett. 1984;175(1):8–12.
[35] Williamson J. Effects of insulin and diet on the metabolism of L (+)-lactate and glucose by the perfused rat heart. Biochem J. 1962;83(2):377–383.
[36] van Hall G. Lactate kinetics in human tissues at rest and during exercise. Acta Physiol. 2010;199:499–508. doi:10.1111/j.1748-1716.2010.02122
[37] Saggerson ED, Mcallister TWJ, Baht HS. Lipogenesis in rat brown adipocytes – effects of insulin and noradrenaline, contributions from glucose and lactate as precursors and comparisons with white adipocytes. Biochem J. 1988;251:701–709.
[38] Fischer B, Schottl T, Schempp C, et al. Inverse relationship between body mass index and mitochondrial oxidative phosphorylation capacity in human subcutaneous adipocytes. Am J Physiol. 2015;309(4):E380–E387. doi:10.1152/ajpendo.00524.2014
[39] Goossens GH, Blaak EE. Adipose tissue oxygen tension: implications for chronic metabolic and inflammatory diseases. Curr Opin Clin Nutr Metab Care. 2012;15(6):539–546.
[40] Ellmerer M, Schaupp L, Sendlhofer G, et al. Lactate metabolism of subcutaneous adipose tissue studied by open flow microperfusion. J Clin Endocrinol Metab. 1998;83:4394–4401.
[41] Sahlin K. Lactate formation and tissue hypoxia. J Appl Physiol. 1989;67(6):2640.
[42] Cadoudal T, Distel E, Durant S, et al. Pyruvate dehydrogenase kinase 4 – Regulation by thiazolidinediones and implication in glyceroneogenesis in adipose tissue. Diabetes. 2008;57(9):2272–2279.

[43] Robinson J, Newsholme E. Some properties of hepatic glycerol kinase and their relation to the control of glycerol utilization. Biochem J. 1969;112(4):455–464.
[44] Medina JM. The role of lactate as an energy substrate for the brain during the early neonatal period. Biol Neonate. 1985;48:237–244.
[45] Roca P, Sáinz F, González M, et al. Energetic components in the unincubated egg fractions of several avian species. Comp Biochem Physiol B. 1982;72(3):439–443.
[46] O'Hea EK, Leveille GA. Significance of adipose tissue and liver as sites of fatty acid synthesis in the pig and the efficiency of utilization of various substrates for lipogenesis. J Nutr. 1969;99(3):338–344.
[47] Weinstock PH, Levak-Frank S, Hudgins LC, et al. Lipoprotein lipase controls fatty acid entry into adipose tissue, but fat mass is preserved by endogenous synthesis in mice deficient in adipose tissue lipoprotein lipase. Proc Natl Acad Sci U S A. 1997;94(19):10261–10266.
[48] Rodbell M. Metabolism of isolated fat cells .I. Effects of hormones on glucose metabolism + lipolysis. J Biol Chem. 1964;239(2):375–380.
[49] Baviskar SN. A quick & automated method for measuring cell area using. Image J Am Biol Teach. 2011;73 (9):554–556.
[50] Ferraz T, Fiuza M, Dos Santos M, et al. Comparison of six methods for the extraction of lipids from serum in terms of effectiveness and protein preservation. J Biochem Biophys Methods. 2004;58(3):187–193.
[51] Florkin M, Duchateau G. Cent manipulations biochimiques simples. 4 ed. Liège: Editions Desoer & Masson; 1968.
[52] Oliva L, Baron C, Fernández-López J-A, et al. Marked increase in rat red blood cell membrane protein glycosylation by one-month treatment with a cafeteria diet. PeerJ. 2015;3:e1101. doi:10.7717/peerj.1101
[53] Grassl M. Determination with GPT and LDH. In: Bergmeyer HU, .editor. Methods of Enzymatic Analysis. Vol. 2. New York: Academic Press; 1974. p. 1682–1685.
[54] Azain M, Kasser T, Baile C. A method for the determination of glucose synthesis in isolated bovine hepatocytes. J Nutr Biochem. 1999;10(4):205–209.
[55] Crespi C, Gastalver G, Palou A, et al. Enzymatic determination of carbon (^{14}C)-labeled glycerol in biological samples. J Biochem Biophys Methods. 1995;30(2):179–183.
[56] Romero MM, Grasa MM, Esteve M, et al. Semiquantitative RT -PCR measurement of gene expression in rat tissues including a correction for varying cell size and number. Nutr Metab. 2007;4:26.

Insulin only partially increases basal ^{14}C-glucose utilization in isolated epididymal rat adipocytes, largely for lipogenesis and glycerogenesis

Ana Cecilia Ho-Palma[1], Pau Toro[1], Floriana Rotondo[1], María del Mar Romero[1,2,3], Marià Alemany[1,2,3], Xavier Remesar[1,2,3] and José Antonio Fernández-López[1,2,3]

[1] Department of Biochemistry and Molecular Biomedicine, Faculty of Biology University of Barcelona, Barcelona, Spain

[2] Institute of Biomedicine, University of Barcelona, Barcelona, Spain

[3] CIBER-OBN Research Web, Barcelona, Spain

Author for correspondence: Dr. José-Antonio Fernández-López; e-mail: josfernandez@ub.edu; telephone number +34 93 4021546; Departament de Bioquímica i Biologia Molecular, Universitat de Barcelona; Av. Diagonal, 645; 08028 Barcelona, Spain.

Abstract

Under normoxic conditions, adipocytes in primary culture were able to convert huge amounts of the medium glucose to lactate and glycerol, essentially fulfilling their energy needs through anaerobic glycolysis. Previously, we postulated that this “wasting” of glucose might help to diminish hyperglycemia. Given the importance of insulin in the metabolism of adipose tissue, we have studied how it affects adipocyte response to varying glucose levels, and whether the known high basal conversion of glucose to 3-carbon fragments is affected by insulin.

Cells were incubated for 24 h in the presence or absence of 175 nM insulin; the medium contained glucose: 3.5, 7 or 14 mM; half of the wells contained ^{14}C-glucose. We analyzed glucose label fate, medium metabolites, and the expression of key genes coding for proteins controlling glucose and lipid metabolism.

Insulin induced a frank anabolic response in adipocytes, increasing both glucose uptake, and the flow of carbon through glycolysis and lipogenesis. The maintained lactate excretion by isolated adipocytes was related to medium glucose levels, which agrees with the purported role of disposing excess (circulating) glucose. The production of glycerol was less dependent on glucose availability. When medium glucose was low, most glycerol came from lipolysis; but when glucose was high, release of glycerol directly via breakup of glycerol-3P was the predominant pathway. Although insulin clearly promotes lipogenesis, it also limited the synthesis of glycerol-3P from glucose and its incorporation into acyl-glycerols, thus preventing unwanted excess triacylglycerol accumulation. We assume that this is a so far not described mechanism of adipose tissue defense to avoid crippling fat accumulation.

Introduction

White adipose tissue (WAT) is a disperse organ [1], distributed in a number of locations in which its basic energy storage activity [2] is complemented by many other physiological functions [3,4,5]. In any case, its main role is to contribute to the defense of energy homoeostasis, helping to control glucose [6], lipid [7] and amino acid [8] metabolism.

Most studies on WAT metabolism have been focused on adipocytes, since they are considered the characteristic and defining cells of WAT. However, adipose tissue structure is far more complex, containing a large number of cell types, not only mature adipocytes, in spite of them occupying most of the space because of their larger size [5]. Between 60% and 80% of the adipose tissue nucleated cells constitute the heterogeneous "stromal vascular fraction", including stem cells, preadipocytes, endothelial cells, and macrophages [9]. Adipose tissue also contains other cell types such as fibroblasts, histiocytes, mast cells, lymphocytes, granulocytes, blood cells and nerve terminals.

An excessive intake of energy may trigger the process that results in chronic insulin resistance and the pro inflammatory state that characterizes metabolic syndrome [10]. In all this succession of events, proinflammatory adipokines, released by adipose tissue, play a significant role, as suggested by the fact that the adipose tissue of obese individuals expresses proinflammatory adipokines in a higher proportion than in those with normal weight [11].

Proinflammatory effects are not limited to increased circulating adipokines released by adipose tissue; excess nutrients such as circulating lipids or glucose may become akin to 'toxic', enhancing oxidative stress and promoting inflammatory responses, whereas food restriction to normal levels reduces both [12].

The development of insulin resistance facilitates the gradual appearance of a proinflammatory state, characteristic of the obese, because of the loss of the important insulin antiinflammatory properties. Insulin inhibits the generation of reactive oxygen species and suppresses a number of proinflammatory transcription factors such as the nuclear factor (NF)-κB, *Egr-1*, and activating protein-1 [13].

We recently found that, under normoxic conditions, adipocytes in primary cultures were able to convert huge amounts of the medium glucose to lactate and glycerol, fulfilling most of their energy needs through anaerobic glycolysis [14,15]. We postulated that this “wasting” of glucose may help diminish hyperglycemia because of the large combined mass of WAT. In *ex vivo* studies we also observed the accumulation of lactate in WAT masses [16], in agreement with the results observed

in cultured cells and the low in vivo WAT oxygen consumption [17,18,19] and high WAT lactate production [19,20] observed in humans.

The 'unnecessary' anaerobic use of glucose by adipocytes (and WAT as a whole), producing large amounts of 3-carbon fragments seems to be intrinsic to the tissue, because the high production of lactate occurred in the absence of external stimuli, and independent of the availability of oxygen. Given the assumed importance of insulin in the metabolism of adipose tissue, and because of its anabolic role, we wanted to discern whether insulin mat indeed favor the accumulation of TAG reserves in the adipocyte, since this way it would hinder the breakup of excess glucose to 3C fragments. The consequence could be the loss of the potential protective action of the tissue modulating hyperglycemia. Consequently, we investigated how insulin intervened on glucose disposal via glycolysis to 3C fragments in contrast to its effect on fatty acid synthesis and increased triacylglycerol storage. , We quantitatively analyzed the fate of labelled glucose, and the metabolic changes induced by insulin on primary cultures of adipocytes. Quantitative analyses of metabolites and key gene expressions allowed us to obtain a wider picture of what is in fact the actual role of insulin in the handling of glucose loads by mature adipocytes.

Materials and Methods

Rats and sampling

All animal handling procedures and the experimental setup were in accordance with the animal handling guidelines of the corresponding European and Catalan Authorities. The Committee on Animal Experimentation of the University of Barcelona specifically authorized the procedures used in the present study.

Healthy adult male Wistar rats (Janvier, Le Genest-Saint Isle, France), weighing 399±64 g were used. The animals were kept in two-rat cages with wood shards as bedding material, at 21-22ºC, and 50-60% relative humidity; lights were on from 08:00 to 20:00. The rats had unrestricted access to water and standard rat chow (#2014, Teklad Diets, Madison, WI USA).

The rats were killed, under isoflurane anesthesia, by exsanguination from the exposed aorta. They were dissected, and samples of epididymal WAT were extracted, and minced with scissors before further processing.

WAT cell isolation and cell incubation procedures

Cells were isolated [21] at 37 ºC for 1 h in a shaking bath using collagenase (LS004196, type I, from Worthington Biomedical, Lakewood NJ USA) in 2.5 volumes of modified Krebs-Henseleit buffer [22]. The cell suspension was filtered through a double layer of nylon hose, transferred to vertical syringes and left standing for 5-6 minutes at room temperature. Adipocytes formed an upper loose cake, floating over a liquid phase that was slowly drained from the syringe; the adipocyte layer was gently re-suspended in fresh buffer (free of collagenase) and the process of mixing and draining was repeated twice, discarding the washing fluids. Aliquots of the adipocyte layer were used for incubation immediately after the final washing. All cell preparations were maintained at room temperature (c. 22ºC), and manipulated within a time as short as possible. Cells were counted, and their spherical (when free) diameters measured using the ImageJ software (http://imagej.nih.gov/ij/).

The complete cell incubation procedure was previously described by us [15,23]. In short: Cell incubations were carried out using 12-well plates (#734-2324VWR International BVBA/Sprl., Leuven Belgium) filled with 1.7 ml of DMEM (#11966-DMEM-no glucose; Gibco, Thermo-Fisher Scientific, Waltham MA USA), supplemented with 30 mL/L fetal bovine serum (FBS, Gibco). The medium also contained 25 mM hepes (Sigma-Aldrich), 2mM glutamine (Lonza Biowhittaker, Radnor, PA USA), 30 mg/mL delipidated bovine serum albumin (Millipore Calbiochem, MA USA) and 100 nM adenosine, 100 U/mL penicillin plus 100 mg/L streptomycin (Sigma-Aldrich). Half of the wells were supplemented with bovine insulin (Sigma-Aldrich): final concentration 175 nM.

The incubation medium was also supplemented with ^{14}C-(U)-D glucose, (#ARC0122B, American Radiolabeled Chemicals, St Louis MO USA; specific radioactivity 11 GBq/mmol). Final glucose concentrations in the wells were, nominally, 3.5, 7 or 14 mM. In the labelled samples the amount of label added per well was about 394 Bq/mmol of glucose.

A ‘parallel’ series of wells was developed, containing the same adipocytes’ suspension and identical medium composition and other conditions than those described above, but in which no label was added. These wells were used for cell gene transcription and medium metabolite analyses.

Each well received 400 µL of the corresponding cell suspension; after initial sampling, the final incubation volume was 2.0 mL. The cells were incubated at 37°C in a chamber ventilated with air supplemented with 5% CO_2, which gave a theoretical pO_2 of 20 kPa [24]. The cells were incubated for 24 h without any further intervention. Then, the wells' contents were transferred with a pipette to small polypropylene tubes, which were left standing for 5 min to pipette out the infranatant and immediately use *in situ* the adipocyte fraction for RNA extraction.

Processing of the incubation media: label distribution

The label-containing samples were used to fraction the label distribution applying a protocol previously described by us [14]. Lactate (including pyruvate) label was determined using centrifuge microcolumns made up with sieve-filter type centrifugation inserts (Ultrafree-MC, Millipore, Bedford, MA USA) containing 250 mg of hydrated, spin dried cationic-form Dowex 1 x 2 ion exchange resin (Sigma-Aldrich) [14]. The retained lactate was eluted with acid and counted.

The medium free of lactate was used in part to convert all glucose to gluconate by incubation with glucose oxidase (type VII from *Aspergillus niger*, Sigma-Aldrich); as well as catalase (from bovine liver, Sigma-Aldrich). Catalase was added to destroy H_2O_2 and to help maintain O_2 availability. The change of nonionic glucose to gluconate allowed its retention (and acidic elution) using microcolumns as described for lactate. The label retained was that of the unaltered glucose remaining in the medium after incubation [14,25].

A second aliquot, of the label-containing medium free of lactate, was treated with glycerol kinase (from *Escherichia coli*, #G6278, Sigma-Aldrich) and ATP for the conversion of glycerol to glycerol-3P. The change in ionization was used to remove the glycerol (as glycerol-3P) from the medium, eluting it with acid and thus counting the label retained in the glycerol moiety [14,26].

Combination of ‘cold’ metabolite measurements and their radioactivity allowed us to calculate the fate of the initial glucose label under all conditions tested and to estimate the specific-C radioactivity for all of them.

Processing of labelled cell components

The procedure for measuring label distribution in the different cell fractions and media have been previously developed, tested and quantified [14]. Briefly, the cells incubated with labelled glucose were weighed, frozen with liquid nitrogen, transferred to glass tubes and immediately extracted with chilled peroxide-free diethyl ether [27]. The aqueous fraction (and interface) was wholly used to estimate the radioactivity. The organic phase, containing essentially TAG, was dried, weighed, re-dissolved in ethyl ether and saponified with KOH in ethanol in the cold. The ether-insoluble potassium soaps were extracted and counted. The aqueous phase, containing all glycerides-glycerol, was also removed and counted [14]. Soap label was that of TAG fatty acids.

Analysis of metabolites in the medium

Medium glucose was measured using a glucose oxidase-peroxidase kit (#11504, Biosystems, Barcelona Spain) to which we added 740 nkat/mL mutarotase (porcine kidney, 136A5000, Calzyme, St Louis, MO USA) [28]. Lactate was measured with kit 1001330 (Spinreact, Sant Esteve d'en Bas, Spain); glycerol was estimated with kit #F6428 (Sigma-Aldrich). NEFA were measured using kit NEFA-HR (Wako Life Sciences, Mountain View, CA USA). Data for medium metabolites was always referred to cell numbers in the well.

Gene expression analysis

Total cell RNA was extracted from all the harvested cells (parallel, non-labelled plates) using the Tripure reagent (Roche Applied Science, Indianapolis IN USA). RNA content was quantified in a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington DE USA). RNA samples were reverse transcribed using oligo-dT primers (Gene Link, Westchester, NY USA) and the MMLV reverse transcriptase (Promega, Madison, WI USA) system.

Real-time PCR amplification was carried out using 10 µL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), 4 ng of reverse-transcribed RNA and 150 nmol of primers. Reactions were run on an ABI PRISM 7900 HT

detection system (Applied Biosystems) using a fluorescent threshold manually set to 0.5 for all runs.

A semi-quantitative approach for the estimation of the concentration of specific gene mRNAs per unit of tissue weight was used [29]. Arbp was the charge control gene [30]. We expressed the data as the number of transcript copies per adipocyte.

The genes analyzed and a list of primers used are presented in Table 1.

Statistical procedures

Statistical analyses and the establishment of significant differences between groups (two-way ANOVAs) were done with the GraphPad Prism 6 program (GraphPad Software, La Jolla, CA USA).

Results

Figure 1 shows the rates of glucose uptake and other metabolite efflux after the incubation of adipocytes for 24 h with 3.5, 7 or 14 mM glucose, and in the presence or absence of insulin. Glucose uptake by adipocytes was slightly affected by initial glucose concentration showing a peak with 7 mM initial glucose. Lactate efflux was also affected by initial glucose concentration, being its efflux greater at 7 and 14 mM glucose; conversely, NEFA, efflux decreased with glucose concentration. Insulin, increased lactate efflux, slightly decreased that of glycerol, and almost abolished NEFA output in adipocytes.

Figure 2 shows the specific carbon radioactivities of the fractions in which direct measurement of cold and labelled compounds were done. The data are shown as percentages of the initial glucose carbon specific activity. As expected, glucose specific activity was maintained independently of insulin or initial glucose concentration; also, that of lactate was maintained on the same range than glucose. In adipocytes, specific radioactivities of free glycerol increased with the amount of glucose in the medium: with elevated concentrations of glucose (14 mM initial glucose) most of the glycerol was obtained from glucose; with lower glucose concentrations, a progressively smaller proportion of glycerol had its origin from glucose.

In adipocytes, the specific activity of glycerides-glycerol was low, and was unaffected by the initial glucose concentration or the presence of insulin. The specific radioactivity of the glycerides-fatty acids was very low, close to one order of magnitude lower than that of glycerol in glycerides, although in this case the presence of insulin increased specific radioactivity.

Figure 3 shows the main metabolites obtained from glucose metabolism. The major products, at least with high glucose concentrations, were lactate and glycerol. In the case of glycerol, the results were different to those obtained in Figure 1, since glycerol production from glucose and its release to the medium increased with the concentration of glucose; however, glycerol (from glucose) incorporated to TAG was constant and independent of both glucose concentration and insulin. On the contrary, insulin stimulated the synthesis of lactate and fatty acids from glucose. Glucose incorporation to glycogen was small and unaffected by insulin.

Figure 4 presents the changes in gene expression of key enzymes and transporters implicated in the glycolytic utilization of glucose by adipocytes. Data were expressed as the number of copies of the corresponding mRNA per cell. Each well contained $6.32 \pm 0.14 \times 10^5$ adipocytes (i.e. 0.34 g of adipose tissue). The glucose transporter gene *Glut1*, showed similar number of copies of its mRNA per cell for all glucose concentration groups, but insulin decreased its expression. The pattern for glucose transporter gene *Glut4* was different, since both insulin and glucose increased its expression; in the case of glucose, the increase only occurred in the presence of insulin. Expression levels of *Glut4* were lower than those of *Glut1*.

The data suggest that insulin increased the activity of pyruvate dehydrogenase, the enzyme responsible of the conversion of pyruvate to acetyl-CoA, by decreasing the expression of its main inhibitor, pyruvate dehydrogenase kinase 4 (*Pdk4*).

The expression of phospho-enolpyruvate carboxykinase (*Pck1*), a main control point for the regulation of gluconeogenesis in liver, was lower in the presence of insulin, but only at 7 mM glucose.

Expressions of hexokinase (*Hk1*), 6-phosphofructokinase liver type (*Pfkl*), lactate dehydrogenase (*Ldha*), pyruvate carboxylase (*Pc*), and malate dehydrogenase (*Mdh1*) were unaffected by glucose concentration and insulin. The monocarboxylate transporter gene (*Mct1*), responsible of lactate (and pyruvate) efflux slightly decreased its expression in the presence of insulin.

The main providers of NADPH in the cytoplasm to sustain lipogenesis are malic enzyme and the reductive part of the pentose-phosphate cycle. There was a tendency to increase the expression of *Me1,* but not *G6pd,* by insulin.

Figure 5 shows the changes in gene expression of key enzymes and transporters implicated in the glycerol and lipid metabolism of adipocytes. When we analyzed the expression of three key points of control of lipogenesis: ATP: citrate lyase (*Acly*), acetyl-CoA carboxylase (*Acaca*) and fatty acid synthase (*Fas*) genes, a similar profile was observed, with higher expressions in the presence of insulin and higher glucose concentrations. This latter effect was only apparent in the presence of both glucose and insulin. Nevertheless, the expression of glycerol-3P acyl-transferase (Gpam), a critical enzyme for TAG synthesis, was inhibited by insulin.

The expression of the main enzymes responsible for endogenous lipolysis (triacylglycerol lipase, *Atgl*, and hormone-sensitive lipase, *Hsl*) was inhibited by insulin. In the case of lipoprotein lipase (*Lpl*) responsible for the degradation of triacylglycerols of exogenous origin, it was not affected by glucose or insulin.

The expression of the enzymes responsible for the synthesis of glycerol 3-phosphate in the adipocyte tended to be inhibited by the presence of insulin. This was the case of the glyceraldehyde-3-phosphate dehydrogenase (*Gdp1*; although the inhibition was not statistical significant, $p = 0.0628$), responsible for the synthesis of glycerol 3-phosphate from dihydroxyacetone-phosphate, and that of glycerokinase (*Gk*) responsible for the phosphorylation of glycerol. The expression of this last enzyme was very low. In the case of the enzymes that use glycerol-3-phosphate as substrate, the expression of phosphoglycolate phosphatase (*Pgp2*), which catalyzes the hydrolysis of glycerol 3-phosphate to glycerol was not affected.

Finally, the expression of aquaporin 7, a transporter playing an important role in glycerol transport, and *Cd36*, that imports fatty acids inside cells, were unaffected by the conditions tested in our study.

Discussion

Under basal conditions, and in absence of insulin, isolated adipocytes, and intact white adipose tissue (WAT) use significant amounts of glucose and secrete high amounts of 3C metabolites as lactate and glycerol. These 3C compounds may be used for hepatic gluconeogenesis [31], lipogenesis [32] or for energy purposes in other tissues.

WAT limits excessive TAG accumulation (excess energy) in the tissue, reducing substrate availability by decreasing blood flow [33]. Additionally, with this high production of lactate and glycerol release (i.e. glucose breakup), WAT defends itself from excess glucose, preventing an inordinate enlargement of its TAG stores [24]. Under insulin-resistant conditions, insulin-stimulated glucose uptake is impaired, and many studies attribute this to a defect in Akt signaling [34]. By releasing 3C metabolites in large proportions, blood glucose levels are reduced, helping lower inflammation and eventual toxic effects of excess glucose. The utilization of 3C metabolites by tissues is unaffected by insulin resistance and circumvents the strict regulation of glycolysis, providing partially metabolized and directly usable energy substrates. The results presented here agree with this interpretation, confirming that most of the glucose taken up by adipocytes is just returned to the medium as 3C metabolites, essentially lactate and glycerol [35], thus helping lower circulating glucose availability.

As expected, insulin elicited an anabolic response in WAT, increasing glucose uptake [36], as well as the flow of carbon through glycolysis and lipogenesis [37]. Figure 6 shows the actions of insulin on these main metabolic pathways in the context of glucose-fatty acid metabolism in the adipocyte.

Glucose uptake was already high, even in the absence of insulin, mainly due to the high expression of *Glut1*, a widely distributed insulin-independent glucose transporter. Insulin increased glucose uptake, despite a lower expression of *Glut1*, compensated by a higher

expression of *Glut4*, which facilitates glucose transport into insulin-sensitive cells. *Glut4* is responsible for the insulin-regulated glucose transport into muscle and adipose cells [38], but a large proportion of glucose enters most body cells by means of Glut1. This is the most widely expressed hexose transporter, which main role is assumed to maintain basal glucose transport in most types of cells [39,40], *Glut1* is regulated through control of gene expression [41,42,43], and only in part via regulative control, which is the main control system for *Glut4* [44].

It has been described that hypoxia increases monosaccharide uptake capacity in adipocytes, contributing to adipose tissue dysregulation in obesity [45]. However, the effects on glucose transporters are the opposite to those induced by insulin: exposure of adipocytes to hypoxia, increase *Glut1* expression, in contrast with no changes in *Glut4* [45].

Although there were no changes in the expression of genes related to the mainstream glycolysis (such as hexokinase and phosphofructokinases), there was a clear increase in the flow of carbon along the glycolytic pathway, as shown by the increased glucose uptake of cultured adipocytes and higher lactate efflux to the medium. The absence of changes in the expression of phosphofructokinases, one of the key regulatory and rate limiting steps of glycolysis, may be explained in part because phosphofructokinase regulation of glycolysis is essentially done through allosteric mechanisms [46].

Lactate and glycerol production (from glucose) help sustain the basic energy needs of the cell through a fully anaerobic (albeit facultative, since it occurs under normoxic conditions) process [47]. The sheer size of adipocytes, and thin cytoplasm layer around its huge lipid vacuole, hamper the intracellular circulation of substrates, thus limiting and compartmentalizing most metabolic activities. In adipose tissue, glucose can be taken up easily by adipocytes from the circulation, and the glucose converted anaerobically to lactate, pyruvate or glycerol, covering the minimal needs of ATP. But the production of acetyl-CoA requires access to mitochondria, few and sparsely distributed in large adipocytes [48]. Additionally, glucose utilization helps lower glycaemia, breaking up 6C to 3C molecules. This way, a portion of circulating glucose was substituted in large proportions by less-regulated 3C substrates, used elsewhere for energy, but also for splanchnic lipogenesis or gluconeogenesis. Lactate secretion by WAT may be considered, thus, primarily a normal consequence of the need to use glycolytic (anaerobic) ATP and to eliminate excess circulating glucose. Consequently it is not a specific indicator of hypoxia, despite the generalized association of lactate to hypoxia [49]. This idea is reinforced by the fact that the high production of lactate (and that of glycerol) from glucose only occurs in the presence of high concentrations of glucose (in our case, 7 and 14 mM).

On the other hand, the global production of glycerol was preserved and sustained, remaining practically constant regardless of the medium initial glucose concentration. This maintained production of glycerol by adipocytes, in amounts similar, or even greater, than those of lactate, together with the different origin of glycerol according to the availability of glucose in the medium, suggest that glycerol release represents a constitutive process, rather independent of external factors. Insulin only slightly decreased glycerol release to the medium, probably a consequence of the inhibition of lipolysis by insulin.Glycerol-3P, derived from glucose at the level of triose-P, is hydrolyzed to glycerol by a glycerophosphatase, as previously postulated by us [50]. This phosphatase has been recently described in liver, and also is present in WAT [51]. The expression of this enzyme (*Pgp2*) was unaffected by both, glucose and insulin, and was neither correlated with the lower efflux of glycerol observed in insulin treated cells. The removal of excess glycerol-3-P under heavy glycolytic pressure (high glucose levels, insulin activation of *Glut4*) may help lower it cell pool, thus helping maintain a lower glycerol esterification with acyl-CoA and thus downregulate TAG synthesis.

The equilibrium between these pathways: a) incorporation to the acyl-glycerol pool or b) direct generation of glycerol from glycerol-3P seems to be a critical point in the regulation of TAG synthesis, regulated by insulin, which lowers the expression of glycerol-P acyl-transferase despite a marked increase in gene expressions along the lipogenic pathway. Glycerol-3-phosphate acyltransferase is the rate-limiting enzyme in the glycerolipid synthesis pathway [52]. Thus, this critically different regulation of glycerol-P acyl-transferase expression, with respect to the other lipogenic enzymes, can be tentatively explained because the induction of glycerol-P acyl-transferase mRNA by insulin is mediated through the expression of SREBP-1, independently of ChREBP [53,54]. Insulin increases SREBP-1 expression both in liver and adipose tissue, but only increases the post-translational activation of SREBP-1c in liver, and decreasing it in adipose tissue [55]; consequently inhibiting the expression of adipose glycerol-P acyl-transferase.

The production of glycerol is more complex than that of lactate. Although its contribution to 3C fragment production from glucose and lowering glycaemia may also be accomplished by lactate, glycerol synthesis is preferred nevertheless. These data agree with our previous findings indicating that adipocytes' glycerol production is a definite objective in addition to being a 3C substrate [15].

The greater incorporation of the glucose carbons to fatty acids of the TAG in the presence of insulin, but without changes in the incorporation of glycerol to the TAG, suggests that insulin induces a higher turnover of TAG. The activation of TAG turnover [15], when glycerogenic flow decreases, is contrary to the assumed focus of insulin-stimulated adipocytes, where lipogenesis and inhibition of lipolysis are trends clearly established through gene expression patterns.

In conclusion, under conditions of high availability of glucose and energy WAT (at least the adipocytes) defend themselves to prevent the pathologic accumulation of excess energy. Part of the panoply of adipose tissue defense systems to prevent this damage, include: a) lower blood flow through the tissue, to decrease the arrival of nutrients [33]; b) the adipocyte massively metabolizes excess glucose to fragments of three carbons that can be used for energy elsewhere, but also helping decrease hyperglycemia. c) WAT, especially under conditions of obesity is able to inactivate large amounts of insulin [56], thus limiting its lipogenic effect; d) last, but not least, as we have shown here, insulin priority rests on the derivation of excess C to the secretion of glycerol rather than on a higher incorporation of glycerol-3P to the synthesis of TAG. In all cases, the measures are short-term, and may have only a limited success, since, anyway, the presence of insulin, induces a higher incorporation of glucose carbon to the adipocyte TAG pool.

References

1. Cinti, S. The adipose organ. in *Prostaglandins Leukotrienes and Essential Fatty Acids* **73,** 9–15 (2005).
2. Romero, M. D. M. *et al.* Treatment of rats with a self-selected hyperlipidic diet, increases the lipid content of the main adipose tissue sites in a proportion similar to that of the lipids in the rest of organs and tissues. *PLoS One* **9,** (2014).
3. Trayhurn, P. Endocrine and signalling role of adipose tissue: New perspectives on fat. in *Acta Physiologica Scandinavica* **184,** 285–293 (2005).
4. Hauner, H. The new concept of adipose tissue function. *Physiology and Behavior* **83,** 653–658 (2004).
5. Alemany, M. & Fernández-López, J. A. Adipose tissue: Something more than just adipocytes. *Curr. Nutr. Food Sci.* **2,** (2006).
6. Rosen, E. D. & Spiegelman, B. M. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* **444,** 847–853 (2006).
7. Arner, P. *et al.* Dynamics of human adipose lipid turnover in health and metabolic disease. *Nature* **478,** 110–113 (2011).
8. Arriarán, S., Agnelli, S., Remesar, X., Fernández-López, J. A. & Alemany, M. Effects of sex and site on amino acid metabolism enzyme gene expression and activity in rat white adipose tissue. *PeerJ* **2015,** (2015).
9. Tchoukalova, Y. D., Sarr, M. G. & Jensen, M. D. Measuring committed preadipocytes in human adipose tissue from severely obese patients by using adipocyte fatty acid binding protein. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **287,** R1132-40 (2004).
10. Johnson, A. R., Justin Milner, J. & Makowski, L. The inflammation highway: Metabolism accelerates inflammatory traffic in obesity. *Immunol. Rev.* **249,** 218–238 (2012).
11. Makki, K., Froguel, P. & Wolowczuk, I. Adipose Tissue in Obesity-Related Inflammation and Insulin Resistance: Cells, Cytokines, and Chemokines. *ISRN Inflamm.* **2013,** 1–12 (2013).

12. Dandona, P., Aljada, A., Chaudhuri, A., Mohanty, P. & Garg, R. Metabolic syndrome: A comprehensive perspective based on interactions between obesity, diabetes, and inflammation. *Circulation* **111,** 1448–1454 (2005).

13. Dandona, P., Chaudhuri, A., Ghanim, H. & Mohanty, P. Insulin as an Anti-Inflammatory and Antiatherogenic Modulator. *Journal of the American College of Cardiology* **53,** (2009).

14. Ho-Palma, A. C. *et al.* A method for the measurement of lactate, glycerol and fatty acid production from^{14}C-glucose in primary cultures of rat epididymal adipocytes. *Anal. Methods* **8,** (2016).

15. Rotondo, F. *et al.* Glycerol is synthesized and secreted by adipocytes to dispose of excess glucose, via glycerogenesis and increased acyl-glycerol turnover. *Sci. Rep.* **7,** (2017).

16. Arriarán, S. *et al.* Evidences of Basal lactate production in the main white adipose tissue sites of rats. effects of sex and a cafeteria diet. *PLoS One* **10,** (2015).

17. Hodson, L. Adipose tissue oxygenation: Effects on metabolic function. *Adipocyte* **3,** 75–80 (2014).

18. Goossens, G. H. *et al.* Increased adipose tissue oxygen tension in obese compared with lean men is accompanied by insulin resistance, impaired adipose tissue capillarization, and inflammation. *Circulation* **124,** 67–76 (2011).

19. Hodson, L., Humphreys, S. M., Karpe, F. & Frayn, K. N. Metabolic signatures of human adipose tissue hypoxia in obesity. *Diabetes* **62,** 1417–1425 (2013).

20. DiGirolamo, M., Newby, F. D. & Lovejoy, J. Lactate production in adipose tissue: a regulated function with extra-adipose implications. *FASEB J.* **6,** 2405–2412 (1992).

21. Rodbell, M. Metabolism of Isolated Fat Cells: I. EFFECTS OF HORMONES ON GLUCOSE METABOLISM AND LIPOLYSIS. *J. Biol. Chem.* **239,** 375–380 (1964).

22. Krebs, H. A. & Henseleit, K. Untersuchungen über die Harnstoffbildung im Tierkörper. *Klin. Wochenschr.* **11,** 757–759 (1932).

23. Rotondo, F. *et al.* Quantitative analysis of rat adipose tissue cell recovery, and non-fat cell volume, in primary cell cultures. *PeerJ* **2016,** (2016).

24. Sabater, D. *et al.* Cultured 3T3L1 adipocytes dispose of excess medium glucose as lactate under abundant oxygen availability. *Sci. Rep.* **4,** (2014).

25. Azain, M. J., Kasser, T. R. & Baile, C. A. A method for the determination of glucose synthesis in isolated bovine hepatocytes. *J Nutr Biochem* **10,** 205–209 (1999).

26. Crespi, C., Gastalver, G., Palou, A. & Roca, P. Enzymatic determination of carbon (14C)-labeled glycerol in biological samples. *J. Biochem. Biophys. Methods* **30,** 179–183 (1995).

27. Ferraz, T. P. L., Fiúza, M. C., Dos Santos, M. L. A., Pontes De Carvalho, L. & Soares, N. M. Comparison of six methods for the extraction of lipids from serum in terms of effectiveness and protein preservation. *J. Biochem. Biophys. Methods* **58,** 187–193 (2004).

28. Oliva, L., Baron, C., Fernández-López, J.-A., Remesar, X. & Alemany, M. Marked increase in rat red blood cell membrane protein glycosylation by one-month treatment with a cafeteria diet. *PeerJ* **2015,** (2015).

29. Romero, M. D. M., Grasa, M. D. M., Esteve, M., Fernández-López, J. A. & Alemany, M. Semiquantitative RT-PCR measurement of gene expression in rat tissues including a correction for varying cell size and number. *Nutr. Metab.* **4,** (2007).

30. Bamias, G. *et al.* Comparative Study of Candidate Housekeeping Genes for Quantification of Target Gene Messenger RNA Expression by Real-Time PCR in Patients with Inflammatory Bowel Disease. *Inflamm. Bowel Dis.* **19,** 2840–2847 (2013).

31. Ross, B. D., Hems, R. & Krebs, H. A. The rate of gluconeogenesis from various precursors in the perfused rat liver. *Biochem J* **102,** 942–51 (1967).

32. Katz, J. & Wals, P. A. Lipogenesis from lactate in rat adipose tissue. *Biochim. Biophys. Acta (BBA)/Lipids Lipid Metab.* **348,** 344–356 (1974).

33. Alemany, M. Regulation of adipose tissue energy availability through blood flow control in the metabolic syndrome. *Free Radic Biol Med* **52,** 2108–2119 (2012).

34. Tan, S. X. *et al.* Selective insulin resistance in adipocytes. *J. Biol. Chem.* **290,** 11337–11348 (2015).

35. Jansson, P. A., Larsson, A., Smith, U. & Lonnroth, P. Glycerol production in subcutaneous adipose tissue in lean and obese humans. *J Clin Invest* **89,** 1610–1617 (1992).

36. Watson, R. T., Kanzaki, M. & Pessin, J. E. Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes. *Endocrine Reviews* **25,** 177–204 (2004).

37. Saltiel, A. R. & Kahn, C. R. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414,** 799–806 (2001).

38. Huang, S. & Czech, M. P. The GLUT4 Glucose Transporter. *Cell Metabolism* **5,** 237–252 (2007).

39. Manolescu, A. R., Witkowska, K., Kinnaird, A., Cessford, T. & Cheeseman, C. Facilitated Hexose Transporters: New Perspectives on Form and Function. *Physiology* **22,** 234–240 (2007).

40. Kaestner, K. H. *et al.* Sequence, tissue distribution, and differential expression of mRNA for a putative insulin-responsive glucose transporter in mouse 3T3-L1 adipocytes. *Proc. Natl. Acad. Sci. U. S. A.* **86,** 3150–4 (1989).

41. Behrooz, A. & Ismail-Beigi, F. Dual control of glut1 glucose transporter gene expression by hypoxia and by inhibition of oxidative phosphorylation. *J Biol Chem* **272,** 5555–5562 (1997).

42. Fandos, C. *et al.* GLUT1 glucose transporter gene transcription is repressed by Sp3. Evidence for a regulatory role of Sp3 during myogenesis. *J. Mol. Biol.* **294,** 103–119 (1999).

43. Hwang, D.-Y. & Ismail-Beigi, F. Control of Glut1 promoter activity under basal conditions and in response to hyperosmolarity: role of Sp1. *Am. J. Physiol. Cell Physiol.* **290,** C337-44 (2006).

44. Leto, D. & Saltiel, A. R. Regulation of glucose transport by insulin: Traffic control of GLUT4. *Nature Reviews Molecular Cell Biology* **13,** 383–396 (2012).

45. Wood, I. S., Wang, B., Lorente-Cebrián, S. & Trayhurn, P. Hypoxia increases expression of selective facilitative glucose transporters (GLUT) and 2-deoxy-d-glucose uptake in human adipocytes. *Biochem. Biophys. Res. Commun.* **361,** 468–473 (2007).

46. Mansour, T. E. Phosphofructokinase. *Curr. Top. Cell. Regul.* **5,** 1–46 (1972).

47. Sabater, D. *et al.* Cultured 3T3L1 adipocytes dispose of excess medium glucose as lactate under abundant oxygen availability. *Sci. Rep.* **4,** (2014).

48. Zhang, Y., Zeng, X. & Jin, S. Autophagy in adipose tissue biology. *Pharmacological Research* **66,** 505–512 (2012).

49. Trayhurn, P. Hypoxia and adipose tissue function and dysfunction in obesity. *Physiol. Rev.* **93,** 1–21 (2013).

50. Del Mar Romero, M., Sabater, D., Fernández-López, J. A., Remesar, X. & Alemany, M. Glycerol production from glucose and fructose by 3T3-L1 Cells: A mechanism of Adipocyte defense from excess substrate. *PLoS One* **10,** (2015).

51. Mugabo, Y. *et al.* Identification of a mammalian glycerol-3-phosphate phosphatase: Role in metabolism and signaling in pancreatic β-cells and hepatocytes. *Proc. Natl. Acad. Sci.* **113,** E430–E439 (2016).

52. Yu, J. *et al.* Update on glycerol-3-phosphate acyltransferases: The roles in the development of insulin resistance. *Nutr. Diabetes* **8,** (2018).

53. Cha, J. Y. & Repa, J. J. The Liver X Receptor (LXR) and hepatic lipogenesis: The carbohydrate-response element-binding protein is a target gene of LXR. *J. Biol. Chem.* **282,** 743–751 (2007).

54. Takeuchi, K. & Reue, K. Biochemistry, physiology, and genetics of GPAT, AGPAT, and lipin enzymes in triglyceride synthesis. *AJP Endocrinol. Metab.* **296,** E1195–E1209 (2009).

55. Boden, G. *et al.* Comparison of in vivo effects of insulin on SREBP-1c activation and INSIG-1/2 in rat liver and human and rat adipose tissue. *Obesity (Silver Spring).* **21,** 1208–14 (2013).

56. Rafecas, I. *et al.* Insulin degradation by adipose tissue is increased in human obesity. *J. Clin. Endocrinol. Metab.* **80,** (1995).

	Protein	5' primer	3' primer	bp
Glut1	glucose transporter type 1	GCTCGGGTATCGTCAACACG	ATGCCAGCCAGACCAATGAG	97
Glut4	glucose transporter type 4	CTTGATGACGGTGGCTCTGC	CACAATGAACCAGGGGATGG	127
Hk1	hexokinase 1	TGGATGGGACGCTCTACAAA	GACAGGAGGAAGGACACGGTA	100
G6pd	glucose-6-phosphate dehydrogenase	GACTGTGGGCAAGCTCCTCAA	GCTAGTGTGGCTATGGGCAGGT	77
PFkL	phospho-fructokinase, liver, b-type	CAGCCACCATCAGCAACAAT	TGCGGTCACAACTCTCCATT	90
Pdk4	pyruvate dehydrogenase kinase 4	CTGCTCCAACGCCTGTGAT	GCATCTGTCCCATAGCCTGA	142
Ldha	L-lactate dehydrogenase a	AAAGGCTGGGAGTTCATCCA	CGGCGACATTCACACCACT	96
Mct1	monocarboxylate Transporter 1	CCCAGAGGTTCTCCAGTGCT	ACGCCACAAGCCCAGTATGT	133
Pc	pyruvate carboxylase	GCCAGAGGCAGGTGTTCTTTG	TTTGGCCCTTCACATCCTTCA	120
Mdh1	malate dehydrogenase 1	GCTGGCTCAAGGGAGAGTTC	TCTCATGTGGTCCGAGATGG	116
Me1	$NADP^+$-dependent malic enzyme	GGAGTTGCTCTTGGGGTAGTGG	CGGATGGTGTTCAAAGGAGGA	143
Pck1	phosphoenolpyruvate carboxykinase 1	CGGGTGGAAAGTTGAATGTG	AATGGCGTTCGGATTTGTCT	142
Acly	ATP citrate lyase	TGTGCTGGGAAGGAGTATGG	GCTGCTGGCTCGGTTACAT	137
Acaca	acetyl-coA carboxylase alpha	AGGAAGATGGTGTCCGCTCTG	GGGGAGATGTGCTGGGTCAT	145
Fasn	Fatty acid synthase	CCCGTTGGAGGTGTCTTCA	AAGGTTCAGGGTGCCATTGT	117
Gpam	glycerol-3P acyl-transferase	GGTGAGGAGCAGCGTGATT	GTGGACAAAGATGGCAGCAG	129
Cd36	platelet glycoprotein 4 [fatty acid transporter]	TGGTCCCAGTCTCATTTAGCC	TTGGATGTGGAACCCATAACTG	154
Lpl	lipoprotein lipase	TGGCGTGGCAGGAAGTCT	CCGCATCATCAGGAGAAAGG	116
Atgl	triacylglycerol lipase (adipose tissue)	CACCAACACCAGCATCCAAT	CGAAGTCCATCTCGGTAGCC	120
Hsl	hormone-sensitive lipase	TCCTCTGCTTCTCCCTCTCG	ATGGTCCTCCGTCTCTGTCC	108
Gpd1	glycerol-3P dehydrogenase (NAD^+)	CTGGAGAAAGAGATGCTGAACG	GCGGTGAACAAGGGAAACTT	113
Gk	glycerol kinase	ACTTGGCAGAGACAAACCTGTG	ACCAGCGGATTACAGCACCA	74
Pgp2	Phosphoglycolate phosphatase [glycerophosphatase]	CCTGGACACAGACATCCTCCT	TTCCTGATTGCTCTTCACATCC	100
Aqp7	aquaporin 7	ACAGGTCCCAAATCCACTGC	CCGTGATGGCGAAGATACAC	127
Arbp	0 S acidic ribosomal phosphoprotein PO [housekeeping gene]	CCTTCTCCTTCGGGCTGAT	CACATTGCGGACACCCTCTA	122

Table 1. Primers used for the analysis of gene expression.

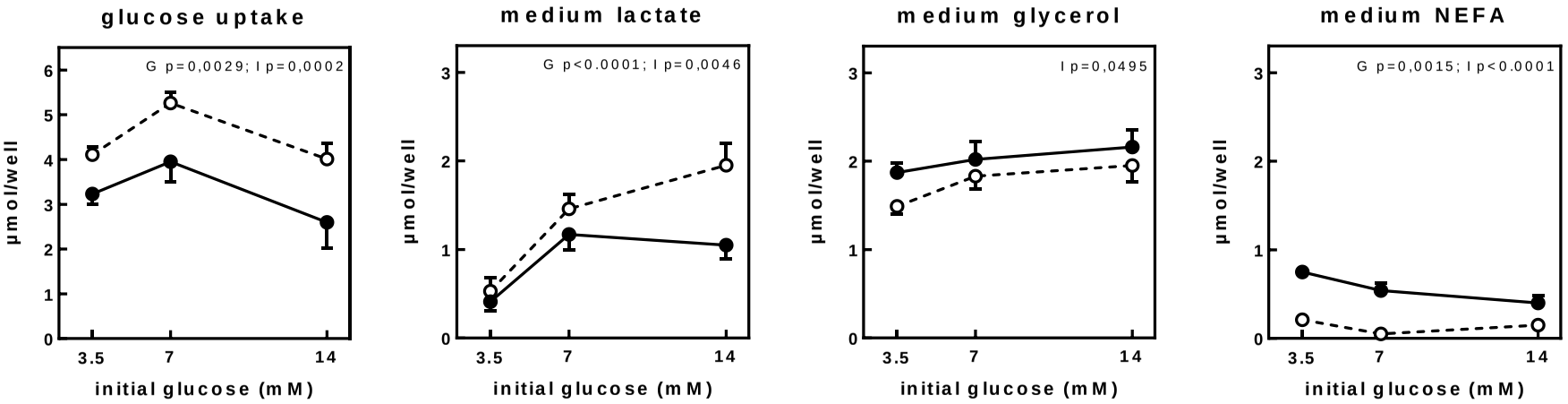


Figure 1. Effect of insulin on the rates of glucose uptake and other metabolite efflux after the incubation of adipocytes for 24 h with 3.5, 7 or 14 mM glucose.

The data are the mean ± sem of 12 different animals. Values are expressed as µmol per well of glucose taken, or lactate, glycerol and NEFA released to the medium after 24 h of incubation. Black circles and continuous line: adipocytes incubated in absence of insulin; white circles and discontinuous line: adipocytes incubated in presence of insulin. Statistical significance of the differences between groups (2-way ANOVA): G correspond to the differences with respect to initial glucose, and I correspond to the differences with respect to the presence or not of insulin. Not significant values (P > 0.05) were not represented.

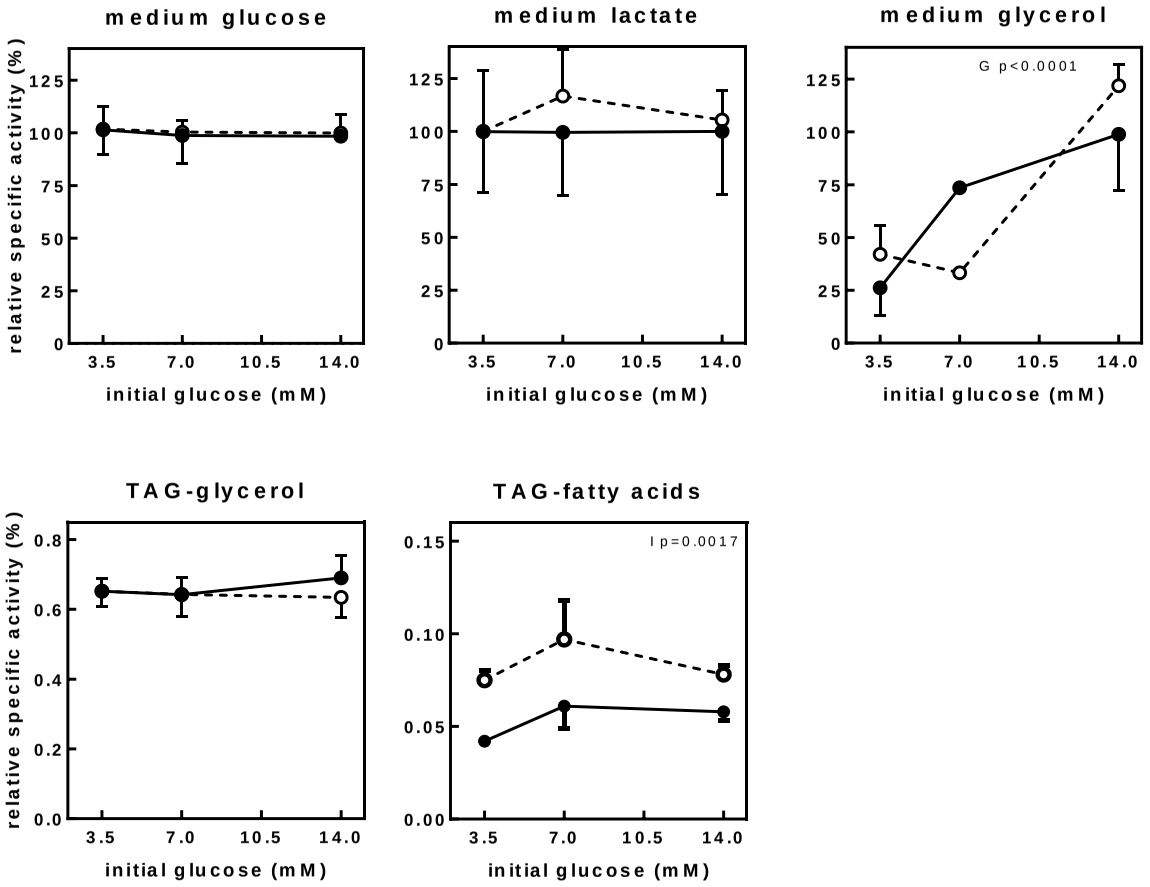


Figure 2. Insulin effect in the carbon specific radioactivity of the main label fractions obtained from ^{14}C-glucose after incubation of epididymal adipocytes in a primary culture.

The data are presented as mean ± sem of 6 different animals. C-specific radioactivity correspond to the quotient of label found in the fraction divided by the molar concentration and the number of carbons the compound contains. In this case, all data have been referred to initial glucose C-specific radioactivity, to which a value of 100 was given. Black circles and continuous line: adipocytes incubated in absence of insulin; white circles and discontinuous line: adipocytes incubated in presence of insulin. The statistical significance data and conventions are the same as in Figure 1.

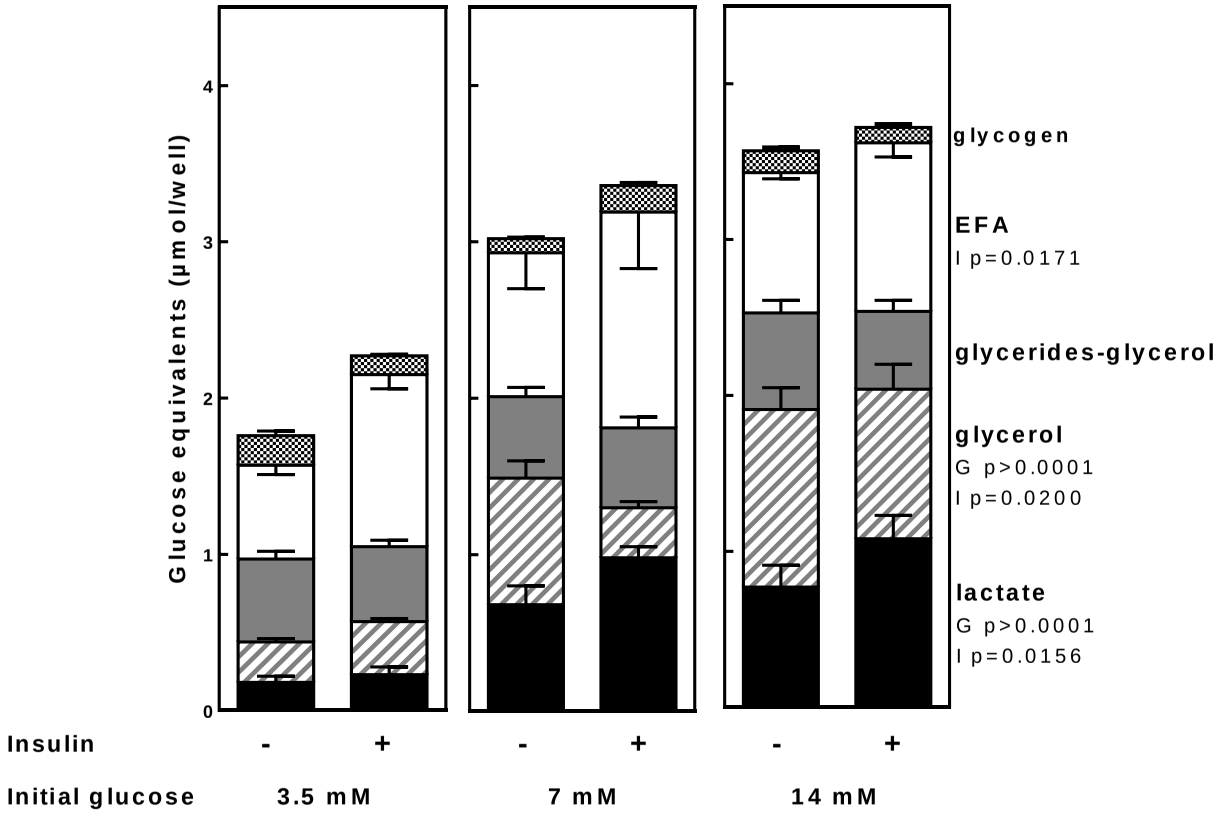


Figure 3. Final distribution of the medium glucose label calculated from the radioactivity incorporated into the different label-containing fractions studied.

The data (calculated as glucose equivalents) are presented as mean ± sem of 6 different animals. EFA = esterified fatty acids (in the cell lipid droplet). The statistical significance data are the same as in Figure 1.

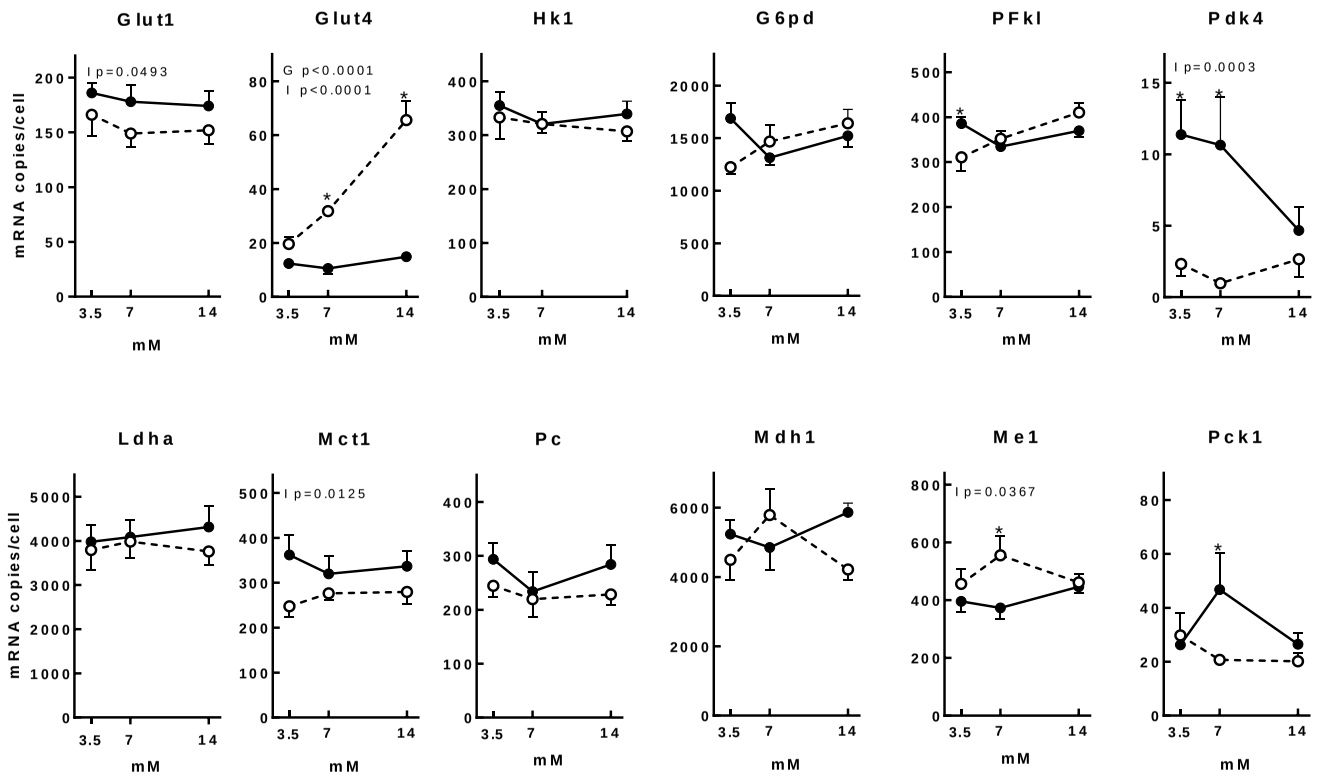


Figure 4. Insulin effect in gene expression of proteins related to glucose metabolism in adipocytes incubated under varying glucose concentration for 24 h.

The data are presented as number of the corresponding mRNA copies per cell, and are mean ± sem of data from six rats. The data were obtained from the “parallel” incubations (i.e. no label). Black circles and continuous line: adipocytes incubated in absence of insulin; white circles and discontinuous line: adipocytes incubated in presence of insulin. The statistical significance data and conventions are the same as in Figure 1. The correspondence between gene names and those of the proteins they code can be seen in Table 1.

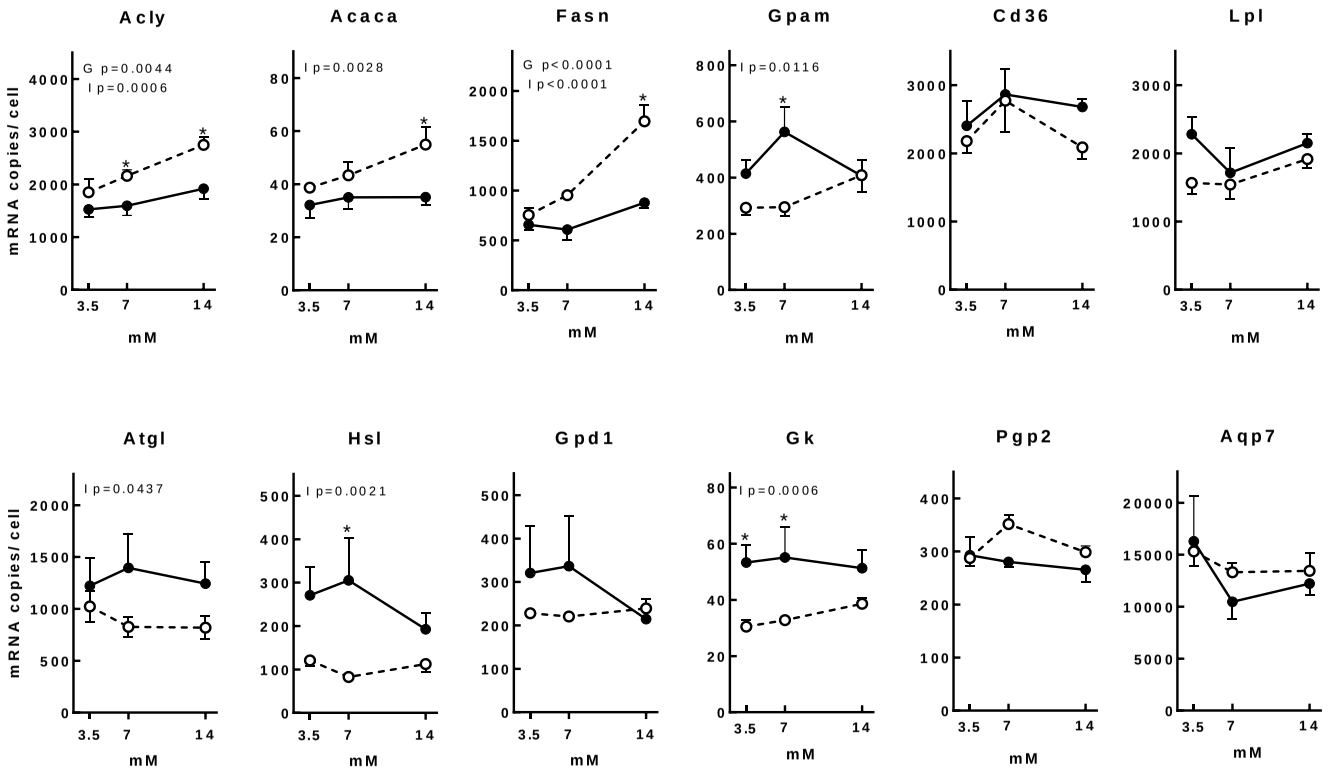


Figure 5. Insulin effect in gene expression of proteins related to lipids and glicerol metabolism in adipocytes incubated under varying glucose concentration for 24 h.

The data are presented as number of the corresponding mRNA copies per cell, and are mean ± sem of data from six rats. The data were obtained from the “parallel” incubations (i.e. no label). Black circles and continuous line: adipocytes incubated in absence of insulin; white circles and discontinuous line: adipocytes incubated in presence of insulin. The statistical significance data and conventions are the same as in Figure 1. The correspondence between gene names and those of the proteins they code can be seen in Table 1.

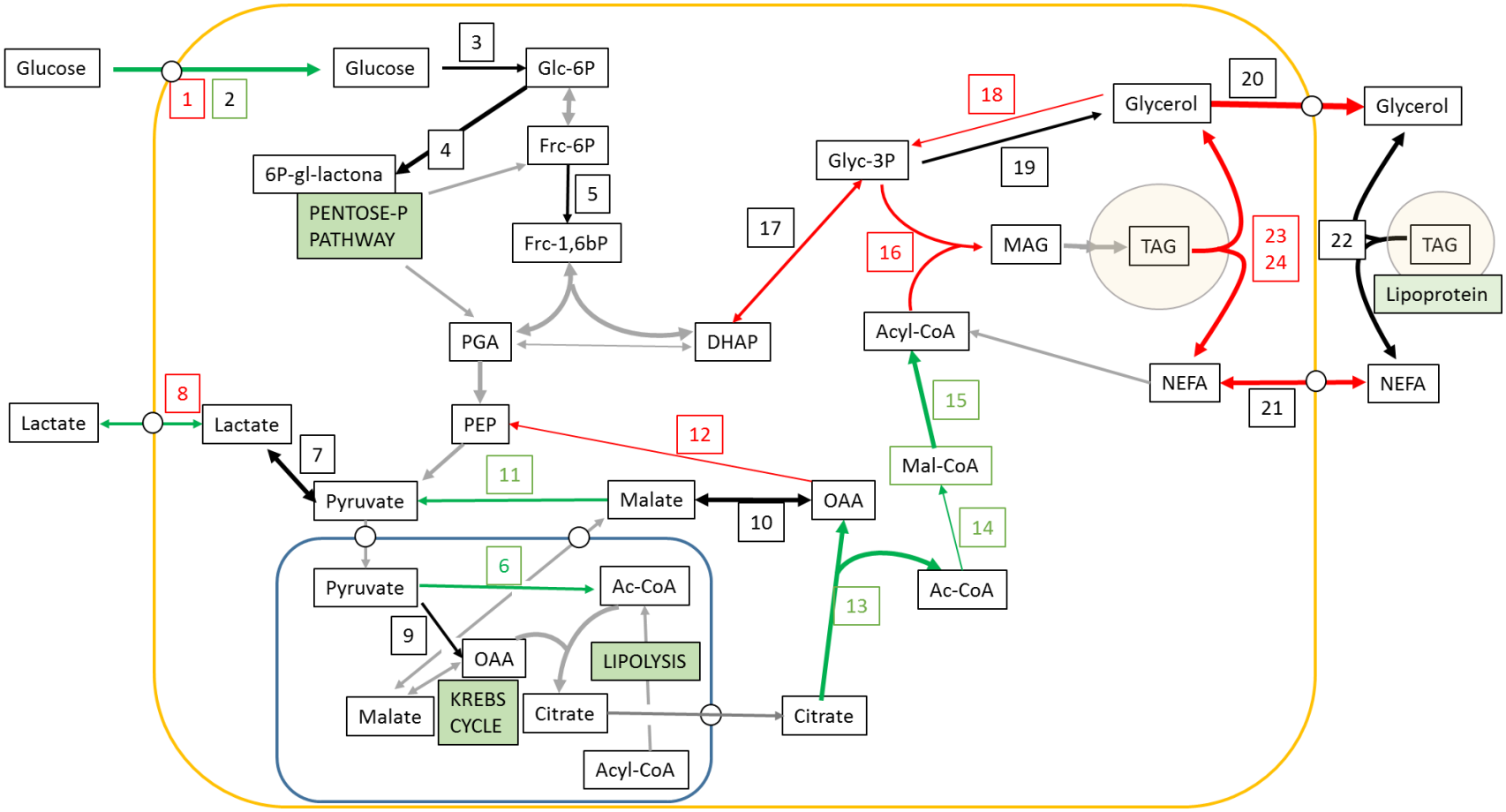


Figure 6. Insulin regulation of the main metabolic pathways in the adipocyte during incubation with glucose.

The graph presents the main intermediate metabolites and substrates. Green lines correspond to pathways activated by incubation with insulin, whilst red lines represent inhibited pathways. The figure incorporates data from label fate, metabolite concentrations, specific radioactivity and gene expression. The squares with numbers represent the proteins/genes controlling the corresponding path. 1- *Glut1* (glucose transporter type 1). 2- *Glut4* (glucose transporter type 4). 3- *Hk1* (hexokinase 1). 4- *G6pd* (glucose-6-phosphate dehydrogenase). 5- *Pfkl* (phospho-fructokinase, liver, b-type). 6- *Pdh* (pyruvate dehydrogenase) [activation resulting from marked inhibition of the expression of *Pdk4*, pyruvate dehydrogenase kinase 4]. 7- *Ldha* (L-lactate dehydrogenase a). 8- *Mct1* (monocarboxylate transporter 1). 9- *Pc* (pyruvate carboxylase). 10- *Mdh1* (malate dehydrogenase). 11- *Me1* (malic enzyme). 12- *Pck1* (phospho-enolpyruvate carboxykinase). 13- *Acly* (ATP: citrate lyase). 14- *Acaca* (acetyl-CoA carboxylase alpha). 15- *Fas* (fatty acid synthase). 16- *Gpam* (glycerol-3P acyl-transferase). 17- *Gdp1* (glycerol-3P dehydrogenase). 18- *Gk* (glycerol kinase).19- *Pgp2* (phospho-glycolate phosphatase). 21- *Cd36* (platelet glycoprotein 4) [fatty acid transpprter]. 20- *Aqp7* (aquoporin 7). 22- *Lpl* (lipoprotein lipase). 23- *Atgl* (triacylglycerol lipase, adipose tissue). 24- *Hsl* (hormone-sensitive lipase).

DISCUSIÓN GENERAL

1. Metodología desarrollada

La primera parte de este proyecto de tesis se enfocó en el desarrollo de la metodología necesaria para determinar el origen bioquímico del lactato y glicerol producidos por el tejido adiposo blanco en cultivos primarios de adipocitos de rata.

Por un lado, el método desarrollado para aislar adipocitos viables y funcionales a partir del tejido adiposo blanco nos ayudó a estimar la recuperación real de estas células luego de su extracción y separación, así como a analizar cuantitativamente la composición del tejido. Sin embargo, hay que tener en cuenta que cuando se usa colagenasa, para liberar el tejido adiposo blanco y que los adipocitos maduros floten individualmente, quedan mezclados otros tipos de células como los macrófagos que contaminan la fracción aislada de adipocitos flotantes y que podrían actuar como variable confundente cuando se analizan los resultados[116]. Además, hay que considerar que los adipocitos más pequeños no flotan como lo hacen los de mayor tamaño, debido a su bajo contenido en lípidos[117]; por ese motivo, la centrifugación en la fase de flotación para separar los adipocitos de la fracción vascular estromal puede sesgar la recuperación de los adipocitos pequeños[118]. Por lo tanto, aunque se omitió el paso de centrifugación, puede que la cantidad de adipocitos pequeños en la fracción aislada sea mínima. Asimismo, el mantener vivos a la mayor cantidad de adipocitos durante las 24h y 48h que dura la incubación fue de vital importancia ya que son muy frágiles y tienden a romperse fácilmente si no se fija el tejido; la adición de adenosina en el medio de cultivo ayudó a disminuir el problema[119]. Ello nos permitió estudiar la funcionalidad y proporción de las células vivas incubadas y nos dio una idea cuantitativa de la composición real del tejido.

La determinación de fragmentos de 3C, como el glicerol, el lactato y la alanina, producto del metabolismo de las células incubadas en el medio de cultivo, es relativamente sencillo, pero no permite distinguir su origen. El uso de precursores marcados radiactivamente es el método clásico empleado comúnmente para este tipo de estudios ya que permite rastrear la producción de los fragmentos de 3C a partir de fuentes de carbono específicas, en este caso la glucosa y por tanto eliminar la incertidumbre asociada con los métodos enzimáticos que no distinguen[120], por ejemplo, el glicerol de glicólisis o el de lipólisis. Tradicionalmente se han utilizado las columnas de cromatografía usando resinas de intercambio iónico (aniónico y catiónico) apiladas para separar compuestos originados a partir de la glucosa como el lactato, el propionato y la alanina[121]. Sin

embargo, los grandes volúmenes empleados para la correcta separación de sustratos limitan el número de muestras que pueden ser analizadas y diluyen inevitablemente el marcaje radiactivo, por lo que no son adecuados para estudios cuantitativos en cultivos celulares ni para comparaciones múltiples.

Por tanto, para analizar cuantitativamente la liberación y procedencia de los fragmentos de 3C a partir de cultivos celulares estándar adaptamos el método descrito con anterioridad a los pequeños volúmenes de incubación utilizados. En este caso una simple centrifugación de los tubos Eppendorf utilizados a manera de mini columnas de intercambio iónico nos permitió recuperar todos los volúmenes empleados sin diluir la muestra. De esta manera analizamos, en el mismo pozo de incubación, el destino de la glucosa para formar lactato y glicerol al mismo tiempo que la incorporación de esta en los adipocitos.

Para la oxidación de glucosa a ácido glucónico, se utilizó glucosa oxidasa y catalasa purificadas[120]. Mientras que la glucosa oxidasa convierte la glucosa a ácido glucónico y peróxido de hidrógeno, la catalasa convierte este último en oxígeno y agua. La catalasa se añadió en exceso para limitar el daño oxidativo en la resina: las resinas de intercambio iónico están hechas de material orgánico y podrían resultar dañadas durante la exposición a fuertes agentes oxidantes como el peróxido de hidrogeno[122], por lo que es importante controlar la concentración del peróxido, así como la temperatura y sobre todo limitar el contacto de este con la resina[123]. Adicionalmente, al reciclar el oxígeno del medio nos aseguramos su disponibilidad para sostener la actividad y la máxima capacidad de la glucosa oxidasa durante toda la reacción.

Inicialmente la incubación con gliceroquinasa[124] se hizo durante toda la noche para asegurar la completa transformación de glicerol a glicerol-3P ya que es posible que el pH óptimo de 9,8 para que la gliceroquinasa funcione al 100% no haya sido preciso. Sin embargo, esta enzima funciona al 80% de su capacidad en un rango amplio de pH (7,6 – 10,4)[125]. Esta larga incubación supuso un problema pues en una de las fracciones separadas recuperamos más radiactividad que su fracción equivalente de la incubación con glucosa oxidasa, posiblemente debido a la ionización o tal vez fosforilación de la glucosa, de forma que la glucosa (ahora cargada negativamente) se quedaba atrapada en la resina junto con el glicerol-3P dando lugar a un exceso de medición. Además, al eliminar el lactato de la muestra en el primer proceso de separación por la columna, se eliminaron los antibióticos presentes en el medio de cultivo, que prevenían el crecimiento de microorganismos. Estos problemas se resolvieron disminuyendo a dos horas la larga incubación y

añadiendo penicilina y estreptomicina durante la separación de la glucosa y del glicerol con glucosa oxidasa y gliceroquinasa, respectivamente.

Para evitar la degradación de las muestras y protegerlas, se añadió ditiotreitol (DTT) y albúmina deslipidada. Aunque, el ditiotreitol, comúnmente utilizado como un agente protector al prevenir la oxidación de grupos tiol, puede interferir con la actividad de la catalasa[126]. No obstante, se ha observado que en dosis de hasta 10 mM de DTT, la catalasa bobina aún conserva al menos un 80% de su actividad[127].

A pesar de los inconvenientes encontrados, el método es versátil y a diferencia del tradicional, mejora considerablemente la recuperación de la radiactividad inicial (90-100%) permitiendo la obtención de datos cuantitativos y sentando las bases para los estudios realizados en la presente tesis. Los resultados preliminares obtenidos en el desarrollo de la metodología sugerían que los adipocitos utilizan mayormente glucosa para producir lactato y glicerol a través de la glucolisis anaeróbica, aunque parte de la glucosa marcada también se incorpora en los ácidos grasos. Todo ello apuntaba a que, bajo las condiciones normóxicas probadas, tanto la glucolisis anaeróbica como la lipogénesis oxidativa coexisten simultáneamente dentro del adipocito. Estos resultados necesitaban ser investigados a fondo.

2. Estudio del metabolismo de la glucosa en fragmentos de 3C

2.1 Respuesta innata de los adipocitos

El tejido adiposo blanco consume muy poco oxígeno[108], y es capaz de subsistir por un largo periodo de tiempo bajo estas condiciones. La elevada producción de lactato procedente de la glucosa a través de la vía glucolítica (anaeróbica) estaría proporcionando la energía necesaria (ATP)[128] para el funcionamiento adecuado de la pequeña cantidad de “citoplasma vivo” de los adipocitos. Esta importante producción de lactato ha sido estudiada ampliamente pero se creía que era a consecuencia de anormalidades metabólicas o de la resistencia a la acción de la insulina, interfiriendo con la utilización de la glucosa en tejidos perifericos[129]. Nosotros planteamos que la producción de lactato es parte de un mecanismo de defensa que contribuye a disminuir la glucemia. En donde el lactato producido, sería utilizado por otros tejidos, puesto que puede ser “reciclado” en el hígado (conversión de lactato a glucosa) y también sirve como sustrato oxidativo para el corazón[130] u otros órganos, al ser una fuente de carbono primario para el ciclo de Krebs y por tanto de energía[131]. Se ha visto que la glucosa radiactiva marca los intermediaros del ciclo de

Krebs, a partir de la captación y utilización del lactato circulante, en todos los tejidos menos el cerebro de ratones ayunados, y cerebro y músculo de ratones alimentados[131]. Además, el flujo de recambio del lactato en la circulación es el más elevado de todos los metabolitos, incluso más que el de la glucosa, de 1,1 a 2,5 veces en ratones alimentados y ayunados, respectivamente[131]. El rápido intercambio del lactato (y piruvato) entre los tejidos y la circulación sanguínea[132] puede ayudar a equiparar la relación entre NAD^+/NADH, permitiendo a todo el organismo amortiguar las perturbaciones del potencial oxidorreductor en cualquier localización[133]. Asimismo, se produciría energía para los tejidos de manera más independiente, ya que la mayor parte de ATP se genera en el ciclo de Krebs, pudiendo el lactato utilizarse preferentemente como sustrato oxidable en lugar de la glucosa[131], reservando esta última para regular funciones más avanzadas del organismo (según requerimientos) como la proliferación celular[134], producción de NADPH a través de la vía de las pentosas fosfato, actividad cerebral[135] y homeostasis de la glucosa sistémica. Asimismo, se ha observado que los niveles de varios intermediarios del ciclo de Krebs están incrementados en el tejido adiposo blanco de obesos, pero no en el hígado ni músculo esquelético[136], por lo que el mayor recambio del ciclo de Krebs podría estar relacionado con el requerimiento de ATP del tejido adiposo para su expansión (hiperplasia) durante la obesidad. También se ha sugerido que concentraciones elevadas de lactato podrían ser un factor inductor del "browning" del tejido adiposo blanco[10], en el que la inducción de la UCP1 constituiría un mecanismo adaptativo para aliviar el estrés oxidativo generado por la acumulación de NADH como consecuencia de la oxidación del lactato dentro de la mitocondria[137].

En cuanto al glicerol, su producción aumentó significativamente con el tiempo, mientras que la actividad específica por carbono disminuyó. Así, inicialmente todo el glicerol producido provenía de la glucosa marcada a través de la vía glucolítica, formando dihidroxiacetona fosfato (DHAP) y luego glicerol-3P. Este último, gracias a la enzima glicerol-3-fosfato fosfatasa (Pgp)[115, 106] es liberado como glicerol y transportado al exterior de la célula por el transportador de glicerol acuaporina 7 (Aqp7). Al parecer, este transportador es modulado por la concentración de glicerol, puesto que en ratones, el ayuno incrementa la cantidad de ARNm de este transportador mientras que la realimentación suprime la expresión en concordancia con los niveles plasmáticos de glicerol[138]. De esta manera, cuando la lipólisis se encuentra aumentada, como ocurre en los adipocitos de obesos[32], los niveles de glicerol se elevan, lo que explicaría la mayor cantidad de Aqp7 a altas concentraciones de glucosa en las primeras 24 horas. Sin embargo, en la segunda mitad de la incubación la mayor parte del glicerol pasó de provenir de la glucosa a proceder de la lipólisis de los triacilgliceroles almacenados, como se observa a partir de la disminución de la

actividad específica por carbono del glicerol en ese periodo, así como por el aumento de la expresión de las lipasas.

Parte de la radiactividad inicial de la glucosa acabó detectándose en los triacilgliceroles presentes en el adipocito, tanto en el glicerol de glicéridos como en los ácidos grasos, apreciándose un aumento significativo de la actividad específica por carbono del glicerol de glicéridos a través del tiempo, pero no de la actividad específica por carbono de los ácidos grasos, manteniéndose esta estable y muy baja en el tiempo. Este último aspecto, junto con el hecho que no hubo cambios en la expresión de los genes marcadores lipogénicos *Acly*, *Acaca*, *Fas*, y junto al aumento en la expresión de *Pdk4*, que bloqueó la función de la piruvato deshidrogenasa, evitando la conversión de piruvato en acetil-CoA, indica que se detuvo la lipogénesis por falta de sustrato. Todo ello confirma la prevalencia de la utilización exógena de ácidos grasos, en este caso provenientes de otros adipocitos, con la incorporación de glicerol recién sintetizado a partir de la glucosa (glicerogénesis) en la lipogénesis[139] y la reducida síntesis *de novo* en adipocitos[140]. También se ha observado que existe una regulación negativa de la síntesis *de novo* a medida que los adipocitos aumentan de tamaño[141], lo que podría explicar que la lipogénesis también disminuya debido a la geometría del adipocito, al ser grandes las distancias que tiene que recorrer el piruvato en el estrecho citoplasma para encontrar alguna de las pocas mitocondrias en donde formar acetil-CoA.

Por otra parte, el aumento de la conversión de DHAP a glicerol-3P a partir de la glucosa marcada necesita la intervención de NADH. No obstante, la transformación de piruvato a lactato también; como consecuencia, el gasto de este NADH citosólico para producir glicerol-3P resultará en la inevitable acumulación de piruvato, cuyo destino principal sería ser oxidado a acetil-CoA en la mitocondria. Este proceso proporcionaría acetil-CoA para la lipogénesis y posteriormente acil-CoA, a la vez que se obtendría glicerol-3P, ambos necesarios para la síntesis de triacilgliceroles. No obstante, durante este proceso, además de la elevación de la expresión de *Pdk4* (inhibidor de la enzima piruvato deshidrogenasa), también se eleva la expresión de la fosfoenolpiruvato carboxiquinasa (*Pck1*), lo que posiblemente sugiere que el piruvato al no poder ser oxidado en la mitocondria se carboxile a oxalacetato[142], retornando este último al citosol probablemente en forma de malato a través de la lanzadera de malato-aspartato[143], donde el malato es nuevamente transformado a oxalacetato; y como no hay necesidad de más NADPH, este oxalacetato citosólico en vez de ser usado vía enzima málico, formará fosfoenolpiruvato gracias a la *Pck1,* reincorporándose así a la vía glucolítica. Posteriormente, el NADH producido por la conversión de malato a oxalacetato puede ser incorporado en la formación de lactato. Por tanto, la clave estaría

en el NADH tanto citosólico como mitocondrial y no en el NADPH necesario en la lipogénesis, proceso inviable por la falta de suficiente acetil-CoA. Además, las expresiones de las enzimas glucosa 6 fosfato deshidrogenasa (*G6pd*) del ciclo de las pentosas fosfato y la enzima málica (*Me1*), indicativas de síntesis de NADPH, permanecen invariables.

La coexistencia contradictoria del aumento de la lipólisis (probada por la disminución de la radiactividad específica del eflujo de glicerol) y el aumento de la síntesis de acilgliceroles (mayor incorporación de ^{14}C en el glicerol), además de la lipogénesis (a partir de glucosa, como lo demuestra el marcaje encontrado en los ácidos grasos de los triacilgliceroles), solo puede explicarse por un aumento en el recambio de triacilgliceroles.

Estos datos apuntan a que pueden coexistir dos procesos diferentes al mismo tiempo ya que, a pesar de la lipólisis, una gran cantidad de ácidos grasos hidrolizados en los adipocitos son reesterificados a triacilgliceroles. Una posible explicación es que la enzima acil-CoA:diacilglicerol aciltransferasa 1 (DGAT1)[144], encargada de catalizar el paso final en la formación de triacilgliceroles, incorporando ácidos grasos a diacilgliceroles y ubicada exclusivamente en el retículo endoplasmático de la célula[145], intervenga en esta reesterificación, no para mantener la cantidad de triacilgliceroles, sino para proteger al retículo endoplasmático de la lipotoxicidad[146].

La carencia de insulina o de cualquier otro estimulo externo, sugiere que la existencia de todos estos procesos al mismo tiempo son un mecanismo automático que el adipocito activa para defenderse del efecto tóxico de la glucosa y de otros sustratos en exceso, independientemente de la concentración de glucosa. Por tanto, se podría decir que son ciclos “fútiles” para gastar energía al liberar fragmentos de 3C reduciendo de esta forma la glucemia y evitando el excesivo almacenamiento de lípidos y sus consecuencias derivadas como la inflamación[11], una de las principales implicadas en el origen del síndrome metabólico. Además, estos metabolitos podrían viajar fácilmente (especialmente lactato y glicerol) por la circulación sanguínea proporcionando energía a otros tejidos sin depender de la glucosa ni de la insulina. También refuerza la idea del tejido adiposo blanco como un activo protagonista en el manejo de la energía y en el control de la glucemia del organismo, especialmente si se tiene en cuenta el gran tamaño del órgano adiposo[147].

2.2 Respuesta metabólica de los adipocitos a la insulina

Bajo las tres condiciones probadas, hipoglucemia o ayuno (3,5 mM), euglucemia (7 mM) e hiperglucemia o estado postprandial sostenido (14 mM), los adipocitos provenientes del tejido epididimal de ratas de 14 semanas de edad, incubados por un periodo largo de tiempo (24 o 48 horas) con glucosa ^{14}C, transformaron una considerable cantidad de esta glucosa en fragmentos de 3C, principalmente lactato y glicerol; mientras que la fracción estromal solo produjo lactato. No obstante, las células estromales, independientemente de la presencia de insulina, captaron glucosa y liberaron incluso más lactato que los adipocitos. El hecho de que estas células produzcan lactato en presencia de oxígeno sugiere que tienen preferencia por el metabolismo anaeróbico, ya que en este caso su geometría no es un obstáculo para el metabolismo aeróbico. Estos resultados podrían corresponderse con la posibilidad de que el flujo sanguíneo esté limitado en el tejido adiposo blanco y sea un mecanismo defensivo contra la disponibilidad en exceso de sustratos[97], ya que la hipoxia derivada no sería un problema para este tejido. Se ha propuesto la idea de la existencia de una "lanzadera de lactato", en la que el lactato producido en condiciones normóxicas es usado como fuente de carbonos[148]. Este concepto se ha aplicado a la producción de energía en células estromales, como fibroblastos y células cancerígenas epiteliales[149], así como también a astrocitos y neuronas[137], en donde la glucosa es captada y liberada como lactato por los astrocitos, siendo este último metabolito utilizado como fuente de energía en las neuronas.

La insulina estimula la captación de glucosa en músculo y tejido adiposo así como el flujo de carbonos a través de la glucólisis y lipogénesis[45]. Los adipocitos al transformar y liberar la glucosa captada en fragmentos de 3C, disminuyen el exceso de esta y, por ende, los efectos tóxicos que produce en el organismo[2]. La ventaja de producir estos fragmentos de 3C es que, a diferencia de la glucosa, no se ven afectados por la resistencia a la acción de la insulina ni están sujetos a la estricta regulación de la glucólisis en la mayoría de los tejidos. Nuestros resultados coinciden con esta interpretación, ya que los adipocitos captaron gran cantidad de glucosa para transformarla en fragmentos de 3C. Idea que se ve reforzada por el hecho de que la producción de lactato se incrementa a medida que las concentraciones de glucosa aumentan.

A pesar de que en el adipocito predomina el transportador de glucosa GLUT4 dependiente de insulina antes que el GLUT1 no insulino dependiente[2], encontramos una mayor expresión de GLUT1 que de GLUT4. La captación de glucosa fue elevada, incluso en ausencia de insulina, aunque dicha captación fue aún mayor en los adipocitos con insulina, a pesar de la baja expresión de GLUT1, en este caso compensado gracias a la mayor expresión de GLUT4.

Por otra parte, la insulina influye en la producción de glicerol y NEFA, los adipocitos incubados con insulina liberaron menos cantidad al medio que sus respectivos controles, una consecuencia del efecto de la insulina que tiende a incrementar la lipogénesis al mismo tiempo que inhibe profundamente la lipólisis[45]. En nuestro experimento la liberación de glicerol fue estable y constante a pesar de las diferentes concentraciones iniciales de glucosa. Sin embargo, cuando las concentraciones de glucosa eran bajas, el principal origen del glicerol fue lipolítico, es decir, que provenía de los triacilgliceroles, mientras que, a concentraciones más elevadas de glucosa (excepto a 7 mM con insulina), su origen fue glucolítico, demostrado por una actividad específica por carbono de glicerol parecida al de la glucosa. También, se puede ver un aumento en la síntesis *de novo* con la presencia de insulina, como se aprecia en la mayor actividad específica por carbono de los ácidos grasos de los triacilgliceroles con insulina. Acompañado, además, por la inhibición de la expresión de *Pdk4* (inhibidor de la enzima piruvato deshidrogenasa), lo que incrementaría el flujo de carbonos hacia acetil-CoA en la mitocondria, así como la activación de las principales enzimas responsables de la lipogénesis: *Acly, Acaca* y *Fas*. Es así como, en el metabolismo del glicerol, el equilibrio entre estas dos vías, la incorporación hacia acil-glicerol o la generación directa de glicerol a partir de glicerol-3P, parece ser un punto crítico en la regulación de la síntesis de triacilgliceroles. En este sentido, la insulina estaría limitando la incorporación del glicerol 3P en los triacilgliceroles, al inhibir la expresión de la enzima glicerol-3P acil-transferasa (*Gpam*). Al mismo tiempo, también se observa el efecto de la insulina en la inhibición de la lipólisis al disminuir la expresión de las lipasas. La mayor incorporación de ^{14}C de la glucosa en los ácidos grasos de los triacilgliceroles en presencia de insulina, aunque sin cambios en la incorporación de glicerol en los triacilgliceroles, sugiere que la insulina induce un mayor recambio de triacilgliceroles. La activación de dicho recambio, cuando disminuye el flujo de glicerol, es contraria al enfoque asumido sobre los adipocitos estimulados por insulina, donde la lipogénesis y la inhibición de la lipólisis son tendencias claramente establecidas a través de los patrones de expresión génica.

En las incubaciones más largas, la insulina sólo acentuó algunos de los cambios observados en las primeras 24 horas, aunque el efecto del tiempo era marcado e importante. Se ha observado que periodos prolongados de incubación con insulina provocan una marcada anormalidad en la habilidad de los adipocitos para transportar la glucosa a su máxima capacidad e inducen la perdida de los receptores de insulina, por lo que el tipo de incubación *in vitro*, como el nuestro, posiblemente acaba simulando un estado de resistencia a la acción de la insulina[150]. Esta resistencia se caracteriza por la disminución de la captación de glucosa en músculo y en

adipocitos[59]; sin embargo, una vez activado el trasportador de glucosa, sigue activo transportando la glucosa a su máxima capacidad sin importar la duración de la incubación y pese a la pérdida del receptor[150].

Uno de los cambios más llamativos en este periodo de tiempo fue la marcada reducción de la expresión de GLUT4, especialmente en los grupos incubados con insulina. Una posible explicación sería que en estados de resistencia a la acción de la insulina, la expresión de GLUT4 disminuye en el tejido adiposo pero no en el músculo[59]. En nuestro experimento la captación de glucosa se mantuvo constante y estable en el tiempo a pesar de la pérdida del transportador GLUT4, no obstante, la expresión de GLUT1 se mantiene o se eleva ligeramente con el tiempo, por lo que la captación de glucosa se mantendría mayoritariamente gracias a este último transportador. Además, en cultivos de células L6 de músculo, se ha visto que la exposición prolongada a la insulina o a un medio privado de glucosa causa una mayor expresión del ARN mensajero de GLUT1 y del mismo GLUT1[53].

Todo ello sugiere que el tejido adiposo funciona de manera autónoma sin necesidad de insulina o que los adipocitos poseen una capacidad de respuesta limitada a la acción de esta[75]. Además, se ha visto que el tamaño de los adipocitos influye, contra más grandes menos sensibles al efecto de la insulina[151]. Por último, se debe tener en consideración que en cultivos celulares la insulina es administrada directamente en el pozo de incubación, de manera continua y fija en el tiempo, sin la posibilidad de utilizar otro sistema de entrega como la portal pulsátil que imita a la fisiológica y que mejora la acción de esta hormona al aumentar la activación de Akt más que en un sistema de entrega continuo y fijo[152].

CONCLUSIONES

1. La puesta a punto de la metodología para aislar adipocitos viables y funcionales a partir del tejido adiposo blanco de rata y la separación en fracciones de los metabolitos resultantes tras la administración de glucosa marcada, ha permitido cuantificar el destino de esta glucosa y por ende determinar el origen de los fragmentos de 3C.

2. En adipocitos blancos maduros, sin la presencia de estímulos externos, la glucólisis y en menor medida la lipogénesis, coexisten al mismo tiempo, lo que junto con el mantenimiento de la lipolisis y de la glicerogénesis, permite sostener el recambio de triacilgliceroles. La presencia de insulina incrementa este recambio puesto que promueve en mayor medida la lipogénesis y disminuye la lipólisis.

3. Los adipocitos, en condiciones aeróbicas, incorporaron glucosa, transformándola mayoritariamente a fragmentos de 3C, principalmente lactato y glicerol, incrementándose dicha producción con el tiempo y con la concentración de glucosa. Todo ello conforma un mecanismo mediante el que los adipocitos pueden defenderse frente al exceso de glucosa.

4. En los adipocitos, el origen del glicerol liberado al medio varía con el tiempo y la concentración de glucosa. En las primeras 24 horas y a concentraciones elevadas de glucosa su origen fue principalmente glucolítico, mientras que a las 48 horas su origen mayoritario fue lipolítico, a partir del mayor recambio de triacilgliceroles.

5. La insulina incrementa la lipogénesis *de novo* en los adipocitos, incorporando más carbonos de la glucosa en los ácidos grasos de los triacilgliceroles. A pesar del incremento de la esterificación, la insulina inhibe la expresión de la glicerol-3P acil-transferasa, como un mecanismo para evitar el excesivo acúmulo de triacilgliceroles.

6. La fracción estromal del tejido adiposo blanco también se comportó de forma anaeróbica en presencia de oxígeno, produciendo incluso más lactato que los adipocitos. Estos cambios fueron independientes de la concentración de glucosa y de la presencia de insulina. A diferencia de los adipocitos, la fracción estromal no produjo y tampoco exportó glicerol ni NEFAs.

REFERENCIAS

1. Goodpaster, B. H. & Sparks, L. M. Metabolic Flexibility in Health and Disease. *Cell Metabolism* **25,** 1027–1036 (2017).
2. Rutkowski, J. M., Stern, J. H. & Scherer, P. E. The cell biology of fat expansion. *Journal of Cell Biology* **208,** 501–512 (2015).
3. Luo, L. & Liu, M. Adipose tissue in control of metabolism. *Journal of Endocrinology* **231,** R77–R99 (2016).
4. Spiegelman, R. and. What we talk about when we talk about fat. *Cell* **156,** 20–44 (2015).
5. Rothwell, N. J. & Stock, M. J. Luxuskonsumption, diet-induced thermogenesis and brown fat: the case in favour. *Clin. Sci. (Lond).* **64,** 19–23 (1983).
6. Peirce, V., Carobbio, S. & Vidal-Puig, A. The different shades of fat. *Nature* **510,** 76–83 (2014).
7. Loncar, D., Afzelius, B. A. & Cannon, B. Epididymal white adipose tissue after cold stress in rats. I. Nonmitochondrial changes. *J. Ultrastruct. Mol. Struct. Res.* **101,** 109–22 (1988).
8. Wu, J. *et al.* Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* **150,** 366–76 (2012).
9. Eto, H. *et al.* Characterization of Structure and Cellular Components of Aspirated and Excised Adipose Tissue. *Plast. Reconstr. Surg.* **124,** 1087–1097 (2009).
10. Jeanson, Y., Carrière, A. & Casteilla, L. A New Role for Browning as a Redox and Stress Adaptive Mechanism? *Front. Endocrinol. (Lausanne).* **6,** 158 (2015).
11. Reilly, S. M. & Saltiel, A. R. Adapting to obesity with adipose tissue inflammation. *Nature Reviews Endocrinology* **13,** 633–643 (2017).
12. Buettner, C. *et al.* Leptin controls adipose tissue lipogenesis via central, STAT3-independent mechanisms. *Nat. Med.* **14,** 667–75 (2008).
13. Marroquí, L. *et al.* Role of leptin in the pancreatic β-cell: effects and signaling pathways. *J. Mol. Endocrinol.* **49,** R9-17 (2012).
14. Friedman, J. M. Obesity in the new millennium. *Nature* **404,** 632–634 (2000).

15. Combs, T. P. *et al.* A Transgenic Mouse with a Deletion in the Collagenous Domain of Adiponectin Displays Elevated Circulating Adiponectin and Improved Insulin Sensitivity. *Endocrinology* **145,** 367–383 (2004).

16. Alemany, M. Adjustment to dietary energy availability: from starvation to overnutrition. *RSC Adv.* **3,** 1636–1651 (2013).

17. Berry, R., Jeffery, E. & Rodeheffer, M. S. Weighing in on adipocyte precursors. *Cell Metabolism* **19,** 8–20 (2014).

18. Foster, M. T., Shi, H., Seeley, R. J. & Woods, S. C. Transplantation or removal of intra-abdominal adipose tissue prevents age-induced glucose insensitivity. *Physiol. Behav.* **101,** 282–288 (2010).

19. Jeffery, E. *et al.* The Adipose Tissue Microenvironment Regulates Depot-Specific Adipogenesis in Obesity. *Cell Metab.* **24,** 142–50 (2016).

20. Tchoukalova, Y. D. *et al.* Regional differences in cellular mechanisms of adipose tissue gain with overfeeding. *Proc. Natl. Acad. Sci.* **107,** 18226–18231 (2010).

21. Jeffery, E., Church, C. D., Holtrup, B., Colman, L. & Rodeheffer, M. S. Rapid depot-specific activation of adipocyte precursor cells at the onset of obesity. *Nat. Cell Biol.* **17,** 376–85 (2015).

22. MacKellar, J., Cushman, S. W. & Periwal, V. Waves of Adipose Tissue Growth in the Genetically Obese Zucker Fatty Rat. *PLoS One* **5,** e8197 (2010).

23. Spalding, K. L. *et al.* Dynamics of fat cell turnover in humans. *Nature* **453,** 783–787 (2008).

24. Jackson, R. M., Griesel, B. A., Gurley, J. M., Szweda, L. I. & Olson, A. L. Glucose availability controls adipogenesis in mouse 3T3-L1 adipocytes via up-regulation of nicotinamide metabolism. *J. Biol. Chem.* **292,** 18556–18564 (2017).

25. Acosta, J. R. *et al.* Increased fat cell size: a major phenotype of subcutaneous white adipose tissue in non-obese individuals with type 2 diabetes. *Diabetologia* **59,** 560–70 (2016).

26. Beutler, B. Innate immunity: an overview. *Mol. Immunol.* **40,** 845–59 (2004).

27. Hotamisligil, G. S. Inflammation and metabolic disorders. *Nature* **444,** 860–867 (2006).

28. Leclerc, V. & Reichhart, J.-M. The immune response of Drosophila melanogaster. *Immunol. Rev.* **198,** 59–71 (2004).

29. Rusten, T. E. *et al.* Programmed Autophagy in the Drosophila Fat Body Is Induced by Ecdysone through Regulation of the PI3K Pathway. *Dev. Cell* **7,** 179–192 (2004).

30. Alemany, M. Steroid hormones interrelationships in the metabolic syndrome: an introduction to the ponderostat hypothesis. *Hormones (Athens).* **11,** 272–89 (2012).

31. Wernstedt Asterholm, I. *et al.* Adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling. *Cell Metab.* **20,** 103-18 (2014).

32. Glass, C. K. & Olefsky, J. M. Inflammation and Lipid Signaling in the Etiology of Insulin Resistance. *Cell Metab.* **15,** 635–645 (2012).

33. Johnson, A. M. F. & Olefsky, J. M. The origins and drivers of insulin resistance. *Cell* **152,** 673–684 (2013).

34. Makowski, L., Brittingham, K. C., Reynolds, J. M., Suttles, J. & Hotamisligil, G. S. The Fatty Acid-binding Protein, aP2, Coordinates Macrophage Cholesterol Trafficking and Inflammatory Activity. *J. Biol. Chem.* **280,** 12888–12895 (2005).

35. Alligier, M. *et al.* Subcutaneous Adipose Tissue Remodeling during the Initial Phase of Weight Gain Induced by Overfeeding in Humans. *J. Clin. Endocrinol. Metab.* **97,** E183–E192 (2012).

36. Henegar, C. *et al.* Adipose tissue transcriptomic signature highlights the pathological relevance of extracellular matrix in human obesity. *Genome Biol.* **9,** R14 (2008).

37. Li, Q., Hata, A., Kosugi, C., Kataoka, N. & Funaki, M. The density of extracellular matrix proteins regulates inflammation and insulin signaling in adipocytes. *FEBS Lett.* **584,** 4145–4150 (2010).

38. Molofsky, A. B. *et al.* Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages. *J. Exp. Med.* **210,** 535–49 (2013).

39. Brestoff, J. R. *et al.* Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. *Nature* **519,** 242–246 (2015).

40. Fujisaka, S. *et al.* Regulatory mechanisms for adipose tissue M1 and M2 macrophages in diet-induced obese mice. *Diabetes* **58,** 2574–82 (2009).

41. Hotamisligil, G. S., Shargill, N. S. & Spiegelman, B. M. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* **259,** 87–91 (1993).

42. Wellen, K. E. & Hotamisligil, G. S. Inflammation, stress, and diabetes. *J. Clin. Invest.* **115,** 1111–1119 (2005).

43. Hotamisligil, G. S., Arner, P., Caro, J. F., Atkinson, R. L. & Spiegelman, B. M. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J. Clin. Invest.* **95,** 2409–2415 (1995).

44. Schmitz, J. *et al.* Obesogenic memory can confer long-term increases in adipose tissue but not liver inflammation and insulin resistance after weight loss. *Mol. Metab.* **5,** 328–339 (2016).

45. Saltiel, A. R. & Kahn, C. R. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414,** 799–806 (2001).

46. Haeusler, R. A., McGraw, T. E. & Accili, D. Biochemical and cellular properties of insulin receptor signalling. *Nat. Rev. Mol. Cell Biol.* **19,** 31–44 (2018).

47. Leavens, K. F., Easton, R. M., Shulman, G. I., Previs, S. F. & Birnbaum, M. J. Akt2 Is Required for Hepatic Lipid Accumulation in Models of Insulin Resistance. *Cell Metab.* **10,** 405–418 (2009).

48. Jiang, Z. Y. *et al.* Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 7569–74 (2003).

49. Titchenell, P. M. *et al.* Direct Hepatocyte Insulin Signaling Is Required for Lipogenesis but Is Dispensable for the Suppression of Glucose Production. *Cell Metab.* **23,** 1154–1166 (2016).

50. Boucher, J., Kleinridders, A. & Kahn, C. R. Insulin Receptor Signaling in Normal and Insulin-Resistant States. *Cold Spring Harb. Perspect. Biol.* **6,** a009191–a009191 (2014).

51. Raught, B., Gingras, A.-C. & Sonenberg, N. The target of rapamycin (TOR) proteins. *Proc. Natl. Acad. Sci.* **98,** 7037–7044 (2001).

52. Samuel, V. T. & Shulman, G. I. The pathogenesis of insulin resistance: Integrating signaling pathways and substrate flux. *J. Clin. Invest.* **126,** 12–22 (2016).

53. Klip, A. & Pâquet, M. R. Glucose transport and glucose transporters in muscle and their metabolic regulation. *Diabetes Care* **13,** 228–43 (1990).

54. Baron, A. D., Brechtel, G., Wallace, P. & Edelman, S. V. Rates and tissue sites of non-insulin- and insulin-mediated glucose uptake in humans. *Am. J. Physiol. Metab.* **255,** E769–E774 (1988).

55. Katz, A., Nyomba, B. L. & Bogardus, C. No accumulation of glucose in human skeletal muscle during euglycemic hyperinsulinemia. *Am. J. Physiol. Metab.* **255,** E942–E945 (1988).

56. Björntorp, P. & Sjöström, L. Carbohydrate storage in man: speculations and some quantitative considerations. *Metabolism.* **27,** 1853–65 (1978).

57. Björntorp, P., Krotkiewski, M., Larsson, B. & Somlo-Szücs, Z. Effects of Feeding States on Lipid Radioactivity in Liver, Muscle and Adipose Tissue after Injection of Labelled Glucose in the Rat. *Acta Physiol. Scand.* **80,** 29–38 (1970).

58. Brüning, J. C. *et al.* A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol. Cell* **2,** 559–69 (1998).

59. Abel, E. D. Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nat. Commun.* **409,** 729–733 (2001).

60. Shepherd, P. R. & Kahn, B. B. Glucose Transporters and Insulin Action — Implications for Insulin Resistance and Diabetes Mellitus. *N. Engl. J. Med.* **341,** 248–257 (1999).

61. Domingo, P. *et al.* Relationship between HIV/Highly Active Antiretroviral Therapy (HAART)–Associated Lipodystrophy Syndrome and Stavudine-Triphosphate Intracellular Levels in Patients with Stavudine-Based Antiretroviral Regimens. *Clin. Infect. Dis.* **50,** 1033–1040 (2010).

62. Magré, J. *et al.* Identification of the gene altered in Berardinelli–Seip congenital lipodystrophy on chromosome 11q13. *Nat. Genet.* **28,** 365–370 (2001).

63. Huang-Doran, I., Sleigh, A., Rochford, J. J., O'Rahilly, S. & Savage, D. B. Lipodystrophy: metabolic insights from a rare disorder. *J. Endocrinol.* **207,** 245–55 (2010).

64. Gavrilova, O. *et al.* Surgical implantation of adipose tissue reverses diabetes in lipoatrophic mice. *J. Clin. Invest.* **105,** 271–8 (2000).

65. Klotz, L.-O. *et al.* Redox regulation of FoxO transcription factors. *Redox Biol.* **6,** 51–72 (2015).

66. Ryder, J. W., Gilbert, M. & Zierath, J. R. Skeletal muscle and insulin sensitivity: pathophysiological alterations. *Front. Biosci.* **6,** D154-63 (2001).

67. Pendergrass, M. *et al.* Muscle glucose transport and phosphorylation in type 2 diabetic, obese nondiabetic, and genetically predisposed individuals. *Am. J. Physiol. Metab.* **292,** E92–E100 (2007).

68. Samuel, V. T. & Shulman, G. I. Mechanisms for Insulin Resistance: Common Threads and Missing Links. *Cell* **148,** 852–871 (2012).

69. Stratford, S., Hoehn, K. L., Liu, F. & Summers, S. A. Regulation of Insulin Action by Ceramide. *J. Biol. Chem.* **279,** 36608–36615 (2004).

70. Cohen, J. C., Horton, J. D. & Hobbs, H. H. Human Fatty Liver Disease: Old Questions and New Insights. *Science.* **332,** 1519–1523 (2011).

71. Corkey, B. E. Banting lecture 2011: Hyperinsulinemia: Cause or consequence? *Diabetes* **61,** 4–13 (2012).

72. Erion, K. A., Berdan, C. A., Burritt, N. E., Corkey, B. E. & Deeney, J. T. Chronic Exposure to Excess Nutrients Left-shifts the Concentration Dependence of Glucose-stimulated Insulin Secretion in Pancreatic β-Cells. *J. Biol. Chem.* **290,** 16191–201 (2015).

73. Kim, M. K., Reaven, G. M., Chen, Y.-D. I., Kim, E. & Kim, S. H. Hyperinsulinemia in individuals with obesity: Role of insulin clearance. *Obesity* **23,** 2430–2434 (2015).

74. Pories, W. J. & Dohm, G. L. Diabetes: Have We Got It All Wrong?: Hyperinsulinism as the culprit: surgery provides the evidence. *Diabetes Care* **35,** 2438–2442 (2012).

75. Czech, M. P. Insulin action and resistance in obesity and type 2 diabetes. *Nature Medicine* **23,** 804–814 (2017).

76. Lackey, D. E. & Olefsky, J. M. Regulation of metabolism by the innate immune system. *Nat. Rev. Endocrinol.* **12,** 15–28 (2016).

77. Holland, W. L. *et al.* Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid–induced ceramide biosynthesis in mice. *J. Clin. Invest.* **121,** 1858–1870 (2011).

78. Muoio, D. M. & Neufer, P. D. Lipid-Induced Mitochondrial Stress and Insulin Action in Muscle. *Cell Metab.* **15,** 595–605 (2012).

79. Corkey, B. E. Diabetes: Have We Got It All Wrong?: Insulin hypersecretion and food additives: cause of obesity and diabetes? *Diabetes Care* **35,** 2432–2437 (2012).

80. Nolan, C. J., Ruderman, N. B., Kahn, S. E., Pedersen, O. & Prentki, M. Insulin resistance as a physiological defense against metabolic stress: Implications for the management of subsets of type 2 diabetes. *Diabetes* **64,** 673–86 (2015).

81. Hoeks, J. *et al.* Prolonged Fasting Identifies Skeletal Muscle Mitochondrial Dysfunction as Consequence Rather Than Cause of Human Insulin Resistance. *Diabetes* **59,** 2117–2125 (2010).

82. Dubé, J. J. *et al.* Effects of acute lipid overload on skeletal muscle insulin resistance, metabolic flexibility, and mitochondrial performance. *Am. J. Physiol. Metab.* **307,** E1117–E1124 (2014).

83. World Health Organization (WHO). Obesity and overweight. *WHO* (2016).

84. Alemany, M. Relationship between energy dense diets and white adipose tissue inflammation in metabolic syndrome. *Nutr. Res.* **33,** 1–11 (2013).

85. Gluckman, P. D. & Hanson, M. A. The developmental origins of the metabolic syndrome. *Trends Endocrinol. Metab.* **15,** 183–187 (2004).

86. Alemany, M. Metabolic Syndrome: A Multifaceted Disease of Affluence. *J. Endocrinol. Metab.* **2,** 155–165 (2012).

87. Alemany, M. The problem of nitrogen disposal in the obese. *Nutr. Res. Rev.* **25,** 18–28 (2012).

88. Barber, T., Viña, J. R., Viña, J. & Cabo, J. Decreased urea synthesis in cafeteria-diet-induced obesity in the rat. *Biochem. J.* **230,** 675–81 (1985).

89. Dijkers, P. F. & O'Farrell, P. H. Dissection of a hypoxia-induced, nitric oxide-mediated signaling cascade. *Mol. Biol. Cell* **20,** 4083–90 (2009).

90. Korhonen, R., Lahti, A., Kankaanranta, H. & Moilanen, E. Nitric oxide production and signaling in inflammation. *Curr. Drug Targets. Inflamm. Allergy* **4,** 471–9 (2005).

91. Vigili de Kreutzenberg, S., Kiwanuka, E., Tiengo, A. & Avogaro, A. Visceral obesity is characterized by impaired nitric oxide-independent vasodilation. *Eur. Heart J.* **24,** 1210–5 (2003).

92. Frisbee, J. C. Vascular adrenergic tone and structural narrowing constrain reactive hyperemia in skeletal muscle of obese Zucker rats. *Am. J. Physiol. Circ. Physiol.* **290,** H2066–H2074 (2006).

93. Frohlich, E. D., Messerli, F. H., Reisin, E. & Dunn, F. G. The Problem of Obesity and Hypertension. *Hypertension* **5,** III71-8 (1983).

94. Barton, M., Carmona, R., Ortmann, J., Krieger, J. E. & Traupe, T. Obesity-associated activation of angiotensin and endothelin in the cardiovascular system. *Int. J. Biochem. Cell Biol.* **35,** 826–37 (2003).

95. Sanz, Y., Santacruz, A. & Gauffin, P. Gut microbiota in obesity and metabolic disorders. *Proc. Nutr. Soc.* **69,** 434–441 (2010).

96. Creely, S. J. *et al.* Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. *Am. J. Physiol. Metab.* **292,** E740–E747 (2007).

97. Alemany, M. Regulation of adipose tissue energy availability through blood flow control in the metabolic syndrome. *Free Radic. Biol. Med.* **52,** 2108–19 (2012).

98. Yin, J. *et al.* Role of hypoxia in obesity-induced disorders of glucose and lipid metabolism in adipose tissue. *Am. J. Physiol. Metab.* **296,** E333–E342 (2009).

99. El-Assaad, W. *et al.* Glucolipotoxicity Alters Lipid Partitioning and Causes Mitochondrial Dysfunction, Cholesterol, and Ceramide Deposition and Reactive Oxygen Species Production in INS832/13 ß-Cells. *Endocrinology* **151,** 3061–3073 (2010).

100. Chaves, V. E. *et al.* Increased glyceride-glycerol synthesis in liver and brown adipose tissue of rat: In-vivo contribution of glycolysis and glyceroneogenesis. *Lipids* **47,** 773–780 (2012).

101. Frayn, K. N. & Humphreys, S. M. Metabolic characteristics of human subcutaneous abdominal adipose tissueafter overnight fast. *Am. J. Physiol. Metab.* **302,** E468–E475 (2012).

102. Herman, M. A. *et al.* A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism. *Nature* **484,** 333–338 (2012).

103. Yore, M. M. *et al.* Discovery of a class of endogenous mammalian lipids with anti-diabetic and anti-inflammatory effects. *Cell* **159,** 318–32 (2014).

104. Letexier, D., Pinteur, C., Large, V., Fréring, V. & Beylot, M. Comparison of the expression and activity of the lipogenic pathway in human and rat adipose tissue. *J. Lipid Res.* **44,** 2127–2134 (2003).

105. Bruss, M. D., Khambatta, C. F., Ruby, M. A., Aggarwal, I. & Hellerstein, M. K. Calorie restriction increases fatty acid synthesis and whole body fat oxidation rates. *Am. J. Physiol. Metab.* **298,** E108–E116 (2010).

106. Mugabo, Y. *et al.* Identification of a mammalian glycerol-3-phosphate phosphatase: Role in metabolism and signaling in pancreatic β-cells and hepatocytes. *Proc. Natl. Acad. Sci.* **113,** E430-9 (2016).

107. Lamontagne, J. *et al.* Identification of the signals for glucose-induced insulin secretion in INS1 (832/13) β-cells using metformin-induced metabolic deceleration as a model. *J Biol Chem* **292,** 19458–19468 (2017).

108. Frayn, K. *Metabolic regulation : a human perspective*. (Wiley-Blackwell Pub, 2010).

109. Frayn, K. Adipose tissue as a buffer for daily lipid flux. *Diabetologia* **45,** 1201–1210 (2002).

110. Choi, S. M. *et al.* Insulin Regulates Adipocyte Lipolysis via an Akt-Independent Signaling Pathway. *Mol. Cell. Biol.* **30,** 5009–5020 (2010).

111. Perry, R. J. *et al.* Hepatic Acetyl CoA Links Adipose Tissue Inflammation to Hepatic Insulin Resistance and Type 2 Diabetes. *Cell* **160,** 745–758 (2015).

112. Brownlee, M. The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* **54,** 1615–25 (2005).

113. Arriarán, S. *et al.* Evidences of Basal Lactate Production in the Main White Adipose Tissue Sites of Rats. Effects of Sex and a Cafeteria Diet. *PLoS One* **10,** e0119572 (2015).

114. Sabater, D. *et al.* Cultured 3T3L1 adipocytes dispose of excess medium glucose as lactate under abundant oxygen availability. *Sci. Rep.* **4,** 3663 (2014).

115. Del Mar Romero, M., Sabater, D., Fernández-López, J. A., Remesar, X. & Alemany, M. Glycerol Production from Glucose and Fructose by 3T3-L1 Cells: A Mechanism of Adipocyte Defense from Excess Substrate. *PLoS One* **10,** e0139502 (2015).

116. Tchoukalova, Y. D. *et al.* In vivo adipogenesis in rats measured by cell kinetics in adipocytes and plastic-adherent stroma-vascular cells in response to high-fat diet and thiazolidinedione. *Diabetes* **61,** 137–44 (2012).

117. *Laforest, S., Labrecque, J., Michaud, A., Cianflone, K. & Tchernof, A. Adipocyte size as a* determinant of metabolic disease and adipose tissue dysfunction. *Critical Reviews in Clinical Laboratory Sciences* **52,** 301–13 (2015).

118. Church, C., Berry, R. & Rodeheffer, M. S. Isolation and study of adipocyte precursors. *Methods Enzymol.* **537,** 31–46 (2014).

119. Londos, C., Honnor, R. C. & Dhillon, G. S. cAMP-dependent protein kinase and lipolysis in rat adipocytes. III. Multiple modes of insulin regulation of lipolysis and regulation of insulin responses by adenylate cyclase regulators. *J. Biol. Chem.* **260,** 15139–45 (1985).

120. Azain, M. J., Kasser, T. R. & Baile, C. A. A method for the determination of glucose synthesis in isolated bovine hepatocytes. *J. Nutr. Biochem.* **10,** 205–9 (1999).

121. Schmidt, S. P., Smith, J. A. & Young, J. W. Rapid determination of (carbon-14) glucose specific radioactivity for in vivo glucose kinetics. *J. Dairy Sci.* **58,** 952–6 (1975).

122. Dow Water Solutions DOWEX™ Fine Mesh Spherical Ion Exchange Resins For Fine Chemical and Pharmaceutical Column Separations.

123. Preventing Biological Growth on Ion Exchange Resins. (2016).

124. Crespi, C., Gastalver, G., Palou, A. & Roca, P. Enzymatic determination of carbon (14C)-labeled glycerol in biological samples. *J. Biochem. Biophys. Methods* **30,** 179–83 (1995).

125. Hayashi, S. I. & Lin, E. C. Purification and properties of glycerol kinase from Escherichia coli. *J. Biol. Chem.* **242,** 1030–5 (1967).

126. Schomburg, Dietmar, Salzmann, M. *The Enzyme Handbook 7*. (Springer-Verlag, 1994).

127. MICHELET, L. *et al.* Down-regulation of catalase activity allows transient accumulation of a hydrogen peroxide signal in *Chlamydomonas reinhardtii*. *Plant. Cell Environ.* **36,** 1204–1213 (2013).

128. Brooks, G. The lactate shuttle during exercise and recovery. *Med. Sci. Sports Exerc.* **18,** 360–368 (1986).

129. M. Digirolamo, F.D. Newby, J. L. Lactate production in adipose tissue: a regulated function with exta-adipose implications. *Faseb* **6,** 2405–2412 (1992).

130. Vergnes, L. & Reue, K. Adaptive Thermogenesis in White Adipose Tissue: Is Lactate the New Brown(ing)? *Diabetes* **63,** 3175–3176 (2014).

133. Hui, S. et al. Glucose feeds the TCA cycle via circulating lactate. Nature 551, 115–118 (2017).

132. Brooks, G. A. Cell-cell and intracellular lactate shuttles. *J. Physiol.* **587,** 5591–5600 (2009).

133. Nocito, L. *et al.* The Extracellular Redox State Modulates Mitochondrial Function, Gluconeogenesis, and Glycogen Synthesis in Murine Hepatocytes. *PLoS One* **10,** e0122818 (2015).

134. Vander Heiden, M. G., Cantley, L. C. & Thompson, C. B. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science.* **324,** 1029–1033 (2009).

135. Schurr, A. & Payne, R. S. Lactate, not pyruvate, is neuronal aerobic glycolysis end product: An in vitro electrophysiological study. *Neuroscience* **147,** 613–619 (2007).

136. Nagao, H. *et al.* Increased dynamics of tricarboxylic acid cycle and glutamate synthesis in obese adipose tissue: In vivo metabolic turnover analysis. *J. Biol. Chem.* **292,** 4469–4483 (2017).

137. Passarella, S. *et al.* Mitochondria and l -lactate metabolism. *FEBS Lett.* **582,** 3569–3576 (2008).

138. Kishida, K. *et al.* Aquaporin Adipose, a Putative Glycerol Channel in Adipocytes. *J. Biol. Chem.* **275,** 20896–20902 (2000).

139. Nye, C. K., Hanson, R. W. & Kalhan, S. C. Glyceroneogenesis is the dominant pathway for triglyceride glycerol synthesis in vivo in the rat. *J. Biol. Chem.* **283,** 27565–27574 (2008).

140. Strawford, A., Antelo, F., Christiansen, M. & Hellerstein, M. K. Adipose tissue triglyceride turnover, de novo lipogenesis, and cell proliferation in humans measured with $^{2}H_{2}O$. *Am. J. Physiol. Metab.* **286,** E577–E588 (2004).

141. Roberts, R. *et al.* Markers of de novo lipogenesis in adipose tissue: associations with small adipocytes and insulin sensitivity in humans. *Diabetologia* **52,** 882–890 (2009).

142. Ballard, F. J. & Hanson, R. W. The citrate cleavage pathway and lipogenesis in rat adipose tissue: replenishment of oxaloacetate. *J. Lipid Res.* **8,** 73–9 (1967).

143. Patel, M. S., Jomain-Baum, M., Ballard, F. J. & Hanson, R. W. Pathway of carbon flow during fatty acid synthesis from lactate and pyruvate in rat adipose tissue. *J. Lipid Res.* **12,** 179–91 (1971).

144. Liu, Q., Siloto, R. M. P., Lehner, R., Stone, S. J. & Weselake, R. J. Acyl-CoA:diacylglycerol acyltransferase: Molecular biology, biochemistry and biotechnology. *Prog. Lipid Res.* **51,** 350–377 (2012).

145. Wilfling, F. *et al.* Triacylglycerol Synthesis Enzymes Mediate Lipid Droplet Growth by Relocalizing from the ER to Lipid Droplets. *Dev. Cell* **24,** 384–399 (2013).

146. Chitraju, C. *et al.* Triglyceride Synthesis by DGAT1 Protects Adipocytes from Lipid-Induced ER Stress during Lipolysis. *Cell Metab.* **26,** 407–418.e3 (2017).

147. Rosen, E. D. & Spiegelman, B. M. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* **444,** 847–853 (2006).

148. Brooks, G. A. Lactate production under fully aerobic conditions: the lactate shuttle during rest and exercise. *Fed. Proc.* **45,** 2924–9 (1986).

149. Pavlides, S. *et al.* The reverse Warburg effect: Aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. *Cell Cycle* **8,** 3984–4001 (2009).

150. Marshall, S., Olefsky, J. M. & We, a B. S. T. R. a C. T. Effects of Insulin Incubation on Insulin Binding , Glucose Transport , and Insulin Degradation by Isolated Rat Adipocytes. *J. Clin. Investig.* **66,** 763–772 (1980).

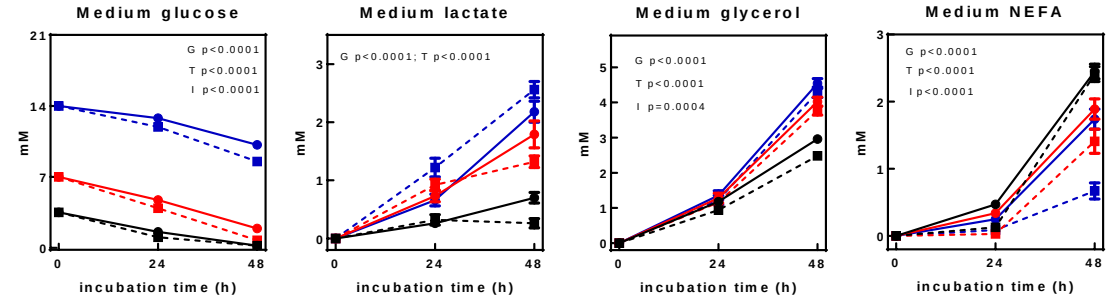
151. Salans, L. B., Knittle, J. L. & Hirsch, J. The role of adipose cell size and adipose tissue insulin sensitivity in the carbohydrate intolerance of human obesity. *J. Clin. Invest.* **47,** 153–165 (1968).

152. Matveyenko, A. V *et al.* Pulsatile portal vein insulin delivery enhances hepatic insulin action and signaling. *Diabetes* **61,** 2269–79 (2012).

ANEXOS

Metabolites levels in medium

A Adipocytes



B Stromal Vascular Fraction

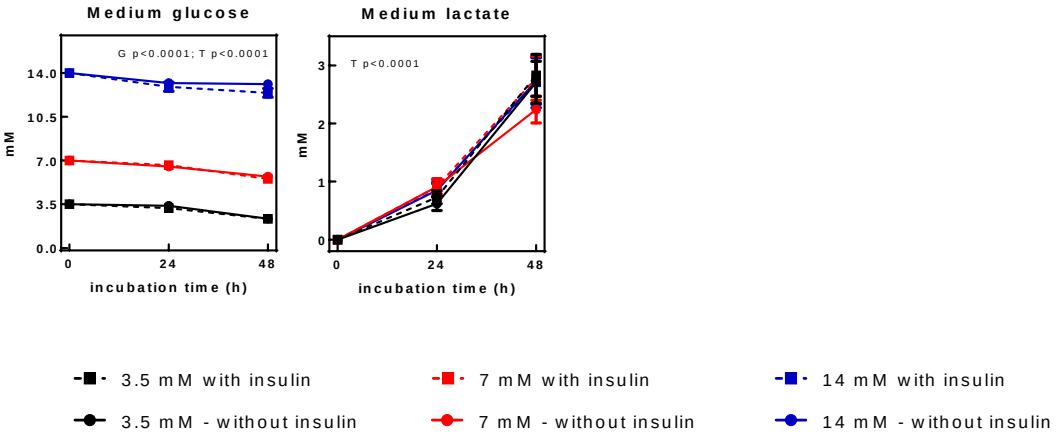


FIGURA A: Efectos de la concentración de glucosa, tiempo de incubación e insulina en la producción de glucosa, lactato, glicerol y NEFA en adipocitos y fracción estromal aislados a partir de tejido adiposo blanco epididimal de rata. Los datos de glicerol y NEFA corresponden sólo a los adipocitos ya que la producción de estos en la fracción estromal fue mínima. Cada pozo contenía aproximadamente 6,32 x 10^5 adipocitos equivalentes a 0,34 gramos de tejido adiposo blanco. Las células fueron incubadas por 24 o 48 horas con insulina (líneas punteadas) y sin insulina (líneas continuas). El color negro corresponde a la concentración inicial de glucosa de 3,5mM, el rojo a 7mM y el azul a 14mM. Los datos representan media ± sem de 12 ratas diferentes. Se realizó un análisis factorial para ver las diferencias estadísticas entre grupos. Sólo se muestran los valores significativos (p≤0,05). La letra G corresponde a las diferencias en la concentración inicial de glucosa, T al tiempo e I a la insulina.

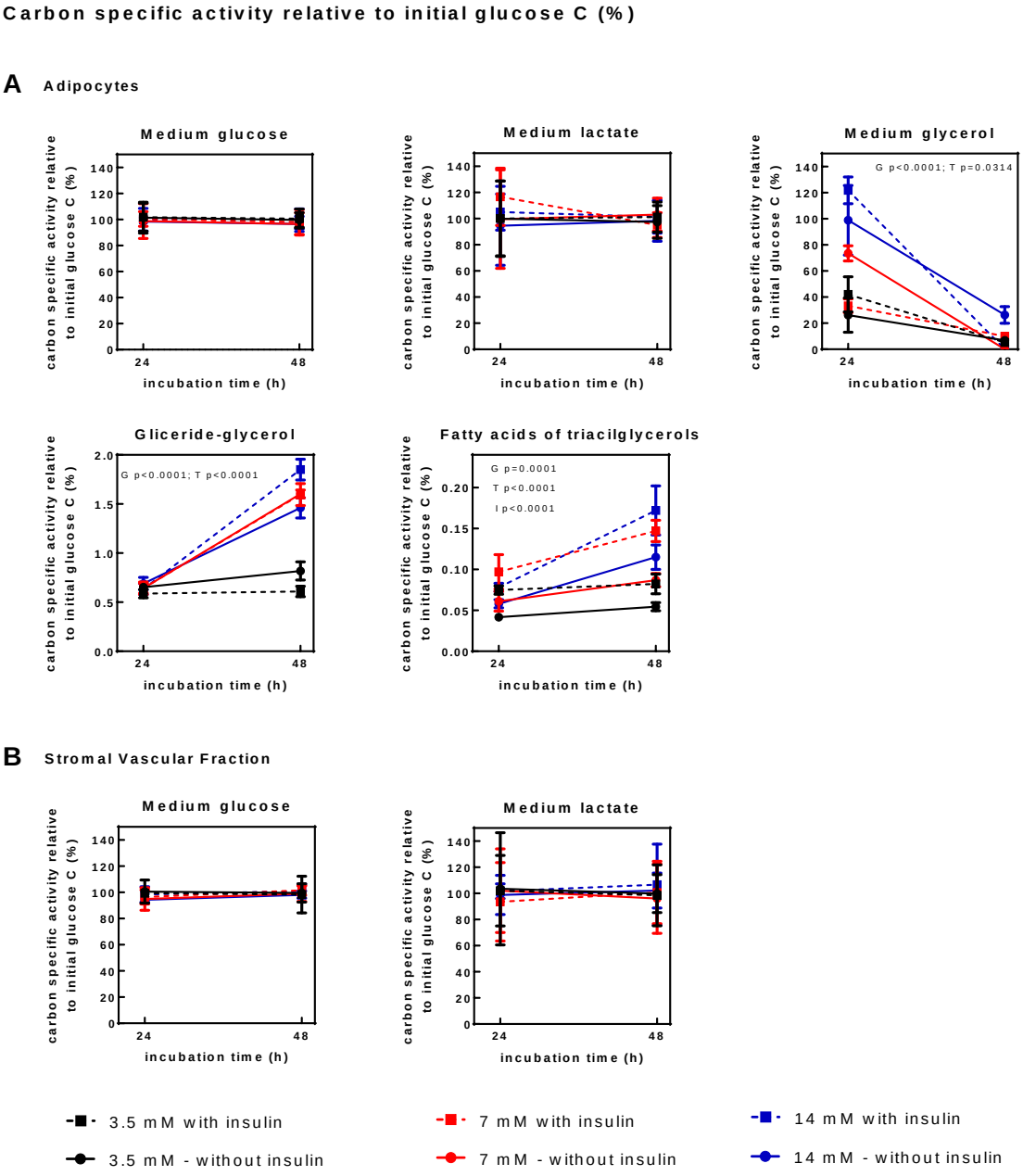


FIGURA B: Porcentajes de las actividades específicas de carbono respecto a la inicial de glucosa de las principales fracciones radiactivas de adipocitos y fracción estromal obtenidas después de 24 o 48 horas de incubación en presencia o ausencia de insulina y con ^{14}C glucosa. Las actividades específicas por carbono corresponden al cociente de la radiactividad encontrada en las fracciones y la concentración molar por carbono que contiene cada compuesto. En este caso, la actividad específica por carbono inicial de glucosa es 100%. Los datos representan media ± sem de 6 ratas diferentes. Se realizó un análisis factorial para ver las diferencias estadísticas entre grupos. Sólo se muestran los valores significativos ($p \leq 0,05$). La letra G corresponde a las diferencias en la concentración inicial de glucosa, T al tiempo e I a la insulina. Los convencionalismos utilizados son los mismos que los de la figura A.

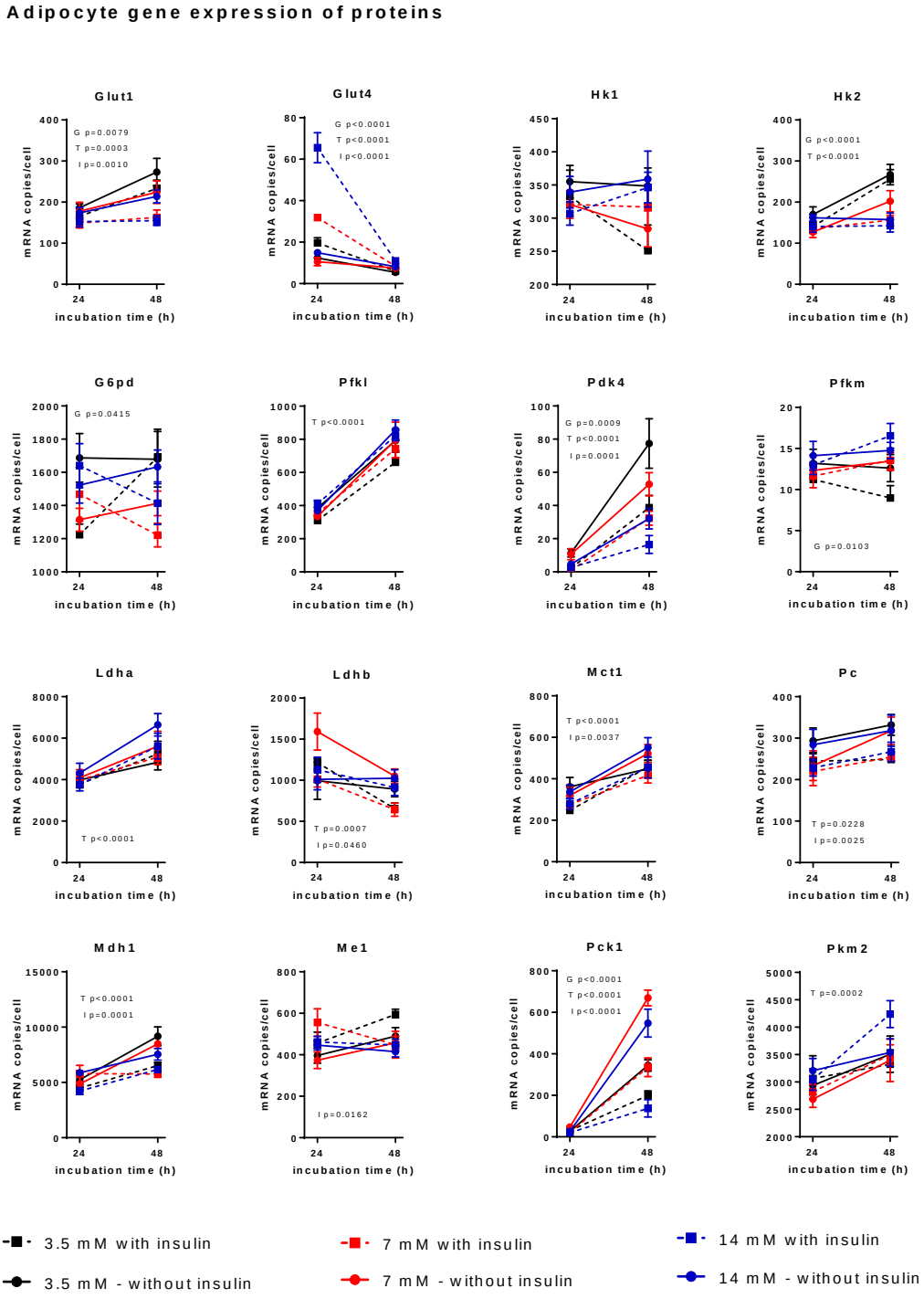


FIGURA C: Expresión génica de las principales enzimas relacionadas con la captación de glucosa y glucólisis del metabolismo de los adipocitos incubados 24 o 48 horas en presencia o ausencia de insulina y con ^{14}C glucosa. Los datos representan media ± sem de 6 ratas diferentes. Se realizó un análisis factorial para ver las diferencias estadísticas entre grupos. Sólo se muestran los valores significativos (p≤0,05). La letra G corresponde a las diferencias en la concentración inicial de glucosa, T al tiempo e I a la insulina. Los convencionalismos utilizados son los mismos que los de la figura A.

Adipocyte gene expression of proteins

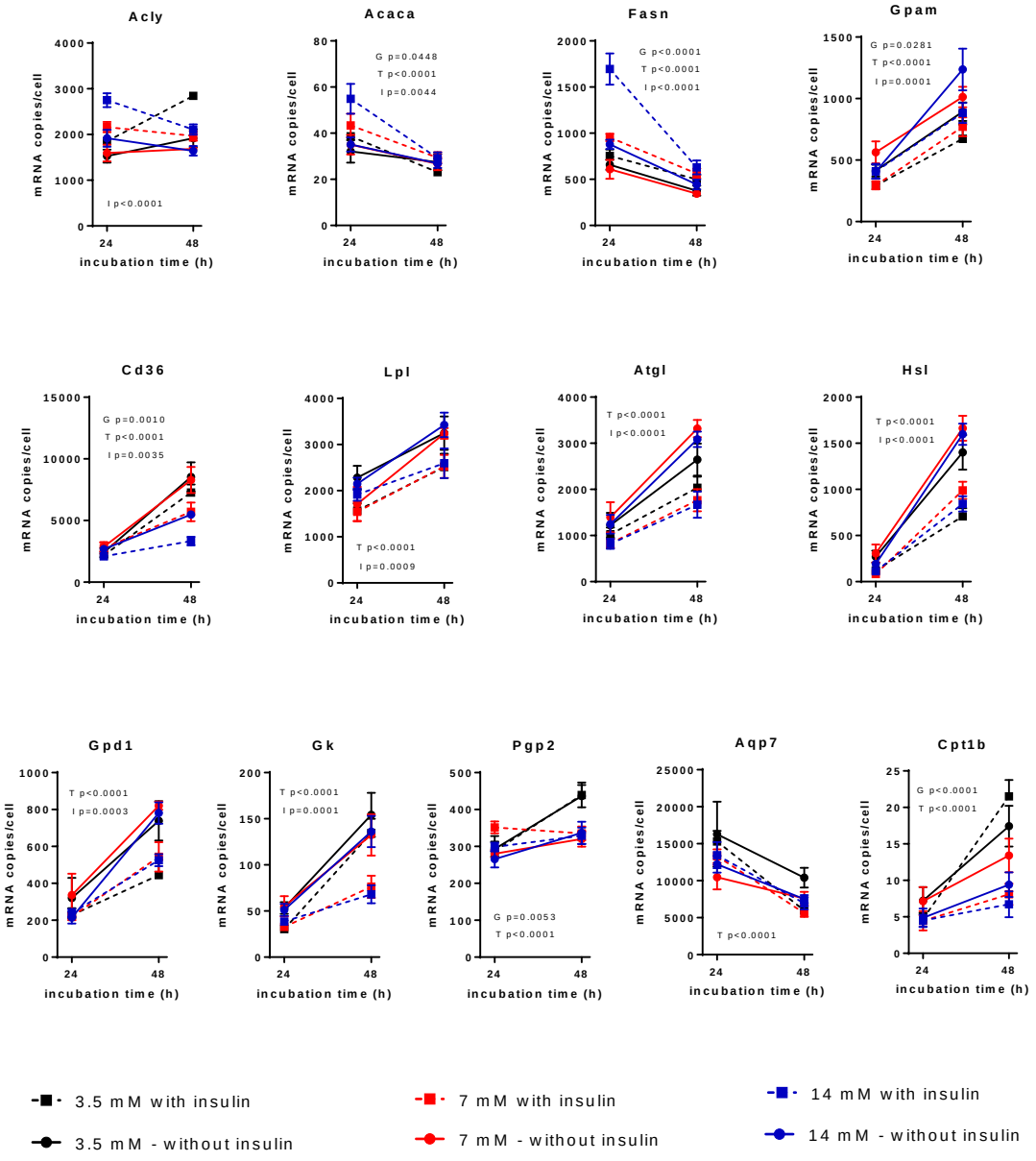


FIGURA D: Expresión génica de las principales enzimas relacionadas con la síntesis o degradación de triacilgliceroles del metabolismo de los adipocitos incubados 24 o 48 horas en presencia o ausencia de insulina y con ^{14}C glucosa. Los datos representan media ± sem de 6 ratas diferentes. Se realizó un análisis factorial para ver las diferencias estadísticas entre grupos. Sólo se muestran los valores significativos (p≤0,05). La letra G corresponde a las diferencias en la concentración inicial de glucosa, T al tiempo e I a la insulina. Los convencionalismos utilizados son los mismos que los de la figura A.

OTRAS PUBLICACIONES

(Pendientes)

Effect of sex on glucose handling by adipocytes isolated from rat subcutaneous, mesenteric and perigonadal adipose tissue

Floriana Rotondo [1], **Ana Cecilia Ho-Palma** [1], **Xavier Remesar** [1, 2, 3], **José Antonio Fernández-López** [1, 2, 3], **María del Mar Romero** [1, 2, 3], **Marià Alemany** Corresp. [1, 2, 3]

[1] Department of Biochemistry and Molecular Biomedicine, University of Barcelona, Faculty of Biology, Barcelona, Spain

[2] Institute of Biomedicine, University of Barcelona, Barcelona, Spain

[3] CIBER OBN, Centro de Investigación Biomédica en Red: Obesidad y Nutrición, Barcelona, Spain

Corresponding Author: Marià Alemany
Email address: malemany@ub.edu

Background. Adult rat epididymal adipocytes covert large amounts of glucose to lactate and glycerol. However, fatty acids efflux is much lower than that expected from glycerol/lipolysis. Use of glucose for lipogenesis is limited, but glycolysis to lactate and other 3-carbon substrates is highly active. In this study, we analyzed whether sex and white adipose tissue (WAT) site may affect the massive utilization of glucose to release of 3-carbon derivatives.

Methods. Mature adipocytes from perigonadal, mesenteric and subcutaneous WAT of female and male rats were isolated, and incubated with 7 or 14 mM glucose during 1 or 2 days. Glucose consumption, metabolite efflux and gene expression of glycolytic and lipogenesis-related genes were measured.

Results. The effects of glucose concentration were minimal on most parameters studied. Sex-induced differences, however, were more extensive.The most marked, distinct, effects observed, were related to the time of incubation. The production of lactate was maintained with time, but that of glycerol increased, shifting from a largely glycolytic origin to its circuitous liberation from triacylglycerols (TAG); i.e. its incorporation was concurrent with TAG selective turnover: lipolytic glycerol was secreted while most fatty acids were recycled. Fatty acid efflux increased with incubation but was, nevertheless, minimal compared with glycerol. Production of lactate and glycerol from glucose was maximal in mesenteric WAT.

Discussion. Female rats showed higher adipocyte metabolic activity than males. In mesenteric WAT, gene expression data (and substrate efflux) suggested that adipocyte oxidation of pyruvate to acetyl-CoA was higher than in males, with enhanced return of oxaloacetate to the cytoplasm for its final conversion to lactate. WAT site differences showed marked tissue specialization-related differences. Utilization of glucose for lipogenesis was seriously hampered when TAG turnover-related lipolysis was activated. We postulate that these mechanisms may help decrease glycaemia and fat storage, producing, instead, a higher availability of less-regulated 3-carbon substrates, used for energy elsewhere.

frontiers

In rat white adipose tissue, lactate production from glucose by adipocytes and nucleated stromal cells is quantitatively comparable, but only adipocytes also release glycerol

Floriana Rotondo [1], Ana-Cecilia Ho-Palma [1], María del Mar Romero [123], Xavier Remesar [123], José Antonio Fernández-López [123] and Marià Alemany [123]*

[1]Department of Biochemistry and Molecular Biomedicine, Faculty of Biology University of Barcelona, Barcelona, Spain

[2] Institute of Biomedicine, University of Barcelona, Barcelona, Spain

[3] CIBER-OBN Research Web, Barcelona, Spain

Abstract

In white adipose tissue (WAT), the global importance of nucleated stromal cells (NSC) has been widely recognized, especially in metabolic regulation, defense, regeneration and WAT control via hormones and cytokines. NSC have different origins, functions and proportions in WAT sites, and change modified by inflammation, starvation and other physiological or pathological conditions. When referring to WAT, the overwhelming size and understood function of adipocytes almost completely obscure the quantitative metabolic contribution of all NSC,. In this study we have analyzed subcutaneous, mesenteric and perigonadal WAT from female and male adult rats. We analyzed separately adipocytes, red blood cells, and NSC. Their ability to use glucose as substrate and produce lactate, glycerol, and fatty acids was measured in front of 7 or 14 mM glucose for 24 and 48 h. The results from these fractions were computed, taking into account their quantitative presence in the original intact healthy mature WAT. More than 2/3rds of WAT cells were erythrocytes, less than 10 % adipocytes and the rest NSC, the contribution of blood cells to lactate production was minimal. NSC produced more lactate than adipocytes as a rule, but only adipocytes secreted glycerol and small amounts of free fatty acids. Glucose consumption was also highest in the NSC fraction. Mesenteric WAT produced more lactate and glycerol than the other sites, but the preeminence of NSC lactate production over adipocytes was maintained in the three sites with little influence of sex. We conclude that the diverse and shifting NSC fraction of WAT has a practically anaerobic metabolism (as in adipocytes), based on glycolysis and steady release of lactate, but its overall quantitative contribution was higher than that of all adipocytes, contributing to the glucose wasting function of WAT. We also concluded that glycerol is the exclusive product of adipocytes, and postulate that its fate is probably to provide energy to the brain, avid consumer of this polyol, and lactate as energy substrates. The differences between WAT sites, with mesenteric WAT taking a key role, are probably related to glucose disposal, but especially to glycerogenesis.

Keywords

Adipose Tissue, Glycolysis, Lactate, Glycerol, Adipocytes, Stromal Cells, glycemia.

Author contribution statement

MA and MMR designed the study. FR, ACHP and MMR did the experimental work. JAFL and XR processed the data and did the statistical analyses. MA wrote the paper draft, which was critically revised by XR, JAFL, MMR, FR and ACHP.