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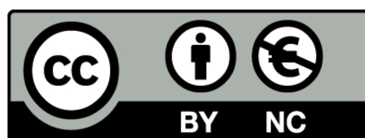
Glucose and white adipose tissue metabolism. Effects of site and sex on the fate of glucose

Floriana Rotondo



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UNIVERSITAT DE BARCELONA

FACULTAT DE BIOLOGIA

PROGRAMA DE DOCTORAT EN BIOMEDICINA

Glucose and white adipose tissue metabolism. Effects of site and sex on the fate of glucose

Memòria presentada per **Floriana Rotondo** per optar al títol de doctor per la Universitat de Barcelona

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INDEX

ABBREVIATIONS 7
1. ABSTRACT 9
2. INTRODUCTION 13
2.1. Evolution of the concept of Metabolic Syndrome 13
2.2. The Metabolic Syndrome as a disease of affluence 16
2.3. Insulin resistance, its consequences, and type 2 diabetes 18
2.3.1. Insulin actions 18
2.3.2. Insulin resistance: a defense mechanism 19
2.3.3. Type 2 diabetes, or diabetes mellitus 20
2.4. Dyslipidemia and hepatic steatosis 21
2.5. Obesity 23
2.5.1. The problem of defining and measuring obesity 23
2.5.2. The ponderostat system 24
2.5.3. Obesity as a disorder of the ponderostat function 26
2.5.4. Obesity and its pathologic consequences 27
2.5.5. The "obesity paradox" 29
2.6. Adipose tissue and inflammation 30
2.6.1. The adipose organ 30
2.6.2. White adipose tissue metabolism 31
2.6.3. Inflammation 34
2.6.4. Chronification of inflammation 35
2.6.5. The glucocorticoid response to inflammation 36
2.6.6. Hypoxia and inflammation 37
3. JUSTIFICATION AND OBJECTIVES 41
[Formal declaration of Autorship by the Thesis co-Directors] 43
4. RESULTS 45
4.1. Development of quantitative methodology 45

4.1.1. Presentation 45

A method for the measurement of lactate, glycerol and fatty acid production from ^{14}C-glucose in primary cultures of rat epididymal adipocytes 47

Quantitative analysis of rat adipose tissue cell recovery, and non-fat cell volume, in primary cell cultures 63

4.2. Adipose cells metabolism: glucose fate and 3C release 89

4.2.1. Presentation 89

Glycerol is synthesized and secreted by adipocytes to dispose of excess glucose, via glycerogenesis and increased acyl-glycerol turnover 91

Utilization 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 107

Influence of sex on substrate handling by adipocytes isolated from subcutaneous, mesenteric and perigonadal adipose tissue of rats 127

In rat white adipose tissue, lactate production by adipocytes and nucleated stromal cells quantitatively comparable, but only adipocytes also release glycerol 145

5. GENERAL DISCUSSION..165

5.1. Development and evaluation of the methodology 165

5.2. Application of methodology: glucose fate and 3C release..166

6. CONCLUSIONS 173

7. BIBLIOGRAFY 175

ABBREVIATIONS

ACEs: angiotensinogen and angiotensinogen-converting enzymes
BAT: brown adipose tissue
BMI: body mass index
CRP: C-reactive protein
DHEA: dehydroepiandrosterone
ER: endoplasmic reticulum
ET-1: endothelin 1
GC: glucocorticoids
GIP: gastric inhibitory polypeptide
GLP1: glucagon-like peptide-1
HDL: high-density lipoproteins
ICAM-1: intercellular adhesion molecule-1
IL: interleukin
iNOS: inducible nitric oxide synthase
IR: insulin resistance
IRS: insulin responsive substrates
LDL: low-density lipoproteins
MCP-1: monocyte chemoattractant protein
MetS: metabolic syndrome
NAFLD: non-alcoholic fatty liver disease
NEFA: free fatty acids
NO·: nitric oxide
PAI-1: plasminogen inhibitor/activator 1
PI3K: phosphatidyl-inositol 3-kinase
RAS: rat sarcoma protein
SH2: *src*-homology-2
T1DM: type 1 diabetes
T2DM: type 2 diabetes mellitus
TAG: triacylglycerol
Th2: T helper 2
TNF-α: tumor necrosis factor-alpha
UCP1: uncoupling protein 1
VCAM-1: vascular cell adhesion molecule-1
VLDL: very-low-density lipoproteins
WAT: white adipose tissue

1. ABSTRACT

The current theories about endothelial inflammation in the adipose tissue in metabolic syndrome point to hypoxia as one of its main causes. Nevertheless, our group has found that both 3T3L1 adipocytes, under normoxic conditions, and WAT, *in vivo*, consume large amount of glucose, with high production of lactate and glycerol irrespective of oxygen availability.

This thesis is based on the hypothesis that adipocytes act essentially as glycolytic cells impervious to hypoxia, and their metabolism may help reduce the blood glucose levels. We assumed that hypoxia, could affect the cells of the stromal fraction, eliciting an inflammatory response. To this purpose, we have studied *ex vivo* the glycolytic and anaerobic capacity of adult adipocytes and stromal vascular cells of both sex and obtained from different WAT sites, developing the methodology needed for a quantitative comparative analysis of data obtained from the same cultured cells well.

We found that adipocytes, despite being the cells present in WAT in lower numbers, occupied almost the whole volume of the tissue, a consequence of their huge size due to their inert fat vacuole. The overall “live cell" volume represented only about 1.5% of the tissue, thus showing a very high metabolic activity of WAT in relative terms.

Adipocytes *ex vivo* incubated with glucose, also took large amounts of the sugar, irrespective of its concentration, releasing instead 3C metabolites, such as lactate and glycerol to the medium. Lactate was fully derived from glucose and was produced at a steady pace, irrespective the presence of oxygen, to form the ATP needed for cell functions. Glycerol efflux increased over time and its origin shifted from glycolitic to glycolytic-lipolytic: new-formed glycerol was incorporated into TAG, by esterification with acyl-CoA, derived from the same TAG lipolisis. The coexistence of these processes appears as a “futile cycle”, with the probable function further to waste excess energy. Lipogenesis was limited because the size of cells limits the access to oxidative mitochondrial pathways. The release of 3C metabolites seems to be a mechanism to lower glycemia, defend WAT against excess of substrate, and provide 3C fragments as more accessible substrates for other tissues.

Mesenteric WAT adipocytes presented the highest metabolic activity, probably to help the hepatic handling of NEFA and reduce the flow of intestinal glucose to the liver. While in almost all WAT sites, excess mitochondrial pyruvate is returned to the cytoplasm to keep forming lactate; in female mesenteric adipocytes it is in part oxidized to acetyl-CoA to fuel lipogenesis.

Stromal vascular cells also released lactate, even more than adipocytes per unit of tissue weight, but not glycerol nor NEFA. Red blood cells produced lactate, but its contribution was quantitatively minimal. Thus, stromal cells, acted in consonance with adipocytes, in all sites and sexes examined, wasting glucose in an anaerobic way, producing high amounts of 3C units. Thus, reinforcing the idea that WAT may be an active protagonist both in energy handling and in the body control of glycemia.

RESUM

Les teories actuals sobre la inflamació al teixit adipós pròpies de la síndrome metabòlica assenyalen la hipòxia com una de les seves principals causes. No obstant això, el nostre grup ha trobat que els adipòcits 3T3L1, en condicions normòxiques i el TAB, *in vivo*, consumeixen molta glucosa, lliurant una elevada producció de lactat i glicerol, independentment de la disponibilitat d'oxigen.

Aquesta tesi es basa en la hipòtesi que els adipòcits actuen essencialment com a cèl·lules glucolítiques resistents a la hipòxia, i que el seu metabolisme pot ajudar a reduir els nivells de glucosa en sang. Suposàvem, però que la hipòxia podria afectar les cèl·lules de la fracció estromal, provocant una resposta inflamatòria. Amb aquest propòsit, hem estudiat la capacitat glucolítica i anaeròbica d'adipòcits adults i cèl·lules estromals, obtinguts de diferents localitzacions del TAB, desenvolupant la metodologia necessària per a una anàlisi comparativa quantitativa de dades obtingudes dels mateixos pous de cultiu.

Vam trobar que els adipòcits, tot i ser les cèl·lules presents menys abundoses al TAB, ocupaven gairebé tot el volum del teixit, com a conseqüència de la seva grandària deguda a la vacuola de greix inert. Quant al volum de tot el, la "part viva", representava sols un 1,5%, aproximadament, demostrant així una activitat metabòlica del TAB molt elevada en termes relatius.

Els adipòcits *ex vivo* incubats amb glucosa també van prendre grans quantitats del sucre, independentment de la seva concentració, alliberant al seu torn metabòlits 3C, com lactat i glicerol. El lactat es va generar completament de la glucosa, a un ritme constant i independentment de la presència d'oxigen, per formar l'ATP necessari per a les funcions cel·lulars. El lliurament de glicerol, però va augmentar amb el temps i el seu origen es va desplaçar de glucolític a glucolític-lipolític; és a dir el glicerol de nova formació es va incorporar als TAG, per esterificació amb acil-CoA, derivat de la mateixa lipòlisi dels TAG. La coexistència d'aquests processos es pot presentar com un "cicle fútil", amb la probable funció de malbaratar l'excés d'energia. La lipogènesi estava limitada perquè la mida de les cèl·lules dificulta l'accés a les vies mitocondrials oxidatives. L'alliberament de metabòlits 3C sembla ser un mecanisme per reduir la glucèmia, defensar el TAB contra l'excés de substrats i proporcionar fragments 3C com a substrats més accessibles per a altres teixits.

Els adipòcits mesentèrics del TAB van presentar l'activitat metabòlica més elevada, probablement per ajudar a la utilització hepàtica dels àcids grassos i reduir-ne el flux de glucosa intestinal. Mentre que en gairebé tots els llocs del TAB, l'excés de piruvat mitocondrial es retornava al citoplasma per seguir formant lactat; En els adipòcits de les femelles, s'oxidava en part a l'acetil-CoA per sostenir la lipogènesi.

Les cèl·lules vasculars estromals també van produeir lactat, fins i tot més que adipòcits, per unitat de pes del teixit, però no glicerol ni àcids grassos. Els glòbuls vermells van produir lactat, però la seva contribució va ser mínima. D'aquesta manera, les cèl·lules estromals van actuar en consonància amb els adipòcits, en tots els llocs examinats i en ambdós sexes, malbaratant la glucosa de forma anaeròbica, produint grans quantitats d'unitats 3C. D'aquesta manera, es reforça la idea que el TAB pot ser un protagonista actiu, tant en la gestió energètica com en el control de la glucèmia de tot el cos.

RESUMEN

Las teorías actuales sobre la inflamación endotelial en el tejido adiposo en contexto del síndrome metabólico apuntan a la hipoxia como una de sus principales causas. Sin embargo, nuestro grupo ha encontrado que tanto los adipocitos 3T3L1, bajo condiciones normóxicas, como el TAB *in vivo*, consumen grandes cantidades de glucosa, con una elevada producción de lactato y glicerol, independientemente de la disponibilidad de oxígeno.

La presente tesis se basa en la hipótesis de que los adipocitos actúan esencialmente como células glucolíticas inmunes a la hipoxia, y su metabolismo puede ayudar a reducir los niveles de glucosa en sangre. Supusimos que la hipoxia podría afectar las células de la fracción estromal, provocando una respuesta inflamatoria. Para este fin, hemos estudiado *ex vivo* la capacidad glucolítica y anaeróbica de adipocitos adultos y células estromales de ambos sexos y obtenidos de diferentes ubicaciones del TAB, desarrollando la metodología necesaria para un análisis cuantitativo comparado de los datos obtenidos de los mismos pocillos de células cultivadas.

Hallamos que los adipocitos, a pesar de ser las células presentes en menor número en el TAB, ocupaban casi todo el volumen del tejido, por su gran tamaño debido a su enorme vacuola de grasa inerte. El volumen total de la parte "viva" del tejido, representaba solo aproximadamente el 1,5% del total, mostrando así el TAB una actividad metabólica muy elevada en términos relativos.

Los adipocitos *ex vivo* incubados con glucosa, también captaban grandes cantidades del azúcar, independientemente de su concentración; liberando al medio, a cambio, metabolitos de 3C, como lactato y glicerol. El lactato procedía en su totalidad de la glucosa y se liberaba a un ritmo constante e independientemente de la presencia de oxígeno, para formar el ATP necesario para las funciones celulares. El eflujo de glicerol aumentó con el tiempo y su origen cambió de glucolítico a glucolítico-lipolítico; así el glicerol neoformado se incorporaba a los TAG por esterificación con acil-CoA, reciclado de la misma lipolisis de los TAG. La coexistencia de estos procesos aparece como un "ciclo fútil", con la función probable de seguir desperdiciando el exceso de energía. La lipogénesis venía limitada por el propio tamaño de las células, que dificulta el acceso a las vías mitocondriales oxidativas. La liberación de metabolitos 3C parece ser un mecanismo para disminuir la glucemia, defender a TAB del exceso de sustratos y también proporcionar sustratos energéticos más accesibles para otros tejidos.

Los adipocitos del TAB mesentérico presentaban la más alta actividad metabólica; probablemente para favorecer la utilización hepática de los ácidos grasos y reducir el flujo de glucosa intestinal. Mientras que en casi todas las localizaciones, el exceso de piruvato mitocondrial regresaba al citoplasma para seguir formando lactato; en los adipocitos de las hembras se oxidaba en parte a acetil-CoA para sostener la lipogénesis.

Las células estromales también liberaban lactato, incluso más que los adipocitos, por unidad de peso del tejido, pero no liberaban glicerol ni ácidos grasos. Los glóbulos rojos produjeron lactato, pero su contribución fue mínima. En definitiva, las células estromales, actuaban en consonancia con los adipocitos, en todas las localizaciones estudiadas y en ambos sexos, rompiendo la glucosa de forma anaeróbica, y produciendo grandes cantidades de unidades 3C. Estos resultados refuerzan la idea de que el TAB puede ser un protagonista activo tanto en el manejo de la energía como en el control global de la glucemia.

2. INTRODUCTION

2.1. Evolution of the concept of Metabolic Syndrome

The word syndrome derives from the Greek σύνδρομον [σύν-(together) and δρομον (running)], literally 'running together, i.e. 'concurrence'. Unlike a disease, characterized by a specific and identifiable pathogenic origin or an alteration of a physiological condition, a syndrome is the association of several pathologies, diseases, or its markers (symptoms), for which the direct and common unique causative agent, process, or mechanism has not been found or is poorly understood; but neither its existence is excluded, since the association is proven [1,2].

The idea of metabolic syndrome (MetS) began its development in the 1920s when an association between hypertension, hyperglycemia and gout was described by Kylin [3]. In 1947, Vague established the implication of sex hormones in obesity [4] and in 1956 found a possible link between gout, visceral obesity, atherosclerosis and diabetes [5]. In 1967, Avogaro and Crepaldi introduced the idea of a multifaceted syndrome, which linked hyperlipidemia, diabetes and obesity [6]. Twenty years later Modan proposed hyperinsulinaemia as the key link between hypertension, obesity and glucose intolerance [7]. This pathological association was then presented as *syndrome X*, a name proposed in 1988 by Reaven, who described it as a triad of diabetes hypertension and cardiovascular risks. Reaven also introduced the concept of insulin resistance (IR) and its centrality in the etiology of diabetes mellitus [8]. Following Reaven's description, the syndrome became a major theme of (mainly clinical) research and medical debate. Shortly afterwards, Kaplan connected the upper body obesity disorder to glucose intolerance, hyperlipidemia and hypertension and named the syndrome as *the deadly quartet* [9]. Different cluster associations of diseases using different names have been also proposed by different authors, as reported in Table 1. Before Kaplan the syndrome was named *metabolic trisyndrome* by Camus [10], *plurimetabolic syndrome* was the denomination proposed by Avogaro et al. [6] , Mehnert and Kuhlmann referred to it with the evoking term *syndrome of affluence* [11]. This variable cluster of diseases, also known as *cardiovascular dysmetabolic syndrome* [12], or *dysmetabolic syndrome* [13], was finally fixed in *metabolic syndrome* in 1999, when the World Health Organization (WHO) Diabetes Group published a clinical definition following, generally, the criteria described by others [14]; they used this name, already coined in 1977 by Haller [15], Singer [16], and later, in 1981 by Hanefeld at al. [17].

From 1999 onwards, a large number of clinical definitions, based on the compliance of a number of clinical parameters established largely from quantitative measures were presented by different Medical Societies, Health Organizations and Committees, which defined MetS as a constellation of interconnected physiological, biochemical, clinical, and metabolic factors.

authors	year	name	diabetes/insulin resistance	obesity	hypertension	dislipidemias	atherosclerosis	hyperuricemia/gout	hepatic steatosis	cardio-vascular risk	calculi	sex hormone implication	micro-albuminuria	inflammation
Joslin [18]	1921		X		X									
Hitzenberger [19]	1921		X		X									
Kylin [20]	1921		X		X									
Marañón [21]	1922		X		X									
Kylin [3]	1923	hypertension-hyperglucemia-hyperuricemia syndrome	X		X			X						
Vague [4]	1947			X								X		
Vague [5]	1956		X	X			X	X			X			
Camus [10]	1966	metabolic trisyndrome	X			X		X						
Avogaro et al. [6]	1967	plurimetabolic syndrome	X	X		X								
Mehnert & Kuhlmann [11]	1968	syndrome of affluence	X		X									
Haller [15]	1977	metabolic syndrome	X	X	X	X	X	X	X					
Singer [16]	1977	metabolic syndrome	X		X			X						
Philips [22]	1977		X	X	X	X				X		X		
Hanefeld et al.[17]	1981	metabolic syndrome	X	X	X	X								
Modan [7]	1985		X	X	X									
Reaven [8]	1988	syndrome X	X		X					X				
Kaplan [9]	1989	the deadly quartet	X	X	X					X				
de Fronzo & Ferrannini [23]	1991	insulin resistance syndrome	X	X	X	X	X			X				
Fagan [12]	1998	cardiovascular dysmetabolic syndrome	X	X	X	X								
Groop & Ortho-Melander [13]	2001	dysmetabolic syndrome	X	X	X	X							X	
Grundy et al. [24]	2004		X	X	X	X								
Reisin & Alpert [25]	2005		X	X	X	X		X						X

Table 1. The evolution of MetS concept and its proposed names.

Main pathologies	Other (or derived) pathologies
Insulin resistance	decreased sensitivity to glucose/ low peripheral tissue glucose uptake type 2 diabetes Alzheimer disease
Hyperlipidemia/ dislipoproteinemia	small, dense LDLs hypercholesterolemia and low HDL cholesterol hypertriacylglycerolemia high ApoB levels large proportions of oxidized lipoproteins
Hepatic steatosis and hepatomegalia	altered hepatic function: hyperbilirubinemia; Increased enzyme leakage altered antioxidant mechanisms altered xenobiotic metabolism
Increased oxidative damage	Increase oxidative damage because of increased free radicals, superoxide, peroxynitrite, etc. increased synthesis and disposal of nitric oxide increased nitrite and nitrate excretion
Hyperuricemia/gout	inflammatory arthritis
Arterial hypertension	increased peripheral blood flow resistance atherosclerosis/ increased vascular micro-damage and enhanced plaque formation altered rheological behavior of red blood cells/ rigidity (and fragility) of red blood cells
Increased cardiovascular risk	atrial fibrillation altered blood coagulation pulmonary resistance/ respiratory insufficiency heart insufficiency higher incidence of ictus
WAT inflammation	obesity/visceral or upper body obesity adipocyte hyperplasia and proliferation high proportion of non-adipocyte cells/ massive macrophage infiltration in WAT altered blood flow/ hypoxia high leptin levels: leptin resistance low adiponectin/ high resistin altered interleukin-6 and adipokine signaling
Altered immune response	asthma psoriasis / *acanthosis nigricans* and other dermic diseases other autoimmune diseases
Sleep apnea	
Altered hypothalamic-pituitary-adrenals axis function	hypercortisolism / Cushing disease-like states disappearance of daily functional rhythms altered thimic states altered gonadotropin secretion, infertility
Altered sex hormone metabolism and function	polycystic ovary syndrome male hypoandrogenism decreased dehydroepiandrosterone production and levels decreased estrogen protection
Altered nervous system functions	decreased cognoscitive abilities, dementia higher incidence of psychiatric alterations depression higher autonomic nervous system activity (selective) peripheral nerve damage, polyneuritis (secondary) eating disorders/ binge eating/ orthorexia, anorexia nervosa
Altered composition of the microbiota	altered immune system control of the biota/presence of nitrate and nitrite increased LPS levels/interaction with the biota
Increased incidence of some types of cancer	colonic, endometrial, renal cell, gallbladder, and upper digestive tracts carcinomas some types of breast and hepatic cancer

Table 2. Main pathology traits associated with the MetS [26].

There is no consensus, yet, on the amplitude of the MetS concept, and, depending on each author's point of view, may include just the most commonly accepted pathologies: insulin resistance, obesity, hyperlipidemia and hypertension; or extend the list to encompass a larger cluster of factors (up to the full list of co-morbidities, shown in Table 2). In general, these pathologies point to an increased risk for the development of cardiovascular disease, type 2 diabetes mellitus (T2DM) and earlier mortality [25,27–29]. Indeed cardiovascular disease mortality has been found to 'increase' with the incorporation of more associated factors to the current MetS definitions [30].

Table 2 presents a list of co-morbidities associated with the MetS. It includes a high number of pathologic traits since we tried to include a list as complete as possible.

2.2. The Metabolic Syndrome as a disease of affluence

MetS has reached today epidemic proportions, affecting most adults in developing and developed countries, where the proportions of obese and overweight people are increasing during the last decades [31,32]. In the year 2000, for the first time, the number of adult humans with excess (according to the current medical view) weight was higher than that of underweight people in the whole world (according to statistical meta-analyses essentially used for information purposes) [33]. The obesity pandemic was first acknowledged in the USA, then in Europe and other formally rich countries, but extends through almost any corner of the world, largely through recently urbanized areas [34].

Today MetS is considered as one of the main causes of death after cancer, infectious disease and accident-related deaths, which nevertheless present higher rates and more immediate danger of death than the pathologies related to the syndrome [26]. MetS is a condition affecting mainly middle-aged and old people: its prevalence progressively increases to a maximum of 40% among those over 70 years [35]; considering that lifespan in rich countries is longer than in the poorer, death in MetS tends to occur at a more advanced age. However the status of MetS as a risk factor for mortality in the elderly population is disputed; since no significant difference in mortality was observed among subjects (with or without MetS) aged 70–79 years [36]. Given the complex interactions of chronic diseases with environmental/lifestyle factors, the effect of MetS on mortality seems to be attenuated by time [36]. The effects on mortality may become even less relevant when The MetS 'benefits', as is the “obesity paradox” [37], are take into account.

Although known as a *syndrome*, with all the constellation of co-morbidities listed above, MetS is increasingly considered to be just a multiform disease with a single basic common origin [1,26]. In fact today the syndrome is well known to be significantly associated with social

development, food availability, sedentarism and excess of body fat [27]. The alterations caused by the inordinate excess of some nutrients convert MetS in a true 'disease of affluence' [26], which has its roots in the development of the basic shared pathogenic mechanism [38] related to what we currently define as *inflammation* [26].

Diet is considered the principal factor responsible for the appearance of obesity and MetS. For eons, human diet has changed deeply; in prehistoric times, such as the Paleolithic, its content just allowed survival. Compared with the actual diets it was hypocaloric, short in lipids, with no salt, based on plant- and small animals-derived food [39]. In the present there is a wide variety (and even excess availability) of food, often rich in high quality protein, sugars, lipids and salt. These four components were always scarce in human history, and the craving for them (needed nutrients: salt, protein; taste: sugar; and high energy density: fats) was ingrained in our collective species 'list of desires'. The importance for these foods is written even in Holy Books (Bible, Quran), with the sweet "*manna"* in the desert or where paradise is described as a place with "*rivers of milk and honey*".

The access to food (in special, that of scarce foods: sweet, salty, proteinaceous and fatty) nowadays is no longer a problem, solved by human ingenuity through efficient agriculture and husbandry. In the present the real problem is posed by the excess of food/energy intake, which unbalances the finely tuned (along eons of evolution and adaptation) strategies for survival amidst scarcity of food and, especially the lack of specific nutrients. Our body, that along evolution developed mechanisms to face up starvation, cannot manage the excess of nutrients contained in our actual diet, and, especially, the 'unnatural' coexistence of excess energy in the form of lipids and glucose, which elicits insulin resistance, the main 'pathogenic' factor associated with MetS [40,41].

Besides lipids and glucose, under conditions of excess food intake, there is often a certain surplus of dietary amino acids. This does not constitute a problem with adequate energy intakes, but an excess of amino nitrogen may become a problem when combined with excess energy availability [42]. Both fatty acids and glucose are essentially energy-providing substrates, which availability spares the oxidation of amino acids [43]. The difficulty of obtaining sufficient dietary protein for growth, reproduction and turnover has made this scarce staple a 'treasured' substrate, which is only oxidized when available in sustained excess (e.g. in carnivorous animals) or when there is nothing else to oxidize (or use as gluconeogenic substrate), as in starvation[43–45]. In any case, amino acid sparing in the presence of abundant energy results in a decrease of the production/excretion of ammonium [46], glutamine trafficking and operation of the urea cycle [47]. The excess of N activates other, so far unknown pathways, which reduce the problem of amino or ammonium accumulation, as observed in nitrogen balances (N) [42]. The presence of large masses of white adipose tissue (WAT)

(obesity) compounds the problem because of its additional implication in amino acid metabolism, contributing to the provision of arginine-family amino acids, which facilitate the excess nitric oxide synthesis which characterize the MetS [42].

Nitric oxide (NO) affects differentially the survival of different groups of bacteria in the microbiota [48,49]. It is formed again in the alimentary canal because of the higher excretion of nitrite or, preferentially, nitrate through the saliva [50,51]. In this way and due to modifications in the immune system, handling of the gut barrier [52] and diet [53,54], MetS induces deep changes in the composition of the microbiota [55,56].

There are other co-morbidities linked to the MetS, but perhaps not clearly a consequence of feeding. They include complicated gallstone disease [57] and a few types of cancer. A statistical association between MetS with colon, gut and some types of breast cancer has been established [58], but the eventual mechanisms of carcinogenesis remain unknown. Liver cancer risk has been also found to increase, with diabetes and obesity [59,60]. The probability of development of hepatocellular carcinoma is increased fourfold in MetS patients [59].

2.3. Insulin resistance, its consequences, and type 2 diabetes

Insulin resistance (IR) and type 2 diabetes *mellitus* (or type 2 diabetes, T2DM) are present in most historical definitions of the syndrome (Table 1) and are considered by many as its main component.

2.3.1. Insulin actions

Under normal conditions, insulin (and other hormones, such as glucagon) regulate blood glucose concentration [61,62]. Insulin is also the main anabolic agent, it stimulates the synthesis of proteins, fatty acids and molecules implied in cell growth and repair [62,63]. Insulin also regulates most of the energy-related processes in the fed state [63]: it controls cell glucose uptake, resulting in lower glycemia [64], and reduces carbohydrate, lipid and protein catabolism [63]. Insulin also sends information to central nervous system on the energy availability [65]. In muscle, insulin promotes the synthesis of glycogen, and facilitates the oxidation of glucose, rather than fatty or amino acids [63].

On the contrary, glucagon (in consonance with other counter-regulatory hormones, such as glucocorticoids and catecholamines) promotes glycogenolysis, gluconeogenesis and ketogenesis under conditions of food deprivation (such as starvation or fasting) [62,63].

In adult humans, under physiological conditions, fasting insulin concentration lies in the range of 18-90 pM [63]. After a meal, when plasma glucose increases, the β-cells response

considerably increases insulinemia. The process is done in two superimposed phases, the release of already synthesized (stored) insulin, and the enhanced synthesis of the hormone [66].

In target organs, at the molecular level, insulin binds the α subunit of its receptor (an hetero-tetramer consisting of 2 α and 2 β glycoprotein subunits), producing a conformational change, which allows the binding of ATP to the β subunit, followed by its phosphorylation [67]. The change confers the receptor a tyrosine kinase activity, resulting in the phosphorylation of the IRS (insulin responsive substrates). The activated IRS bind *src*-homology-2 domain proteins (SH2), thus activating the PI3K (phosphatidyl-inositol 3-kinase)/Akt pathway, main ultimate responsible of metabolic response to insulin, as well as the RAS (rat sarcoma protein) pathway, which stimulates the growth-promoting actions of insulin [67]. This process is summarized in Figure 1.

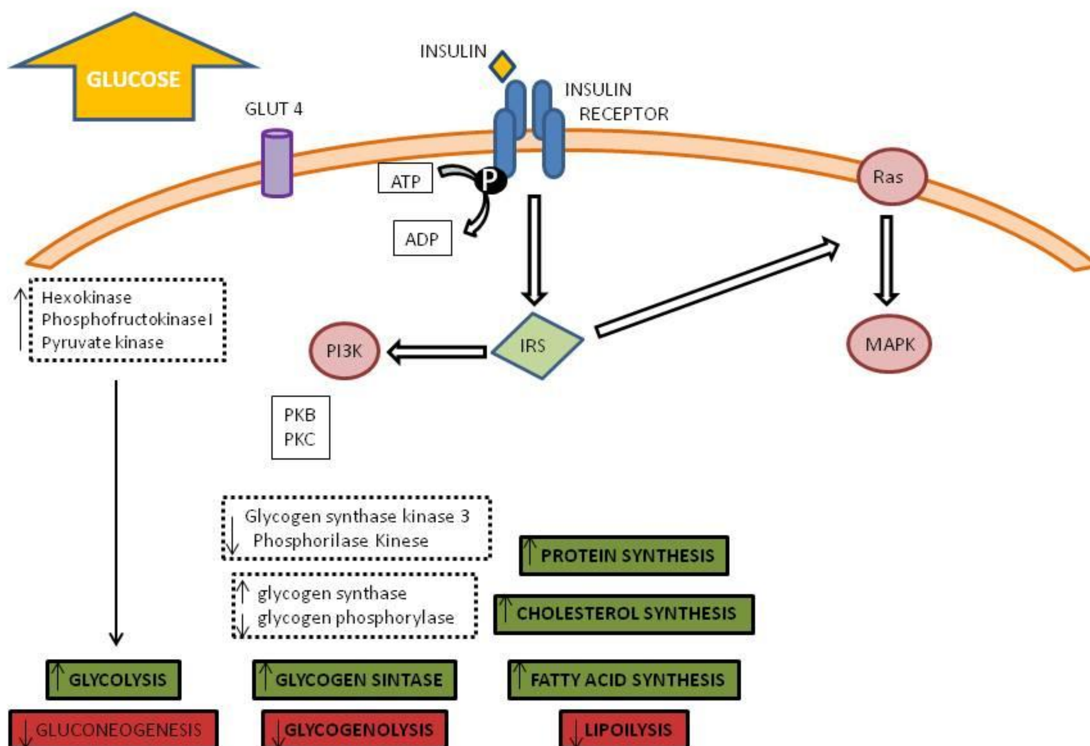

Figure 1. Insulin signaling pathways in the main target organs. Adapted from Wilcox (2005) [63].

2.3.2. Insulin resistance: a defense mechanism

Insulin resistance, widely considered the key component of MetS [68–70], is characterized by decreased sensitivity to glucose and its peripheral uptake [26]. It is the result of the finely-tuned evolution of a mechanism of protection against the temporal lack of glucose during scarcity and/or starvation [71]. Under conditions of starvation, fatty acids released from TAG

(triacylglycerol) reserves substitute glucose as main energy substrate for most tissues [72]. Fatty acid presence in blood is a powerful signal for sparing the scarce glucose available, preserving it for 'exclusive' nervous system and blood cells use, thus blocking its complete oxidation in most tissues [73], but allowing (e.g. in red blood cells) its conversion to lactate. This effect occurs irrespective of glucose abundance, because the coexistence, at the same time, of high lipid (meaning starvation and the use of reserves) and high glucose (i.e. post-prandial or feeding state) is 'metabolically unconceivable'. However nowadays the 'normal' human diets are high in energy (despite low expected energy expenditure), and contain abundant sugars/starch and lipid.

Since a specific mechanism to face this anomaly is absent (it has been unnecessary for all animal species evolutive life), we arrived wholly unprepared to manage this situation, never encountered before. Our body erroneously interprets the mixed signals and fails to manage efficiently the unused glucose. The defense mechanisms prevail, being activated by an availability of (dietary) lipid in gross excess of our needs. This rise in circulating lipid is interpreted by our organs as a signal that we are using our fat reserves, thus activating the preservation of glucose. Then we use the IR 'protective mechanism' to front the situation; a rare event for any organism which main energy staple is starchy carbohydrate[1,41,74].

At the molecular level free fatty acids (NEFA) inhibit the insulin signaling cascade, decreasing the translocation of *GLUT4* to membrane rafts [75]. The ensuing reduction in *GLUT4* effectiveness leads to a further reduction of glucose uptake in spite of the presence of fatty acids [76]. This change is due to an alteration of the PI3K-Akt pathway by IR, while the MAP kinase pathway is not affected [27].

The lack of uptake and oxidation of glucose, results in the reduction of cell glucose availability, but also in increasingly high levels of unused circulating glucose [77]. The excess of glucose induces a reflex increase of insulin secretion by the pancreas, and leads to a non-viable combination of hyperglycemia and hyperinsulinemia [78]. Thus, IR becomes a serious metabolic problem despite being originally an effective mechanism of protection developed to preserve glucose [77,79,80]. This situation, provoked by the automatic, albeit inadequate, response to diet richness leads eventually to T2DM [81].

2.3.3. Type 2 diabetes, or diabetes mellitus

Diabetes is a metabolic disorder, characterized by hyperglycemia. There are two main types of diabetes (i.e. much increased excretion of urine): diabetes mellitus (in which the urine is sweet because it contains glucose excreted because of uncontrolled hyperglycemia) and insipid diabetes in which a failure in the vasopressin system results in the passage of excess

water to the urine. In any case, most cases of diabetes correspond to the mellitus type. There are two quite different types of diabetes mellitus: type 1 diabetes (T1DM), also known as insulin-dependent diabetes, and type 2 diabetes (T2DM), known as noninsulin-dependent diabetes. The first classification date back to 1936, based on the lack of insulin or its "sensitizing factor" [82] , confirmed in 1951 with the finding of insulin in the plasma of human diabetics (*mellitus*) [83].

Most T1DM cases correspond to an autoimmune form of diabetes, characterized by the destruction of pancreatic β cells [84], while in T2DM β-cells are present and can secrete insulin; however insulin action on target tissues is impaired, so that, in the end, its secretion is insufficient [85]. This could be due to an abnormal glucose-stimulated insulin release response [86] or to functional damages in diabetic β-cells [87] .

T2DM is generally considered a consequence of uncorrected and maintained IR. Indeed, the presence of mobilized fatty acids, due to IR, inhibits insulin signaling cascade and prevents glucose uptake from tissue, decreasing the effectivity of insulin [76]. There is also evidence supporting a higher rate of development of Alzheimer's disease [88] through T2DM, to the point that Alzheimer disease has been even postulated as 'type 3 diabetes' [89].

In T2DM, β-cells present a marked resistance to the stimulatory effect of both GLP1 and GIP [90]. This state is associated with impaired insulin secretion and increased levels of fasting plasma glucagon [91]. Basal hepatic glucose production is also increased [92], whilst muscle glucose uptake is impaired [93]. This results in a gross metabolic dysregulation of the utilization of the main energy nutrients: carbohydrate, lipid and protein, and is usually associated with severe hyperglycemia and ketoacidosis [94].

2.4. Dyslipidemia and hepatic steatosis

The relationship of high circulating TAG with hepatic steatosis, IR and hyperlipidic/ hyperenergetic diets is one of the pillars of the development of MetS and of the increasing severity of its complications [38,95].

While IR affects glucose uptake by muscle, glucose uptake is less affected in other organs and tissues, in part because other glucose transporters (such as *GLUT1*, or non-specific transport systems) take up the role of *GLUT4*; this is the case of liver and WAT, where the excess glucose has been widely assumed to be used for lipogenesis[96].

The immediate consequence of this metabolic turmoil is the development of an intense 'abnormal' lipogenic bout, resulting in the disordered deposition of fat, eventually leading to liver steatosis and hepatomegalia [97]. A further complication arising from liver steatosis is

endoplasmic reticulum (ER) stress [98]. ER activity and protein folding may be affected by a number of factors usually related to excess nutrient availability or imbalance [99], such as excess of glucose or amino acids, leading to changes in protein glycosilation, and cell redox state [100]. The overload of ER function, due to the increased demand of protein synthesis, drives to an imbalance between protein folding capacity and fully functional protein requirements. This results in the accumulation of misfolded proteins, damage of hepatic tissue and function and in failure to assembly lipoproteins because of inadequate supply of fully functional apolipoproteins, leading to further fat accumulation [101], and the aggravated alteration of lipid homeostasis [102]. Circulating lipids increase but not in canonic biochemical structures, conformation and proportions, resulting in severe dislipidemia [95].

This hyperlipidemic dyslipidemia situation worsens the inter-organ substrate transfer, a condition which is almost omnipresent in MetS. It is characterized by increased plasma TAG, NEFA and cholesterol. Generally, circulating TAG are increased as a consequence of altered lipoprotein transport, high fatty acid synthesis from excess glucose (unused by most tissues because of IR) and enhanced esterification with glycerol-3P [103].

The liver sends its excess fat to other tissues, to be used there as energy substrate, normally in the form of VLDL (very-low-density lipoproteins) and LDL (low-density lipoproteins), lowering the levels of HDL (high-density lipoproteins) to use its constitutive structural lipids and proteins to build more VLDL and LDL [104]. Cholesterol levels increase because of an imbalance between its synthesis, reutilization, transport and uptake between liver and peripheral tissues [105]. In liver, excess glucose blocks the ketogenic pathway [106], diverting the 3-hydroxy-3-methyl-glutaryl-CoA to mevalonate and then to increase cholesterol synthesis. In peripheral tissues, cholesterol removal from plasma is reduced [107], another consequence of defective insulin signaling [108]. Consequently, plasma high density lipoprotein (HDL)-cholesterol levels decrease [109] and low density lipoproteins (LDL)-linked cholesterol increases [110], which results in smaller and more dense LDLs [111].

Under normal conditions, insulin facilitates the degradation of apolipoprotein B (ApoB). The insulin-elicited stabilization of ApoB, stimulates the assembly and secretion of VLDL [112], which are increased [70], thus contributing to extrahepatic lipid uptake [113].

NAFLD (non-alcoholic fatty liver disease), another name for liver steatosis included in MetS, has a marked synergy, i.e. a negative impact on other hepatic diseases, including hepatitis and fibrosis [60,114,115] which are aggravated. NAFLD also affects other key liver functions such as general detoxification and the removal and inactivation of hormones and metabolites [116,117]. Since insulin clearance is maintained by a healthy liver, NAFLD may also result in unchallenged hyperinsulinemia [118]. This same hyperinsulinemia may stimulate

hepatic CD36 expression, facilitating fatty acid uptake and thus worsening the fat accumulation in the liver parenchyma. [119] .

The increase in oxidative damage, consequence of an excess production of free radicals, markedly affects the oxidation of lipoproteins [120], this process is aggravated by the increased production of NO· from arginine [121,122], in itself probably related to a disordered amino acid catabolism and lower operation of the urea cycle [123].

Gout, which, as indicated above, appeared in the first definitions of the syndrome, is assumed to be a consequence of hyperuricemia and the defective handling of oxidized proteins and purines (i.e. through xanthine oxidase) [124] . But these effects are compounded by the known MetS correlation with arthritis [125].

In addition to liver, fat deposition occurs practically everywhere, in proportions fairly uniform in their distribution in all the body; this distribution has been found to be highly correlated for lipid storage organs and for the rest of body tissues [126]. Obviously, excess TAG deposition eventually reaches WAT, finally resulting in the disordered accumulation of fat of obesity [127].

2.5. Obesity

2.5.1. The problem of defining and measuring obesity

Obesity is a worldwide problem, which incidence is in expansion according to the WHO. The world prevalence of obesity more than doubled between 1980 and 2014, when about 1900 million adults had overweight, and one third of these were considered obese [128]. The same organization defines overweight and obesity as '*abnormal or excessive fat accumulation that may impair health*'. Despite this inconcrete 'definition', it does not exist a way to define which is the “normal” body fat content. This knowledge is essential to define clearly the amount of fat that allows us to define obesity. The problem is complex, since normalcy is not a unique situation, depending on different factors, such as sex, age, race, environmental factors, type of workload, and even diet. One of the few attempts to define obesity using the percentage of body fat was that of the American Society of Endocrinology, defining as obese the women with more than 35 % body fat, and 25% for men [129].

There are a number of methods to estimate body fat, such as bioelectrical impedance, hydrostatic or air plethysmography, isotope dilution techniques, dual x-ray absorptiometry, composite skinfold measurements, and even complex computerized tomography and NMR algorythms [130]. Nevertheless, because of its simplicity, body mass index (BMI) or Quetelet index [131], calculated as body weight (in kg) divided by height squared (in meters), became popular and widely used, despite its very poor sensitivity (42%) and its almost nil diagnostic

performance and narrow window of applicability (height, weight, sex, age, race)[132,133]. In fact, too often individuals with 'normal' body weight according to their BMI have high percentages of body fat, showing a higher risk of developing metabolic syndrome and its co-morbidities. Erroneous high BMI with normal body fat also results in iatrogenic damage because of unnecessary treatments. For these reasons obesity needs to be defined in ways quite different from those used now, based more on adiposity [134] than on the simple use of a number of indexes that are easy to apply to populations, but not to individuals [135]. Any good quantitative measure of obesity needs to be based on a previous definition of normalcy and be modulated by ethnic group, age and sex [136].

2.5.2. The ponderostat system

Obesity is probably the only (albeit inadequate) solution left for the temporal 'elimination' (via deep storage) of the excess energy that cannot be metabolized, excreted or used elsewhere, and which interferes deeply with metabolic regulation. In fact, under conditions of excess energy, and before reaching the full obesity situation, the body tries to compensate this disturbing excess with indirect, non-specific mechanisms, which often produce unwanted effects, aggravating the disorderly situation [137]. The main strategies used are BAT- [138] and muscle-related [139] thermogenesis, increased protein turnover [140,141] and a number of 'futile cycles' (i.e. groups of reactions in which substrate energy is consumed without synthetic or functional benefit, and is lost as heat). We can even include, growth in this list [142]. The objective is always to maintain an optimal, i.e. stable and functional body weight (body fat mass), adjusted to a pre-established set point, since body mass (deeply affected by the weight of stored energy) is homeostatically critical, and its maintenance is of paramount importance for survival [137].

The *ponderostat system*, which controls the amount of body fat, is assumed to be controlled mainly by the hypothalamus [143], and is established on the basis of a homoeostatic system, in which a signal informs the processing system about the mass of body fat (the controlled entity). If this mass is larger than that established by the system, then the system inhibits fat accrual and stimulates its utilization; when the mass is lower than the expected level, then the system operators reverse the situation: accrual is activated (appetite and energy intake are increased), whilst lipolysis, fat oxidation and adaptive thermogenesis are restricted [144,145].

Ponderostat settings are subjected to genetic, epigenetic and developmental influences [146]. Brain responds to neural and metabolic signals, regulating energy balance, through the control of energy intake (largely via control of appetite) and energy expenditure [143,147]. A number of compounds, such as glucose [148], lipid [149], leptin [150] or oleoyl-estrone [151] have been

postulated as metabolic signals for regulation of the ponderostat system. These signals act as indicators of the level of energy reserves, essentially body fat mass. The same role was proposed for resistin [152], ghrelin [153], adiponectin [154], and almost any 'new' cytokine just when it was discovered. Invariably, not fact-sustained hopes soon were checked and the initial assumptions found unjustified. The first limit for a ponderostat signal is its size and chemical nature, since most polypeptides and proteins cannot cross the blood-brain barrier. Smaller or lipophilic compounds do not share this problem, thus oleoyl-estrone has been found to lower the ponderostat setting [155] at pharmacological doses in the rat [156] and in a human [157]. It has been found, however, that oleoyl-estrone is converted to a so far unknown estrone-derived intermediary to accomplish this ponderostat signalling function [158]. Leptin has been found to affect appetite [159] and is able to be transported into a number of critical hypothalamic nuclei [160]. Its levels tend to increase with obesity [161]. For this reason, leptin has been repeatedly proposed for this role [150,162], and the belief (or 'need') of many scientists to consider leptin as the main hormone regulating lipid storage and metabolism [150] has increased, despite the lack of hard data sustaining this claim [163]. Furthermore considerable interest has been invested in the study of insulin-leptin relationships, since insulin is related to energy balance and storage, and has been found to be one of the factors regulating adipocyte expression of leptin [164].

The most known mechanism to waste excess energy regulated by ponderostat system is the thermogenesis. The main components of energy expenditure are the residual heat released in all exergonic (i.e. thermodynamically favored) reactions. Even synthetic (endergonic) reactions are coupled to exergonic reactions (e.g. hydrolysis of ATP) to speed-up metabolism. The proportion of energy lost as residual heat is considerable and helps maintain our temperature. Additional heat is produced from physical activity, and specific cold-induced or excess energy-induced thermogenesis [165]. There are a number of thermogenic mechanisms contributing to maintain body temperature and helping regulate body energy availability under the rule of a ponderostat system [166]. The most common form of increased thermogenesis is just the consequence of increased metabolic activity, especially via enhanced protein turnover [140]. Liver [167,168] and muscle [139] are known to increase thermogenesis under hormonal stimulation. The mechanism is unclear, but there is a clear relationship with the efficiency of conversion of substrates reducing power into ATP [169]. In BAT (brow adipose tissue), a well-controlled and efficient system to generate heat via 'inefficient' use of substrates is based on a inner mitochondrial membrane protein, largely specific of BAT, UCP1 (uncoupling protein 1) [170], which allows entry of cytoplasmic protons bypassing the ATP synthase system, thus breaking the gradient generated by the

mitochondrial respiratory chain [171]. The immediate consequence is the direct release of heat instead of using the reducing energy of NADH and $FADH_2$ to produce ATP.

The central nervous system also influences the secretion of insulin, cortisol, growth hormone, thyroid hormones and catecholamines via autonomous system stimuli and the secretion of hypothalamic (and hypophysis) hormones which activate the main endocrine axes: hypothalamic-pituitary-thyroid, hypothalamic-pituitary-adrenal, or hypothalamic-pituitary-gonadal [172] .

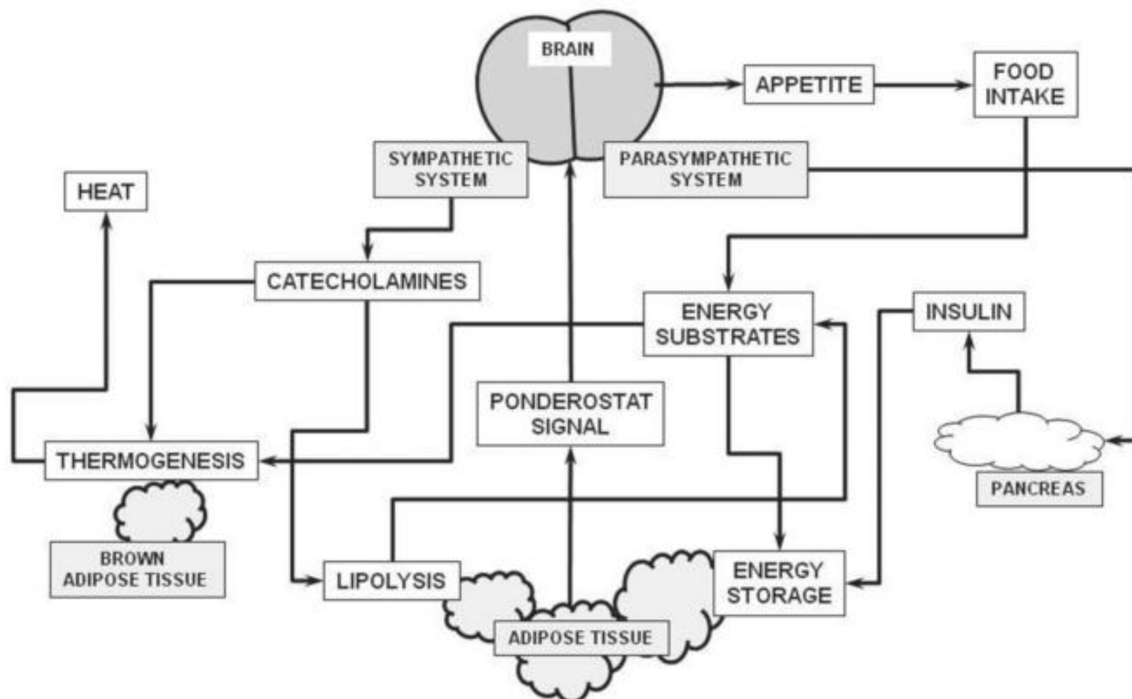

Figure 2. Schematic representation of a ponderostat model [146].

2.5.3. Obesity as a disorder of the ponderostat function

In mammals, feeding is conditioned from visual, olfactory, emotional and cognitive stimuli[173], as well as by learning (from others or by trial-and error) and memory [174]. The influence of genetic and epigenetic conditioning has been repeatedly demonstrated too[175]. The importance of these factors has been already presented as one of the main cause for the hyperphagia that has been postulated as a key pathogenic basis of MetS. These stimuli often can override the ponderostat 'orders', resulting in a damaging and unneeded higher consumption of food [176]. A highly studied example are the case of rodents fed 'cafeteria' diets, which reproduce the variety of desired tastes found in the present-day diet, and which leads to an often massive weight gain [177,178]. The simple addition of fat (not tasty food) allows the ponderostat to act unhindered, decreasing food intake and thus forfeiting the unwanted accumulation of fat (obesity) [179]. This way, when energy intake exceeded energy expenditure

over the capacity of the ponderostat system to compensate, fat deposition in WAT and in most other tissues occurs [176].

For these reasons obesity can be also defined as a disorder of the ponderostat function, since in most cases, the obesity persists even when dietary intake is returned to normalcy [147]. Hypocaloric diets are often ineffective against obesity [180], because the ponderostat setting has been changed, often adjusted to a higher (abnormal) level of body fat [147]. This 'new' disordered level is maintained by a fully operative ponderostat system, which defends the accumulated fat through the control of appetite, fat deposition, lipolysis and thermogenesis [181]. The ponderostat settings are not fixed for life; they change following a developmental blueprint along the life-cycle, following genetic and epigenetic information [146]. The ponderostat settings can be modified by acting through specific hypothalamic site [182]. The problem is that we don't know enough about how the ponderostat is regulated, and we don't even know which are the signals that bring information to it from the whole of our adipose organ [146]. This lack of knowledge elicits the use of inadequate treatments, largely based on the control of appetite or food intake, and not on the ponderostat itself, leaving it to compensate any dietary maneuver we endeavor to decrease boy mass [183,184].

Finally, obesity is elicited by increased energy intake (due to palatability, availability, increased portion size and energy density of the food) [173,177], sedentary lifestyle (decreased muscular exercise for physical work and displacement) [185], environmental conditions (food availability[186], stress[187,188], pollutants and toxic compounds[189], diseases of the body and microbiota [190], etc.). In addition to these known factors the predisposition to obesity is possibly influenced by a growing number of genetic and epigenetic factors [191]. Nevertheless, and despite what is already known, most of the health professionals and the public consider that obesity is "simply" a consequence of ingesting an 'excess' of (food) energy (i.e. the *sin* of gluttony).

2.5.4. Obesity and its pathologic consequences

In the pathological condition of obesity, the disordered increase of adipose tissue leads to the accumulation of fat in non-regular WAT localization, such as liver, as seen above, skeletal/heart muscle and pancreas [192]. Fat accumulation within these organs elicits an impairment of their activity. Intramyocellular lipid accumulation has been attributed both to an excess muscle uptake of fatty acids, limited oxidation (often consequence of the loss of mitochondrial function) and ER stress (induced by excess energy substrates available) [193]. It tends to reduce the efficiency, timely and power of contractility in muscle and heart, with the expected consequences of loss of function [194,195].

In heart muscle the accumulation of very long chain fatty acids leads to fatty hypertrophy and decreases the transmission of electric contracting signals along the myocardium, resulting in the loss of heart efficiency [196], one of the main cardiovascular risks associated with obesity [144]. To make the situation worse for the ailing heart, obesity increases volemy because of higher peripheral tissue volume. This larger tissue mass to irrigate brings the heart to work always in the upper limit of its capabilities, and maintains a condition of arterial hypertension, one of the principal traits of MetS [197,198]. A situation that cannot be maintained indefinitely, and which enhances the risk of cardiovascular events, often compounded by catecholamine damage on the heart electric signalling structures [199]. There is a close association between the repeated catecholamine surges of sleep apnea and the disarrangement of contraction signals in the heart, often driving to maintained atrial fibrillation [200]. This condition decreases further the ability of the heart to maintain a viable blood flow, compounded by the loss of electric conductivity of myocardium and the His bundle, rapidly worsening the condition of the heart and the peripheral ravages of hypertension [200,201]. The MetS diabetic-related hyperinsulinemia contributes to this 'perfect storm' increasing the activity of the heart renin-angiotensin system, sympathetic activation, worsening vasoconstriction and hypertension at the same time [202,203].

Obstructive sleep apnea is clearly associated to obesity and MetS, but we don't know yet how, and neither its causes [204,205]. Its influence on heart function (arrhythmia) via continued catecholamine surges has been already discussed.

The obesity-created disproportion between lung size and the grossly increased body mass, leads to a chronic impairment of lung function, and to pulmonary venous hypertension [206,207].

TAG accumulation and ER stress also affect the functions of the nervous system, largely due to damage to neural transmission and peripheral nerves [208]. Probably, for this reason MetS/obesity are closely correlated with a number of psychological and central nervous system-related traits which affect personality [209]. But MetS also increases the incidence of a number of nervous/psychiatric alterations; thus MetS is about twice more common in patients with bipolar disorder [210]. MetS is also associated with other cognitive impairment states and dementia [211,212], as well as with depression, which is already becoming one of the main defining characteristics of MetS [213–215].

Excess circulating lipids may lead to the alteration of platelet function, contributing to the formation of plaque material as well as its attachment to the arterial wall and its maintenance [216]. The possible role of perivascular WAT and its production of proinflammatory cytokines [217], plus endothelin-1, the expression of vascular cell adhesion molecules and the mitogenic stimulus to vascular smooth muscle cells facilitate the

aggregation of platelets, resulting in the deposition of atheroma plaque [27,218]. The presence of these depots decreases the effective diameter and confers rigidity to the vessels, contributing to a lower flow capacity (with increased blood flow velocity), risk of generalized blood cell breakup (possibly driving to thrombosis and *ictus*) [219]. These pathological traits are compounded by higher blood pressure, not attenuated because of the loss of flexibility of the arterial wall because of plaque-derived atherosclerosis [216].

The combination of all these factors markedly increases cardiovascular risk, and explain its close relationship with obesity and associated diseases within the context of MetS, increasing the risk disability and advancing the age of death [220].

2.5.5. The "obesity paradox"

Nevertheless, obesity presents also some advantages. It has been observed that in patients with coronary artery disease who undergo percutaneous coronary interventions, the outcomes depend on their body fat mass [221]. Those who are overweight or slightly obese fare better than those who have normal weight and those extremely thin. These results have been defines as "the obesity paradox" [37]. The risk for in-hospital complication and cardiac death is lower for overweight or obese people [37]. The probability to survive heart failure is also increased in those with some degree of excess body fat [222]. A possible explanation for this 'paradox' may lie in nitric oxide (NO·) [42], which mediates vasodilation and inhibits thrombocyte aggregation [223]. A number of studies support the increased activity, in the obese, of the inducible nitric oxide synthase (iNOS) [122], as well as the increased sensitivity to NO· of coronary circulation observed in obese rats [224] .

Other statistically significant benefits, part of the "obesity paradox" include patients with atrial fibrillation treated with oral anticoagulants, who show more favorable prognosis [225], due to reduced mortality risk and decreased severity of acute stroke for obese people [226]. This long-term prognosis of stroke is better in overweight people, because of their maintenance of body weight [227].

Likewise, more "obesity paradoxes" have been reported for a number of chronic diseases, most of them within the umbrella of MetS, including chronic obstructive pulmonary disease [228], chronic kidney disease [229], rheumatoid arthritis [230], and others.

2.6. Adipose tissue and inflammation

2.6.1. The adipose organ

White adipose tissue is loosely organized to from a large, disperse organ, made up of different size (and function) depots [231]. They are distributed in/below the skin subcutaneous depots, including the gluteal and abdominal fat, and associated with other organs and between them, filling space and helping maintain their integrity. The largest masses of WAT include the mesenteric/omental, retroperitoneal, epididimal, mediastinal, perirenal, perigonadal masses [231]. WAT also covers the heart, blood vessels and other organs (prostate, heart), which helps control and regulate. In addition, we can find smaller masses of adipocytes (WAT?) interspersed between muscle masses, and infiltrated within organs [126]. These cell groups function in addition to the fat depots present in other sites, as is the case of steatotic liver fat, intramyocellular fat depots [192]. The sum of these cells constitute the adipose organ, endowed of vascularization, innervations, complex cytology and high physiological plasticity [231], with the unitary function of sharing energy contained in food for storage, thermogenesis (with brown adipose tissue) and to sustain the whole body metabolic needs via control of substrate transformation, availability, partition and storage [232].

Adipose tissue has marked plasticity and adaptability to the environmental conditions. Thus cold exposure and physical exercise may induce "browning", i.e. the appearance of brown adipocytes interspersed between the white, plating a thermogenic function [233,234]. Subcutaneous breast tissue is modified into milk-secreting glands during lactation [235]. In bone marrow, there is a large proportion of WAT, but the limits/differences with the hematopoietic tissue are diffuse and point to a direct dependence or transformation [236]. It has been postulated that these processes are reversible, but there is also evidence that stem cells may evolve in a number of cell types, including the differentiated secretory or thermogenic types described [236]. A high potential for differentiation, the coexistence of several lines of stem cells [237] and the protean flexibility of WAT may explain these rapid changes of cell composition and differentiation of WAT. This include its high capacity for tissue regeneration, reparation and wound healing [238]. It has been proposed that brown adipose tissue can transdifferentiate to white, under conditions of gross excess of energy and body fat, to help WAT to store even more energy [231]. However, this is improbable, since the cell lines developing WAT are different from those of most WAT lines and in any case the transformation could not be complete because it also (essentially) implies modifications on mitochondria structure and function [239].

Adipocytes are not the only cells in adipose tissue, and are neither the most abundant or metabolically active [240]. WAT contains a stromal vascular fraction, composed by epithelial

cells constituting the endothelium of capillaries and small vessels, where it is assumed that critical inflammatory processes occur [241]. This fraction also contains a large number of stem cells, responsible of most processes of regeneration and repair in which WAT participates [238]. When they differentiate into adipocytes, the pre-adipocytes constitute a subpopulation of young growing cells in the process to differentiation to full mature adipocytes [242]. There are also blood cells, as in any other tissue, and a large number of specialized cells, not all of which have been identifies, and neither are present in all WAT locations. Many of them play necessary ancillary roles such as maintenance of the fiber (largely collagen) tissue frame and even synthesize specialized products or cell types. We can found, then, fibroblasts, histiocytes, lymphocytes, granulocytes and mast cells [243]. An important component of WAT cells are those implied in the defense mechanisms, i.e. cells from the immune system. Their presence may be subjected to the conditions of aggression, metabolic challenge or inflammation [244,245]. These cells include macrophages, lymphocytes, T cells and others [246]. Although white adipose tissue has been usually considered just as an energy depot, the dumping place for excess energy, at present this impression has deeply changed because of more information proving otherwise [247].

2.6.2. White adipose tissue metabolism

Despite the scarce cytoplasm in white adipocytes, WAT is metabolically very active: it participates in Cori cycle [248], in glucose-alanine cycle [249] and in Randle cycle [250]. Moreover in our group a full urea cycle was found in four localization of rat WAT [251]. Notwithstanding WAT is still poorly studied, except for its function of storage and mobilization of energy according to body demand, contributing to its homeostasis [252]. The main diet- or reserve-derived body energy staples are fatty acids, largely stored as TAG. The process of TAG storage/hydrolysis in WAT is highly regulated by hormonal (mainly insulin, catecholamines, glucocorticoids etc.), metabolic (glucose, NEFA), and nutritional (energy intake) factors [253].

Lipid storage is stimulated by insulin; binding of the hormone with its receptor inhibits lipolysis, and stimulates lipogenesis [254]. This is accomplished first by increasing the uptake of glucose [255], and second via activation of lipogenesis [254]. Insulin decreases the availability of glucose and fatty acids in plasma [61], facilitating *GLUT4* translocation to the plasma membrane, thus promoting glucose uptake from the bloodstream [255]. Pyruvate is oxidized to acetyl-CoA and insulin activates lipogenesis through increased expression and/or dephosphorylation of ATP citrate lyase, acetyl-CoA carboxylase, fatty acid synthase and glycerol-P acyl-transferase [256].

Insulin enhances LPL (lipoprotein lipase) secretion, allowing the hydrolysis of fatty acids from plasma lipoproteins [257]: TAG in VLDL and chylomicra (essentially in the postprandial state), hydrolyzed by LPL in the capillaries of WAT, release fatty acids. These are transported into adipose cells by CD36 transporter and activated with CoA by the acyl-CoA synthase, to be finally reesterificated into TAG [253].

In fact, only a fraction of WAT TAG fatty acids are synthesized *de novo*, since most dietary fatty acids and those from hepatic lipogenesis are re-esterified, to form TAG, via adipocyte fatty acid transporters, acyl-CoA synthase and final coupling to glucose-derived glycerol·3P [258].

During starvation fat reserves are hydrolized to fatty acids and glycerol by WAT lipases; the hormone-sensitive lipase is activated by phosphorilation, elicited by catecholamines (largely via noradrenaline secreted by synpathetic terminals) or glucagon [257]. Lipolysis is also activated by glucocorticoids [259,260], but not too often *in vivo* [261], and a number of cytokines, such as TNFα and the natriuretic peptides [260]. Both fatty acids and glycerol are released to plasma: glycerol is used as energy substrate elsewhere [262,263], or used for gluconeogenesis or even reesterified to TAG by the liver [263,264], while fatty acids are used as energy substrates by peripheral tissues [260], in order to spare glucose for brain and erythrocytes. Liver may use the surplus of fatty acids to form TAG, later exported as lipoproteins [265] or synthesize ketone bodies via ketogenesis to supply the energy needs of other tissues [266].

WAT amino acid metabolism in WAT is sparsely known, but it is able to use a number of amino acids (i.e. branched-chain) for lipogenesis, energy supply, with a possible role in adipocyte differentiation [267], but also as a way to export 3C units (alanine) [268] or preformed structures (citrulline, arginine) [269]. WAT amino acid metabolism is related to the overall body availability of energy and N [270]. It has been postulated that WAT plays a potentially significant role in the disposal of excess amino N, since it contains a complete functional urea cycle [251].

Glucose could be used by WAT to form glycogen, to synthesized fatty acids (and then TAG), or may just be glucolyzed into lactate, which is released to the bloodstream [271]. In comparative terms, the synthesis of glycogen represents only less than 5% of metabolized glucose [272] and the *de novo* synthesis of TAG, is limited in mature adipocytes [273]. However, glycolysis to lactate has been proven to take up a significant share of all glucose processed [274]. According to DiGirolamo et al. WAT produces a considerable amount of lactate both under starvation and in the postprandial state [275]. WAT is able to incorporate glucose even under IR conditions [276]. There are some differences in the lactate production: mature WAT, with larger adipocytes converts more glucose to lactate than younger WAT containing smaller adipocytes [275], more prone to lipogenesis, instead [273]. However, this

general rule is not universal, since lactate production depends on site, thus, mesenteric WAT, despite having smaller cells, converts more glucose to lactate than epididymal WAT, with larger cells [277].

The lactate produced by adipose tissue can be used as substrate for energy almost anywhere, including the heart [278] and brain [279], or for lipogenesis (liver, WAT) [263,280]. The gluconeogenic potential of lactate [281] and glycerol [262] (another 3C product of WAT) may be critical in starvation, since they are re-converted to glucose to provide for special organ needs and to maintain glycemia [275].

The obvious primary target of the peripheral lactate inter-organ flow seems to be the liver, where it may be used for gluconeogenesis, glycogen storage [275] or lipogenesis [263]. A critical point for substrate partition is that under conditions of excess glucose availability, glycogen synthesis and gluconeogenesis are blocked, which leaves only lipogenesis as main emergency pathway for disposal of excess lactate [282]. Under these conditions, the excess glucose may be broken down to 3C units by WAT [275], with limited lipogenesis in part due to limited oxygen and the geometry of cell disposition in WAT [273]. The excess lactate ends up in the liver, with powerful oxidative capacity, which activates lipogenesis [263] and exports TAG as lipoproteins [265]. These are broken up by WAT, taking up the released fatty acids, which are incorporated to its own TAG stores [253]. The consequence is the increase of TAG stores in WAT, which fatty acids do not come from WAT lipogenesis but from the transformation of excess 3C fragments to fatty acids by the liver. This way, some sort of glucose-fatty acid or Randle cycle [250] is established [283].

This use of glucose is paradoxical, but provides at least two additional operative advantages: 1) so much metabolic transformation is energetically ineffective and helps in part to lose a small but significant amount of substrate energy as heat. And 2) there is an abundance of 3C fragments in circulation, substituting glucose; they can be used for energy by almost all tissues, with the advantage of not being so tightly controlled by the insulin-glucose system. Furthermore, critical organs for survival, such as the nervous system use efficiently lactate and glycerol [284] as main substrate, in addition and in preference to glucose [285]. The glia being responsible for the generation of sufficient lactate to feed the neurons [279]. This role of WAT opens a wide range of possibilities for regulation, sustenance and survival that probably will be studied in depth along the next years.

We know little about adipocyte metabolism, however, even less is known about the combined (i.e. WAT) metabolism of the cells in the stromal fractions. No 3C efflux from WAT stromal cells has been identified, for instance. A problem shared by adipocytes and stromal cells, in fact almost everything related to WAT metabolism lacks the presentation of

quantitative easy to compare data. Most analyses and studies are simply descriptive and not comparable even in a same individual between different WAT sites. In many cases, no comparisons at all are done, assuming that all WAT from a given individual 'should' be identical in function, structure and metabolic behavior.

2.6.3. Inflammation

In obesity, adipose tissue function is altered, since it reacts to the constant energy surplus by decreasing cell substrate handling, in order to limit the incorporation of more energy to its TAG stores. In addition it secretes cytokines to signal the condition of metabolic aggression [286]. This results in depression of cell metabolism, alteration of hormone signaling, increased apoptosis, and the blockage of tissue differentiation and growth. In sum, adipose tissue loses a large part of its functions [287].

Adipose tissue, through the secretion of cytokines, tries to restore homeostasis, inducing the response of the immune system and inflammation [1]. The term “inflammation”, which derives from the Latin “inflammatio” (literally 'in flames'), refers to the defensive body response to an aggression, with the aim to destroy the agent responsible of this state. When it cannot be eliminated, inflammation may switch to a chronic state. The classical inflammation, described by Celsus, is characterized by four principal signs: *rubor*, *turgor*, *calor* and *dolor*; meaning respectively, redness, turgidity, heat and pain [288]. Later the fifth sign *functio lesa* (loss of function) was added to the tetrad [289]. These signs derive largely from an increased blood flow, the consequent edema, and the presence of cytokines, prostanoids and histamine [290]. Despite being defined as “inflammation”, the kind of inflammation found in deranged tissues in MetS is deeply different from the classical one: in this case, the aggressor is not an invader (organism, antigen, toxic, etc.), but a generalized defect of substrate handling, which makes ineffective the defense reaction and increases the intensity of the response [1,291]. The failure to restore a physiologically normal condition, leads to the chronification of inflammation[1]. Primary inflammatory responses take place in microvascular endothelium of most tissues, including WAT, liver, pancreas, muscle, intestine, and brain [292].

An overload of dietary fat induces adipocyte hypertrophy and hyperplasia [293]. The size of adipocytes affects the seriousness of WAT inflammation. The immune system defends the body in part via WAT, by means of the incorporation of macrophages and T lymphocytes, tuned with a shift from anti-inflammatory to pro-inflammatory state [244]. Macrophages' profile change from M2, which secrete anti-inflammatory cytokines, like IL (interleukin)-10, to M1, responsible of the secretion of pro-inflammatory cytokines, such as IL-1b, IL-6, TNF-α (tumor necrosis factor-alpha) [294]. In fact large adipocytes produce considerable amounts of MCP-1

(monocyte chemoattractant protein), which drives the infiltration of activated M1 macrophages into WAT and their secretion of pro-inflammatory agents [295]. The infiltrated M1 macrophages express TNF-α, which have a direct role in adipose tissue IR development, and its levels are increased in obesity [296]. The M1 macrophages, together with other adipose tissue cells, produce IL-6 [297]. Both TNF-α and IL-6 prevent the normal pre-adipocyte development and differentiation, promoting lipid storage in other tissues, and impair insulin signaling, by decreasing tyrosine phosphorylation of key signaling molecules [298]. Other pro-inflammatory agents involved in the development of WAT inflammation, are IL-1β, PAI-1 (plasminogen inhibitor/activator 1) [299], angiotensinogen, ET-1 (endothelin 1), implied in thrombosis and hypertension [300], prostaglandins, lipoxins and resolvins that activate the inflammatory response [301,302]. On the other side, there is a reduction in adiponectin that inhibits inflammation, enhances insulin sensitivity, and also increase glucose transport and fatty acid oxidation [303].

Hypertrophy may result in adipocyte necrosis, releasing the cell contents to the extracellular space, a situation that spurs inflammation even more [304]. This process is related to the formation of ‘‘crown-like structures’’ by macrophages surrounding doomed or dead adipocytes [304]. This process is envisaged as a cleaning system, which function is eliminate non-functional or damaged cells, but which also help to prevent uncontrolled growth by culling cells to maintain proliferation at bay [305].

The CD8+ (cytotoxic T) lymphocytes can precede the infiltration of macrophages, and contribute to modify the macrophages' number and state through recruitment and activation [306]. On the other side, Th2 (T helper 2) cells, and eosinophils, can inhibit the migration and polarization of pro-inflammatory macrophages, and induce M2 polarization [307]. The number of anti-inflammatory regulatory T lymphocytes tends to decrease in adipose tissue with obesity [308,309].

In addition to macrophages and lymphocytes, neutrophils and mast cells also are known to promote inflammation and IR [309,310]. Furthermore, activated T cells from WAT migrate (and provoke damages) to blood vessels, kidney, brain, and other organs [303].

2.6.4. Chronification of inflammation

The above described inflammatory state, with time, can extend from WAT to the whole body, becoming a chronic condition. Its extension and chronfication is a critical event for the development of MetS [311,312].

In the obese with liver steatosis, the own liver specialized macrophages, the Kupffer cells, are those which change their activation state, producing inflammatory mediators [313]. A similar

process occurs in skeletal muscle, where the macrophages of inter-muscular adipose tissue exhibit a pro-inflammatory M1 phenotype [244]. In vascular tissue, TNF-α initiates the inflammatory changes; IL-6 is directly implicated in atherogenesis; PAI-1 promotes clotting, CRP (C-reactive protein) reduces the synthesis and biological activity of NO·, upregulates ET-1 and activates cell adhesion molecules, leading to an unbalanced endovascular homeostasis and, from here to a pro-atherogenic state [303]. Also ACEs (angiotensinogen and angiotensinogen-converting enzymes), produced by adipose depots and associated to vasoconstriction, contribute to hypertension and to increase the endothelial expression of the adhesion molecules, VCAM-1 (vascular cell adhesion molecule-1), ICAM-1 (intercellular adhesion molecule-1) and MCP-1, promoting the infiltration of inflammatory cells in the arterial wall [298].

Inflammation arrives to pancreas because of the increase of non-esterified free fatty acids and glycerol in plasma, which drives to the deposition of excess lipid in pancreas, damaging the β-cells [314]. The affected islets show amyloid deposits, fibrosis and macrophage infiltration, with increased levels of pro-inflammatory cytokines and chemokines [315]. The accumulation of misfolded proteins, due to ER stress in β–cells, and the activation of pro-inflammatory signaling pathways [316], may even lead to the initiation of the apoptotic cascade, resulting in cell death and causing diabetes [317,318].

MetS is associated with WAT inflammation, forming the pathological basis of obesity, which is clearly related to a deeply altered immune response [319]. Despite most physicians tend to consider diseases such as psoriasis and asthma as relatively unrelated to WAT, obesity and the MetS, their relationship is progressively becoming more clear [320,321]. There are also other skin disorders more or less associated to the syndrome, such as lichen *planus*, androgenic alopecia, systemic *lupus erythematosus*, warts or skin tags, and *acanthosis nigricans* [322], in all cases, the relationship with immune-driven defense mechanisms (lupus), viral infections (warts) or altered melanocortin-related tissue responses have been evidenced [323].

2.6.5. The glucocorticoid response to inflammation

Inflammation is characterized by an increased secretion (and metabolic intervention) of glucocorticoids (GC). In MetS, the hypothalamus-pituitary-adrenals axis is more active [324]. Moreover the same adipose tissue presents the enzyme 11β-hydroxysteroid dehydrogenase 1 [325], responsible of the interconversion of relatively inactive cortisone to its active form, cortisol. One of the main functions of GC is to maintain 'metabolic order', i.e. to prevent derangements, loss of control or to correct damages incurred by the operation of body systems [326]. GC are miracle drugs because their administration helps to recover

normalcy in disordered systems, producing a re-adjustment of control systems and a normalization of substrate handling. Curiously, as a powerful therapeutic tool, in most of the cases, their administration is tentative and the results, often positive have a considerable flair of serendipity [327]. This is just the consequence of multiple pathway modulation, and several-tier regulation, encompassing from whole body structures to specific protein control of expression, activation or transport [328].

The higher GC activity in MetS is a logical consequence of the changes elicited by gross incorporation of substrates, alterations in the rate of utilization of substrates and storage of lipids. Glucocorticoids are secreted to lower the immune response and to re-establish 'order'. This may result in enhanced liver gluconeogenesis, theoretically for glycemic maintenance [329], but in fact raising an already high glycaemnia. Moreover GC mobilize protein [330] to fuel gluconeogenesis, and minerals from the bone [331], inducing in this way protein and mineral wasting, which could lead to emaciation and osteopenia [332]. Consequently, excess CG secretion turns itself in a serious problem, since in trying to correct the damages of inflammation; the responses elicited may create a deeper havoc and affect even more deeply the chiasm in the control of glucose, amino acid and fatty acids metabolism [333].

CG inhibit the synthesis and activity of androgens [334] which are, together with insulin, the main anabolizing agents, enhancing protein synthesis [335]. In adipose tissue, especially in enlarged adipocytes, the aromatization of androgens to estrogens is improved [336], leading in obesity to the increased of hypoandrogenemia. GC have a synergistic effect with estrogens, reducing endothelial inflammation [337]. Nevertheless lower levels of DHEA (dehydroepiandrosterone) precursor lead to a decrease of availability of estrogen and their protective affects [338], resulting in the reduction of bone mineral density [332], as observed even in overweight adolescents with MetS [339]. On the other side, in postmenopausal women with MetS, lumbar bone density is higher, hinting at a protective effect of MetS on bone, which has been attributed to estrogen secreted by WAT [340,341].

Glucocorticoids also may induce resistance to insulin, leptin and other regulatory factors, affecting even the secretion of gonadotropins [342,343]. Altered sex hormone metabolism and function, lead to oligomenorrhea or amenorrhea, and, especially, polycystic ovary syndrome (POCS) [344].

2.6.6. Hypoxia and inflammation

Today the theory of hypoxia being the initiating event in adipose tissue dysfunction, fibrosis and inflammation is widespread [345,346]. Part of this assumption relies on the poor oxygenation

of WAT and the abundant presence of lactate (a telltale of anaerobic glycolysis) both *in vivo* and in cell cultures of adipocytes [347]. According to this generalized hypothesis, hypoxia would be the initial factor eliciting WAT inflammation [346]. Many studies, often unawares of the ability of WAT to produce huge amounts of lactate under normoxic conditions [283], i.e. actually not related to a state of hypoxia, keep on publishing studied focused on the role of hypoxia, and studying the molecular mechanisms implied, in the development of WAT inflammation [346,348].

One of the evidences to support the hypoxic hypothesis of inflammation is the absence of a significant increase of WAT overall blood flow, corresponding to lower flow per unit of tissue weight, when WAT mass is enlarged (inflammation, obesity) [349]. These differences tend to disappear when the non-cell mass of WAT (largely fat) is discounted from the equation, since in obesity, adipocytes tend to be larger than in normal weight [350].

WAT contains several different mechanisms to modulate its own blood flow (and that of the tissues it covers and controls) [218]. In addition to containing a complete rennin-angiotensin system [351] and endothelin [352]. Vasodilatation is often achieved by the control of NO· production and release [353], a mechanism altered in obesity as explained above. Vasoconstriction usually involves catecholamines [354] and, during inflammation, ET-1 [355]. The blood flow reduction may be a mechanism to limit the entry of blood-carried energy substrates into the tissue. Consequently, blood flow is well controlled (and adjusted to needs) in WAT and the lower relative blood flow observed in obesity being attributed to a limitation of the supply of energy substrates as part of WAT's own mechanisms of defense against energy overload [218]. Direct measurement of pO_2 in WAT *in vivo* also proved that, even in severe obesity, the tissue receives sufficient oxygen to sustain its metabolism, because of the low adipocyte oxygen needs, resulting in low WAT oxygen consumption[356]. This suggests that the inflammation development in WAT is not necessarily related to oxygen supply, at least in a cause-effect way. Furthermore, in humans, no correlation has been found between obesity and hypoxia [356], experimental data just pointed to a reverse situation [350].

Moreover the association between hypoxia and inflammation was also justified by the co-localization of hypoxic areas and infiltration of macrophages [349] and by the fact that hypoxia can enhance oxidative and endoplasmic reticulum stresses: both mechanism being directly correlated with the progress of the inflammatory response [357,358]. However, immune cells entry in a given tissue (WAT being a well-known example) is elicited by signals of aggression, being it hypoxia, alien proteins or antigenic compounds, changes in redox of pH homeostasis, excess energy substrates or signaling molecules imbalance [244]. The implication of macrophages could not be exclusively pinned to hypoxia alone, and consequently could not be used as telltale proof of hypoxia. In any case, there is a considerable dispersal of data and results in the literature; this way, it has been reported that limited WAT oxygenation

strongly affects lipolysis and lipid oxidation [359,360], the change the expression of adipokines and inhibits insulin signaling, thus being postulated as a primary cause for IR [347,361,362].

Nevertheless, the main "proof" used to attribute WAT an hypoxic state and to establish a correlation between obesity and the development of hypoxia, is the utilization of glucose through the cytoplasmic glycolytic pathway to lactate [363]. Its production and release in WAT of obese subjects increase [364]. As expected' larger (and older) adipocytes produce more lactate from glucose than smaller or plurivacuolar ones [274,275]. Lactate tends to lower blood pH, which because of the Bohr effect [365], allows more oxygen release from oxyhemoglobin, thus compensating -at least in part- the postulated hypo-oxygenation caused by the reduction of blood flow.

Muscle lactate production has been assumed to be part of a Cori cycle [248]; WAT lactate synthesis can be also considered in this category, because of the large WAT mass and the also considerable amounts of glucose and lactate involved.

In our group we found that adipocyte production of lactate is not "per se" a consequence of hypoxia, but that adipocytes are probably ''obligatory glycolytic'' cells (at least in most of its extra-vacuolar space) [283]. 3T3L1 adipocytes under conditions of normoxia produce lactate from glucose and proportionally to its levels. Glucose is used at extremely high rates: most of the glucose is converted to lactate with only part of it being used to synthesize fat [283]. Moreover WAT produces lactate, practically irrespective of site, diet or sex, and its ability to do it is not directly dependent of WAT metabolic state [271]. Lipogenesis need mitochondria and sufficient oxygen to proceed. The reason why mature adipocytes are glycolytic cells is supported by their mass, shape and composition, since these cells are just made up of an enormous lipid droplet covered by a thin layer of cytoplasm, where mitochondria are sparse [231] and the most immediate way to use glucose is anaerobic glycolisis. These data indirectly support the notion that hypoxia could not be a critical factor in the regulation of lactate production. On the contrary, it seems that for WAT with large adipocytes, to help dispose of excess glucose may be part of a, more general strategy to help lower glycemia and maintain glucose homeostasis [271].

However, we know little about adipocyte metabolism and the overall physiolocigal function of the adipose organ, adipose tissue stands as, probably, the main battlefield where the battle for knowledge of inflammation is being fought. The consequences of any breakthrough in our knowledge in this field may help us understand better and treat effectively MetS. The never-ending struggle of WAT against the "invisible enemy" created by misdirected defense mechanisms and the ingenuity of humans against the blueprint acquired during evolution must end. Metabolic quantitative data about 3C origin and metabolism could change the way

to see MetS origins and metabolic foundations. We do not actually know why WAT thrives in low oxygen, and why so much lactate (and, especially) glycerol are produced. We do not know if this is really a way to eliminate glucose and lower glycemia or if the objectives go ever farther. We are aware whether obesity is part of another strategy to defend our organism.

There is a gap of decades in the bibliography about 3C metabolites utilization, metabolic meaning and functions. Because of this lack of quantitative information of WAT and its components (adipocytes and stromal vascular cells), and with the long-term purpose to a better understanding of MetS, we decided to focus the research on 3C fragment WAT metabolism under different conditions; sexes and location, always including a systematic quantitative approach to establish lines of comparison.

3. JUSTIFICATION AND OBJECTIVES

This thesis was developed in the Nitrogen-Obesity research group of the University of Barcelona, within a line of studies focused in the mechanisms related to the control of body weight, insulin resistance and the metabolic syndrome. In the last years, the group centered its research especially on white adipose tissue metabolism, its utilization of substrates and the active role it plays in this syndrome.

This thesis project was carried out in parallel with Cecilia Ho Palma, another PhD student of the group. Both theses were focused on the same theme, the handling of glucose and 3C unit production by white adipose tissue, but worked on different aspects: Cecilia's centered on lactate and mine on glycerol as metabolites. The project needed the development of new methodology, including a method to isolate, incubate and analyze the metabolism of adipose tissue cells; this part constituted the start of the line I developed, and a quantitative tracer method to account for the fate of glucose, which constituted Cecilia's main line of work.

Most current theories on metabolic syndrome, with respect to endothelial inflammation produced in the adipose tissue point to hypoxia as one of its main causes. In spite of these assumptions, our group has found that both 3T3L1 murine adipose cells (under normoxic conditions) and *in vivo* WAT consume large amounts of glucose, with the consequent production of lactate [271,283] and glycerol (in amounts not justifiable only by lipolysis) [369]. Upon these antecedents lies the hypothesis that adipocytes act essentially as glycolytic cells, whose metabolism would affect blood glucose levels, contributing to the control of glycemia. The reduction of blood flow in the metabolic syndrome, despite the higher NO· production, would protect adipose tissue against the excess of substrates but, generate hypoxia at the same time. This state, thus innocuous for adipocytes, may affect the cells of the stromal fraction, leading to intra-tissular competition for oxygen and substrates.

Consequently, the main **objective** of this thesis (and Cecilia's) was to quantitatively verify this hypothesis by studying *ex vivo* the glycolytic (anaerobic in spite of normoxia) capacity of mature WAT (adipocytes and stromal) cells, identifying their differences in metabolic function, and the effect on them of sex and location.

Thus, the following partial objectives have been developed:

1- Adjustment of existing, and development of methods to carry out the experiments obtaining safe and repetitive quantitative results.

2- Analysis of glucose's fate and production of 3C metabolites, especially lactate and glycerol, by rat epididymal WAT's adipocytes, to check their glycolytic *ex vivo* capability.

3- Comparison of the effects of sex and WAT site on adipocytes' handling of glucose, by studying cells isolated from perigonadal, mesenteric and subcutaneous WAT from rat of both sexes.

4- Evaluation of the quantitative contribution of stromal vascular cells (in comparison to adipocytes) to the generation of 3C from glucose through the glycolytic pathway under normoxic conditions in different WAT sites.

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INFORME DELS DIRECTORS DE LA TESI DOCTORAL SOBRE L'AUTORIA DEL TREBALL REALITZAT

Amb relació a la tesi doctoral de Floriana Rotondo: *"Glucose and white adipose tissue metabolism, effects of site and sex on the fate of glucose"*, de la que els sotasignats som co-directors, d'acord amb la normativa vigent, fem constar que:

a) Bona part del treball de la present tesi doctoral ha estat feta per la candidata a doctora Floriana Rotondo en col·laboració amb una altra candidata del nostre departament, Ana Cecilia Ho-Palma. Com s'indica en la pròpia tesi, ambdues han compartit bona part del seu treball de tesi, amb un objectiu comú que fa necessària la inclusió de la tasca realitzada plegades per una millor comprensió dels resultats. El treball no hagués estat possible de realitzar sense aquesta col·laboració. Entenem que la inclusió de quatre dels treballs generats en ambdues tesis doctorals permetrà una més adequada avaluació de les tesis i podrà donar al Tribunal avaluador una visió molt més general dels plantejaments estudiats.

b) Cal remarcar que els directors (Dr. Xavier Remesar Betlloch i Dr. José Antonio Fernández López) de la tesi doctoral d'Ana Cecilia Ho Palma (i, òbviament, ella mateixa) accepten plenament aquest plantejament, que esperem repetir en la presentació de la tesi d'Ana Cecilia.

c) La normativa ens demana que indiquem, de manera aproximada, però, en quina proporció ha contribuït en cada treball cadascuna de les estudiants de doctoral. Fetes les consultes pertinents, i d'acord amb els co-directors de la tesi d'Ana Cecilia Ho-Palma, entenem que per a cada un dels treballs que es presenten en aquesta tesi doctoral, la participació és la que indiquem en percentatges del treball global:

1) Ho-Palma AC, Rotondo F, Romero MM, Memmolo S, Remesar X, Fernández-López JA. Alemany M. A method for the measurement of lactate, glycerol and fatty acid production from ^{14}C-glucose in primary cultures of rat epididymal adipocytes. Analytical Methods 2016, 8: 7873-7885, Q2

20 % Floriana Rotondo 80 % Ana Cecilia Ho-Palma

2) Rotondo F, Romero MM, Ho-Palma AC, Remesar X, Fernández-López JA, Alemany M. Quantitative analysis of rat adipose tissue cell recovery, and non-fat cell volume, in primary cell cultures. PeerJ 2016, 4: e2725, Q2

80 % Floriana Rotondo 20 % Ana Cecilia Ho-Palma

3) Rotondo F, Ho-Palma AC, Remesar X, Fernández-López JA, Romero MM, Alemany M. Glycerol is synthesized and secreted by adipocytes to dispose of excess glucose, via glycerogenesis and increased acylglycerol turnover. Scientific Reports 2017, 7: 8983 Q1

50 % Floriana Rotondo 50 % Ana Cecilia Ho Palma

4) Utilization 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. AC Ho-Palma, F Rotondo, MM Romero, JA Fernández-López, X Remesar and Marià Alemany. Submitted to Biochim Biophys Acta – Mol Cell Biol Lipids in 11/2017

50 % Floriana Rotondo 50 % Ana Cecilia Ho Palma

5) Influence of sex on substrate handling by adipocytes isolated from subcutaneous, mesenteric and perigonadal adipose tissue of rats. F Rotondo, AC Ho-Palma, X Remesar, JA Fernández-López , MM Romero and M Alemany. Submitted to Scientific Reports in 8/2017

100 % Floriana Rotondo

6) In rat white adipose tissue, lactate production by adipocytes and nucleated stromal cells is quantitatively comparable, but only adipocytes also release glycerol. F Rotondo, AC Ho-Palma, MMar Romero, X Remesar, JA Fernández-López and M Alemany. To be submitted to Adipocyte in 12/2017

100 % Floriana Rotondo

En conjunt, el treball de Floriana Rotondo seria "equivalent" a quatre treballs sencers dels Q1-Q2.

Dra. María del Mar Romero Romero Dr. Marià Alemany Lamana

Barcelona, 14 de desembre de 2017.

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1 / 1

4. RESULTS

4.1. Development of quantitative methodology

4.1.1. Presentation

The methods were critical because they had to be adapted for use, from a quantitative point of view, in minimal amounts of sample. Since no such methodology existed, the first step in our work was to adapt, modify and check specific procedures for the development of the experiments conducted during the thesis.

The first of these methods was developed in order to establish in a definitive way the metabolic fate of glucose in primary cultures of rat adipocytes, and to clarify the reasons behind the massive production of lactate. We used ^{14}C-labelled glucose and adapted to the small volume of a single incubation well. The procedures allowed us to analyze, using the same cells of a standard culture well, the fate of glucose to form both lactate and glycerol, as well as its incorporation into the lipogenic path and to TAG.

The second method presented was a consequence of the need to isolate and obtain viable cells from rat WAT, for subsequent incubation, allowing a quantitative analysis of their distribution in the tissue. According to this approach, we had to adapt the already existing methods to our necessities, determine the viability and functionality of cells up to 48 hours incubation and find the better way to count and analyze the different cell types.

Originally the method was thought to separate all kinds of WAT cells, but the difficulty to obtain enough viable cells of each type, lead us to focus just on adipocytes and on the whole cells of stromal vascular fraction. In the latter, we found a large number of red blood cells, despite the fact that the animals were killed by exsanguination. For this reason, we had to differentiate these cells and count them separately. After using cytometric analysis to evaluate the percentage of erythrocytes, we started to use the Scepter 2.0 cell counter, employing the combination of two kind of sensor (each sensor specific for a different range of cells size) to count both nucleated and not nucleated stromal cells. To do that we had previously established a red blood cells Scepter profile. For mature adipocytes' counting we used microphotographs of cells within a Neubauer chamber in combination with the ImageJ software, to measure their size.

We used a classical collagenase tissue digestion to liberate cells; different kinds of filter meshes were tested and observed through microscope, to find the better to obtain the maximal yield in adipocytes recuperation. The quantification of adipocytes recovery, through the analysis of lipid, led us to another challenge: the analysis of the proportion of “live” cell space. This has been possible through the combination of cell counting and size analysis

with the measures of tissue and lipid density, water and lipid content of adipocyte fractions compared with the whole tissue. Additional analyses of protein and DNA content were done but then discarded for the nuclear study, because of the limitations uncovered.

Usually, the methodology available can deeply condition the data obtained from different experiments, driving to a large variability between them and complicating the possibility to refer them to the whole live tissue. For this reason, a deeper knowledge of WAT composition (accumulation of homologous data from the same source) and cell recovery have been a necessary step for direct comparison of data from different sources and for a quantitative approach.

The first method was mainly developed by Cecilia Ho Palma, while the second one was mainly developed along my work’s line. My participation to each study was respectively of about 20% in the first and in the range of 80% in the second. My colleague and friend Cecilia, reversing the percentages for the two 'symbiotic' methodologies developed.

The methods were combined in the later experiments, allowing the quantitative analysis of the different data obtained from the same source, for different times and conditions, facilitates comparison and leading to a complete information of the systems analyzed.

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

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

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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.

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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

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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

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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 ± 46 × 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

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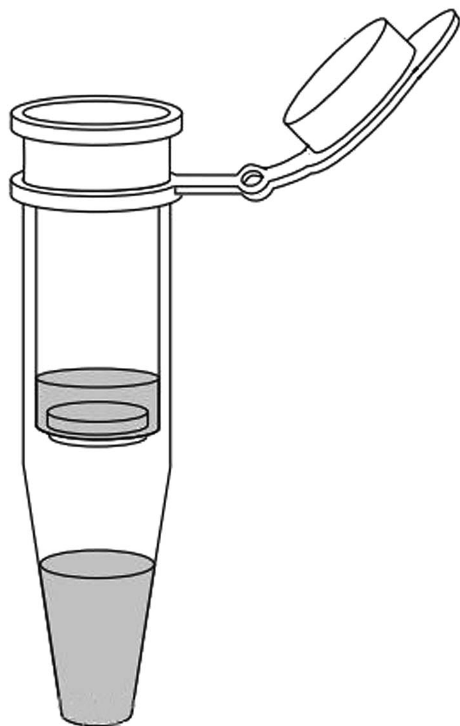


Fig. 1 Centrifuge-separation tube with plastic insert used in the preparation and use of ion-interchange columns.

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.

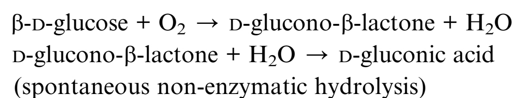
In order to check the effectiveness of the columns, an analysis of lactate label distribution was carried out under otherwise standard conditions (but no cells). We used cold (5 mM) and labeled L-lactate [317 Bq per well] (^{14}C-(U)-L-lactate; #NEC-599, Perkin-Elmer, Boston, MA USA; specific activity 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)

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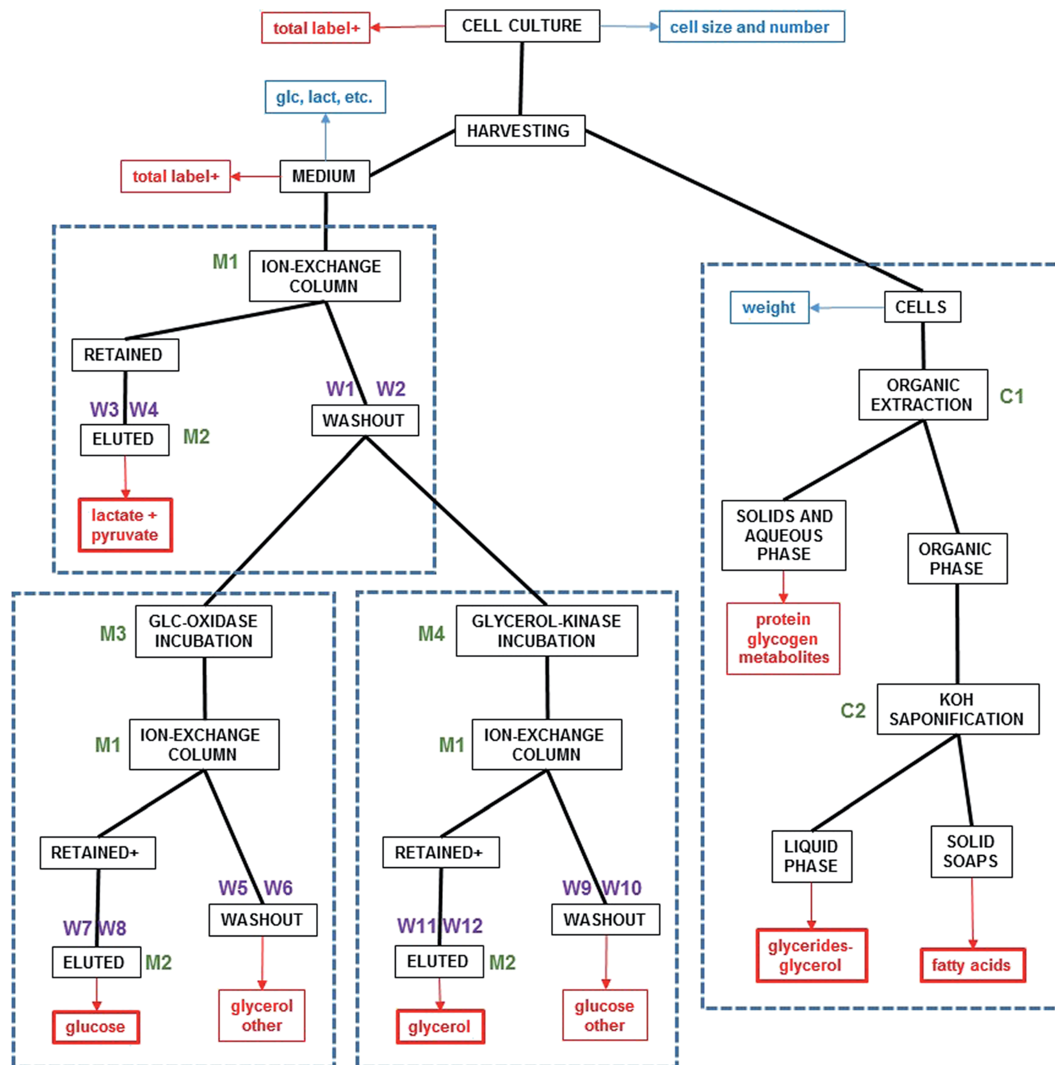
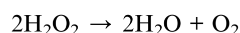


Fig. 2 Flow diagram of the process of analysis of the fate of ^{14}C-glucose label in the medium and cells of rat epididymal WAT incubated for 24 hours. The process is marked in black, red fractions refer to the counting of the fraction radioactivity, and blue is reserved for non-radioactive measurements (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.

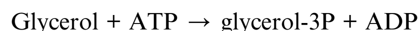
The label retained in the column was eluted following the same procedure described above for lactate, which yielded washouts W7 and W8 (total volume of 1 mL), which were counted using several scintillation vials. They contained the eluted gluconic acid whose label was that of the original glucose present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

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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.

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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.

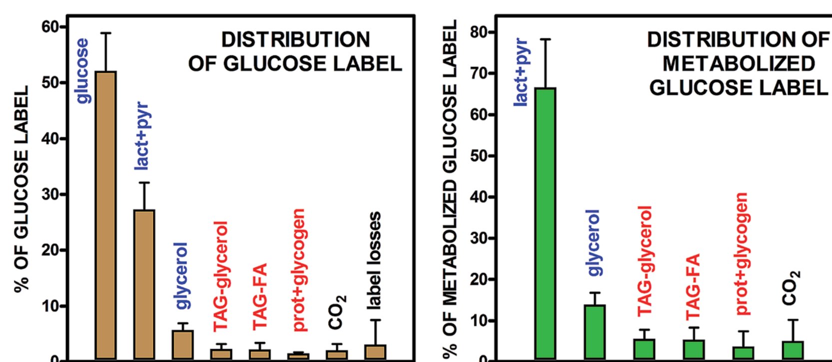

Fig. 3 Percent distribution of labeled glucose (total label and metabolized glucose) after 24 h of incubation by epididymal adipocytes. The results are the mean ± sem of four different rats. TAG = triacylglycerols; FA = fatty acids; prot = protein; lact + pyr = lactate + pyruvate.

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,

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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.

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

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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

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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

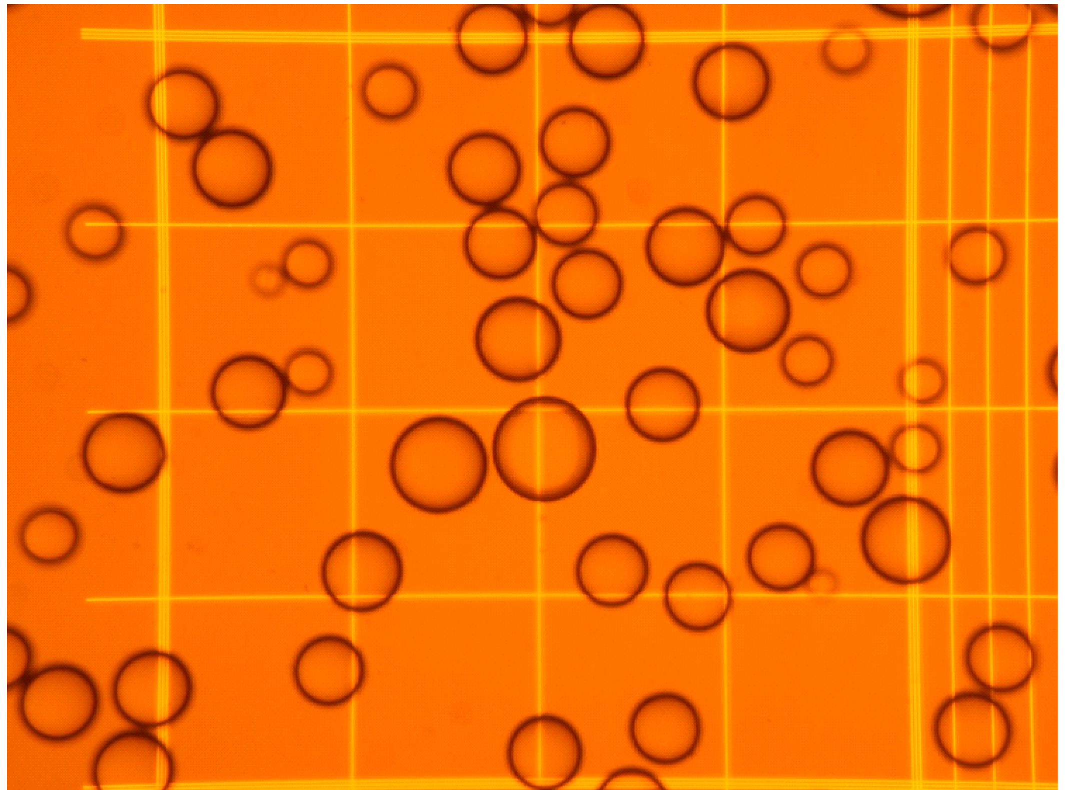
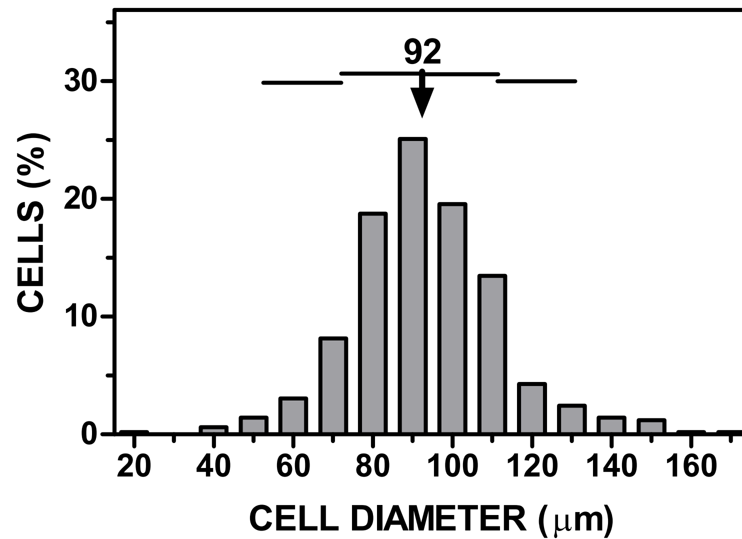


Figure 1 Representative microphotography of an adipocyte preparation observed at the microscope using a Neubauer chamber. The squares in the grid have a width of 250 µm, and correspond to a volume of 6.25 nL.

± 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



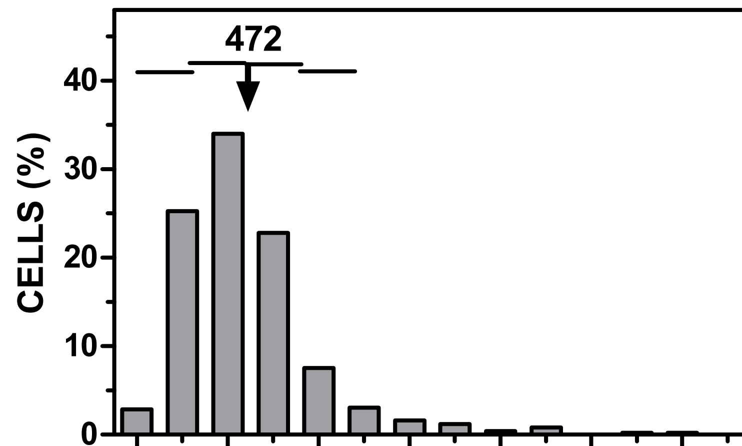


Figure 2 Representative graph of cell size (diameter, volume) *vs.* cell numbers representation obtained applying the cell extraction procedure described in the text to a sample of epididymal adipose tissue. The data have been grouped to facilitate the presentation. The arrow (and the number above) represent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

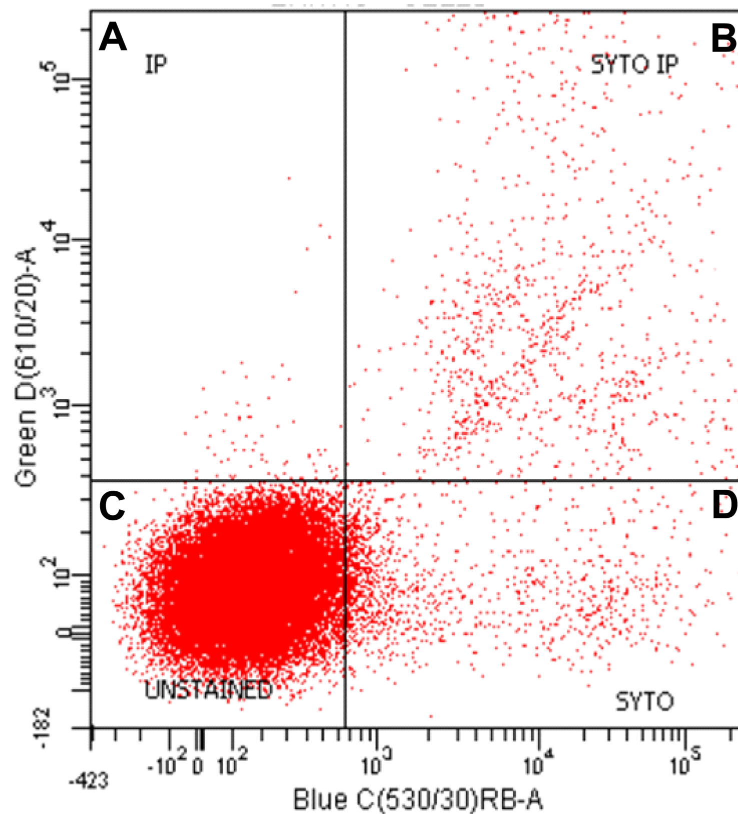


Figure 3 Representative graph of flow cell analysis of stromal fraction of epididymal rat WAT to discriminate nucleated from non-nucleated cells. Both propidium Iodide (IP) and Syto-13 (SYTO) bind DNA-positive and double positive particles (i.e., cells). The dots in the (A), (B) and (D) correspond to 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 $\times 10^6$	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 $\times 10^6$	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.

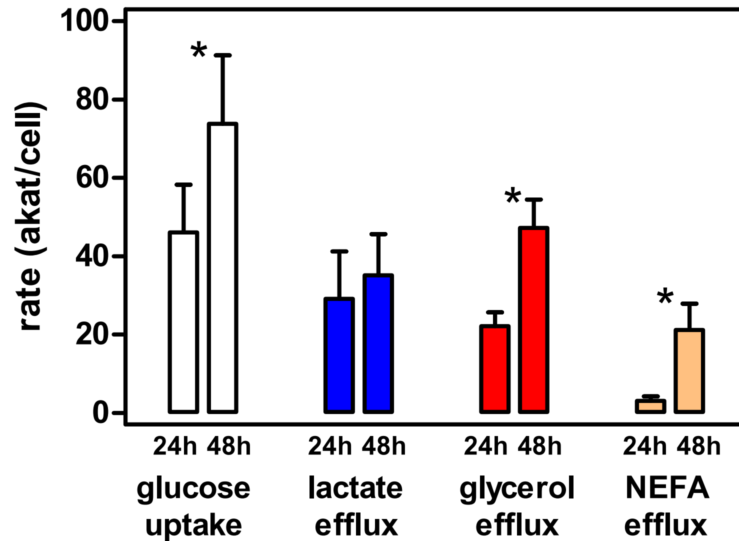


Figure 4 Metabolic activity of epididymal WAT adipocytes in primary culture at 24 h and 48 h of incubation. The data represent the mean ± SD of four different rats (triplicate wells). The data are presented as rates of uptake (glucose, white bars), or efflux to the medium (lactate, blue bars, glycerol 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.

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ADDITIONAL INFORMATION AND DECLARATIONS

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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.

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4.2. Adipose cells metabolism: glucose fate and 3C release

4.2.1. Presentation

The second part of this thesis employed the methods developed and described in the previous chapter. They were used to study the fate of glucose and the source of the carbon in the 3C substrates analyzed, mainly lactate and glycerol because of their high production. This was a pathfinding study, using newly developed quantitative methodology combined with semi-quantitative analysis of gene expression applied to the pathways under investigation. Obviously, our first try with this powerful tool was to analyze the basal conditions. For this reason, the studies included in the present thesis were conducted in the absence of any external stimulus such as hormones or drugs, checking only the effects of glucose availability.

The first series of experiments dealt with mature large adipocytes isolated from rat epididymal WAT. The investigation of Cecilia Ho Palma was centered principally on lactate and mine mostly on glycerol. Thus, our participation in the two papers included was roughly equal for both studies, since the two of us worked on cell incubation and metabolites analyses, then Cecilia did most of the work on label distribution analysis and I completed the data on expression.

We started using epididymal white adipose tissue because it is widely used as "representative" of WAT, easy to dissect without contamination of other tissue, and quite abundant to allow for both radioactivity and gene expression analyses.

In these studies we combined the information of specific activity (from radioactivity data) with medium metabolites efflux and gene expression, all obtained from the same source and under identical conditions. Thus, we could establish a rather complete picture and overall information of glucose fate, in which all (quantitative) data have concordant origins, are methodologically coherent and reinforce the conclusions using different sources, methods and strategies.

After analyzing the large number of results emerging from these studies, the rest of my thesis was focused on a second group of experiments, largely consequence of the results obtained. The objective was now to find out the effects of site and sex in the handling of glucose, as well as the contribution of stromal vascular cells to WAT metabolism. The latter study was done by analyzing these cells under the same conditions used for adipocytes, and the results we obtained were, again, new and even surprising.

Due to its localization, WAT functions are in part specialized, but they are also affected by sex. In fact sexual distribution of the main fat masses, and even the body percentage of lipid

is dependent on sex. In order to combine these two factors: site and sex, we selected three sites: a) perigonadal WAT (i.e. periovaric in females and epididymal in males, anatomically and -we hope- functionally analog), considered as an example in the storage WAT; b) subcutaneous WAT, the WAT most studied in human (in this case we used a uniform piece of this WAT tissue, the inguinal cordons); and c) mesenteric WAT, because of its close relationships with intestine and liver. Mesenteric WAT is a genuine representative of visceral WAT, which is often considered responsible of most of the co-morbidities associated to obesity.

To carry out these studies (which I fully developed), the quantitative analysis of WAT composition for the different male WAT “depots” in both sexes was repeated. Moreover, because of the erythrocytes' large presence in the stromal fraction, we incubated, separately, red blood cells in the presence of glucose, to quantify their possible contribution to the metabolite efflux in medium (gene expression analysis posed no problems because of the absence of nuclei). We also incubated tissue slices to analyze gene expression, glucose uptake and metabolites' release from whole tissue. This part of the study was stopped after processing all samples and performing gene expression analysis of perigonadal WAT, because of the need for a time and funds we lacked.

In parallel to these groups of experiments, the research group followed, with Cecilia Ho Palma, another line of study, continuing on the search of how insulin affects the fate of labelled glucose, with the question of how insulin could affect glucose wastage, lipogenesis and the release of glycerol and lactate. This line was now her sole responsability.

The obvious continuation of the overall study would be to test other hormonal stimuli (such glucocorticoids or catecholamines, but also the own adipokines), a path followed in part by Cecilia. However, the last paper presented opened an even larger window for study, the so far ignored conjoint metabolic role of nucleated stromal cells and adipocytes, probably centered on their influence on adipocyte equilibrium between 3C handling and lipogenesis. First checking the eventual presence of specialized oxidative cells, and those attached to the surface of adipocytes, but also analyzing the proportions of cell types and their contribution to overall WAT metabolism under hormonal modulation and, especially inflammation. Too much for a thesis to carry on, but at least the unwritten objective to look at adipose tissue somewhat differently, with more respect and, perhaps, limiting the verdict of guilty for inflammation and metabolic syndrome, has been advanced. The near future will clarify further this question.

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

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

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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,

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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.

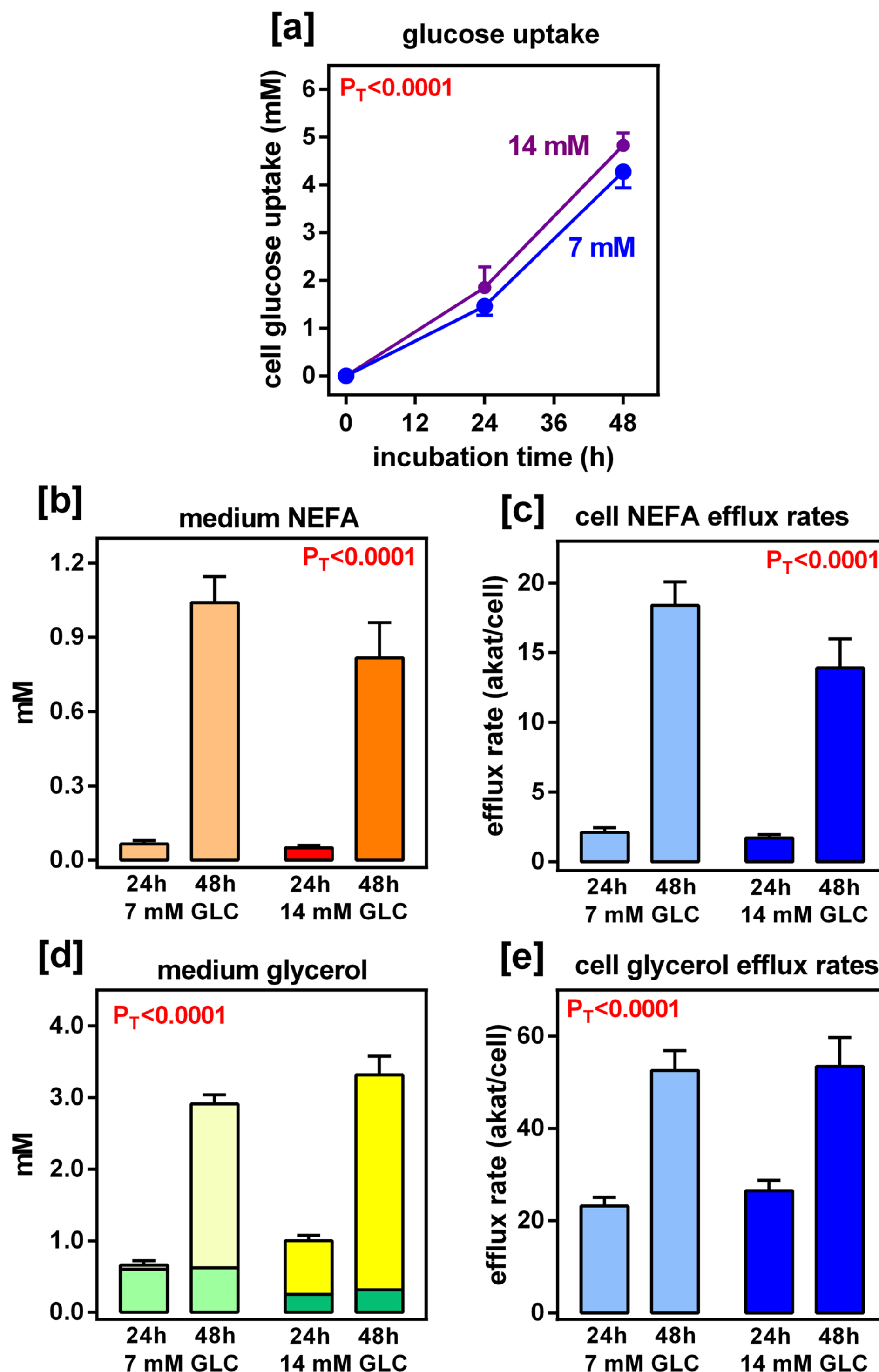

Figure 1. Effects of medium glucose concentration and incubation time on glucose uptake, and on the efflux of NEFA and glycerol, by primary cultures of rat epididymal adipocytes. The data are the mean ± sem of four different pairs (pooled) of rats; [**a**]: Glucose uptake vs. time. Blue circles: nominal 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 ($P_i = 0.0021$).

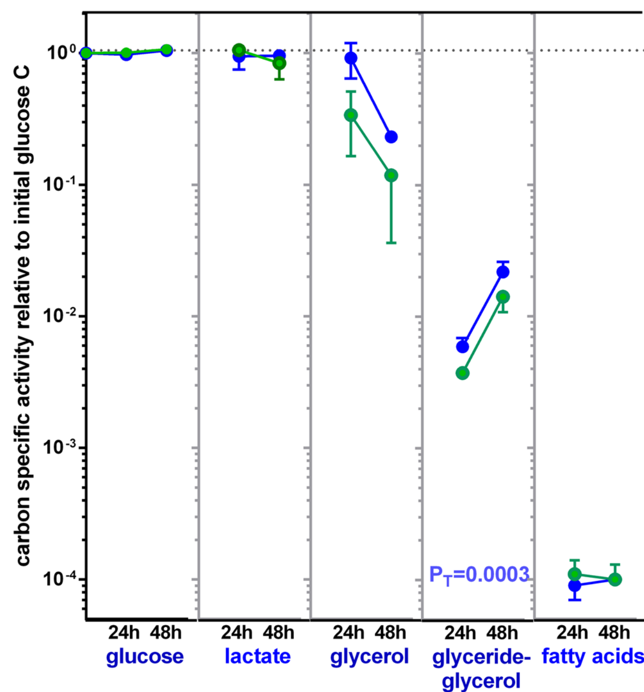

Figure 2. Carbon specific radioactivity of the main label fractions obtained after incubation of epididymal adipocytes in a primary culture in the presence of ^{14}C-glucose. The data are presented as mean ± sem of four different rats, and are presented in a log scale to show the wide differences between 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 epidydimal 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

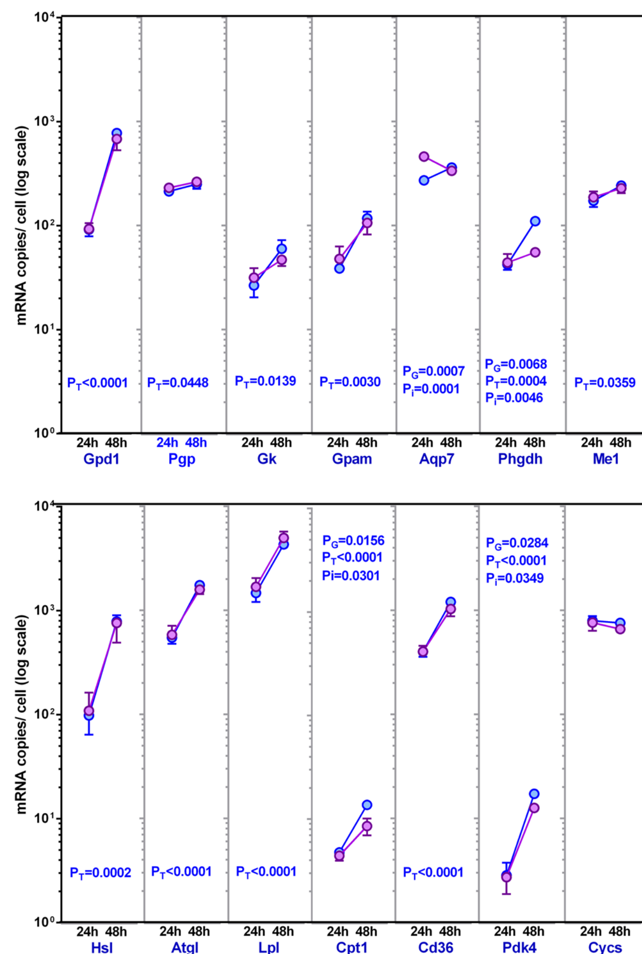

Figure 3. Gene expression of proteins related to glycerol metabolism in adipocytes incubated under varying glucose concentration for up to 48 h. The data are presented as number of copies of the corresponding mRNA per cell, and are mean ± sem of data from four rats. The data were obtained from the "parallel" 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.

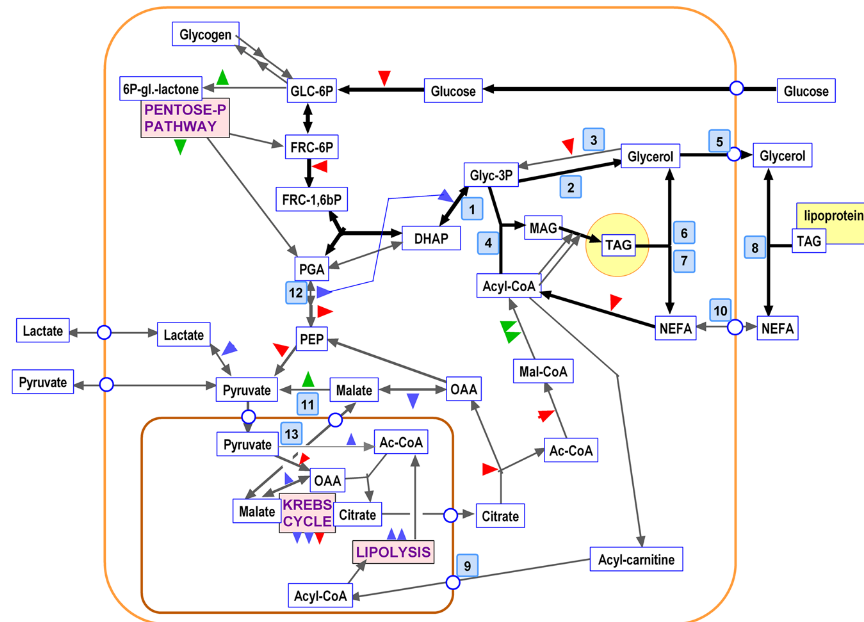

Figure 4. Main metabolic pathways affecting glycerol in the context of glucose-fatty acid metabolism in the adipocyte. The graph presents the main intermediate metabolites and substrates. Wide lines correspond to main pathways activated by incubation with glucose, whilst thin lines represent inhibited 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).

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Utilization 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

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Abbreviations used:

FBS = fetal bovine serum; NEFA = non-esterified fatty acids; TAG = triacylglycerols; WAT = white adipose tissue

Abstract

White adipose tissue converts large amounts of glucose to glycerol and lactate. In the present study, we quantitatively traced the label from glucose to lactate, glycerol and fat in primary cultures of mature rat epididymal adipocytes. Cells were incubated with 7/14mM ^{14}C-glucose for 24/48 h. Medium metabolites and their label and that of cells' components were measured. Parallel non-labelled incubations were used for gene expression analysis of key proteins glucose catabolism. Glucose concentration had little effect on the results. Lactate efflux was essentially unaltered. But glycerol efflux increased after 24h, coinciding with an arrest of lipogenesis. The results showed a steady glycolytic production of lactate, for cell maintenance, but also a marked glycerogenesis, largely in the 24-48h period. The adipocyte metabolism change was elicited/paralleled by gene expression. Only a small fraction of glucose was used for lipogenesis, blocked after 1-day incubation, when glycerol efflux increased amidst an active triacylglycerol turnover. Lactate steady efflux showed that anaerobic glycolysis was the main adipocyte source of energy. Factoring in the large mass of adipose tissue, we can assume that it plays an important quantitative effect on the maintenance of glycaemia, removing blood glucose and returning 3C fragments, thus avoiding lipogenesis.

Key words: Adipose tissue; adipocyte; lactate; glycerol; lipogenesis; anaerobic glycolysis

1. Introduction

WAT is a disperse organ, considered for a long time 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 insufficient or cumbersome because of extreme dispersion. Cytoplasm continuity within a mature adipocyte is severely constrained by the huge fat vacuoles, i.e. physically limiting the metabolic interaction between thinly stretched cytoplasm and mitochondrial powerhouses, which conditions its metabolism [7].

These considerations portrait a unique, complex and often misunderstood organ made up from quite a number of 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 [8], into deadly components of metabolic syndrome molecular inflammation [9]. We have not had evolution time to evolve methods to cope with the derangement caused by affluence [9]. However, there are responses to this epidemic 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 [10].

WAT, as main storage space (albeit not unique [11]) also defends itself from this 'excess energy aggression', first limiting blood flow, but also through hypertrophia and inflammation [12], making difficult lipogenesis because of the sheer size of the cells [13]. Isolated adipocytes and intact white adipose tissue (WAT) secrete significant amounts of lactate, glycerol, and other 3C fragments derived from glucose, under basal conditions [14]. They may be used for hepatic gluconeogenesis [15], for lipogenesis [16] or, more probably, for energy elsewhere.

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 end product of cytoplasmic glycolysis, massively reduced to lactate as a way to eliminate excess cytoplasmic NADH. Alanine is also a common 3C export product from peripheral tissues [17] 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 by glycerol dehydrogenase and NADH, yielding *sn*-glycerol-3P, which can produce free glycerol by the action of a phosphatase [18] or esterified with acyl-CoA to form acyl-glycerols. It has been assumed for a long time that glycerol released by WAT is just a byproduct of lipolysis of TAG. It is also known that WAT capacity to recycle glycerol is limited [19].

Despite pyruvate and lactate being potentially good lipogenic substrates for WAT [16], neither alanine [20] 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 [21].

In WAT, the steady supply of glycerol-3P sustains the synthesis of acylglycerols by the incorporation of acyl-CoA provided by the lipogenic pathway, which depends on the availability of glucose [22] or other sources of acetyl-CoA [16]. Acyl-CoA may be also produced via activation, with ATP, of free fatty acids, usually taken up from the extracellular space. The lipogenic pathway is more active in small young adipocytes [23] than in large mature adipocytes, which tend more to incorporate preformed fatty acids [24].

3T3L1 cells can convert large amounts of glucose into lactate through (anaerobic) glycolysis in the presence of abundant oxygen [25]. Similarly, when studied *in vivo,* rat WAT [26], also produces large amounts of lactate in normoxic conditions. Despite sufficient oxygen availability, in WAT, glucose was also converted to other 3C units, such as alanine, and glycerol [13, 27]. In normal cells, with sufficient proximity to mitochondria, pyruvate is oxidized to acetyl-CoA in a process, which, in contrast, is fully aerobic. 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 [13, 27] 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 [28]. The low proportion of active cell content is, thus, stretched thin, severely hampering the free interchanges between cytoplasm and mitochondria, and, as a consequence, fully oxidative metabolism is limited to the cell sections close to the (often scarce) mitochondria. In addition to hamper (oxidative) energy production from glucose, lipogenesis is also severely reduced [13, 29]. Cell size, thus, has a deep influence on adipocyte function.

The situation described is not observed in smaller, younger, adipocytes, which show a comparatively remarkable lipogenic capacity [23] in contrast with the mature cells [24]. Small plurivacuolar cells, such as 3T3L1 fibroblast-derived adipocytes, show both active glycolysis [25] and lipogenesis [30]. The proximity of mitochondria allows for their full oxidative potential to be extended practically to the whole cell, essentially allowing the function of mitochondria as energy and acetyl-CoA provider for lipogenesis, to complete the glycolytic and NADPH synthesis processes of cytoplasm in small adipocytes [23]. Lipogenesis and glycolysis run parallel in 3T3L1 cells, but larger WAT adipocytes could not maintain this relationship, and lipogenesis is decreased [24]. Since lactate-pyruvate production through anaerobic glycolysis is maintained in basal conditions [26, 27], most of the ATP should be provided by the inefficient glycolysis [25], which favors the hacking of huge amounts of glucose to pyruvate, and then to lactate.

Glycerol is released without a parallel efflux of NEFA (i.e. the products of lipolysis) to justify its appearance [27]. 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 [13]. The glycerol secreted by incubated adipocytes is generated from glucose via glycerol-3P [27]. However, the direct, phosphatase-mediated path, massively responsible of the initial production of free glycerol was largely substituted, with longer incubations, by glycerol from increased cell TAG turnover [13, 27]. Most glycerol was released to the medium via aquaporin 7 [31], but fatty acids were recycled, minimizing the actual NEFA (non-esterified fatty acids) efflux [13].

The present study was carried in parallel and in part superimposed to the paper by Rotondo *et al.* [13] cited above and centered on glycerol. Here we have focused our attention on the quantitative fate of glucose and overall massive conversion of the sugar into 3C units, checking the proportion of glucose that is mobilized through lipolysis. This is the key factor of our analysis because of the extended notion that lipolysis is a main path to dispose of excess glucose in WAT. However, the purposeful synthesis of glycerol, even at the expense of accelerated TAG turnover [13, 27] represents a considerable change in our view of this tissue. Because of the quantitative nature of our study, we have also analyzed the lesser components of the 3C glycolytic family: pyruvate and alanine. This process may be part of the mechanisms of WAT to defend itself from excess substrates [2, 27], such as glucose, under conditions of insulin resistance, by means of a self-regulated mechanism to prevent the excessive accumulation of TAG and to help lower glycaemia. The use of ^{14}C-glucose allowed us to establish the proportions of glucose used by adipocytes to produce 3C units in comparison with its oxidation and lipogenesis [29].

2. Methods

2.1 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 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, Harkan) and water at any time, and were kept in two-rat cages with wood shards as bedding material, at 21.5-22.5ºC, 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, and was used immediately for adipocyte isolation.

2.2 Isolation, measurement and incubation of adipocytes

Adipocytes were isolated by incubation with collagenase as described in a previous paper [28], essentially following the Rodbell procedure [32]. Cells were counted, and their (spherical) diameters measured using the ImageJ software (http://imagej.nih.gov/ij/) [33]. The yield with respect to WAT sample mass was estimated in a number of randomly selected samples as previously described [28]. 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, 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), 1 mM pyruvate (Gibco), 30 mg/mL delipidated bovine serum albumin (Millipore Calbiochem, MA USA), 100 U/mL penicillin and 100 mg/L streptomycin and 100 nM adenosine (Sigma-Aldrich).

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 of different size [29]. 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 measured under the same conditions [25]. The cells were incubated for 24 or 48 h without any further intervention, as previously described [28]. 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 additional medium metabolite analyses.

2.3 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 [29]. 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 easy to use, non-reactive, and is highly effective for TAG [34]. 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 in the cold with KOH in ethanol [35]. The ether-insoluble potassium soaps were extracted and counted. The aqueous phase, which contained essentially glycerides-glycerol was also removed and counted [29]. 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 those of fatty acids (soaps) and glycerides-glycerol. The cells of the 'parallel' wells were used to extract their RNA for analysis of gene expression.

2.4 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 mutarotase (porcine kidney, 136A5000, Calzyme, St Louis, MO USA) [36]. 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 [37] in 1.5 mL of tris-HCl buffer 92 mM pH 7.2, containing 100 nM 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 OD 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 OD (i.e. consumption of NADH) [29]. When the OD was stabilized, alanine was measured [37] with the addition to the cuvettes of 20 µL (170 nkat) of alanine transaminase (porcine heart #G8255 Sigma-Aldrich), and comparing the rates of OD decrease *vs.* time in the samples against alanine standards.

TABLE 1 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	

2.5 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 [29]. 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 1x2 ion exchange resin (Serva

Electrophoresis GmbH, Heidelberg, Germany) as previously described [29]. 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 [29, 38].

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 [29, 39].

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.[29]). The label present in TAG fatty acids allowed us to calculate the amount of glucose to be oxidized to CO_2 in that synthesis; since the ratio was constant, label in CO_2 was calculated from that found in the cell (soaps fraction) fatty acids.

2.6 Gene expression analyses

Total cell RNA was extracted from the harvested cells ('parallel' wells) using the Tripure reagent (Roche Applied Science, Indianapolis IN USA), being 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.

We used a semi-quantitative approach for the estimation of the concentration of specific gene mRNAs per unit of tissue weight [40]. *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 1.

It was not feasible to use a meaningful 'zero time' for gene expression data because the cells were 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 minimal [28], and there were no changes in the levels of oxygen during the 2-day incubation [25].

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

3. Results

3.1 Metabolite efflux

Figure 1 shows the concentrations of metabolites in the medium after incubation of adipocytes for 24 or 48 h. The fall in glucose levels was steady and similar for 7 mM and 14 mM being directly related to the time of incubation. When the data were analyzed as percentage of initial values (data not shown), no differences were found between the two glucose concentrations tested. Medium lactate increased steadily

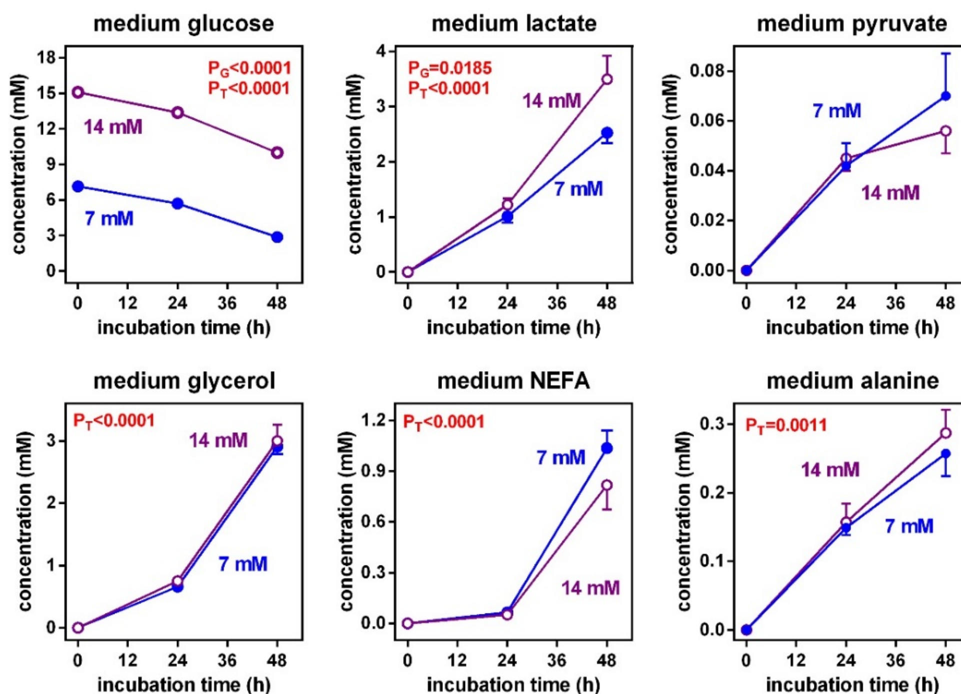
and almost linearly with time, showing small (albeit statistically significant) differences between the glucose groups. Pyruvate levels were much lower than those of lactate, and no statistically significant differences were observed between the groups, in fact, the presence of pyruvate practically did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

Table 2 Rates of glucose uptake and efflux of, lactate, pyruvate, alanine, glycerol and NEFA from isolated epididymal WAT adipocytes in primary culture.

process (attomol/cell·s)	7 mM glucose		14 mM glucose		P values
	24 h	48 h	24 h	48 h	
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. 1 and/or described in the text. The rates are shown in uniform units: attomoles per second and cell (comparable to akat/cell).

* Data also presented in reference [13]



[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

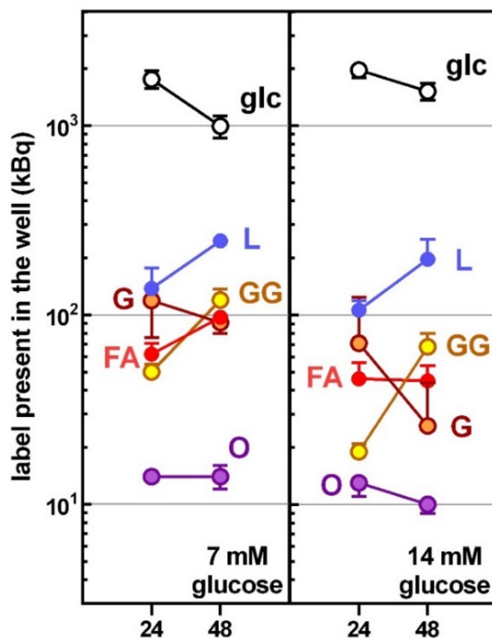
Concentrations of glucose, lactate, pyruvate, glycerol, NEFA and alanine in the medium of adipocytes isolated from rat epididymal WAT incubated for 24 or 48 h.

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. 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 and the effect of time of incubation by P_T.

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.

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.



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.

The data correspond to the mean ± sem of 4 different 2-rat pools, and are presented in a log scale.

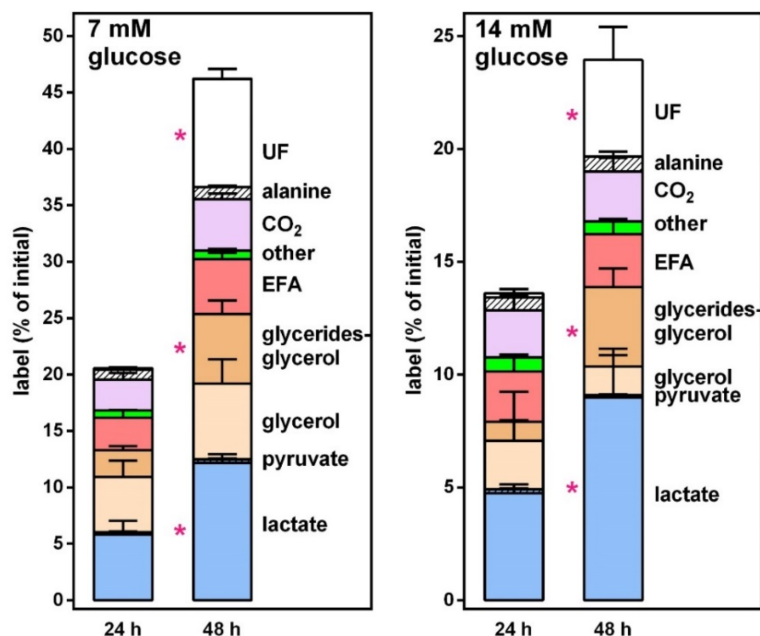
The statistical analysis (2-way-ANOVA) results are shown in the embedded Table; NS = not statistically significant.

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 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 rather stable in 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 2; these rates 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 may yield two lactates, 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 remained fairly uniform with time and glucose concentration.

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



	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 fate, calculated from the radioactivity incorporated into the different label 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 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.

3.2 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. Figure 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 not accounted for label. 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.

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 less error) which maintained a similar amount of label. The rise in glycerides-glycerol label was considerable, significant for time and glucose. Changes in the 'others'

(glycogen) fraction did not show effects of time or glucose, in addition, the label present in this fraction was extremely low

When the data in Figure 2 were tabulated and adjusted to the actual amount of glucose label we obtained the stacked histograms of Figure 3, in which the fate of glucose label actually 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); 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 showed little effect on its 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 50-60 % of all glucose metabolized, and that of fatty acids only about 10-12% in most groups. The proportion of preserved 3C (plus glycogen) vs. lipogenic products was (at 48 h) in a range close to 6.

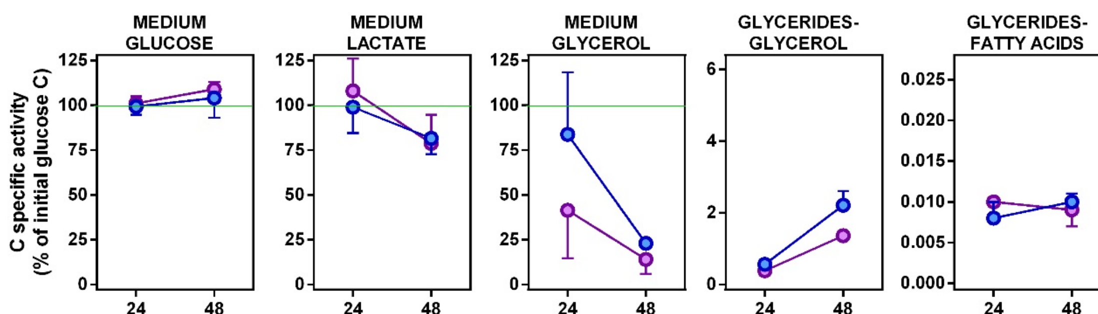


Figure 4 Carbon-specific radioactivity of medium glucose, lactate, glycerol and the cell glycerides components: glycerol and fatty acids.

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. Colour conventions are the same as in Figure 1.

Statistical analysis of the differences between groups (2-way ANOVA): The only significant data correspond to time: $P_T = 0.0003$ (glycerides-glycerol). Glycerol fractions showed no significant differences with respect to glucose.

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 specific activity. In spite of the considerable variability of individual data, the simple differences in scale of the specific radioactivity of the fractions, all derived from glucose (the only initial source of label) shows that, as expected, glucose specific activity remained unchanged. That of lactate, was also maintained (no statistically significant effects of glucose or time were observed) on the same range than glucose

In medium glycerol, the specific radioactivities clearly decreased with time and, at 48 h 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 in the limit of significance but was not statistically significant. The glycerides-glycerol values, however, were much lower, at least one order of magnitude at 48 h, than those of medium 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.

3.3 Protein gene expression

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 per cell of its mRNA 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 (genes *Pfkj* and *Pfkm*) showed the same pattern (i.e. no effects of glucose concentration and increased expression with incubation), but the *Pfkl* isozyme showed a more powerful increase response and had a much higher level 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 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 glycolytic enzymes described above, 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 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.

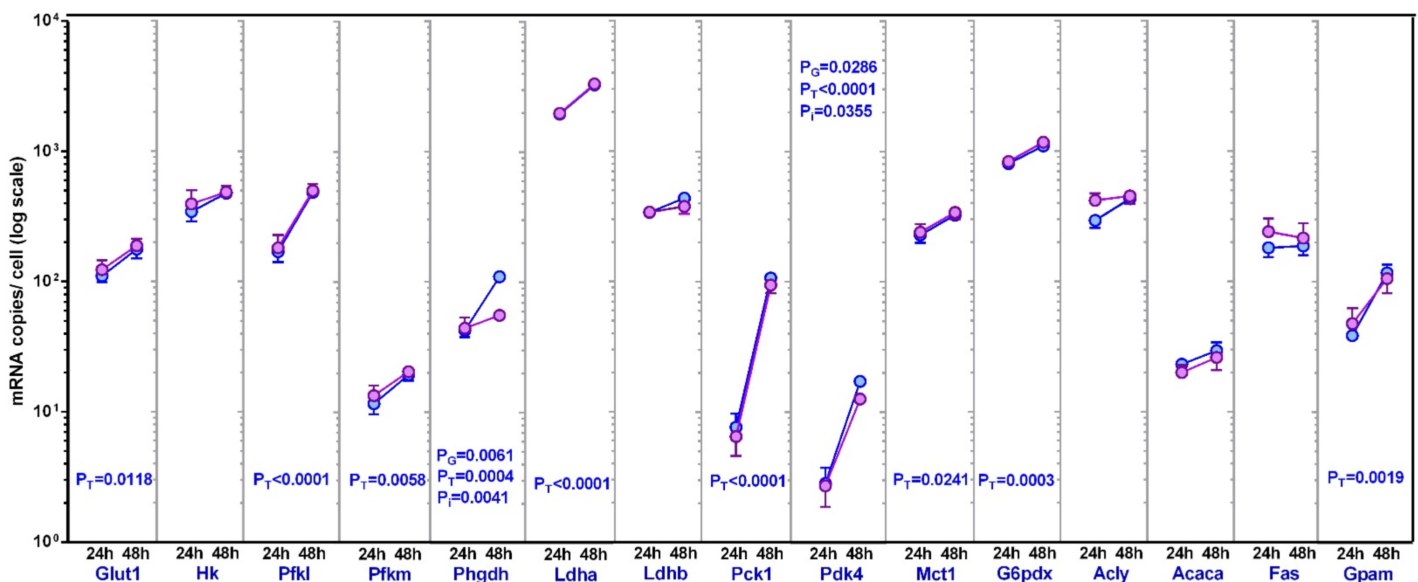


Figure 5 Level of expression, in adipocytes, of the main enzymes related to glucose uptake and glycolysis shown in Figure 1, and are expressed as the number of copies of the corresponding mRNAs per cell

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: P_T (incubation time) P_G (initial glucose) and P_i (interaction between both). Data for *Pck1* and *Pdk4* were taken from reference [13].

The enzyme P-enol-pyruvate carboxy-kinase gene (*PcK1*) at 24 h of incubation was 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 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 first 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 incubation time, 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).

4. Discussion and Conclusions

Adipocytes (or WAT) take up excess glucose, when confronted with high glucose levels, converting a large proportion of it into 3C fragments, such as lactate [41], pyruvate , alanine [42] and glycerol [13, 14], which may be used as energy substrate elsewhere, or, largely, by the liver in the gluconeogenic [15] and/or lipogenic pathways [43]. 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 [25], a process that enhances the limitation of blood flow as defense system against excess energy substrates [12]. 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 excess glucose toxic effects. The entry of 3C fragments in most tissues goes unhindered by insulin resistance and/or hexose uptake control [44] and provides, instead, directly usable energy substrates, which are already partially metabolized. These fragments are massively used by liver, muscle, heart, brain and other organs [45], including the adipose tissues (WAT, BAT) [46].

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 fragments, essentially lactate and glycerol, thus markedly decreasing the levels of glucose. However, this process practically was 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 hacking of glucose to 3C fragments was proceeded during a 2-day incubation, 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 into 3C fragments (and, to a minor extent, fatty acids) was not elicited by external signals and neither by glucose concentration itself. Consequently, we can assume that it may be a pre-established innate process, which potentiates glucose break up by defect. One of the most relevant consequences, which our label tracing data proved, is the limited importance of lipogenesis in the disposal of glucose by WAT.

A critical finding of this study is the unwavering reliance of adipocytes on glucose to provide energy (and release lactate) via glycolysis, independently of the availability of glucose in the medium. This may be a consequence of the mass of cells, which limit access of most of the cytoplasm to more efficient oxidative processes in mitochondria [47], another consequence of the cell geometry. However, this factor has deep consequences: 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 the limitation of incoming blood flow to limit excess energy unloading [2]. This way, lipogenesis (an aerobic process) may be reduced by controlling oxygen availability. Oxygen levels are usually low already in WAT under *in vivo* conditions [48], probably because they are needed only for oxidative processes such as lipogenesis: low oxygen, and limited access to mitochondria may become essential factors limiting lipogenesis. There is no hypoxia because adipose tissue oxygen consumption is low [49]. The widely assumed relationship between supposed WAT hypoxia, often justified by lactate production, [50] and inflammation needs to be revised [26]. Perhaps the low WAT blood flow, which we link to a defense system preventing substrate loading, may help, also to limit the conversion of glucose to lipid favoring, instead its return as 3C fragments.

Since there were no external stimuli or environmental changes differently affecting the cells during the two consecutive days of incubation, the changes observed should be elicited by internal factors

developed during incubation. In fact, glucose consumption and gene expression increased in the second day with respect to the first. The data showed that the influence of glucose concentration was indeed minimal. The alterations in substrate handling were clearly correlated with gene expression data. They also defined two different successive incubation time-related groups, as previously observed [25]. The only process practically unchanged along both periods was the production of lactate (i.e. the pace of the glycolytic pathway and its production of the ATP needed for the cell maintenance) [13, 25, 27].

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 [13], lowering its specific radioactivity. However, on day 2, most of the medium glycerol came from TAG turnover [13], 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 [51], preventing the conversion of pyruvate into acetyl-CoA. The lack of substrate resulted in the maintenance (not increase) of label, or 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. 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 [27]; 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 [13] that resulted in accelerated TAG turnover, in which most of fatty acids were recycled and glycerol excreted [13].

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 lactates, 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 [13].

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 (probably as malate) [13]. 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 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 [52] . 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 [53]. Glycerol is essentially the only carbohydrate in the avian egg, and sustains the live and development of birds [54]. Both 3C compounds can fully substitute glucose for most biochemical functions.

The quantitative estimation of glucose conversion into 3C fragments or fatty acids (x2C), however, established that adipocytes (and by extension WAT) actively participate in the control of glycemia [13], lowering glucose levels and contributing to limit its pro-inflammatory effect via insulin resistance. However, this glucose is largely recycled to 3C fragments, i.e. usable as energy substrate by almost any tissue, as indicated above. These 3C fragments 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 massively converted into fatty acids 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 fragments would be used by other organs or tissues, such as the liver, for lipogenesis [55], 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 [56]. But, as presented here, this widely accepted irreversible conversion of 3C to 2C units (linked to lipogenesis) is not massively carried out by mature (i.e. not growing) adipocytes.

The metabolic prowess of WAT, a tissue with so small proportion of 'live' cytoplasm [28], does not cease to surprise us with a widespread and powerful participation in the overall control of body energy. Also because of the growing number of functional metabolic pathways it contains and 'hides' in between so much fat. Perhaps we should look more keenly, beyond this fat and its assumed perils, probably to discover that WAT may be a main actor in the fight against the ravages of excess energy intake. Perhaps using inadequate tools but achieving, nevertheless, a remarkable effectivity.

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Influence of sex on substrate handling by adipocytes isolated from subcutaneous, mesenteric and perigonadal adipose tissue of rats

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Influence of sex on substrate handling by adipocytes isolated from subcutaneous, mesenteric and perigonadal adipose tissue of rats

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Abstract

The effects of sex and site on the largely anaerobic disposal of glucose by adipocytes was analyzed. Mature adipocytes from perigonadal, mesenteric and subcutaneous adipose tissue (WAT) of female and male rats were incubated with 7 or 14 mM glucose. The consumption of glucose, efflux of metabolites and gene expression of glycolytic and lipogenic-related genes were estimated after 24 or 48 h. Glucose concentration effects were minimal, in contrast with sex, site and, especially, the time of incubation. Lactate production was unchanged by time, but that of glycerol shifted from a direct glycolytic origin to its generation by selective triacylglycerol turnover: fatty acids were recycled, but not glycerol, especially in mesenteric WAT. Lipogenesis was limited. Females showed a higher overall metabolic activity, and in mesenteric WAT, oxidation of pyruvate to acetyl-CoA was more marked than in males, which forced, in these, a higher recycling of oxaloacetate to the cytoplasm for its conversion to lactate. WAT site differences showed a marked specialization. Adipocytes' use of glucose for lipogenesis was seriously hampered, wasting instead more glucose into lactate and glycerol. We postulate that these mechanisms help decrease glycaemia, producing less-regulated 3-carbon substrates which could be used for energy elsewhere.

Introduction

Adipose tissue is a large and disperse organ, found around and within most specialized organs and in separate large masses traditionally considered sites for storage of fat reserves. The marked differences between different adipose tissue sites have been widely described [1,2], up to the point that their main (unique?) characteristic is the massive accumulation of fat (essentially triacylglycerols, TAG) in intracellular vacuoles, taking most of the cell space [3]. However, the marked oxidative function of brown adipose tissue with often multiple lipid vacuoles and a large number of mitochondria, contrasts with the predominantly glycolytic activity of the often enormous single-vacuole adipocytes in white adipose tissue (WAT) [4]. In between there are a number of intermediate (or specialized from different cell lines [3]) adipocytes (i.e. cells in which most of its weight/volume is taken up by lipid stores) [3], such as beige [5] or pre-adipocytes [6]. This wide array of cell models, which retain a number of similar characteristics, result in the considerable variability of functions of BAT and WAT (including beige and *brite* or brown-in-white [7]) tissue masses which develop highly specialized functions depending on their placement within the body and also carry on general functions that identify the adipose organ as a critical element in the control of energy handling [8,9].

During decades, the prevailing idea of WAT as just storage organ, the perils of its excessive mass, related to the development of metabolic syndrome [10], the permanent state of inflammation that characterizes most obesities [11] and the widely generalized attribution to "visceral" WAT of many of the metabolic dangers associated with obesity co-morbidities [12] has shown that site is a critical factor for WAT. However, the close control exerted by smaller masses of adipose tissue (perivascular [13], pericardial [14], intramuscular [15], etc.) and the active presence of a large number of other cell types interspersed between adipocytes, such as stem cells [16], immune system cells [17], stromal vascular cells [18] and even hematopoietic

cells [19], contribute to extend, multiply and specialize the function of WAT from the stage of control of energy to that of defense and control of the function of other organs [20,21]_ENREF_20, via hormones [22] and cytokines [23].

The distribution of body fat is a well-known sex-related character, clearly marked in humans [24] (and less so in rodents). The differences in distribution are directly related to their function, and in the case of obesity [25], with marked differences in overall metabolic function, including the inordinate hypertrophic and hyperplasic growth of some specific WAT masses [26]. The sex-related differences in WAT function have been widely studied, but either analyzing the function of specific depots [25] or considering the overall effects on body energy budget or health markers [27].

We have recently studied the marked effect of sex on rat WAT amino acid metabolism [28,29], we have also found that WAT, and adipocytes are able to convert glucose in almost anaerobic conditions (despite oxygen availability), enormous amounts of lactate and glycerol [4,30], despite its tiny percentage of active cytoplasm with respect to tissue weight [3]. In line with this same line of study, we have investigated whether the massive conversion of glucose to 3C substrates, largely lactate and glycerol of epididymal adipocytes of adult rats (unpublished results) were just a peculiarity of males or was a general trend of WAT, affecting other sites. The difficulties inherent to the study of a disperse organ [31] (or system of groups of cells having in common perhaps only its high fat content) limit the comparative possibilities of the available methodology. Consequently we studied the use of glucose consumption, as well as lactate and glycerol release in adipocytes isolated from three different WAT sites. The objective was to find out whether the marked metabolic site differences were largely a question of adipocyte specialization of the additional functions of the non-adipocyte cells in WAT.

Adipocytes were isolated from perigonadal (PG, i.e. epididymal or periovaric) masses. This site contains large cells, and probably the highest percentage of fat per g of tissue [32]; it is considered essentially an example of storage WAT [33]. In rats, mesenteric (MES) WAT is more disperse, lax, structurally complex in its connections, in part bridging the gap between intestine (and dietary nutrients) and liver [34]. In humans it accounts for most of "visceral" WAT. Our third option, subcutaneous (SC) WAT, is, in itself another example of diversity with extreme differences between places: from masses of WAT surrounding those of BAT, to the common dermal mix of adipose and conjunctive tissue binding together the skin to the serous membranes. However, subcutaneous WAT is taken as one of the most studied adipose tissues in humans [35], but much less often in rats because of sampling problems. We used the inguinal cordons, which provide a clearly distinguishable site and sufficient material for analysis.

Thus, our main objective in carrying out this study was to determine the possible balance between the defining characteristics and relative metabolic uniformity of adipose tissue (the adipose organ [36]); but also the specific metabolic needs related to the tissue mass placement. The influence of sex was included in the equation and the key parameters to measure were handling of glucose and 3C fragments.

Results

Cells, glucose uptake and metabolite efflux

Mean adipocyte volume for the suspensions of cells used are shown in Figure 1. There were significant differences in size depending on site (the largest cells were found in PG WAT), but not for sex. Despite their relative uniformity, we wanted to compare quantitatively the effects of sex, site, glucose concentration and exposure (incubation time) to the hexose using otherwise uniform conditions. Thus, we converted the metabolite efflux (or uptake in the case of glucose) into rates akin to the enzyme activity unit katal, and taking the cell as unit for comparison. The data, thus, were converted into attomoles per second and cell, and are shown in Figure 2. We used a three-tiered statistical ANOVA analysis: four way, as indicated in the Figure itself; three way, independently for each site, and two-way for sex in each site.

Four-way ANOVA shows the general trend: differences between sites affected all parameters, sex also affected everything except lactate efflux. On the other side, doubling glucose concentration affected only glucose uptake, whereas time of incubation affected all parameters except glucose uptake. The latter parameter was closely linked to the incubation time, with limited effects of sex (except in PG WAT).

Lactate efflux was remarkably uniform and not related to glucose concentration or time of incubation (except in PG WAT), but there was an overall significant effect of sex on SC (albeit not in the other sites). Glycerol and NEFA efflux showed a pattern closely similar, despite glycerol efflux being much larger than that of NEFA in all cases. There was a significant effect of sex in all WAT sites (except for NEFA in PG WAT), and a practically nil effect of glucose concentration, which contrasted with the marked effect of incubation time, raising the efflux of both glycerol and NEFA in all sites, irrespective of sex, with the sole exception of female adipocytes of MES WAT, which were unaffected by glucose and/or incubation time. It

is remarkable that glycerol (and NEFA to a lower extent) efflux by MES WAT cells was higher than those of SC and PG.

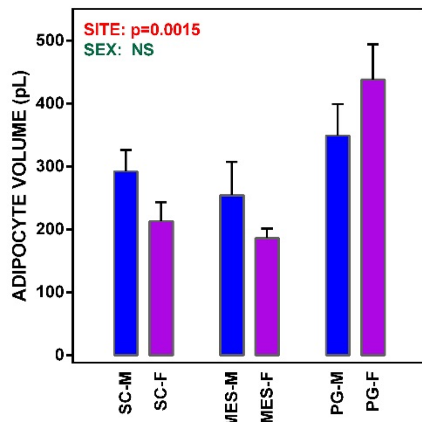
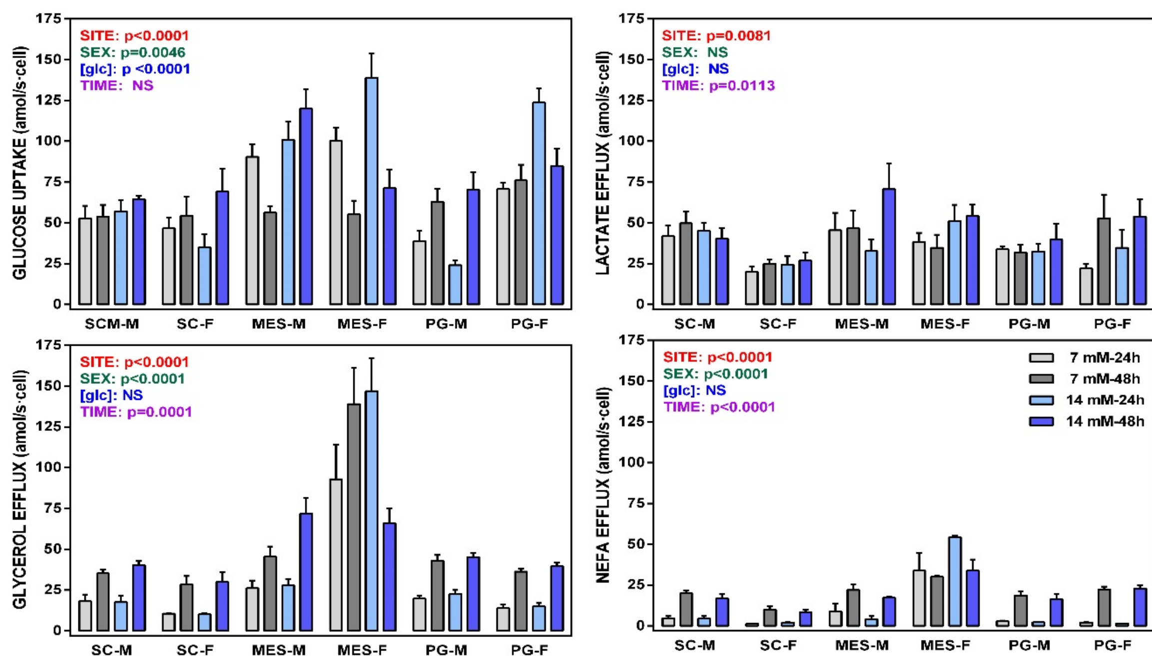


Figure 1

Mean adipocyte volume in WAT sites of female and male adult Wistar rats

The data are the mean ± sem of 4 groups of 2 rats-each for sex and site. SC = subcutaneous WAT; MES = mesenteric WAT; PG perigonadal (epididymal – males, periovaric – females) WAT; blue bars, M = male rats; purple bars, F = female rats. Statistical comparison between groups: two-way ANOVA; the p values for sex (green) and site (red) are included in the Figure. NS = not significant (p>0.05).



glucose uptake							lactate efflux						
3-way	SC		MES		PG		3-way	SC		MES		PG	
sex	NS		NS		<0.0001		sex	<0.0001		NS		NS	
[glc]	NS		0.0005		0.0488		[glc]	NS		NS		NS	
time	0.0383		0.0005		NS		time	NS		NS		0.0351	
2-way	M	F	M	F	M	F	2-way	M	F	M	F	M	F
[glc]	NS	NS	0.0018	0.0283	NS	0.0040	[glc]	NS	NS	NS	NS	NS	NS
time	NS	NS	NS*	0.0003	0.0006	NS*	time	NS	NS	NS	NS	NS	0.0368
glycerol efflux							**NEFA efflux**						
3-way	SC		MES		PG		3-way	SC		MES		PG	
sex	0.0092		<0.0001		0.0007		sex	<0.0001		<0.0001		NS	
[glc]	NS		NS		NS		[glc]	NS		NS		NS	
time	<0.0001		NS		<0.0001		time	<0.0001		NS		<0.0001	
2-way	M	F	M	F	M	F	2-way	M	F	M	F	M	F
[glc]	NS	NS	0.0481	NS	NS	NS	[glc]	NS	NS	NS	NS	NS	NS
time	0.0008	0.0003	0.0003	NS	<0.0001	<0.0001	time	<0.0001	0.0001	0.0010	NS	<0.0001	<0.0001

* Statistically significant Interaction [glc]-time

Figure 2

Glucose uptake rates and lactate, glycerol and NEFA efflux rates (all in attomoles per second and cell) of adipocytes obtained from three WAT sites of male and female adult Wistar rats.

The data are the mean ± sem of 4 wells containing the pooled adipocytes of two rats, incubated for 24 or 48 h. The abbreviations for site and sex are the same as in Figure 1. The Figure quarters include a 4-way ANOVA analysis of the overall differences for site (red), sex (green), initial glucose concentration in the medium (blue) and time of incubation (purple); additional data for site: glucose uptake: all sites were different; lactate, glycerol and NEFA efflux: MES was different from SC and PG, which were not between them. The embedded Table shows the results of 3- and 2-way ANOVA analysis of the data (p values).

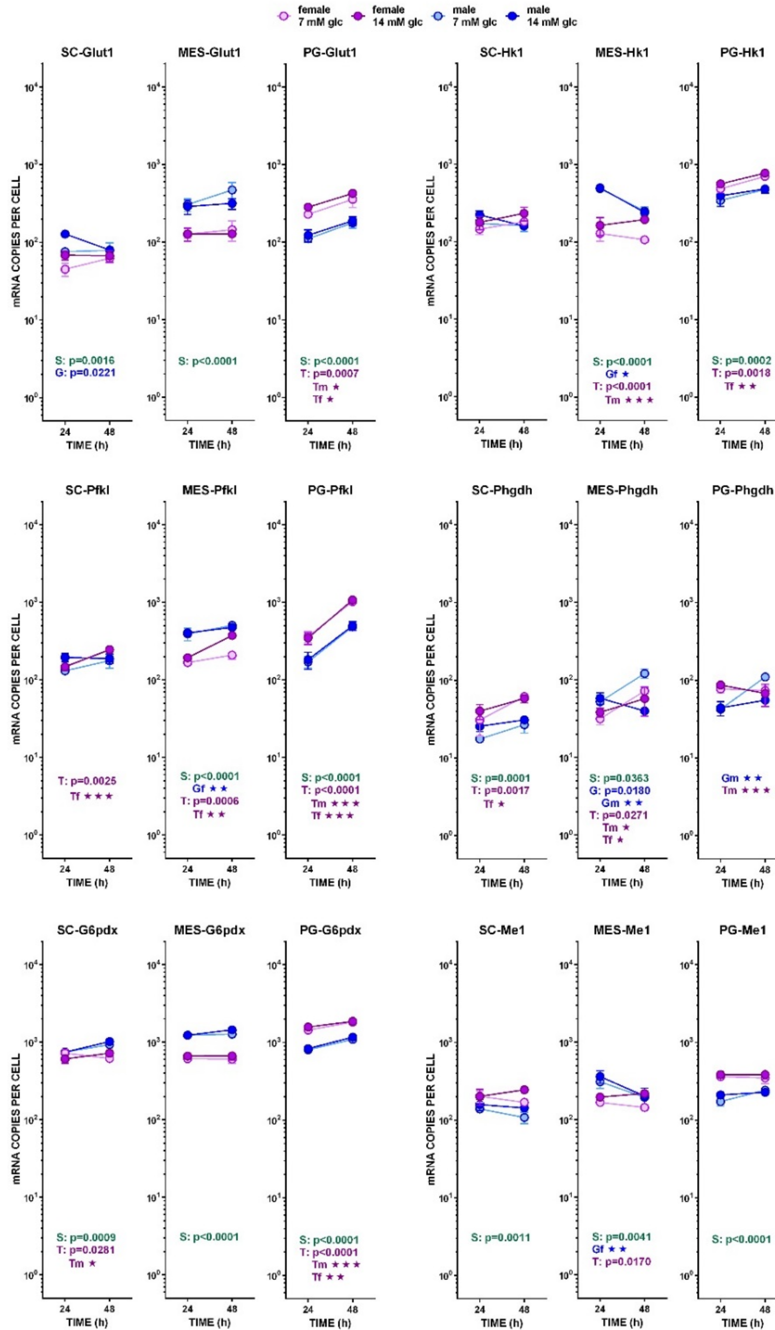


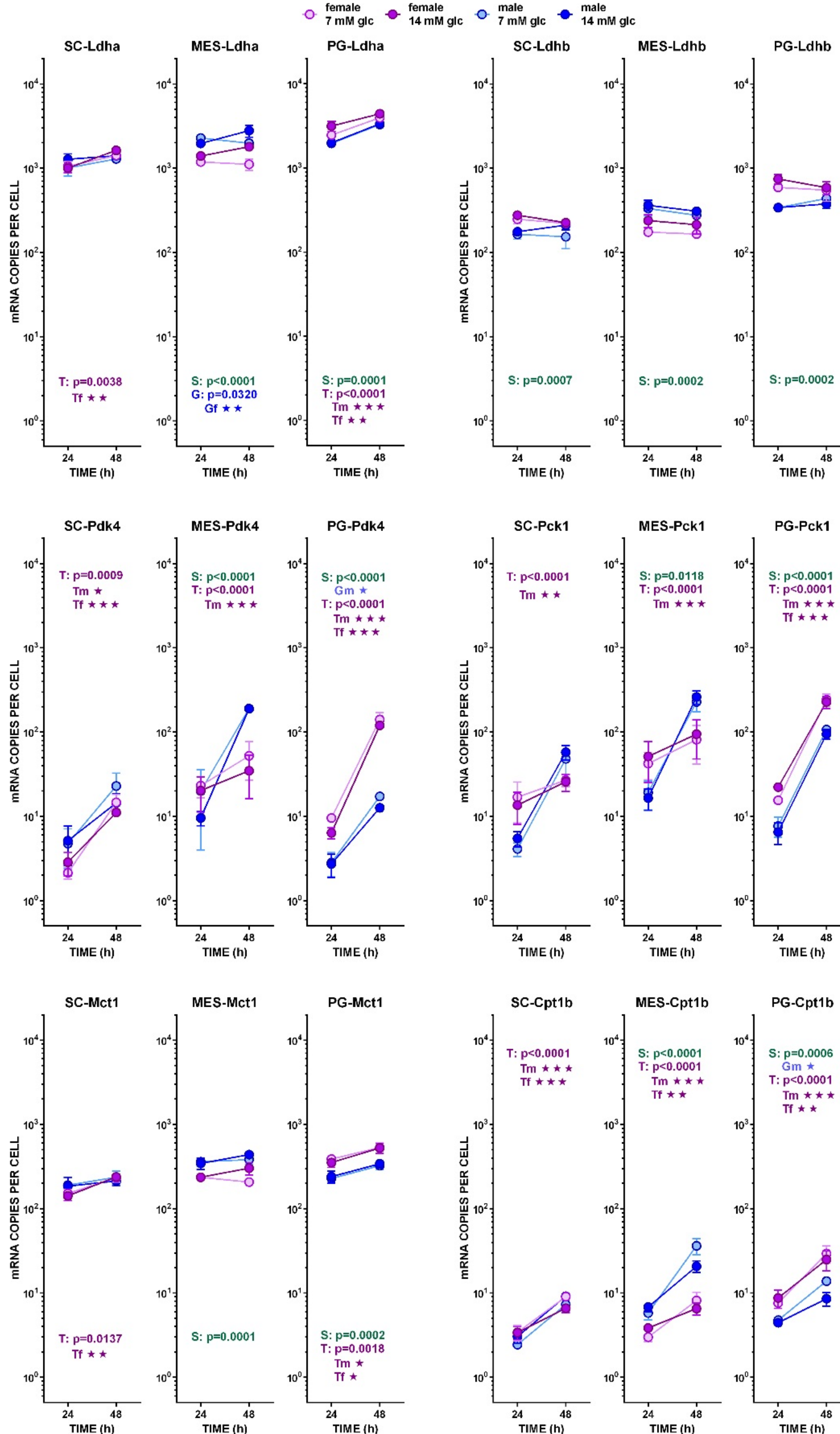
Figure 3

Expression of genes related with the metabolism of glucose, lipogenesis and 3C handling in adipocytes isolated from subcutaneous, mesenteric and perigonadal WAT of male and female adult rats incubated for 24 h or 48 h in the presence of 7 mM or 14 mM glucose (I)

The data are the mean ± sem of 3-4 different pools of 2 animals each, and represent the number of mRNA copies per cell of each gene. The data are represented on a log scale. Blue circles represent males: deep blue 14 mM glucose, light blue 7 mM glucose; purple circles represent females: violet-purple 14 mM glucose, mauve 7 mM glucose. SC = subcutaneous, MES = mesenteric and PG = perigonadal WAT

Statistical analysis of the differences between groups: The figures contain the p values for a 3-way ANOVA analysis (sex [S in green], glucose [G in blue] and time of incubation [T in purple]) applied to each site. Results of two-way ANOVA for these paramers are also represented: the letters and colors are the same, adding **m** for male and **f** for female subgroups; for 2-way ANOVAs, the p values are represented by up to three stars, corresponding to three levels of statistical significance of the differences (p<0.05, p<0.01 and p<0.001). Only significant differences have been represented. The results for 4-way ANOVA analyses are shown in the embedded Table for each gene studied. The conventions used here are the same as in the rest of the Figure and in Figure 2. NS (i.e. not statistically significant) corresponds to p>0.05.

gene	site		sex: S	[glucose] G	time T
Glut1	<0.0001	SC≠(MES,PG)	NS	NS	0.0023
Hk1	<0.0001	SC≠MES≠PG	NS	0.0371	NS
Pfkl	<0.0001	SC≠MES≠PG	0.0006	NS	<0.0001
Phgdh	<0.0001	SC≠MES≠PG	NS	0.0164	<0.0001
G6pdx	<0.0001	SC≠MES≠PG	NS	NS	<0.0001
Me1	<0.0001	SC≠ME≠PG	0.0007	0.0206	NS

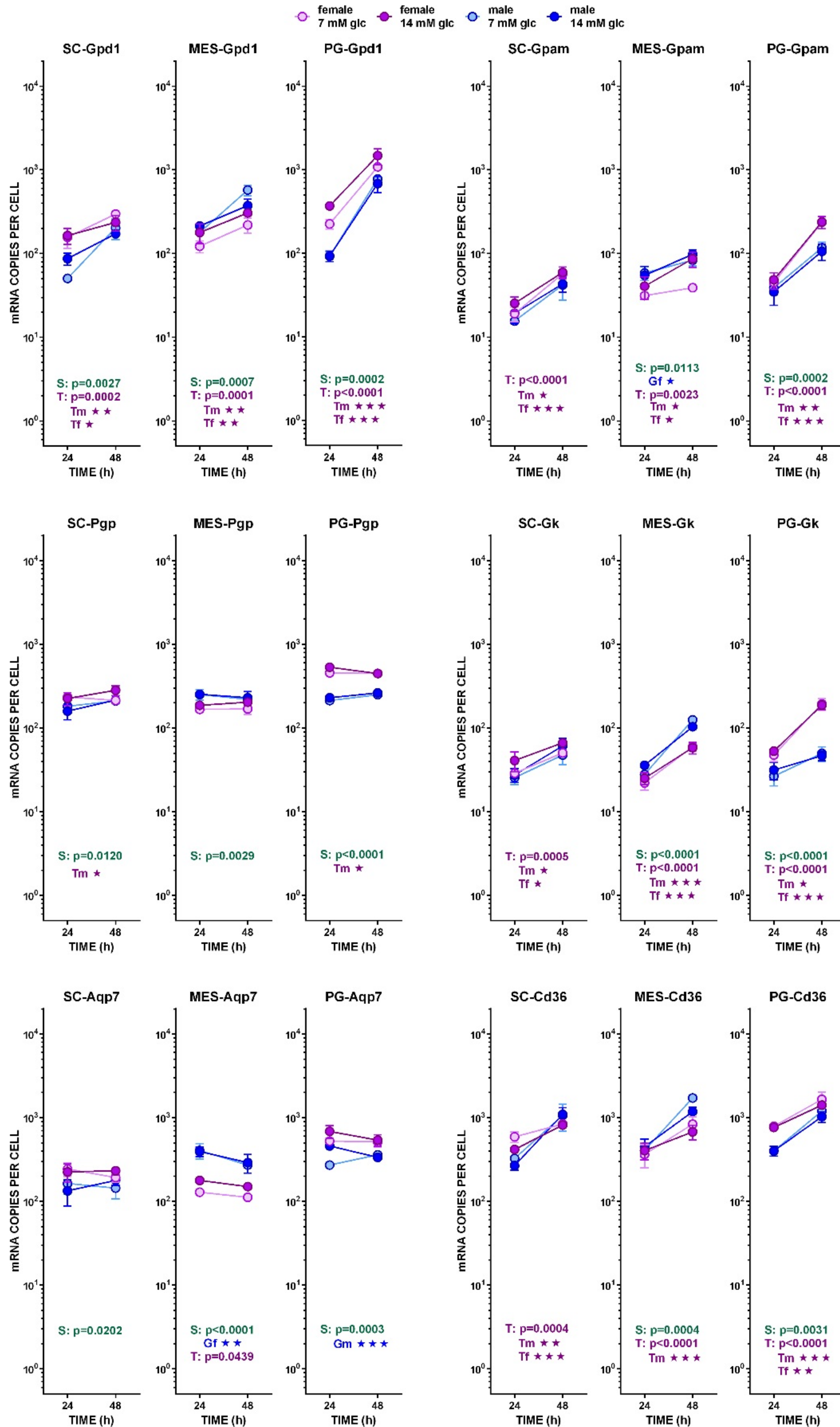


gene	site		sex: S	[glucose] G	time T
Ldha	<0.0001	SC≠MES≠PG	NS	0.0037	<0.0001
Ldhb	<0.0001	(SC,MES)≠PG	0.0063	NS	NS
Pdk4	<0.0001	SC≠MES≠PG	NS	NS	<0.0001
Pck1	<0.0001	SC≠(MES,PG)	NS	NS	<0.0001
Mct1	<0.0001	SC≠MES≠PG	NS	NS	<0.0001
Cpt1b	<0.0001	SC≠(MES,PG)	NS	NS	<0.0001

Figure 4

Expression of genes related with the metabolism of glucose, lipogenesis and 3C handling in adipocytes isolated from subcutaneous, mesenteric and perigonadal WAT of male and female adult rats incubated for 24 h or 48 h in the presence of 7 mM or 14 mM glucose (II)

The data are the mean ± sem of 3-4 different pools of 2 animals each, and represent the number of mRNA copies per cell of each gene. The distribution figure setup, and other conventions are those described in Figure 3



gene	site		sex: S	[glucose] G	time T
Gpd1	<0.0001	SC≠MES≠PG	0.0004	NS	<0.0001
Gpam	<0.0001	SC≠MES≠PG	0.0027	NS	<0.0001
Pgp	<0.0001	(SC,MES)≠PG	<0.0001	NS	NS
Gk	<0.0001	SC≠MES≠PG	0.0001	NS	<0.0001
Aqp7	<0.0001	SC≠MES≠PG	NS	NS	NS
Cd36	0.0030	(SC,MES)≠PG	NS	NS	<0.0001

Figure 5

Expression of genes related with the metabolism of glucose, lipogenesis and 3C handling in adipocytes isolated from subcutaneous, mesenteric and perigonadal WAT of male and female adult rats incubated for 24 h or 48 h in the presence of 7 mM or 14 mM glucose (III)

The data are the mean ± sem of 3-4 different pools of 2 animals each, and represent the number of mRNA copies per cell of each gene. The distribution figure setup, and other conventions are those described in Figure 3

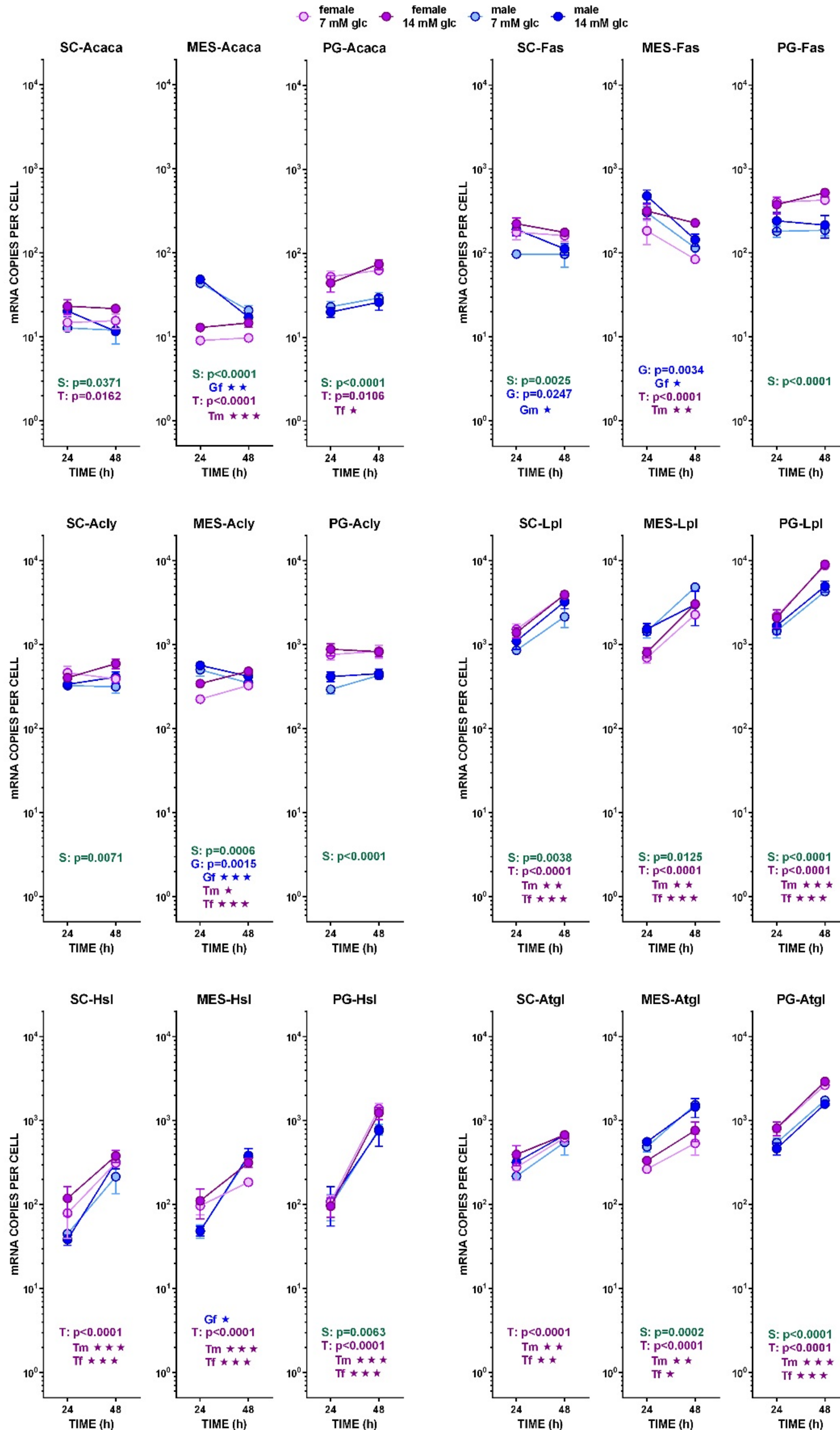


Figure 6

Expression of genes related with the metabolism of glucose, lipogenesis and 3C handling in adipocytes isolated from subcutaneous, mesenteric and perigonadal WAT of male and female adult rats incubated for 24 h or 48 h in the presence of 7 mM or 14 mM glucose (IV)

The data are the mean ± sem of 3-4 different pools of 2 animals each, and represent the number of mRNA copies per cell of each gene. The distribution figure setup, and other conventions are those described in Figure 3

gene	site		sex: S	[glucose] G	time T
Acaca	<0.0001	SC≠MES≠PG	0.0020	NS	NS
Fas	<0.0001	SC≠MES≠PG	<0.0001	0.0003	0.0014
Acly	<0.0001	(SC,MES)≠PG	<0.0001	0.0118	NS
Lpl	<0.0001	(SC,MES)≠PG	0.0001	NS	<0.0001
Hsl	<0.0001	(SC,MES)≠PG	0.0043	NS	<0.0001
Atgl	<0.0001	SC≠MES≠PG	NS	NS	<0.0001

Analysis of gene expression

We used the same comparative approach described above for metabolites to analyze the changes in gene expression at 24 and 48 h of incubation. Here we present the data as copies of the gene mRNA transcripts per cell. Figures 3 to 6 show the gene expression of main enzymes and transporters affecting the metabolism of glucose, lipogenesis and 3C fragment handling in the adipocyte. The statistical analysis

of the differences between sites, sexes and the medium glucose concentration, as well as the duration of cell incubation are presented in part in the graphs themselves (site-centered 3-way ANOVA analyses for sex, glucose concentration and time of incubation), as well as in embedded Tables: 4 way-ANOVAs, including site, and two-way ANOVAs for each sex (for the remaining comparison factors: glucose and incubation time). The presentation –and initial analysis– of the data in the present section will be done along three main lines: a) Glucose and glycerol, b) Fatty acid metabolism, c) Pyruvate, lactate and oxaloacetate. Figure 7 shows a schematic view of the genes studied superimposed to the main metabolic pathways of carbohydrate-lipid relationships in the adipocyte.

a) Glucose and glycerol

In MES, the expression of *Glut1* was higher in males than in females and lower in PG WAT; it was practically unaltered by glucose and incubation time. In SC WAT, the *Glut1* expression was lower than in the other sites. The pattern of expression of *Hk1* was similar *Glut1* in significant effects of sex, but not in SC WAT; in male adipocytes MES WAT the effects of sex were more marked, decreasing its gene expression with incubation time. Sex differences were generalized, also in *Pfkl*, repeating the pattern of higher male expression in MES and lower in PG WAT. The effect of glucose levels was only observed in female MES WAT, but incubation time increased the expression of this gene, especially in PG WAT. The expression of *Phgdh* (not a pathway control enzyme) were rather uniform, and lower than the other glycolytic enzymes analyzed. There was a trend to increase expression with incubation time, and to maintain the effects of sex described for the other enzymes. In sum, no marked changes were observed to be influenced by the conditions of the study, suggesting a fluid and fairly uniform operation of glycolysis down to pyruvate with practically no effects of external glucose concentration, and increased expressions with incubation time.

Glycerol efflux was paralleled by a marked trend to increase the already high expression of *Gpd1* (i.e. compared with *Phgdh*) with incubation time, but --again– not by the concentration of glucose in the medium. The maximal increase, not linked to sex, was observed in PG WAT. The phosphatase pathway (*Pgp*) showed changes only for sex (higher values for females) in PG and SC WAT. This was not the case for the enzyme catalyzing the reverse reaction, glycerokinase, which gene (*Gk*) maintained the differences between sexes (MES, PG), but showed a higher increase of its expression with incubation time. The overall number of copies per cell of *Gk* was, however, about one order of magnitude lower than that of *Pgp*. The patterns for Aquaporin 7 gene (*Aqp7*) expression closely resembled those of *Pgp*, including its range.

The assumed incorporation of newly formed glycerol-3P into acyl-glycerols, judging from the expression of *Gpam*, increased with incubation time, in a pattern comparable to that of *Gpd1*, deeply affected by time but not by medium glucose levels. Sex differences were maintained (higher values for females in SC, and, especially, PG WAT, and higher for males in MES.

b) Fatty acid metabolism

Lipogenesis did not seem to represent a quantitatively significant process under the conditions tested because of the limited possibility of producing acetyl-CoA from the main medium substrate, glucose (down to pyruvate). Pyruvate dehydrogenase activity seems to be limited both because of scarce number of mitochondria and because of the relatively elevated expression of the main controller of pyruvate dehydrogenase, kinase 4, the expression of its gene (*Pdk4*) showed a marked effect of incubation time, increasing (in all three sites) differently according to sex. Males and females' *Pdk4* expression in SC and PG WAT increased about one order of magnitude in 24 h. In MES WAT, males followed the same pattern, but no significant increase with incubation time was observed in females (despite its spectacular climb in PG WAT). Under these conditions, pyruvate dehydrogenase could not fully operate at 48 h, but the effects should be less marked in the MES WAT of females.

The transport to the cytoplasm of acetyl-CoA via citrate: ATP lyase was not affected by the treatment received by adipocytes as shown by the little change found in the expression of its gene *Acly*. Carboxylation of acetyl-CoA to malonyl-CoA was probably unchanged (or decreased in male adipocytes of MES WAT) from the expression of *Acaca*. A similar pattern, with higher number of copies per cell, can be found for *Fas*, fatty acid synthase. As for the availability of NADPH in the cytoplasm, the pentose phosphate pathway indicator gene *G6pdx* showed high numbers of copies and a marked sexual differentiation in MES and PG WAT, but no changes induced by glucose or incubation time, suggesting no changes in the main provider of NADPH in parallel to the glycolytic and pyruvate-handling pathways already analyzed. The other main NADPH generator, malic enzyme (*Me1*), also showed little change in its expression, with a tendency to lower the number of copies in MES-WAT with incubation time.

Probably, the uptake of medium fatty acids (from cell remnants) was, at least, activated, since the expression of one of the main transporters *Cd36* increased with time. Sex affected MES and PG WAT, and no effects of glucose levels were observed at all. Mitochondrial utilization of Acyl-CoA was assumed to be low (if any), first because of the nature of the tissue and its energy needs (expected low oxidative metabolism) , second because of the ample availability of glucose, and third because the expression of *Cpt1b* was very low in all three sites, thus making difficult the entry of Acyl-Coa into the mitochondria). The

already described differences because of sex were maintained and there was a marked increase in the expression of this gene in both sexes with time.

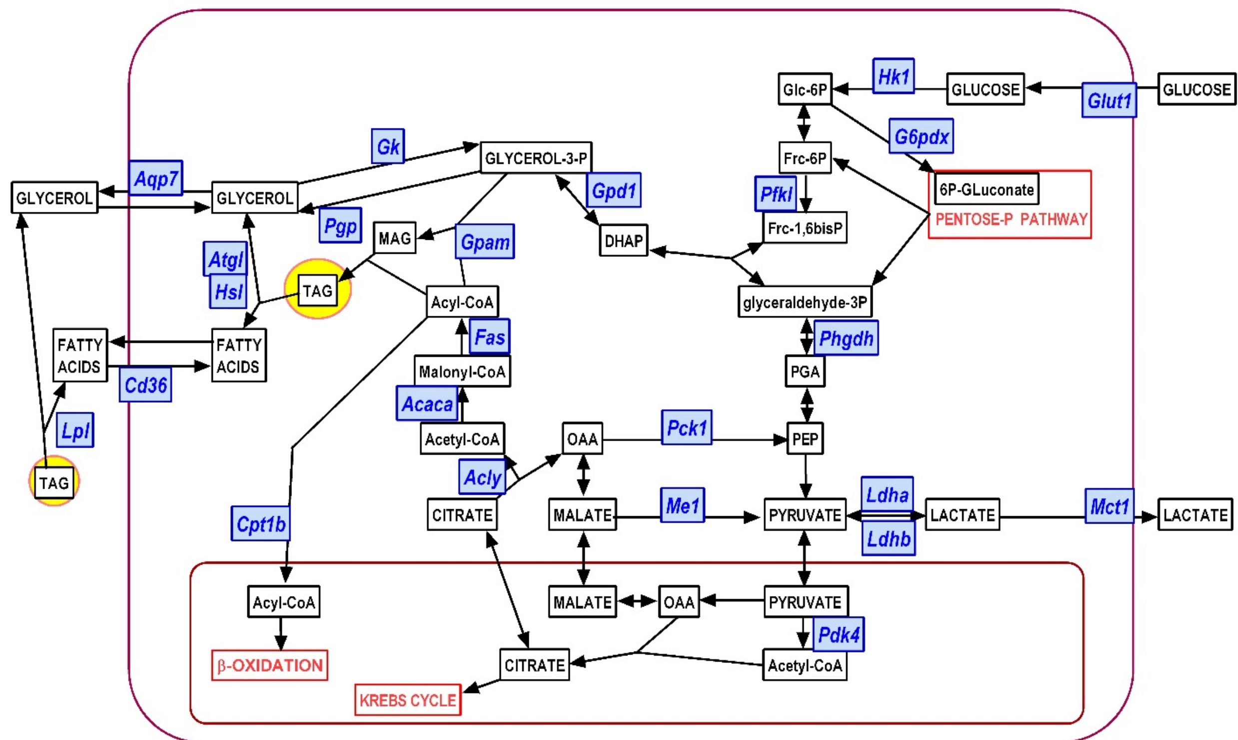


Figure 7

Diagram showing the position of the genes investigated on a scheme of the main adipocyte energy metabolism pathways

Blue: genes (for enzymes and transporters); white: metabolites.

The main lipases of adipose tissue: the external lipoprotein lipase (*Lpl*), and the internal adipose TAG lipase (*Atgl*) and hormone-sensitive lipase (*Hsl*) showed essentially the same trends. All showed a generalized effect of sex, and increases in gene expression with incubation time, but no effects of glucose concentration; the sole exception being female MES adipocytes marked influence of glucose concentration but not of incubation time. *Lpl* showed the highest number of copies per cell found in this study. In the case of MES WAT, females' increase of expression with time was less marked than that of males, a reminiscence of the discordance described for *Pdk4*. The high coordinated increase in lipase activity was not correlated to the limited release of NEFA into the medium, and these levels were far from being correlated with any of the expressions of the lipases studied.

c) Pyruvate, lactate and oxaloacetate

In addition to the different sex-related response of *Pdk4* expression of adipocytes as a consequence of incubation, limiting the synthesis of Acetyl-CoA in most of the conditions analyzed, the obviously major outlet for excess pyruvate generation in the cytoplasm was its conversion to lactate. The number of copies of *Ldha* was high, with overall (4-way ANOVA) effect of glucose, a trend to increase its expression with time of incubation, and no effects of sex. Glucose only affected more intensely the MES values, where no effect of time of incubation. There was a marked differentiation by sex (higher values for male sin MES abut not in PG WAT). *Ldhb* showed much less change (induced by sex) and also lower number of copies per cell. The ratio of expression of both lactate dehydrogenase isoform genes (*Ldha* / *Ldhb*) was fairly constant, with a mean value of 6.5 for both sexes. The monocarboxylate transporter gene (*Mct1*) expression presented a pattern closely similar to that of *Ldha*, except for the influence of sex, suggesting a partially coordinated regulation. *Me1* showed only limited changes in expression as described above, which agrees with the lower needs for NADPH (to be used mainly for lipogenesis) observed under the conditions of incubation. Last, but not least, the key marker enzyme gene *Pck1* (P-enol-pyruvate carboxy-kinase), a critical enzyme bridging the pyruvate kinase gap to favor the arrival of oxaloacetate to the triose-P pool under conditions of scarcity of glucose showed a marked sex-related difference in its response to incubation. Irrespective of glucose concentration, both sexes in PG and only males in SC and MES WAT increased dramatically its expression with time of incubation, suggesting the need for the conversion of

excess cytoplasmic oxaloacetate into P-enol pyruvate. This change was not observed in female adipocytes of SC and MES WAT, in a pattern closely resembling that of MES *Pdk4*.

Discussion

The main outcome of this study is a reinforcement of the widely accepted notion that WAT sites are both remarkably uniform [29] from a qualitative point of view, and they play different metabolic functions depending on their location [2]. The differences between sites being mainly quantitative. This idea is not new, but the comparative studies analyzing metabolic pathways are too few and incomplete to support it. We hope that this contribution may help reinforce the assumption that WAT plays a critical function on glucose handling and, consequently in the maintenance of glycaemia [4,29]. We also present additional evidence that key metabolic functions of WAT are deeply affected by sex [37,38]. This is more clearly observed in MES and SC WAT than in the fat attached to reproductive organs, the epitome of anatomic sex differences. Our data also reinforces the postulated role of glycolysis to lactate as provider of enough energy (ATP) for white adipocyte normal function [39-41], reducing its need for oxygen [42], in line with the scarcity of mitochondria and oxidative metabolism [43,44]. Glucose is taken up as needed, and lactate is released not as a function of glucose availability (and uptake) but, as we previously postulated, as a 3C metabolite, easily used for energy or carbon source elsewhere [30,41,45,46], and providing direct ATP availability [41] to the thin layer of cytosol surrounding the huge fat droplet that constitutes most of the living fraction of the adipocyte.

Our previous studies were carried out using only male rats' epididymal adipose tissue [46], a classical depot WAT site; the comparison done here with periovaric WAT of female rats showed little differences between them, even on potential fatty acid synthesis. These results were not expected, given the higher tendency to fat accumulation of adult males versus females (in rats, but also in other species such as humans) [25,47]. However, as a rule, the number of copies per cell for most of the genes studied was higher in females than in males, in contrast with the smaller (NS) size of their adipocytes, hinting at a possibly higher overall metabolic activity of female WAT [48].

MES WAT showed a markedly higher efflux of glycerol, and NEFA (to a proportionally lower extent in molar terms), compared with the other sites investigated. Our previous analysis of glucose carbon fate in epididymal WAT showed that a sizeable proportion of glucose ended forming part of glyceride-glycerol [4,49], especially after incubation of adipocytes for more than one day in the presence of glucose [46]_ENREF_3, and that there was a significant lipase-driven triacylglycerol turnover, in which most of fatty acids were recycled to TAG [50], but glycerol was excreted [51,52]. The data of the present study agree with this interpretation, but since no labelled C has been used, we can only deduce the origin of glycerides-glycerol in the adipocytes. We know, however, that the rates of glycerol release to the medium by MES WAT were much higher than those of epididymal (and periovaric) WAT. We can, thus, assume that glycerol production in MES WAT may be higher than that of the only tissue quantitatively analyzed in detail with tracers, male PG WAT [4]. Since MES WAT plays an ancillary energy handling role to the liver, its massive production of glycerol (to our knowledge not previously described) may help facilitate the hepatic handling of NEFA (arriving from intestine mixed in the same portal blood with MES-WAT efflux, i.e. those not used for TAG carried out by lymph, as well as excess of systemic blood NEFA); but also to lower the portal intestine-derived glucose load carried to the liver as shown by the higher glucose uptake and 3C fragment efflux rates in MES than in SC and, especially, PG WAT. We can speculate that this function may buffer the impact of large digestive glucose loads arriving to the liver, and thus facilitate its hepatic handling.

The differences in response between female- and male-derived MES adipocytes facing periodic exposure to excess energy (in this case, glucose) seem minimal, but far-reaching. In addition to higher overall glycerol efflux, in female rats, pyruvate dehydrogenase inhibition by its kinase 4 seems not to be altered by either glucose levels or time of incubation, in contrast with the high increases elicited in males [46] in the number of copies of *Pdk4*, a powerful inhibitor of the dehydrogenase [53], which is mainly regulated via transcription [54]. This increase was also observed in SC and PG WAT of both male and female rats, being, thus, a unique effect restricted to (female) MES WAT. This assumed "lower potential inhibition" of pyruvate dehydrogenase hints at a potentially higher flow of 3C (pyruvate) into mitochondrial acetyl-CoA, thus facilitating either its oxidation or incorporation into the lipogenic pathway. This could not proceed so easily in males (and in other WAT sites of females), which *Pdk4* expression increases with time and exposure to glucose, preventing the decarboxylation of mitochondrial pyruvate to acetyl-CoA. Since in male MES, excess mitochondrial pyruvate could not be processed to 2C units, it must be returned to the cytosol; lacking acetyl-CoA it could not be derived through the Krebs cycle, which does not oxidize it in any case). Thus, the most probable way of utilization is via carboxylation [55] and its transfer to the cytoplasm through the pyruvate/malate shuttle, partly using the machinery of fatty acid synthesis [56]. After malate is transferred into the cytosol, it is either used by the malic enzyme to provide NADPH (improbable in this case, as shown in Results) or oxidized to oxaloacetate by malate dehydrogenase, providing NADH [57]. Cytoplasmic oxaloacetate can be converted into P-enol-pyruvate by P-enol-pyruvate carboxy-kinase [58]. In males, the expression of its gene, *Pck1*, was raised with incubation time in parallel to *Pdk4*, but was stabilized in females following the same pattern than the kinase, an effect extended to SC-WAT. The differences in the

cytosol-mitochondria handling of pyruvate suggest a deep sex-related metabolic pathway deviation of pyruvate fate, which ultimately may help explain the known different metabolic handling of lipids (and, probably glucose) by visceral WAT depending on sex [59].

Curiously, the alternatives for disposal of the oxaloacetate extracted from the mitochondria by the malate shunt point only to the regeneration of P-enol pyruvate, which could not go further up the glycolytic pathway because of the overproduction of pyruvate; the generation of large amounts of lactate at a constant rate is proof of the unequivocal direction of glycolysis in the adipocytes, at least under the conditions and cell sizes used in this study, leaving open only the conversion (again) of P-enol-pyruvate to pyruvate by pyruvate kinase. However, the difference (in males) lies, precisely, in the provision of cytoplasmic NADH by malate dehydrogenase (malate shunt), which allows the conversion of this pyruvate to lactate and its release to the medium. We know that the generation of glycerol-3P in parallel to pyruvate drains the NADH produced by the triose-P dehydrogenase for glycerogenesis [46]. As a result there should be an excess of pyruvate (or a deficit of NADH) in the cytoplasm [46]. The obvious alternative for pyruvate disposal would be lipogenesis, implying the malic enzyme; but the expression of its gene, *Me1*, remains unchanged, not following the pattern of *Pdk4* or *Pck1*, i.e. the cytosolic production of NADH is favored over that of NADPH (i.e. oxaloacetate and then pyruvate-lactate pathway gains over oxaloacetate and then acetyl-CoA).

Since pyruvate is a good substrate for lipogenesis [60] and WAT is the largest and specialized depot tissue for body lipid storage, one can expect this pathway to be activated, starting with the massive production of acetyl-CoA, its transfer to the cytoplasm via citrate and then a full activation of lipogenesis, including necessarily higher expressions of *Acaca* and *Fas* and the activation of NADPH provision (via *G6pdx*, *Me1*). None of these signs was detected, no effects of glucose or incubation affected their expressions of the genes. Probably, there was no significant synthesis of fatty acids under the conditions of high glucose described, in agreement with previous studies using labelled glucose [46].

In addition to the paradoxical apparent inactivity of lipogenesis, and despite a high increment with time (but not with higher glucose availability) of lipase gene expressions [3,41,46]: *Lpl* and *Atgl*; (in MES *Hsl* we observed a tendency to "restraint" resembling those of *Pdk4* and *Pck1*), the expected massive efflux of NEFA did not occur. In any case, NEFA were released to the medium in much smaller proportions than the canonical 3-to-1 molar ratio *vs*. glycerol expected from straight lipolysis; as described for the initial phase of catecholamine-elicited WAT lipolysis [61]. The results were, however, fully compatible with the activation of TAG turnover, a critical regulatory system [62], as a mechanism to selectively release glycerol as a 3C unit ultimately derived from glucose, as we have previously postulated [30,46] _ENREF_30_ENREF_67. The highest number of lipase gene mRNA copies per cell, and the steepest increase with time corresponded to PG tissue [3,46], with even higher values for females. However, the glycerol release rates of this site were smaller than those of MES.

The higher female number of copies for *Gk* (glycerol kinase gene) may suggest the existence of an additional restrain (free glycerol being recycled to *sn*-glycerol-3P [63]) thus potentially decreasing the actual release of glycerol in parallel to lower NEFA liberation; or unbalancing the equilibrium between glycerol-3P synthesis and hydrolysis [64].

Regardless of the fairly uniform pattern of gene expressions, the limited changes observed and the actually small amount of "live matter" in the adipocytes used in the incubations, the proportion of glucose that was converted to 3C units, largely lactate, the convoluted mechanisms to produce and release glycerol, especially addressed to the liver (at least in the highest glycerol producer, MES WAT), and the considerable restraint observed in the actual synthesis of fatty acids are, again, not completely new, but its concatenation is. The production of glycerol by WAT is known for a long time [51,65,66], and has been linked to glucose [67,68] but also (mainly) to lipolysis [69,70], within the context of the glucose-fatty acid cycle [71]. However, glycerol and fatty acids efflux seldom have been analyzed together [61]. The quantitative approach used here shows a relation between glycerol and NEFA release, but not the expected, and points to lipolysis as part of TAG turnover. This turnover was accelerated with time, apparently to release glycerol. Nevertheless, the main factor affected by these changes, and that most modulated by sex (at least in MES adipocytes) was the equilibrium between NADPH and NADH in the thin layer of adipocyte cytoplasm. Intercellular cooperation, perhaps close neighboring cells acting as providers of mitochondria oxidative power, is required to fully understand how WAT works, and also to uncover the intricate effects of sex on WAT operation [71].

In fact, the resilience of adipocytes to produce more fatty acids even under excess glucose availability, breaking up most of the glucose instead, and releasing lactate and glycerol, is remarkable, and makes us wonder whether the current opinion of WAT as the last in the queue, having to take up and use for fat synthesis (and hence induction of obesity) the remaining excess glucose available, remains true.

Methods

Animals and sampling

The experimental design and the rat handling procedures were applied following the animal treatment guidelines established by the corresponding European, Spanish and Catalan Authorities. The

Committee on Animal Experimentation of the University of Barcelona specifically authorized the procedures used in this study.

Wistar rats (Janvier, Le Genest-Saint Isle, France), 14-week old (8 male and 8 female), were used after an acclimation period of at least 7 days. The animals were kept in two-rat cages under standard conditions: i.e. 21.5-22.5ºC, and 50-60% relative humidity; lights were on from 08:00 to 20:00. The rats had continuous free access to water and standard rat chow (#2014, Teklad Diets, Madison WI USA).

The animals were killed, under isoflurane anesthesia, at the beginning of a light cycle. Females were in the proestrus phase. After complete anaesthesia, the animals were exsanguinated with syringes from the just-exposed aorta. They were rapidly dissected, excising samples of mesenteric (MES) WAT, cleaned of attachments and pancreatic tissue; epididymal /periovaric (i.e. perigonadal PG) WAT, and both cordons of inguinal SC WAT. Tissue samples of a pair of same-sex rats with similar weight were coarsely minced and pooled prior to the separation of adipocytes. In all, four 2-rat samples were used for each tissue and sex adipocyte extraction.

Preparatin and incubation of adipocytes

Adipocytes were isolated by incubation with collagenase as described in a previous paper [3], essentially following the Rodbell procedure [72]. Adipocytes were counted from the final washed suspensions. Their (spherical when free) diameters were measured using serial microphotographs and the ImageJ software (http://imagej.nih.gov/ij/) [73]. The recovery of adipocytes with respect to the mass of WAT used was estimated in a number of randomly selected samples, as previously described [3]. Incubations were carried out using 12-well plates (#734-2324VWR International BVBA/Sprl., Leuven Belgium). The incubation medium consisted of 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 contained added glucose at a final nominal concentration of 7 mM or 14 mM. The medium also contained 25 mM hepes (Sigma-Aldrich, St Louis MO USA), 2mM glutamine (Lonza Biowhittaker, Radnor, PA USA), 1 mM pyruvate (Gibco), 30 mg/mL delipidated bovine serum albumin (Millipore Calbiochem, MA USA), 100 nM adenosine, 100 mU/mL penicillin and 100 mg/L streptomycin (all from Sigma-Aldrich).

Each well contained 400 µL of the cell suspension. Since 0.1 mL of medium was used for initial measurements (zero values), 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. The cells were incubated for 24 h or 48 h, without any further intervention, as previously described [3,4].

The incubation of adipocytes was stopped by pipetting out the whole contents of the well, allowing the adipocytes to float and form a defined layer, which was taken out. The infranatant medium was centrifuged (to eliminate the remaining adipocytes), mixed, aliquoted and frozen.

Glucose was measured using a glucose oxidase-peroxidase kit (#11504, Biosystems, Barcelona Spain) containing 750 nkat/mL mutarrotase (porcine kidney, 136A5000, Calzyme, St Louis, MO USA) [74]. 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).

Gene expression analyses

Total cell RNA was extracted from the packed washed adipocytes using the Tripure reagent (Roche Applied Science, Indianapolis IN USA), and were quantified using a Nanodrop 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 and measurement of evolved fluorescence were developed in an ABI PRISM 7900 HT detection system (Applied Biosystems),

A semi-quantitative approach for the estimation of the concentration of specific gene mRNAs per unit of tissue weight was used [75]. *Arbp* was used as the charge control gene [76]. The results were expressed as the number of transcript copies per cell, in order to obtain comparable data between the groups, and thus forfeiting the eventual influence of large amounts of fat and interstitial protein matrix. The genes analyzed and a list of primers used are presented in Table 1 (and on Figure 7).

Statistics

Statistical analyses and comparisons between groups (four-, three- or two-way ANOVAs) were applied using the Stratgraphics program (Statpoint Technologies, Warrington, VA USA)..

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In rat white adipose tissue, lactate production by adipocytes and nucleated stromal cells is quantitatively comparable, but only adipocytes also release glycerol

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ABSTRACT

In white adipose tissue (WAT), the global importance of nucleated stromal cells (NSC) has been widely recognized, especially its implication in metabolic regulation, defense, regeneration and even tissue control via hormones and cytokines. These cells have different origins, functions and proportions in adipose tissue, and change with inflammation, starvation and a number of physiological and pathological conditions. When referring to WAT, the overwhelming size and understood function of these cells almost completely obscure the quantitative metabolic contribution of all types of NSC, together to WAT function, in addition to their diverse specific immune, regenerative of paracrine functions. 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 mM 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.

INTRODUCTION

Adipose tissue, and more especially white adipose tissue (WAT) is one of the most peculiar animal tissues. It has been proposed that its well-known dispersion and variability in site size, distribution and function is, precisely, a characteristic expression of its adaptability, diversity of functions and complementarity [1], constituting a real adipose organ [2]. The adipose organ is anatomically disperse, and it is also more than just adipocytes [3]; it is responsible of a growing number of physiological, metabolic and regulative functions. Within this concept of adipose organ we should also include the small portions of adipose tissue

cells interspersed in and between other organs and tissues such as muscle [4], achieving a symbiotic-like metabolic complementarity for handling energy substrates [5].

After decades of assuming that WAT was essentially an energy (in the form of triacylglycerols, TAG) dump, the finding of its multiple, complex regulatory [6] and defense [7] functions has increased our attention of WAT, especially focused on the roles of specific masses of tissue, such as perivascular [8], intramuscular [9], or epicardic [10] WAT. Their direct implication on the metabolism of estrogen [11, 12], androgen [12, 13] and corticosteroids [13, 14], adds to their ability to inactivate insulin [15], modulate thyroid hormone action [16], produce GH releasing hormone [17], and secreting a growing number of cytokines, both pro-inflammatory, anti-inflammatory, appetite regulators and energy controllers [18, 19].

WAT may elicit its conversion into a number of other tissues, via stem cells [20], participating in repair and regeneration [21]. WAT includes a large number of immune cells, such as macrophages [22] and lymphocytes, as well as stromal epithelial cells [23, 24]. The latter play a key role in the control of blood flow to WAT itself [25, 26] and neighboring tissues [27], largely via nitric oxide metabolism [28, 29]; in this line, WAT also contains a complete renin-angiotensin system [30] and endothelin [31]. The versatility of WAT is shown by its special relationship with bone marrow [32], mammary tissue [11] and skeletal integrity (largely via collagen synthesis [33]) and its capacity to generate brown [34] / beige [35] adipocytes in addition to its own thermogenic ability [36]. By the way, WAT *also* stores TAG.

When analyzed in depth, most WAT sites contain between 50 and 90 % fat [37], with as little as 1.3-1.5 % of "live" adipocyte cell volume (i.e. that of the cell minus fat vacuoles) [38]. Other cells may be attached to adipocytes or vessels, form a fiber scaffold [39] or roam free in the interstitial space of WAT. Their variety is considerable, in accordance with the functions and origins described above, but this composition changes under 'aggression' and the ensuing stimulation of defense systems [40, 41], implicated in the inflammatory response which lies at the basis of metabolic syndrome [42]. The pathological importance of knowing WAT specific functions is paramount, given its mass, distribution, cell plurality, protean capacity to change, adapt and respond, and the dramatic consequences observed on these factors in human disease, as is the case of metabolic syndrome.

In spite of these abundant known antecedents, our knowledge of WAT metabolism remains incomplete and largely limited to glycolysis, lipogenesis and TAG handling. It is a consequence, in part, of the practical difficulties of comparison. Adipocyte sizes may differ by several degrees of magnitude, essentially because of accumulation of fat, which mass dilutes, and interferes separation and analysis procedures; thus hampering any comparison of tissues from different origins. The large mass of collagen and other protein fibers with respect to the tiny mass of 'live cytoplasm' makes inviable to establish comparisons based on tissue protein content. Specific mRNA, but also DNA or nuclei measurements, are not reliable either because of the variable number and type of cells which may be present in a given sample of WAT, often with gross differences with those of other, sites, subjects or even due to sampling.

It is already known that WAT behaves, in the presence of glucose and oxygen, as a normally anaerobic tissue [43, 44], with low oxygen consumption [45] and large production of lactate [46] and glycerol [47]. Primary cultures of adipocytes have related lipogenesis, and this enhanced glycolytic activity, to the size of the cell [48]. In recent papers, we have related enzyme activities, metabolite efflux or gene expression [49, 50] data to the number of cells, but this procedure requires considerable manipulation: isolation, counting and analysis of recovery of the cells [38]. This necessary step undoubtedly may hamper the rapid comparative (and quantitative) analysis of WAT sites under different conditions.

Most of our studies have been devoted to adipocytes, since adipocytes are the characteristic and defining cells of WAT. However, the abundance of other cell types, despite its small 'live mass' per gram of tissue have seldom been investigated from a metabolic point of view. In the present study we have checked, using a quantitative approach, whether the sum of nucleated and non-nucleated (i.e. red blood cells RBC) actually contributes significantly to the active glycolytic-based production of 3C units from glucose. In the present study, we investigated whether the generalized assumption that adipocytes alone are the backbone and main metabolic actors of WAT remains true.

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 426±12 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. They 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 (EP), mesenteric (MES) and subcutaneous (SC; both inguinal cordons were taken) WAT were extracted. In order to obtain sufficient material, the same-site samples of two rats were pooled, minced with scissors and thoroughly mixed before further processing.

WAT cell isolation and preparations

Samples of whole WAT were reserved and used for lipid and water content analysis as well as for density estimation as previously described [38]

Cells were isolated [51] 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 [52]. Then, the cell suspension was filtered through a double layer of nylon hose. The retained debris was recovered and weighed. The cell suspension was 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. The latter was slowly drained from the syringe and the upper adipocyte layer was left in it as previously described. The adipocyte layer was gently suspended again in fresh incubation buffer (free of collagenase) and the process of mixing and draining was repeated twice, discarding the washing fluids [38]. Aliquots of the adipocytes containing layer were used for cell size estimation, lipid content (for analysis of recovery), and for incubation as described below [50].

The first washing contained most of the non-attached stromal cells. It was used directly for incubations, cell number estimation and analysis of red blood cell content, as previously described [38]. All cell preparations were maintained at room temperature (c. 22ºC), and manipulated for a time as short as possible; adipocytes were used immediately after the final washing.

The stromal cell space in the isolated cell suspension was used to relate their numbers and volumes to initial tissue weight; it was estimated as the sum of the volume of the lower phase of adipocyte separation (extracted in the syringe), plus a part of the volume of the adipocyte phase not occupied by the adipocytes themselves. This latter volume was the difference between the volume of the phase and that of adipocytes, calculated from their numbers and cell volumes [38]. 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 free 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.

Measurement of isolated cell parameters

Adipocyte suspensions were examined using a Neubauer chamber (#717810 Neubauer improved bright line, Brand Gmbh, Wertheim, Germany). At least 16 fields for sample were photographed using an inverted microscope. Cells were identified, counted, and their size analyzed (under the conditions used, all cells adopted a spheroid form), using the *FIJI ImageJ* software (http://imagej.nih.gov/ij/) [53].

Total stromal cells (i.e. including RBCs) were analyzed in each sample with the Scepter 2.0 cell counter (EDM Millipore Corp, Billerica, MA USA) hand-held cell sizer, 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). RBCs were estimated from the counting of particles with volumes between 25 fL and 60 fL, since these limits included 90 % of total RBC (experimental data not shown). The larger particles were considered nucleated stromal cells (NSC), as previously described [38]. Particles smaller than 25 fL were also counted; they were considered to be, essentially, fat droplets and other small cell or fiber agglomerate debris.

Cell incubation procedures

The complete procedure was described previously by us [38, 50]; shortly: 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), 1 mM pyruvate (Gibco), 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). The cells were supplemented with glucose (Sigma-Aldrich) to a final concentration of either 7 mM or 14 mM.

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 CO_2 (5%), which gave a theoretical pO_2 of 20 kPa [43]. 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, in the case of adipocytes, were left standing for 5 min to pipette out the infranatant and immediately use *in*

situ the adipocyte fraction for RNA extraction. In the case of NSC (containing the original tissue RBCs), the tubes were centrifuged for 8 min at 500xg. Supernatant medium was extracted, and the cell precipitates (RBC and NSC) were then used for immediate RNA extraction. All supernatants were aliquoted, frozen and stored at -20ºC until processed.

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 mutarrotase (porcine kidney, 136A5000, Calzyme, St Louis, MO USA) [54]. 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 the number of cells in the well.

Estimation of RBC lactate production under the conditions of incubation

The variable presence (affecting cell counts) of RBC in the samples (despite the rats being exsanguinated), and their glycolytic nature (affecting glucose uptake and lactate output) were possible sources of interference for the analysis of non-adipocyte production of lactate. Since no practical method was available to remove RBCs from the non-adipocyte cell preparations without producing unknown damage or modification to the other cells, we opted for the estimation of the eventual capability of RBCs to produce lactate using RBCs (from blood) alone. Two different preparations of RBCs were obtained from the pooled fresh (heparinized) blood of two 'WAT donors'. In one, blood was suspended (diluted) in WAT cell isolation medium; then, the cells were centrifuged and washed in incubation medium and re-suspended (i.e. treated as RBCs from WAT. This suspension was compared with the direct dilution of blood in incubation medium. Using the Scepter cell counter as described above, the number of cells in both suspensions was counted. Three cell concentrations were prepared: 5, 25 and 50 million cells per well. The RBCs were then incubated under the same conditions than the other cells for up to 48 h. The cells were harvested and discarded, and the media lactate was measured using the procedure described above.

Gene expression analysis

Total cell RNA was extracted from all the harvested cells 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 (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 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 [55]. *Arbp* was the charge control gene [56]. We initially expressed the data as the number of transcript copies per nucleated cell.

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

Data presentation and statistical procedures

Since a main objective of the study was to evaluate the eventual contribution of non-adipocyte cells to the efflux of glycolytic and lipolytic efflux, we presented the data in two complementary forms. The direct values of substrate efflux (uptake for glucose) and number of copies of specific protein mRNAs were presented per cell (in Tables); whereas, these same values corrected by the number of cells present in each tissue cell fraction per unit of whole tissue weight were shown in Figures. This way, in a theoretically reconstituted WAT each cell type: adipocytes, NSC and RBC showed its comparative effect on each of the parameters measured.

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

RESULTS

WAT cell distribution

Table 2 shows the number of cells (adipocytes, NSC and RBC) present in 1 g of WAT from subcutaneous, mesenteric or epidydimal sites of adult male rats. No statistically significant differences in adipocyte of RBC numbers were observed, but in EPI WAT, the number of NSC per g was about half that found in MES, SC

TABLE 1 List of primers used

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	
Ldha	L-lactate dehydrogenase a	5' >	AAAGGCTGGGAGTTCATCCA	96
		> 3'	CGGCGACATTCACACCACT	
Ldhb	L-lactate dehydrogenase b	5' >	GCGAGAACTGGAAGGAGGTG	145
		> 3'	GGGTGAATCCGAGAGAGGTTT	
Pfkl	Phospho-fructokinase, liver, b-type	5' >	CAGCCACCATCAGCAACAAT	90
		> 3'	TGCGGTCACAACTCTCCATT	
Pfkm	Phospho-fructokinase, muscle	5' >	CATCCCATTTGTGGTCATTCC	149
		> 3'	TAAACACTCGCCGCTTGGT	
Phgdh	Phospho-glycerate dehydrogenase	5' >	CTGAACGGGAAGACACTGGGAA	138
		> 3'	AACACCAAAGGAGGCAGCGA	
Gpam	Glycerol-3-phosphate acyl-transferase, mitochondrial	5' >	GGTGAGGAGCAGCGTGATT	129
		> 3'	GTGGACAAAGATGGCAGCAG	
Pdk4	Pyruvate dehydrogenase kinase, isoenzyme 4	5' >	CTGCTCCAACGCCTGTGAT	142
		> 3'	GCATCTGTCCCATAGCCTGA	
Fas	Fatty acid synthase	5' >	CCCGTTGGAGGTGTCTTCA	117
		> 3'	AAGGTTCAGGGTGCCATTGT	
Hsl	Lipase, hormone sensitive	5' >	TCCTCTGCTTCTCCCTCTCG	108
		> 3'	ATGGTCCTCCGTCTCTGTCC	
Atgl	Adipose triacylglycerol lipase	5' >	CACCAACACCAGCATCCAAT	120
		> 3'	CGAAGTCCATCTCGGTAGCC	
Lpl	Lipoprotein lipase	5' >	TGGCGTGGCAGGAAGTCT	116
		> 3'	CCGCATCATCAGGAGAAAGG	
Arbp	0S acidic ribosomal phospho-protein PO [housekeeping gene]	5' >	CCTTCTCCTTCGGGCTGAT	122
		> 3'	CACATTGCGGACACCCTCTA	

WAT showing intermediate values. No differences were found in the mean cell volume for NSC. But adipocytes in MES WAT had about half the volume of those in EPI WAT.

The number of RBC may indicate a rough approximation to the volume of blood vessels present in the tissue, despite exsanguination, and assuming a standard hematocrit value of 45%, the number of RBCs found in MES WAT represent about 17 µL/g, whilst the values for SC WAT and EPI WAT were 7.8 µL/g and 10.6 µL/g, respectively. The data agree with a higher blood irrigation of MES WAT compared with the other sites. Despite their different localization and function, MES and SC WAT seem more structurally closer than when compared with EPI WAT. a) The ratio of cell volumes (adipocytes/NSC) was 600 and 624, respectively for MES and SC, but double, 1207 (all are mean values) for EPI WAT. b) The ratio of number of nucleated cells (NSC/adipocytes) was 13.7 for SC, 13.0 for MES and 9.6 for EPI. Nevertheless, the closeness of these data suggest that under standard (i.e. no inflammatory conditions, as is the case), the numbers of NSC and adipocytes, despite their sizes were rather uniform. The proportion of fat in the tissue agrees with the larger adipocytes in EPI WAT, which translates into significant differences in the proportion of fat in the tissue between sites. Curiously, this results in probably smaller (no statistical calculation was done because the data were derived from measurements derived from transformed data) interstitial space for EPI WAT compared with the smaller-cell WAT sites.

RBC lactate production

Figure 1 presents the production of lactate by fresh blood RBC incubated under the same conditions than the cells extracted from WAT. The number of RBC present in the actual stromal cell suspensions used were in a range comparable to the intermediate concentration of cells depicted here. The results obtained with washed cells were similar to these, albeit slightly lower (no significant differences anyway), and thus are not represented. The production of lactate was marked and depended on incubation time and the number of cells. The production of lactate was not altered by glucose concentration in the 7/14 mM range studied (data not shown).

TABLE 2 WAT site cell characteristics of young male Wistar rats

parameter	units	SC WAT	MES WAT	EPI WAT	p
cell counts					
adipocyte	cellsx10^6/g WAT	3.19 ± 0.84	4.59 ± 0.98	2.90 ± 0.35	NS
nucleated stromal cells	cellsx10^6/g WAT	43.8 ± 7.7	59.8 ± 3.8	27.9 ± 7.3	0.0220
red blood cells	cellsx10^6/g WAT	108 ± 51	236 ± 57	150 ± 61	NS
cell fragments	10^6/g WAT	151 ± 50	103 ± 22	105 ± 18	NS
recovery of WAT adipocytes	%	70.4 ± 7.6	83.0 ± 8.6	77.2 ± 0.1	--
cell volumes					
adipocyte volume	pL/cell	251 ± 30	159 ± 33	309 ± 7	0.0065
nucleated stromal cells	fL/cell	419 ± 28	255 ± 79	256 ± 44	NS
red blood cells	fL/cell		32.4 ± 1.9		--
red blood cells	% stromal cells	62.8 ± 12.3	79.4 ± 3.3	84.4 ± 2.9	NS
cell fragments and droplets	fL/fragment		<5		--
other tissue data					
debris (dry weight)	mg/g WAT	37	59	22	--
water in intact tissue	mg/g WAT	220 ± 63	160 ± 37	64.4 ± 11.2	NS
water in tissue minus debris	mg/g WAT	213 ± 62	152 ± 41	62.9 ± 10.2	NS
fat in intact tissue	mg/g WAT	740 ± 20	744 ± 24	871 ± 12	0.0014
fat in tissue minus debris	mg/g WAT	713 ± 29	701 ± 62	852 ± 1	0.0440
tissue density	g/g WAT	0.9246 ± 0.0130	0.9208 ± 0.0129	0.9282 ± 0.0048	NS
fat density	g/mL WAT		0.922 ± 0.022		--
debris and interstitial space	µl/g WAT	237 ± 97	275 ± 92	145 ± 106	--

The data are the mean ± SD of different [N: PG 2; MES 3; SC 4] 2-rat tissue pools. Statistical significance of the differences between groups (1-way anova). The column P represents the p values corresponding to the effect of WAT site. NS = $p>0.05$

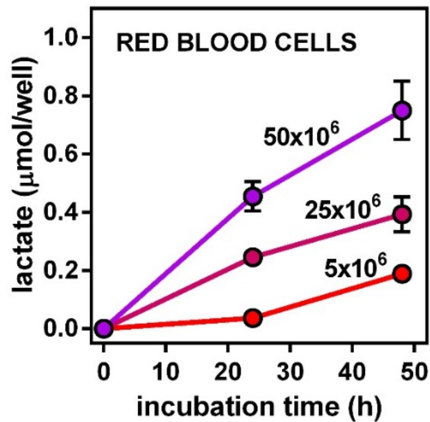

Figure 1 Production of lactate by RBCs incubated in medium with 14 mM glucose

The data are the mean ± sem of 4 samples of adult rat blood. The data on the lines indicate the number of RBC per well.

Glucose uptake and metabolite efflux from incubated adipocytes and NSC

Table 3 shows the glucose uptake and lactate, glycerol and NEFA efflux from incubated adipocytes, NSC and RBC incubated for 24 h in 7 mM or 14 mM (initial values) of glucose. The values for SNC shown are the result of discounting the values of the incubated crude tissue extract preparations minus the calculated contribution of RBCs. The only statistically significant difference between groups (taking as comparative factors site and initial medium glucose) was the effect of WAT site on glucose uptake, maximal in MES-WAT. No effects of glucose concentration were observed at all on adipocyte or NSC lactate efflux. The RBC lactate efflux was practically the equivalent of the glucose taken up, as expected. Ant the values for lactate were closer to this relationship for NSC, and less for adipocytes.

A remarkable finding was the negligible secretion of either glycerol or NEFA by NSC, and neither, as expected for RBC.

When the individual cell data were adjusted by the number of cells of each type studied, in order to show the values corresponding to all cells present in 1 g of WAT, we obtained the results depicted in Figure 2. These data apparently reflect a minimal quantitative importance of RBC both with respect to glucose uptake and lactate efflux. For this reason, all comparisons have been limited to NSC and adipocytes. With respect to glucose uptake, as well as lactate and glycerol efflux, adipocytes showed a clear effect of site, with higher values in MES WAT. This effect was also significant for lactate efflux on NSC, with, again, a higher production of lactate in MES WAT. No significant changes were observed in NEFA efflux (only in adipocytes). Taken as a whole, and contrary to what we expected, in all three sites, lactate efflux by all NSC was higher than that produced by the adipocytes. It is remarkable to add, that there were no statistically significant effects of initial glucose concentration on any of the parameters analyzed.

Comparative gene expression in WAT sites

Table 4 presents the copies per cell of the specific mRNA for genes that are related to the production of 3C fragments from glucose. All genes, except *Pdk4* and adipocyte *Hsl* and *Lpl* showed a significant effect of site in their expression. In general, the highest values were found in MES WAT. The effects of glucose were much more limited: NSC *Glut1* and *Ldhb*, and adipocyte *Fas*, no other effect was observed, and those listed were close to the limit of significance. Some of the data presented may be partially artefactual, because of the low number of copies presented, in some cases lower than 1 per cell. This is possible because the NSC is a complex mixture of very different types of cells, a difference magnified in this case by the different levels found in different sites. However, the lowest values of copies per cell in NSC correspond to lipid metabolism. (*Gpam*, *Hsl*, *Fas*, *Atgl* and *Lpl*), but also includes a critical regulatory enzyme *Pdk4* and *Ldhb*, much more abundant in adipocytes.

As in the case of medium metabolites, Figures 3 and 4 show the contribution of adipocytes and NSC to the total number of copies for the genes studied in 1 g of WAT. The differences between the contributions of the limited number of adipocytes compared with the NSC were similar for most glucose-lactate metabolism-related genes (Figure 3). In the six genes depicted: *Glut1*, *Hk1*, *Pfkm*, *Phgdh*, *Ldha* and *Ldhb*, the effect of 'type of cell' was statistically significant, and in all cases and in both cell types the effect of site was also significant. In addition, in NSC, Glut 1 and *Ldhb* showed a significant effect of glucose. In most cases MES WAT showed the highest contributions (and SC the lower) in adipocytes and NSC, with global gene expression values markedly parallel in all case except for lactate dehydrogenase b (*Ldhb*), which expression was much lower than the a isozyme (*Ldha*), and even lower values for NSC compared with adipocytes, the only 'discordance' in that series of data.

TABLE 3 WAT site cell glucose uptake and metabolite efflux of young male Wistar rats

parameter	-cells	units	SC WAT		MES WAT		EPI WAT	
			7 mM	14mM	7 mM	14mM	7 mM	14mM
glucose uptake	adipocytes	pmol/cell in 24h	4.56 ± 0.68	4.94 ±0.58	7.81 ± 0.68	8.73 ± 0.99	3.36 ± 0.52	4.02 ± 1.95
	nucleated stromal cells	pmol/cell in 24h	0.344 ± 0.085	0.63 ± 0.29	0.447 ± 0.077	0.811 ± 0.082	0,784 ± 0.150	1.06 ± 0.45
	red blood cells	fmol/cell in 24h	5.71 ± 1.70 [*]					
lactate efflux	adipocytes	pmol/cell in 24h	3.63 ± 0.56	3.90± 0.42	3.93 ± 0.91	3.88 ± 1.20	2.47 ± 0.48	2.81 ± 0.42
	nucleated stromal cells	pmol/cell in 24h	0.647 ± 0.214	0.504 ± 0.183	0.796 ± 0.141	0.876 ± 0.196	0.550 ± 0.222	0.564 ± 0.217
	red blood cells	fmol/cell in 24h	11.4 ± 3.4 [*]					
glycerol efflux	adipocytes	pmol/cell in 24h	1.56 ± 0.35	1.52 ± 0.35	2.26 ± 0.42	2.40 ± 0.33	1.71 ± 0.16	1.95 ± 0.21
	nucleated stromal cells	fmol/cell in 24h	not detected					
NEFA efflux	adipocytes	pmol/cell in 24h	0.401 ± 0.109	0.385 ± 0.119	0,752 ± 0.407	0.360 ± 1.67	0.242 ± 0.038	0.178 ± 0.023
	nucleated stromal cells	pmol/cell in 24h	not detected					

The data are the mean ± SD of (N=4or 3) 2-rat –pooled samples; [*] calculated value (from data shown in Figure 1). Statistical significance of the differences between groups (2-way anova). The only significant ($p=0.0008$) relationship was that of glucose uptake by adipocytes with respect to WAT site.

TABLE 4 WAT site expression (in copies per cell) of genes related with glycolysis and glycerol metabolism of young male Wistar rats

gene		cells	SC WAT		MES WAT		EPI WAT		p	
			7 mM	14mM	7 mM	14mM	7 mM	14mM	site	glucose
Glut1	glucose transporter 1	adipocytes	75.7 ± 5.0	128 ± 14*	304 ± 56*	287 ± 61	111 ± 11	123 ± 22	<0.0001	NS
		stromal	20.0 ± 4.8	26.3 ± 9.2*	31.1 ± 4.3*	52.6 ± 5.6*	16.3 ± 5.6*	23.3 ± 5.5	0.0050	0.0322
Hk1	hexokinase 1	adipocytes	172 ± 15	222 ± 29*	488 ± 52*	500 ± 35*	343 ± 54	394 ± 108	0.0011	NS
		stromal	11.9 ± 2.6	6.71 ± 4.04	26.0 ± 1.6	32.4 ± 0.1*	18.8 ± 6.7	20.5 ± 4.7	0.0007	NS
Ldha	type a lactate dehydrogenase	adipocytes	1003 ± 196	1274 ± 202*	2271 ± 260*	1963 ± 214	1959 ± 157	1988 ± 154	0.0003	NS
		stromal	95.6 ± 11.6	77.8 ± 11.8*	222 ± 9*	265 ± 38*	189 ± 20	168 ± 13	<0.0001	NS
Ldhb	type b lactate dehydrogenase	adipocytes	164 ± 21	176 ± 8*	332 ± 62*	362 ± 51*	339 ± 32	340 ± 23	0.0003	NS
		stromal	1.15 ± 0.18	0.66 ± 0.37*	3.63 ± 0.48*	1.96 ± 0.69*	3.94 ± 0.48	3.66 ± 0.32	<0.0001	0.0345
Pfkl	phospho-fructo-kinase liver	adipocytes	131 ± 11	195 ± 24	392 ± 73*	404 ± 24*	170 ± 29	182 ± 46	<0.0001	NS
		stromal	15.1 ± 2.6	7.56 ± 3.24	34.1 ± 1.2	41.2 ± 7.3*	17.2 ± 4.8	16.9 ± 3.3	<0.0001	NS
Phgdh	3-phospho-glycerate DH	adipocytes	17.5 ± 1.5	25.5 ± 3.7*	52.1 ± 11.6*	58.0 ± 9.8*	42.3 ± 4.7	44.0 ± 9.2	0.0013	NS
		stromal	2,64 ± 0.33	2.44 ± 0.53*	7.23 ± 0.96	8.14 ± 0.81*	5.40 ± 1.46	5.67 ± 1.60	0.0013	NS
Gpam	glycerol-3P acyl-transferase	adipocytes	15.6 ± 1.1	19.3 ± 4.7*	58.3 ± 10.7*	54.7 ± 15.6	38.8 ± 2.4*	34.7 ± 10.7*	0.0018	NS
		stromal	*0.478 ± 0.100*	*0.439 ± 0.115**	*1.12 ± 0.25*	*1.32 ± 0.54**	0.982 ± 0.137	0.904 ± 0.136	0.0298	NS
Pdk4	pyruvate DH kinase 4	adipocytes	4.76 ± 2.36	5.16 ± 2.52*	19.9 ± 15.9*	9.50 ± 1.81*	2.83 ± 0.92	2.72 ± 0.85	NS	NS
		stromal	*0.061 ± 0.007**	*0.024 ± 0.024**	*0.050 ± 0.016*	*0.047 ± 0.002**	*0.063 ± 0.009**	*0.013 ± 0.003**	NS	NS
Fas	fatty acid synthase	adipocytes	97.2 ± 6.3	191 ± 30*	302 ± 54*	478 ± 84*	181 ± 27	241 ± 63	0.0004	0.0166
		stromal	5.42 ± 0.83	3.49 ± 0.99*	11.2 ± 1.8	19.7 ± 5.1*	8.62 ± 1.35	10.4 ± 1.9	0.0012	NS
Hsl	hormone-sensitive lipase	adipocytes	45.0 ± 5.5	38.4 ± 5.5*	48.6 ± 8.6*	48.4 ± 6.5	98.3 ± 34.1	109 ± 54	NS	NS
		stromal	*0.739 ± 0.282*	*0.364 ± 0.079*	*0.966 ± 0.182*	*1.01 ± 0.15**	*0.566 ± 0.050**	*0.599 ± 0.011*	0.0195	NS
Atgl	adipose TAG lipase	adipocytes	218 ± 20	320 ± 45	488 ± 65*	560 ± 56*	547 ± 71	465 ± 74*	0.0005	NS
		stromal	*5.69 ± 0.85*	*5.22 ± 0.67**	11.8 ± 2.8	18.4 ± 1.03*	6.08 ± 1.48	6.08 ± 0.92	<0.0001	NS
Lpl	lipoprotein lipase	adipocytes	859 ± 42	1106 ± 228*	1437 ± 222*	1537 ± 238*	1466 ± 262	1681 ± 358	NS	NS
		stromal	2.62 ± 0.87	0.89 ± 0.20*	3.50 ± 0.38	3.51 ± 1.37*	3.32 ± 0.42	3.53 ± 0.26	0.0412	NS

The data are the mean ± sem of 4 different (* n=3) 2- rat pooled. Data in *italics* were obtained with 30 or more cycles. Statistical significance of the differences between groups (2-way anova). The far right columns show the p values for site and initial glucose concentration. NS = p>0.05

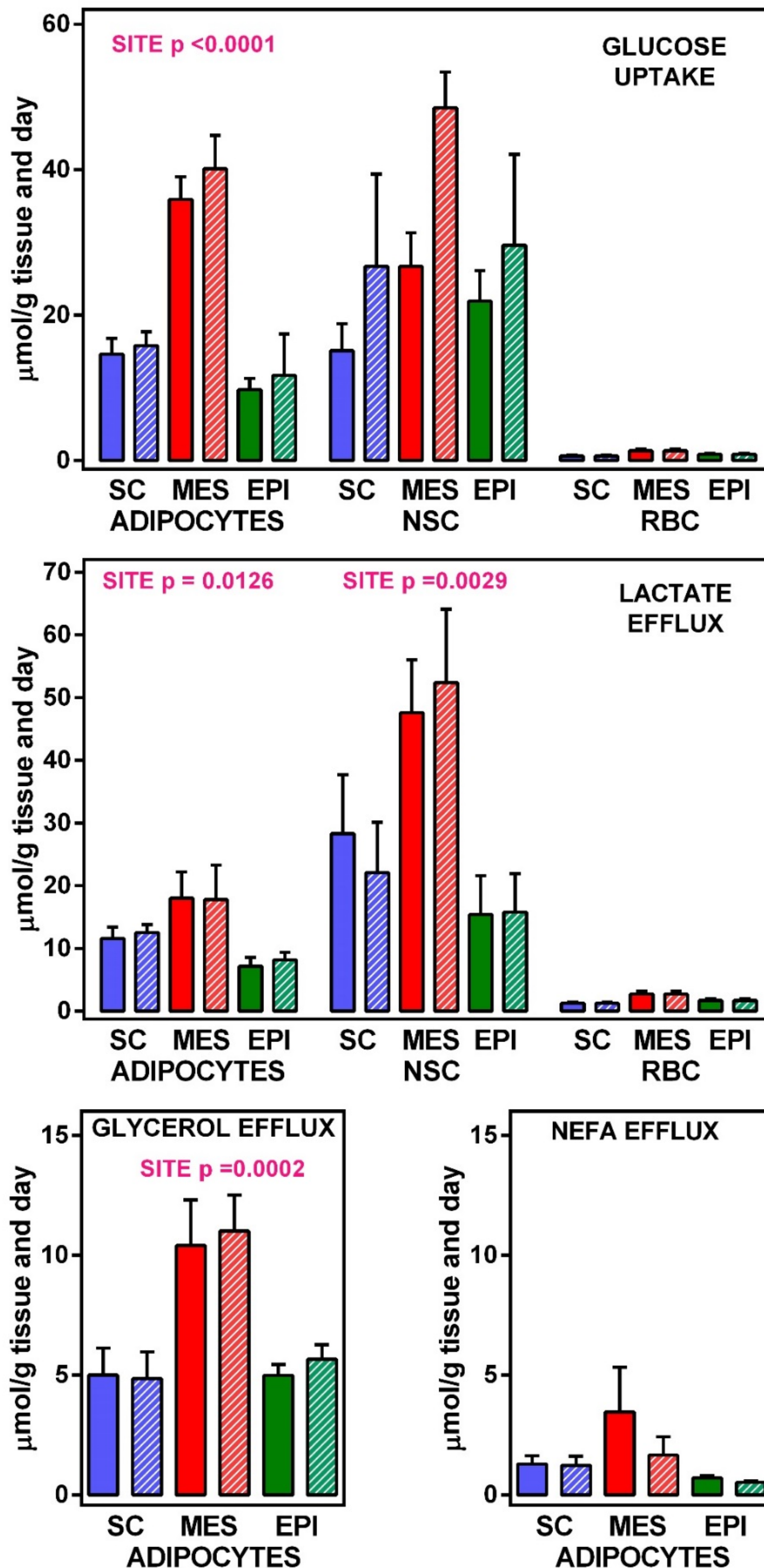

Figure 2 Glucose uptake, and lactate, glycerol and NEFA efflux from WAT component cells corresponding to 1 g of tissue

SC = subcutaneous WAT; MES = mesenteric WAT; EPI = epidydimal WAT. Full columns = 7 mM glucose, dashed columns = 14 mM glucose.

The data are the mean ± sem of (N=4 or 3) 2-rat pooled samples obtained from adult male rats. The data for glucose uptake and lactate efflux correspond to the number of adipocytes, NSC and RBC contained in 1 g of tissue (Table 2) multiplied by their individual uptake/efflux data (Table 3). The data for glycerol and NEFA correspond exclusively to the adipocytes present in 1 g of tissue, since no efflux of either was observed in NSC or RBC.

Statistical significance of the differences between groups (2-way anova): the data for site in adipocytes and NSC are indicated in the Figure itself, the absence of data indicates that the differences, if any, were not significant (P>0.05); no significant differences were observed for the effect of initial glucose concentration

The trend shown by *Ldhb* is partially repeated (Figure 4) in *Gpam*, *Pdk4*, *Hsl*, *Atgl*, and *Lpl*, which represent a limited view of lipid metabolism. In all genes studied, the effect of type of cell was, again, significant, the effects of site were significant for *Gpam*, *Fas*, *Atgl* and *Lpl*, for all cells, as well as in NSC *Hsl*. An effect of initial glucose concentration was observed only in adipocyte *Fas*.

The expressions per unit of tissue weight were clearly higher in adipocytes than in NSC, one or two orders of magnitude lower. The primacy of MES was maintained, but the differences between SC and EPI WAT were less

marked, interchanging positions, in clear divergence with the glycolytic data of Figure 3. In adipocytes, *Lpl* expression in EPI WAT was more than one order of magnitude lower than in SC and MES. The expression of *Pdk4*, normally low, wass even lower in NSC, with a marked trend to decrease with glucose concentration (anyway, the results were not significant), which tend to agree with the reverse trend on *Fas*, since lipogenesis requires the formation of acetyl-CoA and the decrease in *Pdk4* hints at a lower inhibition of pyruvate dehydrogenase.

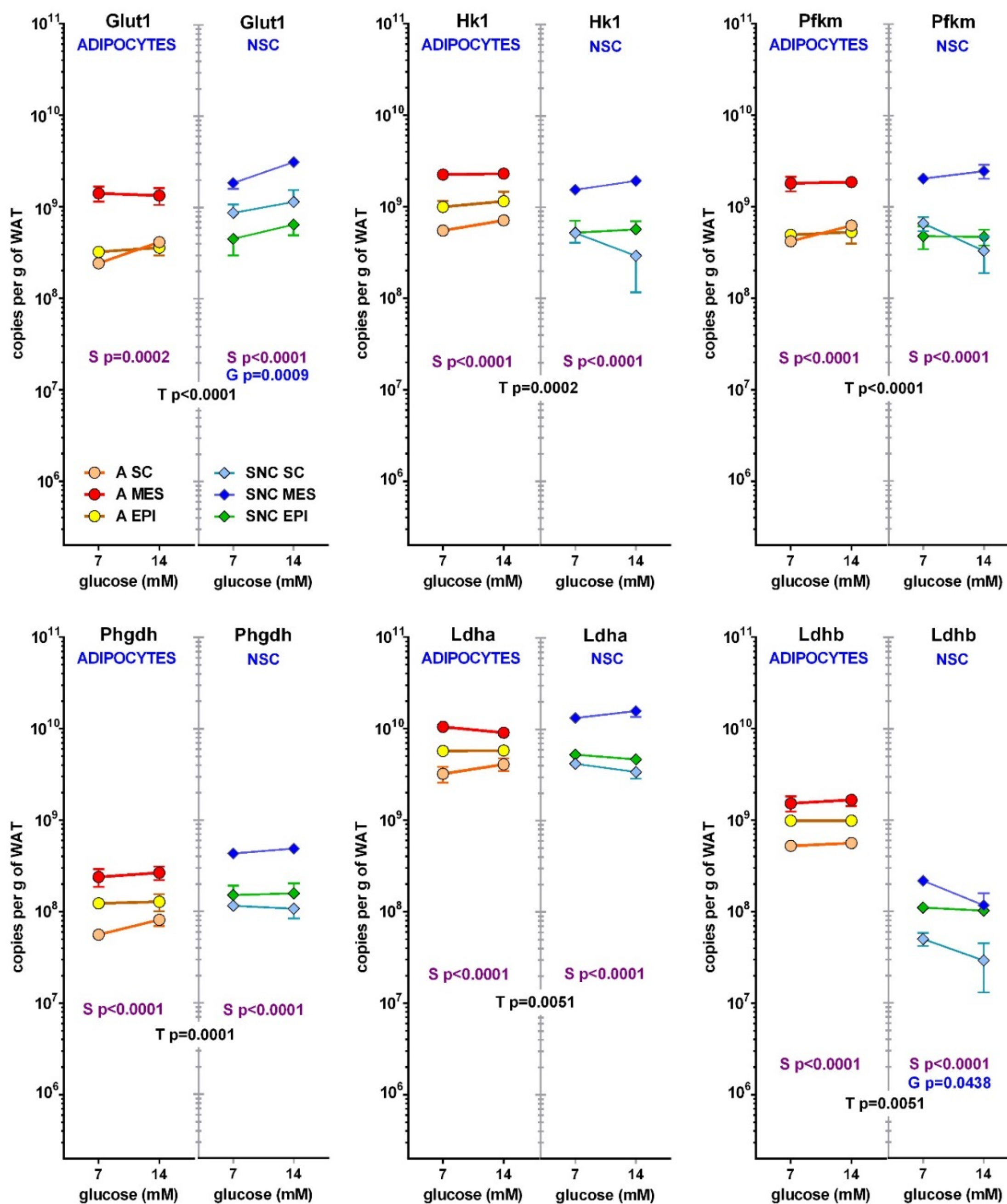

Figure 3 Number of copies of glycolysis-related genes in adipocytes and NSC contained in 1 g of WAT from different sites (SC, MES and EPI) or male adult rats

The data correspond to the mean ± sem of (N=4 or 3) 2- rat pooled samples. The data have been presented in a 5+ orders of magnitude logarithmic scale for easy direct comparison of the abundance of all gene transcripts. The values presented were calculated from the data presented in Tables 2 and 4. Statistical significance of the differences between groups (3-way anova). T: corresponds to differences between the 'type' or cell (adipocyte vs. NSC), S: refers to the differences between 'sites' (SC, MES, EPI) within the same cell group, and G represents the statistically significant differences in expression induced by the initial 'glucose' concentrations.

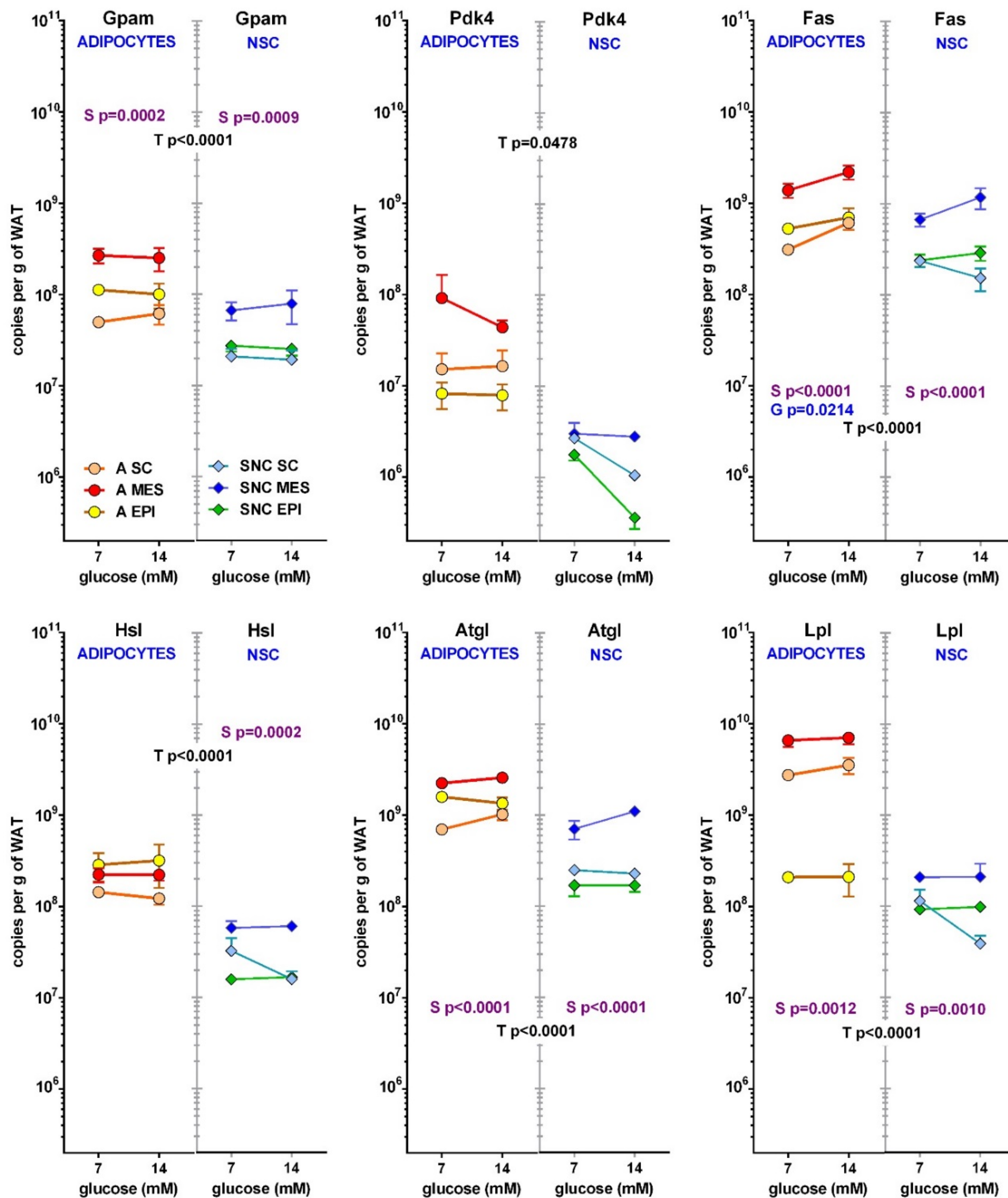

Figure 4 Number of copies of glycerol and lipid metabolism-related genes in adipocytes and NSC contained in 1 g of WAT from different sites (SC, MES and EPI) or male adult rats

The data correspond to the mean ± sem of 4 different 2-pooled rat samples. All conventions are the same described for Figure 3.

DISCUSSION

In WAT, the global importance of NSC has been widely recognized, especially its implication in questions of regulation [57], defense [58], regeneration [59] and even metabolic control [60] via hormones and cytokines. However, and despite its also accepted variety and diversity depending on WAT site, to our knowledge, no actual analysis of its overall metabolic contribution to the handling of substrates has been so far analyzed nor established. The results presented here are only the tip of an iceberg that needs to be acknowledged, measured, known and recognized in its true magnitude.

We are aware that the methodology we used should be refined further to gain sufficient insight on the quantitative role of NSC in substrate handling, but the basic concepts have been already established [50], and the isolation,

incubation, viability and analyses needed are available, often being standard and commonly used. The data presented here continues what has been developed in a on a series of preceding methodological and quantitative studies that drove us to the findings shown here [38, 43, 44, 50, 61, 62].

The data presented, support the idea that WAT is even more complex than we usually believe. Adipocytes represent less than 10 % of nucleated cells, despite each containing about 10-fold the 'live cell' volume of NSC, overall, a few microliters per gram of WAT [38]. It has been assumed that WAT reason to exist is essentially defined by adipocytes and their TAG stores [63], metabolic activity being centered on lipogenesis from glucose and lipolysis of TAG. This may be largely true for the largest and more deposit-prone sites, such as EPI WAT, but not as much for the more metabolically active MES WAT [64] or the smaller control-related deposits such as perivascular [65] or pericardic [66] depots. However, and with respect to glycolysis to lactate our data show that NSC glycolytic activity was higher than that of adipocytes in all three quite different sites studied. Nevertheless, the difference was magnified even more for MES WAT.

This high lactate production was unrelated to oxygen availability (abundant in the incubation medium), in a parallel way to that shown by adipocytes [67]. This relative independence of NSC with respect to oxygen agrees with its limited WAT consumption *in vivo* [45],

The question is to know whether the observed glycolytic function is intrinsic or adopted. It may be postulated that the presence of large adipocytes, with most of their thin layer of cytoplasm too far away from mitochondria that become, necessarily, glycolytic [68], despite a sufficient availability of oxygen, because what is needed for glucose oxidation and lipogenesis are functional mitochondria [50], not always close to where the energy substrates are taken up. In any case, the proximity of adipocytes could not be a determinant factor, since in our system, the NSC were incubated in the absence of adipocytes. There were no functional adipocytes in the NSC preparations, since the patterns of gene expression are quite different, and more related to site than to cell type, and, over all, no glycerol, neither NEFA were freed to the medium. There was *Lpl* and there was a large amount of cell fragments, essentially fat micro-droplets measured by the particle counter (Table 1), and yet no glycerol nor NEFA were present. The possibility of uptake and oxidation could not be ruled out, but if it were active, then, why use not fatty-acid derived acetyl-CoA for energy, and resort instead to the inefficient production of lactate? Even more, if the fatty acids were accumulated, then the cells density would be lower and would not sediment by centrifugation as easily.

There is a long list of WAT NSC types, and their dependence on oxygen is patent when studied from other sources [69]. Thus, it is difficult to attribute this WAT-inbred trend simply to an adaptation to anaerobiosis, to the ability to survive with the few ATPs that glycolysis to lactate can provide.

In studies of WAT non-adipocyte cells, lactate production has been often associated to hypoxia [70]. The results found complicate the widely acknowledged relationship of hypoxia in WAT with the negative consequences of inflammation [71]. How can hypoxia become a key factor eliciting a pathologic inflammatory response in a tissue which 'main' cells, adipocytes, live off the ATP generated by glycolysis [43], consuming oxygen (and depending on mitochondrial abundance) for lipogenesis [72] ? Now, the other WAT cells, stromal structural, immune system cells, stem cells, fibroblasts and others, may thrive also using the glycolytic pathway for energy in spite of having sufficient oxygen available to use more efficient mitochondrial oxidative metabolism. These data clearly hint at a WAT adaptation to low oxygen and/or high glucose hacking that runs in parallel for adipocytes and NSC; this trend is maintained even when oxygen is widely available (but glucose is available too).

The regulation of WAT blood supply has been postulated as a mechanism of defense against excess substrate availability [26], but it reduces the supply of oxygen and promote hypoxia, even counting with Bohr Effect which facilitates the release of oxygen from RBC when lactate is high [73]. Probably, the adaptation to low oxygen renders the blood oxygen supply as secondary for WAT cells function (few cells, low intrinsic needs) leaving blood flow control as a critical element for relation of substrate supply [74]. Our own data presented here also hint to MES WAT having a higher blood irrigation than the other sites, but lactate production is highest there.

The gearing of adipocytes and NSC for glycolysis is fully apparent in Figure 3, with a marked parallelism on the quantitative expression of these two halves of active WAT: adipocytes and NSC. However, a number of differences (lipid metabolism, lactate dehydrogenase ratio) show their different origin and function, but the fact that both participate of WAT glycolytic activity raises the question of the metabolic importance of these cells with respect to whole-body energy handling.

The capability to thrive with low oxygen may be of paramount importance for a tissue implicated in tasks of regeneration and defense, being able to grow in hypoxic niches where angiogenesis is a critical tool for repair and normalization [75]. The parallelism of this facet of WAT and tumors ends here, since the density of substrate and oxygen consumption per unit of weight are quite different, as are the rates of glucose consumption (i.e. the Warburg Effect [76]) and the unique property of WAT to produce large amounts of glycerol alone, without the concourse of NEFA [50]. Here we have found that this is a peculiarity of adipocytes, since NSC did not release either glycerol or NEFA. We were unsure that the adipocyte preparation was free of attached or bound NSC, but we are fairly certain that the NSC preparations were not contaminated with enough adipocytes to produce detectable levels of glycerol. Thus, glycerol efflux remains a truly specific function of adipocytes. Glycerol is a 3C substrate, easily used for gluconeogenesis [77], but which may be incorporated easily into any cell

metabolism via glycerokinase, forming glycerol-3P which may be esterified with acyl-CoA to form glycerides, such as TAG [78] or incorporated into the glycolytic pathway for energy via glycerol-P dehydrogenase [79]. Neurons use both lactate [80] (in part provided by the glia from glucose [81]) and glycerol [82] for energy [80, 82] and development [83]. Glycerol levels and uptake are not as strictly regulated as is insulin, thus WAT breaking up of glucose to 3C units has the advantage to eliminate excess glucose (and thus low glycemia) [84] without creating a problem of energy supply to the brain, which can be sustained by both lactate and glycerol [85, 86]. We have not yet sufficient proof to establish a direct metabolic connection between WAT and brain, helping to assuage the ravages of metabolic syndrome, but most of what we know now suggest that the protective role of WAT extends beyond the immune system into the maintenance of specific energy supply to the brain.

In sum, we found that NSC can match the glycolytic activity of adipocytes, being responsible for a significant portion (which may be a major part) of glycolytic conversion of glucose to lactate. However, NSC do not seem to participate in glycerol metabolism (there is no glycerol nor NEFA efflux ftom NSC). We also conclude 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 [86] and lactate [87], as substrate. There are marked differences between WAT sites, with mesenteric WAT taking a key role, probably related to glucose disposal and glycerogenesis.

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5. GENERAL DISCUSSION

5.1. Development and evaluation of the methodology

With the methodology developed in the first part of my thesis project, we obtained a method that permits the extraction and the separation of viable and functional WAT cells. This method allows also the estimation of the actual recovery of these cells and the quantitative analysis of tissue composition. The methodology was checked in epididymal WAT of 18 weeks old rat, but this approach could be used for any WAT localization and allows to obtain data which could be referred to live tissue. They could be also applied to compare different WAT location and individual situation, such as physiological and pathological one.

Intact adipocytes were separated with a mean recovery of 76%, basing the calculation on lipid content. These cells float on the top of digested suspension, while the stromal cells were distributed uniformly in all the suspension and between adipocytes. Three washes, needed to enrich adipocytes fraction, resulted in a loss of an additional 3% of these cells. The first 24 hours of incubation results in the loss of about 4% of incubated adipocytes, while in the second period the combined total loss was 13% of cells.

The functionality of adipocytes recovered with this method was confirmed by glucose uptake and metabolites (lactate, glycerol and NEFA) efflux, during 24 and 48 hours incubation. While lactate efflux was constant, glucose uptake and the efflux of glycerol and NEFA increased during the second day, demonstrating the cells functionality not only at 24, but also at 48 hours of incubation, in agreement with the bibliography [367–369].

Adipose tissue, due to its high fat content, is a tissue difficult to work with. Adipocytes, which most volume is filled by lipids, are often considered the representative cells of the tissue, despite the stromal cells compound for immune, stem, blood, endothelial, stromal cells and other types of cells, and are implicated in a lot of process, such defense [370], regeneration [371], differentiation [372,373] and others functions [374,375]. For this reason the study of the actual composition of the tissue, as well as the proportion of “live cell”, gave us critical information about the real predominant component of this tissue.

Adipocytes were about three orders of magnitude larger than stromal cells, and fill 90% of WAT space. If we discount their inert lipid vacuoles, adipocytes still represented the principal source of cell cytosol of WAT. In fact, adipocytes “live cell” volume represented 1.3% of total tissue volume, against 0.2% of the nucleated stromal cells, one order of magnitude higher when comparing cell to cell. Nevertheless, when we consider cell numbers, nucleated stromal cells predominated over adipocytes (27% of total cells against 7%), while the most abundant cells in tissue were red blood cells (66%), despite tissue samples were obtained

after rats' exsanguination. Their volume, 2 orders of magnitude lower than adipocytes "live volume", corresponds to just 1 µl of blood in one gram of tissue.

When considering the whole (combined) "live cell" volume of the tissue, it represented just 1.5% of the total. This is about 50-fold lower than liver cell volume, pointing to a very metabolic active role of a tissue (the adipose) always considered just a dump for excess energy. This agree with the remarkable metabolic activity of the whole adipose organ [269,271], its considerable glycolitic capability [271,366] and its important contribution to amino acid metabolism [251,376,377], in addition to the already well known ability to store fatty acids taken from plasma lipoproteins [378].

Despite the fact that our data came a) from different animals, and b) were obtained from measurements done on small amounts of cell primary cultures, they were consistent with results published in the literature. In fact the interstitial space found is close to that described in previous reports, and is in the range of other tissues [379]; number and volume of adipocytes also were in agreement with those already described [380,381]. In addition, in our calculations, the sum of cells' volumes and interstitial space computed for almost the totality of tissue volume, which is an indirect control of the results obtained.

On the other hand, we developed a miniaturized, radioactive method for the analysis of the fate of glucose, which could be applied to systems just like ours: cultured primary adipocytes. In fact the procedure, complex but doable, was adapted to small volume and allows, starting from the same incubation medium, the separation and quantification of radioactive lactate, glycerol and glucose. Moreover, the method allowed to use incubated cells to obtain and quantify cytoplasmic radioactivity (radioactivity in glycogen and protein) and fat radioactivity, discriminating fatty acids and glycerides-glycerol radioactivity.

5.2. Application of methodology: glucose fate and 3C release

In primary culture, epidydimal mature adipocytes from rat 14 weeks old, incubated (for 24 and 48 hours) with glucose at physiological and hyperglycemic conditions (7 and 14 mM), took up large amount of glucose to release 3C metabolites, such as lactate and glycerol.

Glucose uptake, irrespective of its concentration, was high, dependent on incubation time, and independent from insulin, since it is allowed by the high expression of transporter *Glut1* (glucose transporter type 1) and phosphorylation by *Hk* (hexokinase).

Lactate carbon had its origin in glucose, as first postulated [283], and now demonstrated by the use of ^{14}C-labelled glucose. The rates of lactate production were rather constant: the amount of the metabolite increased with incubation time, and its efflux remained unchanged over

time. This results in the anaerobic production of ATP, probably in quantity sufficient to cover for the cell needs.

On the contrary, glycerol production changed over time, as its efflux was higher during the second day of incubation. The pathway through which glycerol was released also changed with time. Initially (first 24 hours) glycerol was synthesized directly from glucose, which, via glycolysis, forms dihydroxyacetone phosphate and then glycerol-3P. The enzyme phospho-glycerol phosphatase (*Pgp*), previously postulated by our group [366] and later described by Mugabo et al. [382] was highly expressed in our experimental system, and can break glycerol-3P to free glycerol, rapidly removed by transporter aquaporin 7 [383] (also highly expressed). This enzyme, by controlling the availability of glycerol-3P, it is a key element to regulate the synthesis of TAG [382]. However after the first day of incubation, probably because of the continuous exposure to glucose, the origin of glycerol shifted from glycolytic to lipolytic, proven by a marked reduction in the specific radioactivity of released glycerol (despite being released in higher amounts).

The levels of radioactivity found in adipocyte TAG stores (both in the glycerol and fatty acid moieties), the stoichiometry of the reaction, and gene expression, suggest that newly synthesized glycerol-3P (from glucose) was incorporated into TAG with the concourse of acyl–glycerols. Activation of lipases resulted in TAG lipolysis; glycerol was excreted, but fatty acids were recycled again to form TAG with fresh glycerol-3P. The huge mass of unlabeled TAG provided non-radioactive glycerol which lowered the specific activity of glycerol efflux, whilst increased that being stored in TAG.

The conversion of dihydroxyacetone-P to glycerol-3P needs the reducing power of NADH. The use of glycolytic NADH to this purpose and its consequent absence for the conversion of pyruvate to lactate, may result in the transient accumulation of pyruvate, which usual fate is to be oxidized to acetyl-CoA. This mechanism would provide both glycerol, in its activated form, and acetyl-CoA, used for lipogenesis, and thus acyl-CoA ready to form TAG. But, to form a 18C fatty acid 9 acetyl-CoA are needed; and a molecule of TAG needs one single glycerol-3P for 3 fatty acids (i.e. 27 acetyl-CoA). Definitively, lipogenesis is not coupled to the lipolytic release of glycerol. These data confirm the prevalence of using exogenous fatty acids, with new synthesized glycerol, on lipogenesis [384] and the few synthesis de novo in adipocytes [258]. Lipogenesis was low in the system studied, since at most only about 10 % of glucose C ended in the form of fatty acids. It is not the lack of oxygen, but probably the huge cell-scale distances between most of the cell cytoplasm around the fat drop and the mitochondria (few and disperse) able to form acetyl-CoA. This could explain the metabolic differences between small and mature adipocytes [273].

In addition, after 24 hours incubation this lipogenic mechanism ceased, a fact proven by a lack of changes not only in fatty acids specific activity, but also in lipogenic marker genes *Acly*, *Acaca*, *Fas*. On the contrary, *Pdk4 (*pyruvate dehydrogenase kinase), *Gpam* (glycerol-3-phosphate acyltransferase) and lipases levels rose. The increase in *Pdk4* expression indicated a probably complete inhibition of pyruvate dehydrogenase, thus cutting off the production of acetyl-CoA and with it both lipogenesis and total glucose oxidation. *Pdk4* rise with incubation time in parallel with *Pck1* (P-enol-pyruvate carboxykinase), suggesting that the inviable oxidation of mitochondrial pyruvate elicits its carboxylation to oxaloacetate [385] and return to the cytoplasm, where oxaloacetate is used to form P-enol-pyruvate, which reincorporates to the glycolytic pathway. The exit from mitochondria may take place, probably via the malate shuttle and in the form of malate [386]. Cytoplasmic oxaloacetate does not seem to be used via malic enzyme (no need for additional NADPH), but converted to P-enol-pyruvate and then yielding again pyruvate; the NADH provided by malate conversion to oxaloacetate may complete the operation by producing lactate. The key, thus, lies on cytoplasmic NADH, not on NADPH needed by lipogenesis (inviable because of the lack of sufficient acetyl-CoA). The expressions of enzymes indicative of NADPH synthesis remain unchanged: *G6pdx* (glucose-6-phosphate dehydrogenase) for the pentose-P cycle or *Me1* (malic enzyme). These data indicate that even when glucose is abundant, at least when lipolytic glycerol is being produced, lipogenesis is stopped.

The operation of these mechanisms at the same time can be taken as a "futile cycle", with the function (probably secondary to its original meaning) to waste excess of energy. This may be a mechanism comparable to those controlled by the ponderostat, but unlike these futile cycles, the energy-wasting system described seems to be an intrinsic mechanism of the adipose tissue itself.

Thus WAT, with the release of 3C metabolite [387], may help lower glycemia and defend itself from excessive storage of fat, helping to reduce the negative effects of high plasma glucose, as in inflammation, at the base of metabolic syndrome. Moreover these metabolites (i.e. lactate and glycerol), in blood flow could reach most tissues, bypassing insulin control, and could be used by liver [263,264], muscle [388], heart [262,278] and especially by brain [279,284], where they could maintain a vital line of energy supply irrespective of glucose and insulin.

Lactate production by WAT has long been known, but under the belief that its high production is the consequence of metabolic abnormalities and insulin resistance, interfering with peripheral glucose utilization [275]. In our system (lacking insulin, and in part comparable to a situation of IR) we postulate a quite different situation, in which lactate production contributes to lower glycemia, being also used as alternative substrate by other tissues. It

could be even used for hepatic lipogenesis [280], "returning" again to adipose tissue in the form of LDL-TAG, completing a Randle cycle [250].

Glycerol, whose production from WAT is also already known [387,389,390], has the additional advantage, as 3C substrate, with respect to lactate or alanine, to have no charge, nor N. Moreover, glycerol has been proposed as ponderostat signal, due to its capacity to decrease body weight [391], but this idea has not been further developed.

The different patterns show by lactate and glycerol efflux highlight their different functions: the uniformly and efficient lactate production appears as a way to produce energy (and is not a consequence of hypoxia), while glycerol production seems a definite objective of cell metabolism in itself. In this scenario, the limited lipogenesis may appear more as a secondary pathway.

These mechanisms were spontaneously activated by adipocytes in the absence of external stimuli, solely depending on the bulk flow of substrate through glycolytic pathway, as is glucose presence (independently of its concentration). For this reason, it appears as an automatic mechanism used by adipocytes to maintain some sort of control against excess of substrate.

The size of white adipocytes could play a key role in their metabolism: the presence of the thin cytoplasm layer, covering the central lipid drop, difficult the communications between cytoplasm and mitochondria to carry out aerobic metabolism.

All adipocytes (from both sexes and different sites) show the same pattern: high uptake of glucose, unaffected by its concentration, steady efflux of lactate and increasing liberation of glycerol, related to incubation time.

The female periovaric WAT adipocytes act in the same way, in spite of adult males presenting a higher tendency to accumulate fat [392], but female gene expression patterns showed, in general, a higher number of copies of mRNA per unit of tissue weight that than in males. This is in agreement with a higher metabolic activity in female adipocytes, despite (or perhaps because of) its lower size.

The higher expression of female *Gk* (glycerol kinase), point to a possible recycling of glycerol to glycerol-3P, reducing glycerol release, thus equilibrating its synthesis and hydrolysis, and leaving more substrate for acyl-CoA esterification. However, the high effectivity of aquaporin 7 removing glycerol from WAT makes this possibility doubtful [393].

Mesenteric WAT adipocytes presented a higher activity, with higher efflux of glycerol and NEFA. Considering the relationship between this adipose tissue and liver, this could be a manner to help with the hepatic handling of intestine-released fatty acids. In addition this

WAT may help to reduce the important amount of glucose coming from digestive tract. In this site, adipocytes from males seemed more active than those of females, but the latter differed in the way excess pyruvate was used. Compared with other tissues, here the pyruvate dehydrogenase complex was not as severely inhibited by the kinase, probably allowing higher rates of lipogenesis. These changes could help understand at least part of the metabolic sex differences in visceral WAT handling of lipids and glucose [394].

When WAT was quantitatively analyzed with the methodology described above, the number of stromal vascular cells in epidydimal WAT was about half than in other two WAT sites and its fat content was significatively higher. Epididymal WAT adipocyte size doubled that of mesenteric, and the size of subcutaneous adipocytes was intermediate between both. Adipocytes represented less than 10% of nucleated cells, but contained 10-fold their "live cell" volume.

This knowledge allowed the direct comparison between adipocytes and stromal vascular cells (nucleated and not) in all the tissues analyzed and between tissues. According to our results, glucose uptake was higher in mesenteric WAT and lower in epididymal WAT, showing a similar pattern in lactate efflux.

Stromal cells produced large amounts of lactate, higher than that produced by adipocytes, but on the other hand they did not produce glycerol, nor NEFA. Red blood cells did not contribute in a significant way to the overall WAT production of lactate, since their efflux of lactate was 10 to 50-fold lower. Once again, mesenteric WAT was the most active in the production of lactate. These data agree with the finding of Newby et al. [277], which confirm the higher lactate production by mesenteric adipocytes with respect to other WAT locations. Nevertheless, our data show that stromal cells also parallel this behavior.

We found that mesenteric adipose tissue has, probably, a higher blood irrigation than the other sites; thus, once again, the idea that lactate is a consequence of hypoxia seems forced. Moreover, in the case of stromal cells, their geometry does not impede aerobic metabolism, thus it seems a "choice" of these cells to prefer anaerobic metabolism, making even more difficult to justify WAT inflammation as consequence of hypoxia.

The idea that lactate is formed under fully aerobic conditions as a mechanism to share the carbon source has been already proposed long ago, under the name of "lactate shuttle" [395], and has been applied to the energy relationships between astrocytes and neurons [396], as well as to stromal cells, such as fibroblasts and epithelial cancer cells [397]. In our experimental set up, stromal cells did not produce lactate to share carbon for adipocytes, but generate the 3C units in parallel to them.

Finally, we demonstrated here that stromal cells act in synergy with adipocytes: both cell type, in the different sites examined, and in both sexes, acted promoting the waste of glucose and producing high amounts of 3C units, in the presence of oxygen. This outcome could be in agreement with the postulated regulation of WAT blood supply as a defense mechanism against excess substrate availability [218], since the resulting hypoxia would not be a problem for the adipose cells. It also reinforces the idea that WAT could be an active protagonist in energy handling and in the body control of glycemia, especially considering the large size of the adipose organ [398].

6. CONCLUSIONS

1. We developed a method for the isolation of viable and functional WAT cells, which allows the estimation of their actual rate of recovery and size, and the quantitative analysis of tissue composition.

2. Red blood cells were the most abundant cell type in mature adipose tissue (about 2/3rds of total), followed by nucleated stromal cells (short of 1/3rd) and adipocytes (less than 10 %). Most of WAT volume was occupied by fat (70-90 %), which conditions cell to cell relationship and even the functionality of cell metabolism.

3. White adipose tissue "live cell" volume represented just about 1.5% of total tissue. When compared with liver, and despite the "low" global metabolic activity of WAT, the adipose organ showed a comparatively higher metabolic activity (when fat was discounted). Per g of WAT, adipocytes' "live cell" volume was, nevertheless, about 1 order of magnitude higher than that of nucleated stromal cells and 2 orders higher than that of erythrocytes.

4. Adipocytes incorporated medium glucose (in our experimental setup) independently of its concentration, and converted most of it to 3C metabolites (largely lactate and glycerol). Lactate was produced steadily through anaerobic glycolysis (in the presence of oxygen), to obtain the ATP needed to sustain the cell metabolism.

5. Adipocyte's glycerol efflux was also of glycolytic origin; not being just a byproduct as lactate is, but a specific export product of adipocytes, synthesized from glycerol-3P via phosphatase and, mainly through selective TAG turnover (i.e. recycling the fatty acids).

6. In mature white adipocytes both lipolysis and lipogenesis coexist, the latter highly limited by the cell geometry and size (proximity to mitochondria). Lipolysis was highly active, together with glycerogenesis to sustain TAG turnover in what may be considered a "futile cycle" helping to waste part of the excess of energy.

7. WAT sites adipocytes metabolism was found rather uniform from a qualitative point of view, since their differences were mainly quantitative. Thus, mesenteric WAT produced more glycerol (and NEFA) than the other WAT sites analyzed, probably to contribute to the splanchnic handling of dietary fats and, essentially, to reduce the huge load of glucose released from the intestine and directed to the liver.

8. From the analysis of substrate levels and enzyme gene expressions, we postulate that periovaric adipocytes are more active than the epididymal ones. In addition, the adipocytes of female rats mesenteric WAT, tend to use more easily pyruvate through lipogenesis

compared to the cells of the other sites, where most of the unused pyruvate was recycled again, via oxaloacetate and P-enol-pyruvate to be finally exported as lactate.

9. The stromal vascular cells of WAT showed, in consonance with adipocytes, a full anaerobic profile, producing (per g of tissue weight) even more lactate than the adipocytes. However, stromal cells did not produce nor export glycerol as the adipocytes do. Red blood cells contribution to overall production of lactate was almost negligible.

10. Both adipocytes and stromal cells, in the sites examined (and in both sexes), wasted large amounts of glucose. In epididymal WAT, $2/3^{rds}$ of this glucose, approximately, were converted / released in the form of 3C units (glycerol, lactate, alanine and pyruvate). Lipogenesis accounted, at most, for about $1/10^{th}$ of the glucose used. These data support the hypothesis of WAT being an active protagonist of energy partition and overall control of glycemia; providing, in addition, less-regulated 3C substrates for easier utilization by other tissues and organs.

11. In sum, the data presented hint at mature WAT as an organ which protects us from hyperglycemia, with little incidence in body lipogenesis and far from being (at least directly) a sink for unwanted glucose energy.

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