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

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Disclosure of interests

The Authors report no competing interests

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Abstract

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

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

Introduction

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

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

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

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

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

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

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

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

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

Results

Metabolite efflux

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

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

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

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

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

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

Label distribution in the cells and medium

We used the incubation wells in a way similar to a closed system, in which the glucose label added was distributed in the fractions later analyzed and compared. 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 label not accounted for. Since the label remaining in glucose was much higher than the small fraction going into 'other' cell fractions (protein, metabolites and, especially glycogen), we presented the data on a log scale to include all fractions in a single graph.

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

When the data in 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 used is shown. Since the label in 7 mM and 14 mM groups was the same (but there was twice as much glucose in the 14 mM group); to facilitate comparison, the scale for 14 mM has been halved with respect to that of 7 mM. Each group contains two columns, for 24 and 48 h. The considerable similitude in height and distribution of both 7 mM and 14 mM glucose groups

attests to the practically nil effect of doubling glucose levels in the medium. Its consumption and utilization showed little effect on its utilization and fate. The only fractions with significant differences between 24 h and 48 h were lactate, glycerides-glycerol and 'unaccounted for' label for both glucose groups. At 48 h, the 7 mM glucose group used slightly more than 46 % of all glucose available in the well, whereas that of 14 mM consumed about 24 % of the glucose available, that is 5.8 to 6.4 μmol glucose, respectively. If the 'unaccounted for' label were discounted, the final amount of glucose consumed would remain practically the same in both groups.

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

Figure 4 shows the specific carbon radioactivities of the five fractions in which direct measurement of cold and labelled compounds were done. The data are shown as percentages of the initial glucose carbon specific activity. In spite of the considerable variability of individual data, the simple differences in scale of the specific radioactivity of the fractions, all derived from glucose (the only initial source of label) shows that, as expected, glucose specific activity was maintained. That of lactate, was also maintained (no statistically significant effects of glucose or time were observed) on the same range than glucose.

The specific radioactivities of free glycerol in the medium decreased with time. At 48 h, they were only a fraction of the initial glucose values. The data for glycerides-glycerol showed an opposite pattern, from practically zero at 24 h the values increased steadily to about 2 % of the initial glucose specific radioactivity at 48 h, also showing a significant effect of incubation time. The effect of glucose concentration in both glycerol groups was not statistically significant (but was in the limit of significance). The glycerides-glycerol values,

however, were much lower at 48 h, at least by one order of magnitude, than those of medium free glycerol. The specific radioactivity of the glycerides-fatty acids was very low, close to four orders of magnitude lower than initial glucose, and did not change either with time or glucose concentration. Despite forming part of the same TAG molecules than glycerides-glycerol, their C specific radioactivity was more than two orders of magnitude lower.

Protein gene expression

The high adipocyte production of lactate may be related to the high number of copies of the gene for main isozyme of lactate dehydrogenase (*Ldha*), which repeated the same pattern of the 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, nevertheless, relatively high, in the same range of *Hk* and *Pfkl*. The monocarboxylate transporter gene (*Mct1*), responsible of lactate (and pyruvate) efflux showed the same pattern of change already described for glycolytic enzymes and lactate dehydrogenase.

Figure 5 presents the changes in gene expression of key enzymes and transporters implicated in the glycolytic and lipogenic utilization of glucose by adipocytes. The glucose transporter gene *Glut1*, showed similar number of copies 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 (isozyme genes *Pfjk* and *Pfkm*) showed the same pattern (i.e. no effects of glucose concentration and increased expression with longer incubations), but the *Pfkl* isozyme showed a more powerful increase response and higher levels of gene expression than *Pfkm*. The glyceraldehyde-P dehydrogenase gene

(*Phgdh*), despite catalyzing a fully reversible path showed a marked difference in expression induced by glucose availability, with a higher increase at the lower glucose levels.

The enzyme P-enol-pyruvate carboxy-kinase gene (*Pck1*) was, at 24 h of incubation, poorly expressed in adipocytes, but in the next 24 h its expression increased steeply. Again, no effects of glucose were observed. This dramatic increase in expression was paralleled by *Pdk4*, the gene controlling pyruvate dehydrogenase kinase 4, main inhibitor of pyruvate dehydrogenase activity. The effect of glucose was in the limit of significance. This strong activation during the second day of incubation can be interpreted as a blockage of the oxidation of pyruvate to acetyl-CoA, thus preventing its incorporation to lipogenesis or the Krebs cycle.

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

Discussion

Adipocytes (or WAT) take up excess glucose, when confronted with high levels, converting a large proportion of it into 3C metabolites, such as lactate [32], pyruvate, alanine [33] and glycerol [12, 13], which may be used as energy substrate elsewhere, or, largely, by the liver in the gluconeogenic [14] and/or lipogenic pathways [34]. But with this action,

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

The results presented here agree with this interpretation, showing, in quantitative terms, that most of the glucose taken up by adipocytes is just returned to the medium as 3C metabolites, essentially lactate and glycerol, thus lowering the circulating glucose availability. However, this process was practically not influenced by glucose in a range going from normal plasma levels, 7 mM to twice this figure (akin to postprandial state or sustained hyperglycemia). It must be noted that the hacking of glucose to 3C metabolites proceeded during a 2-day incubation of the cells, in which no external hormones or signals (including those of other WAT cells not firmly attached to adipocytes) were able to affect the processes described and quantified. Thus, we can conclude that isolated adipocytes' conversion of glucose to 3C metabolites (and, to a minor extent, fatty acids) was not elicited by external signals and neither by glucose concentration itself. Consequently, we can assume that this active conversion of 6C glucose to 3C metabolites may be a pre-established innate process, which potentiates glucose break up by defect. One of the most relevant consequences of this response, which our label tracing data proved, is the limited importance of lipogenesis in the disposal of glucose by mature adipocytes.

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

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

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

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

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

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

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

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

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

The quantitative estimation of glucose conversion into 3C fragments or fatty acids (x2C), established that adipocytes (and by extension WAT) actively participate in the control of glycemia [12], lowering glucose levels and contributing to limit its pro-inflammatory

effect via insulin resistance. This glucose is largely recycled to 3C metabolites, i.e. usable as energy substrate by almost any tissue, as indicated above. The 3C substrates are not subjected to the same strict controls as glucose (insulin), and can be easily reconverted again (if needed) to glucose via hepatic gluconeogenesis.

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

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

Methods

Rats, housing, handling and sampling

All animal handling procedures and the experimental setup were in accordance with the animal treatment guidelines set forth by the corresponding European, Spanish and Catalan

Authorities. The Committee on Animal Experimentation of the University of Barcelona specifically authorized the procedures used in the present study (procedure DAAM6911).

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

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

Isolation, measurement and incubation of adipocytes

Adipocytes were isolated by incubation with collagenase as described in a previous paper [25], essentially following the Rodbell procedure [48]. Cells were counted, and their (spherical) diameters measured using the ImageJ software (<http://imagej.nih.gov/ij/>) [49]. The yield with respect to WAT sample mass was estimated in a number of randomly selected samples as previously described [25]. The adipocytes recovered were in the range of 73-75 % of those present in the tissue. Cell incubations were carried out using 12-well plates (#734-2324VWR International BVBA/Sprl., Leuven Belgium) filled with 1.7 ml of DMEM (#11966-DMEM-no glucose; Gibco, 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 (Sigma-Aldrich). Adenosine (Sigma-Aldrich) 100 nM was also added to help maintain the integrity of the cells.

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

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

Cell harvesting and processing of labelled cell components.

The incubation of adipocytes was stopped by harvesting the cells after the medium was extracted, mixed, aliquoted and frozen. The procedure for measuring label distribution in the different fractions of cells and media have been previously developed, tested and quantified [26]. Briefly, the cells of wells incubated with labelled glucose were weighed, frozen with liquid nitrogen, transferred to glass tubes and immediately extracted with chilled peroxide-free diethyl ether, since it is non-reactive, and is highly effective for TAG [50]. The

aqueous fraction contained small remnants of medium, most cell metabolites and glycogen. The interphase contained most of the cell proteins. This aqueous (and interface) fraction was wholly used to estimate the radioactivity. The organic phase, containing essentially TAG, was dried, weighed, re-dissolved in ethyl ether and saponified with KOH in ethanol in the cold [51]. The ether-insoluble potassium soaps were extracted and counted. The aqueous phase contained all glycerides-glycerol; it was also removed and counted [26]. Soap label was that of TAG fatty acids. Total cell label was estimated from the harvested cells suspension. TAG label was taken as the sum of fatty acids (soaps) and glycerides-glycerol. The cells of the 'parallel' wells were used to extract their RNA for analysis of gene expression.

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

Processing of the incubation media: metabolites.

We used both labelled and parallel well media to estimate the levels of glucose, lactate, glycerol and non-esterified fatty acids (NEFA). Glucose was measured using a glucose oxidase-peroxidase kit (#11504, Biosystems, Barcelona Spain) to which we added 740 nkat/mL mutarotase (porcine kidney, 136A5000, Calzyme, St Louis, MO USA) [52]. Lactate was measured with kit 1001330 (Spinreact, Sant Esteve d'en Bas, Spain); glycerol was estimated with kit #F6428 (Sigma-Aldrich); NEFA were measured using kit NEFA-HR (Wako Life Sciences, Mountain View, CA USA).

Pyruvate and alanine were measured sequentially [53] in 1.5 mL of tris-HCl buffer 92 mM pH 7.2, containing 100 nM mM NADH (Calbiochem San Diego CA USA) and 1 mM 2-ketoglutarate (Sigma-Aldrich), to which 25 μ L of incubation medium (adequately diluted

with Krebs-Ringer bicarbonate buffer) were added. In standards, samples were substituted by different concentrations of alanine and pyruvate. The decrease in 5 min of the absorbance at 340 nm was measured after the addition of 20 μ L (6 μ kat) of lactate dehydrogenase (rabbit muscle #427217 Calbiochem). Pyruvate was estimated from the fall in absorbance (i.e. consumption of NADH) [26]. When the lecture was stabilized, alanine was measured [53] with the addition to the cuvettes of 20 μ L (170 nkat) of alanine transaminase (porcine heart #G8255 Sigma-Aldrich), and comparing the rates of absorbance decrease vs. time in the samples against alanine standards.

Processing of the incubation media: label distribution

The label-containing samples were used to fraction the label distribution applying a protocol previously described by us [26]. Lactate (including pyruvate) label was determined using centrifuge microcolumns made up with sieve-filter type centrifugation inserts (Ultrafree-MC, Millipore, Bedford, MA USA) containing 250 mg of hydrated, spin dried cationic-form Dowex 1x2 ion exchange resin (Serva Electrophoresis GmbH, Heidelberg, Germany) as previously described [26]. The retained lactate was eluted with acid and counted.

The medium free of lactate was used in part to convert all glucose to gluconate by incubation with glucose oxidase (type VII from *Aspergillus niger*, Sigma-Aldrich); as well as catalase (from bovine liver, Sigma-Aldrich). Catalase was added to destroy H₂O₂ and to help maintain C₂ availability. The change of nonionic glucose to gluconate allowed its retention (and acidic elution) using microcolumns as described above for lactate. The label retained was that of the unaltered glucose remaining in the medium after incubation [26, 54].

A second aliquot, of the label-containing medium free of lactate, was treated with glycerol kinase (from *Escherichia coli*, #G6278, Sigma-Aldrich) and ATP in a medium adequate for the complete conversion of glycerol to glycerol-3P. The change in ionization

was used to remove the glycerol (as glycerol-3P) from the medium, eluting it with acid and thus counting the label retained in the glycerol moiety [26, 55].

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

Carbon dioxide production along the lipogenic process was estimated by the calculation of NADPH needed to synthesize one (~C18) acyl-CoA molecule (equivalent to one fatty acid residue in TAG) and assuming that 1 mole of CO₂ was produced in the pentose-P pathway for each 2 moles of NADPH generated (explained in more detail in Ho-Palma et al.[26]). The label present in TAG fatty acids allowed us to calculate the amount of glucose oxidized to CO₂ to allow for that synthesis; since the ratio was constant, label in CO₂ was calculated from that found in the cell (soaps fraction) fatty acids.

Gene expression analyses

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

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

A semi-quantitative approach for the estimation of the concentration of specific gene mRNAs per unit of tissue weight was used [56]. *Arbp* was used as the charge control gene.

We expressed the data as the number of transcript copies per cell, in order to obtain comparable data between the groups, given the uniformity of the samples in that aspect. The genes analyzed and a list of primers used are presented in Table 2.

It was not feasible to use a meaningful 'zero time' for gene expression data because the cells 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 [25], and there were no changes in the levels of oxygen during the 2-day incubation [22].

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

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

process and units (attomol/cell·s)	7 mM glucose		14 mM glucose		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. 2 and/or described in the text. The rates have been presented in uniformed units: attomoles per second and cell (comparable to akat/cell).

Table 2 List of primers used in the present study

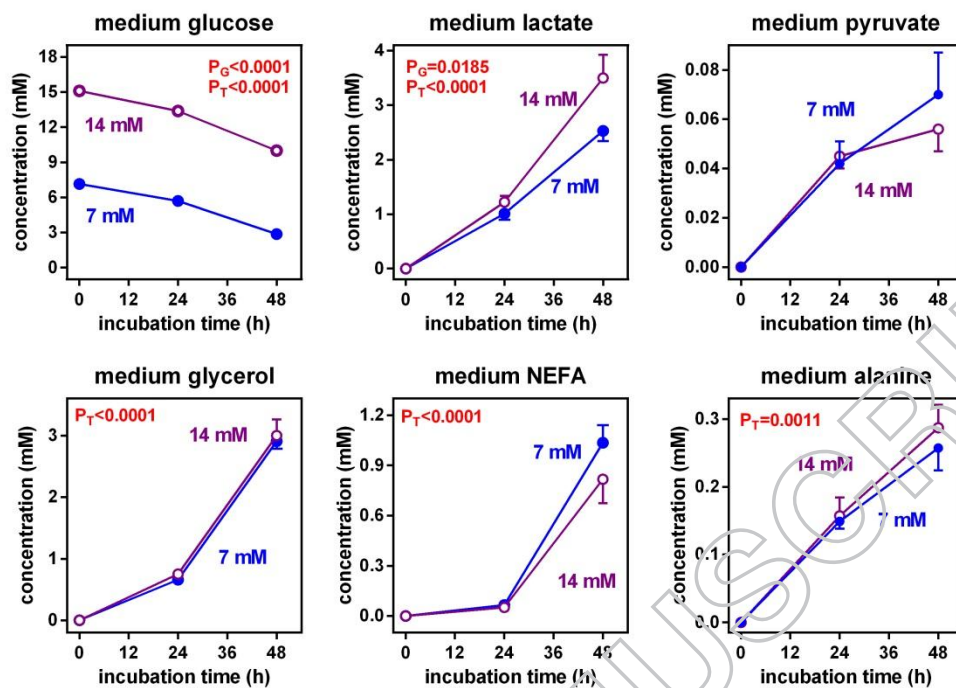
gene	protein	direction	sequences	bp
<i>Glut-1</i>	glucose transporter type 1, erythrocyte/ brain	5' >	GCTCGGGTATCGTCAACACG	97
		> 3'	ATGCCAGCCAGACCAATGAG	
<i>Hkl</i>	hexokinase type 1	5' >	TGGATGGGACGCTCTACAAA	100
		> 3'	GACAGGAGGAAGGACACGGTA	
<i>Pfkl</i>	phospho-fructokinase, liver, b-type	5' >	CAGCCACCATCAGCAACAAT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

Figure 1 Concentrations of glucose, lactate, pyruvate, glycerol, NEFA and alanine in the incubation medium of adipocytes isolated from rat epididymal WAT

The data are the mean \pm sem of 8 different 2-rat pools (4 for alanine and pyruvate). Each well contained about 4.7×10^5 adipocytes, equivalent to those present in 0.25 g of WAT. The cells were incubated 24 or 28 h. Blue dots and lines correspond to initial 7 mM glucose, and mauve represents the 14 mM data.

Statistical significance of the differences between groups (two-way ANOVA); only significant values are shown. The P values corresponding to the effect of initial glucose concentration are shown under P_G ; the effect of time of incubation by P_T and their interaction by P_i .

The inserted Table shows the metabolite concentration ratios for lactate/pyruvate, alanine/pyruvate and glycerol/NEFA, as well as its statistical analysis. These ratios were calculated from the data shown in the graphs; NS = not statistically significant.

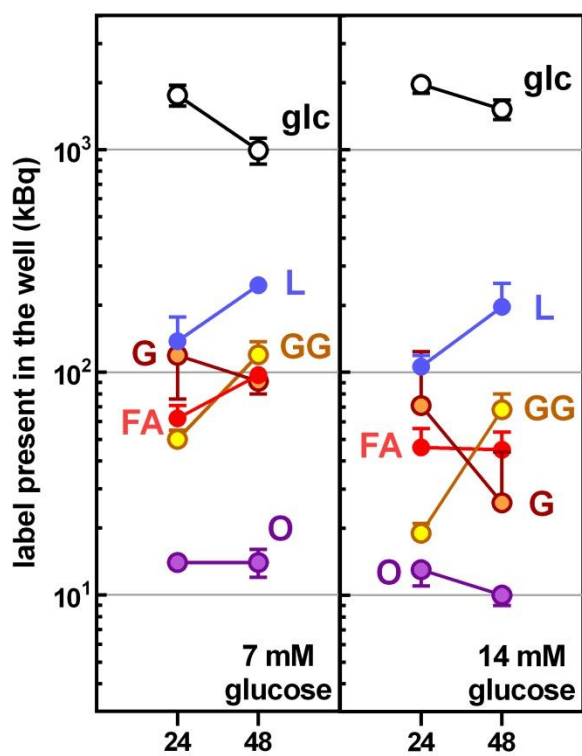


[glucose] mM	incubation time	METABOLITE RATIOS		
		lactate/ pyruvate	alanine/ pyruvate	glycerol/ NEFA
7	24	28±6	4.4±1.5	20±3
	48	49±11	4.1±0.9	3.6±0.4
14	24	31±7	3.6±0.7	30±10
	48	64±16	5.7±1.2	5.6±0.6
"glucose" P		NS	NS	NS
"time" P		0.0233	NS	0.0015

Figure 2 Well label distribution in absolute values for the analyte compartments of mature epididymal rat adipocytes incubated 24 h or 48 h in a medium with 7 mM or 14 mM glucose.

glc = glucose; L = lactate; G = free (medium) glycerol; GG = glycerides-glycerol; FA = glycerides-fatty acids; O = other cell fractions (protein, metabolites and, mainly, glycogen).

The data correspond to the mean \pm 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.



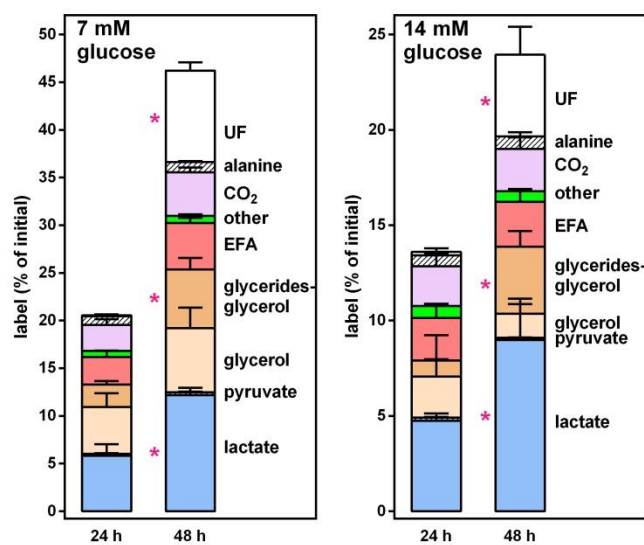
2-way ANOVA		"time" P	"glucose" P
glc	glucose	<0.0001	<0.0001
L	lactate	0.0003	NS
G	glycerol	NS	NS
GG	glycerides-glycerol	0.0010	0.0171
FA	glycerides-fatty acids	NS	NS
O	other (glycogen)	NS	NS

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

The data have been stacked up to show the total glucose label not recovered in the intact glucose fraction at the end of the study, i.e. the glucose taken up by the cells. The values presented correspond to the mean \pm 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; CO₂ represents an estimation of (only) the carbon oxidized during the process of lipogenesis. EFA = esterified fatty acids (in the cell lipid droplet).

The 2-way-ANOVA statistical analysis data of the results is presented in the embedded Table. Red asterisks represent statistically significant ($P < 0.05$) differences between the 24 h and 48 h data; NS = not statistically significant.



	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			

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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 \pm sem of 4 different 2-rat pools. The data are shown as percentages of the initial labelled glucose added to the medium. The medium components (glucose, lactate and glycerol) are drawn at the same scale; cell components scales are grossly extended. The conventions are the same as in Fig. 1.

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

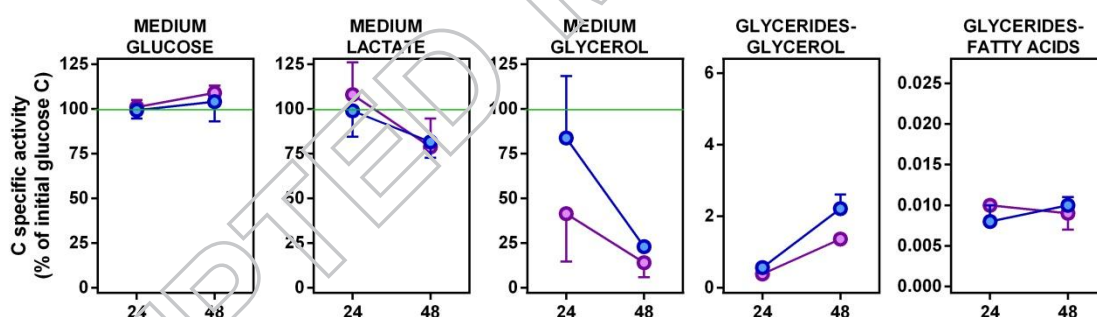


Figure 5 Level of expression, in adipocytes, of the main enzymes related to glucose uptake and glycolysis shown in Figure 1, and are expressed as the number of copies of the corresponding mRNAs per cell

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

