J Physiol Biochem (2010) 66:245–253
DOI 10.1007/s13105-010-0031-9

ORIGINAL PAPER

Enhanced utilization of glycerol for glyceride synthesis in isolated adipocytes from early pregnant rats

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Received: 4 March 2010 / Accepted: 30 June 2010 / Published online: 22 July 2010

Abstract Adipose tissue normally has low glycerol kinase activity, but its expression is enhanced under conditions of augmented insulin sensitivity and/or obesity. Since these conditions occur during early pregnancy, the comparative utilization of glucose or glycerol by isolated adipocytes from rats at 0, 7, 14, or 20 days of pregnancy was studied. Incubations were carried out in the presence of [U^{14}C]-glucose or -glycerol in medium supplemented or not with 5 mM glucose and 100 nM insulin. The conversion of glucose into esterified fatty acids and glyceride glycerol was greatest in adipocytes from 7-day pregnant rats, the effect being further enhanced by insulin. Both the amount of aquoporin 7 and the in vitro conversion of glycerol into glyceride glycerol were greatest in adipocytes of 7-day pregnant rats, the later being unaltered by insulin. In the presence of glucose, the overall glycerol utilization was lower than in its absence and glycerol conversion into glyceride glycerol was further decreased by insulin, the effect only being significant in adipocytes from 7-day pregnant rats. It is proposed that the enhanced utilization of glycerol for glyceride glycerol synthesis in adipose tissue contributes to the net accumulation of fat depots that normally takes place in early pregnancy.

Keywords Insulin · Glycerol kinase · Lipogenesis · Adipose tissue · Aquoporin 7

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Introduction

During early pregnancy, there is an increase in white adipose tissue mass, followed by a stable or even decreased fat mass during the late phase of gestation [19, 24, 31, 53]. These two stages are associated with major changes in fat cell functions. Whereas during early pregnancy, there is an enhanced lipogenesis [37] and augmented lipoprotein lipase activity [20, 28], during late pregnancy an augmented adipose tissue lipolytic activity, especially under lipolytic stimulus conditions (i.e., catecholamines), has been consistently found [4, 10, 16, 27]. The net anabolic condition present in adipose tissue during early pregnancy seems to be driven by insulin, which pancreatic concentration and secretion are enhanced [35, 45], and the tissue responsiveness to insulin is augmented, as found in rat [39]. Furthermore, clinical studies suggest that, during early pregnancy, the mother has an increased insulin response [25, 34]. The situation drastically changes during late

pregnancy, when both hyperinsulinemia and insulin resistance are consistently present [12, 43]. This insulin resistance appears to contribute to the lower antilipolytic actions of insulin in late pregnancy [39, 40], thus actively contributing to the catabolic condition found in fat cells at this stage.

Plasma free fatty acid (FFA) levels result from the balance between their release from triacylglycerols in adipocytes during lipolysis and their intracellular reutilization by re-esterification. This apparent "futile cycle" results in the preservation of fatty acids and creates an important mechanism for energy homeostasis [15, 52]. The re-esterification process requires glycerol-3-phosphate as a substrate for triacylglycerols synthesis. Although glucose is the major precursor for glycerol-3-phosphate in adipose tissue, its supply to the tissue may be limited under conditions of insulin resistance, hypoglycemia, and/or active lipolytic activity. In these conditions, glycerol-3-phosphate must be synthesized from other precursors such as lactate, pyruvate, and amino acids throughout the pathway termed glyceroneogenesis [15, 36]. Classically, it has been considered that glycerol itself cannot be used by white adipose tissue because, under physiological conditions, significant glycerol kinase activity was not found [23, 54]. However, by 1967 Robinson and Newsholme had already described the presence of glycerol kinase in rat adipose tissue [41], and we have consistently reported the capability of the tissue to utilize glycerol under basal conditions [1, 2, 7–9, 17, 18]. However, an enhanced capacity of white adipose tissue to utilize glycerol [6, 26, 33] and an augmented glycerol kinase activity [22, 29, 30, 48] have both been reported in obese rats and mice, the change being correlated to plasma insulin levels [29, 49]. Furthermore, in both rats and humans, it has been demonstrated that glycerol kinase is stimulated by insulin [38, 47]. Although these antecedents were practically ignored for over 30 years, white adipose tissue glycerol kinase has been revisited in the present decade, and it has been shown that both its expression and activity are induced by thiazolidinediones [13, 46, 50] and that its activity is enhanced in aquaporin 7-deficient mice, this being associated with their development of adult-onset obesity [21]. As commented above, as there are transitory changes in adipose tissue mass, circulating insulin levels and tissular insulin sensitivity throughout pregnancy, the possibility exists that they are also followed by changes in the capacity of the tissue to metabolize glycerol. Therefore, the present study addressed the determination of the comparative utilization of glucose and glycerol as a source for fatty acids and glyceride glycerol in white adipose tissue of pregnant rats at different stages of gestation. The study was further extended to determine their comparative responsiveness to insulin.

Materials and methods

Animals

Female Sprague-Dawley rats from our own colony were housed at 22-24°C on 12:12-h light-dark cycles (0800–2000 h), with free access to water and fed a chow diet (RMM from Harlan Interfauna IBERICA S. A., Barcelona). Some animals were mated when weighing 190–220 g, and day 0 of pregnancy was determined by the presence of spermatozoids in vaginal smears. Animals were studied under fed conditions on days 7, 14, or 20 of pregnancy, and age- and sex-matched virgin rats (day 0) were always studied in parallel. The experimental protocol was approved by the Animal Research Committee of the University CEU San Pablo, Madrid, Spain. After CO_2 anesthesia, animals were decapitated.

Isolation of adipocytes

The lumbar adipose pads were rapidly dissected, placed in 0.9% NaCl and cut into small pieces. Adipocytes were prepared according to the method of Rodbell [42] with minor modifications. Briefly, freshly isolated adipose tissue pad pieces were digested by collagenase A (10 mg/mL; Roche Diagnostics¸ Barcelona, Spain, activity 0.21 U/mg) in Krebs-Ringer bicarbonate buffer (KRB), pH 7.4 (0.7% NaCl, 0.035% KCl, 0.009% $CaCl_2$, 0.016% KH_2PO_4, 0.03% $MgSO_4.7\ H_2O$, and 0.043% $NaHCO_3$) containing 4% (wt/vol) bovine serum albumin (BSA; fatty acid free, fraction V; Sigma-Aldrich, Madrid, Spain) and 5 mM glucose (KRB-buffer), for 30 min at 37°C in an O_2-CO_2 atmosphere (95%-5%) with shaking (60 cycles/min). Subsequently, fat cells were dispersed and filtered through a silk screen, washed three times with KRB to eliminate collagenase, and resuspended in the same buffer at a concentration of approximately 0.4×10^6 cells/ml. Total cell lipid

content was determined gravimetrically after organic extraction [5].

Glycerol and glucose incorporation into adipocytes

To determine the incorporation of labeled substrates into tissue lipids, freshly isolated adipocytes were incubated at 37°C in KRB-BSA buffer for 90 min with shaking as above in the presence of 0.5 μCi/tube of either D-[U-^{14}C]-glucose (specific activity 12.5 mCi/mmol) or D-[U-^{14}C]-glycerol (specific activity 40 mCi/mmol; from New England Nuclear, Madrid, Spain). In the incubations performed in the presence of labeled glucose, the media contained 5 mM glucose. The incubations with labeled glycerol were done in the presence or absence of 5 mM glucose, and no cold glycerol was added to the media. Lipids were extracted from adipocytes in chloroform-methanol (2:1, by vol) by the method of Folch et al. [11] with a few modifications [17]. Aliquots of total lipids were saponified in five equivalents/L of ethanolic KOH for 1 h at 100°C, and, after acidification with H_2SO_4, fatty acids were extracted with heptane. Radioactivity in the glyceride glycerol fraction was estimated by the difference between radioactivity in total lipids and in the fatty acids fraction, which was measured by liquid scintillation (Opthifase "Hifase 2", Perkin Elmer, Spain) in a Beckman β-counter (LS 650 Multi-purpose scintillation counter, Beckman, Madrid, Spain).

Measurements of lipolysis

To analyze the insulin responsiveness, freshly isolated adipocytes were incubated as above in KRB-BSA medium containing or not 5 mM glucose in the presence or not of 100 nM insulin (from bovine pancreas, Sigma). After 30 min of incubation, 0.5 μCi/tube of the corresponding labeled tracer was added and incubated for a further 90 min at 37°C with shaking as above.

To estimate the basal lipolytic activity, the tubes were placed on ice after 90-min incubation, and aliquots of 50 μL of the infranatant were removed for the enzymatic determination of glycerol (GPO-Trinder, Sigma-Aldrich, Madrid, Spain). Glycerol released into the incubation medium was taken as an index of lipolytic activity and expressed as nmol glycerol released/min/100 mg of cell lipids.

Protein extraction from adipose tissue

Of the frozen lumbar adipose tissue, 100 mg were powdered in liquid nitrogen in a mortar, pre-cooled previously to −80°C, and lysed for 30 min in an ice-cold 30 mM HEPES buffer (pH 7.4), containing 5 mM EDTA; 1% Triton X-100, 0.5% sodium deoxycholate, and 2 mM protease inhibitor (Pefablock, Roche). Cellular debris was pelleted and discarded following centrifugation at 17,000×*g* for 30 min at 4°C. Supernatant was collected and protein concentration was determined by the BCA protein assay from Pierce (Rockford, IL, USA).

Immunoblotting

Of the tissue protein, 25 μg were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk and then incubated at 4°C overnight with the indicated antibodies specific for rat proteins: anti-aquoporin 7 (rabbit polyclonal, Chemicon-Millipore, Madrid, Spain), anti-Glut4 (goat polyclonal, Santa Cruz Biotechnology, Germany), anti-β-actin (mouse monoclonal, Sigma-Aldrich, Madrid, Spain); horseradish peroxidase-conjugated secondary antibodies were used as appropriate. Immunoreactive bands were visualized using the enhanced chemiluminescence system and quantified by densitometry. Amounts of protein were normalized to expression levels of β-actin.

Statistical analysis

Results are expressed as means±SE of four to ten animals per group. Statistical comparisons were made by analysis of variance followed by a Newman-Keuls Multiple Comparison Test with 95% confidence limits, using the GraphPad Prism 5 for Mac. Values were log transformed to equalize variances between conditions.

Results

Adipocytes from lumbar fat pads of pregnant and virgin rats were incubated in vitro in the presence of

5 mmol/L ^{14}C-glucose. Basal lipolytic activity by adipocytes did not differ between 0-, 7-, 14-, and 20-day pregnant rats (1.69±0.11, 1.65±0.14, 1.58±0.16, and 2.07±0.26 nmol/L glycerol/min^{-1}. 100 mg cell lipids^{-1}; p=0.1984). As shown in Fig. 1, the incorporation of glucose into total lipids was significantly higher in adipocytes of 7-day pregnant rats than in those from either 0 (p<0.01), 14 (p<0.05), or 20 days of pregnancy (p<0.05). A similar change was found in the incorporation of glucose into the fatty acids fraction, whereas the incorporation of glucose into glyceride glycerol was higher in adipocytes from 7-, 14-, or 20-day pregnant rats than in those from virgin rats (day 0).

When adipocytes were incubated in the presence of labeled glycerol in medium containing 5 mmol/L glucose, approximately half of the tracer incorporated into lipids appeared as fatty acids whereas the other half appeared as glyceride glycerol (Fig. 2a). Values of glycerol incorporated into total lipids, fatty acid or glyceride glycerol in adipocytes of 7-day pregnant rats appeared higher than in any of the other groups, although the differences were not statistically significant in the case of the incorporation into fatty acids, due to the variation of the values. Neither of the variables differed in cells from virgin (0-), 14-, or 20-day pregnant rats (Fig. 2a). When incubations were carried out in the absence of glucose (Fig. 2b), the absolute incorporation of labeled glycerol into adipocyte lipids was always higher than in the presence of glucose, and the whole label in lipids appeared in the form of glyceride glycerol, whose value was much higher in adipocytes from 7-day pregnant rats than in those from any of the other groups, where no differences were found.

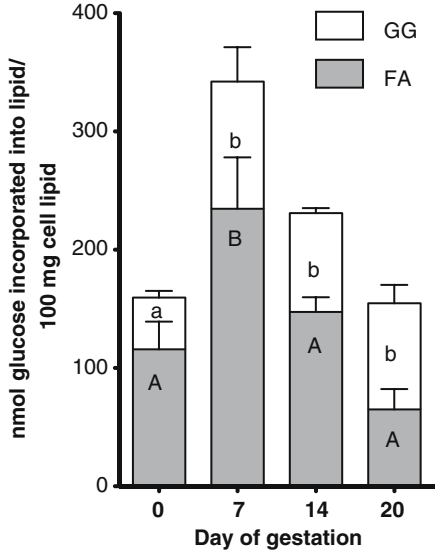

Fig. 1 Incorporation of glucose into both fatty acids (FA) and glyceride glycerol (GG) in adipocytes isolated from virgin (0), 7-, 14-, and 20-day pregnant rats. Cells were incubated in vitro during 90 min in Krebs Ringer bicarbonate buffer containing BSA and 5 mM D-[U-^{14}C]glucose. Statistical comparison between groups is shown by *letters* (*capital letters* fatty acids, *small case letters* glyceride glycerol): different letters indicate statistical significant difference between the groups (p<0.05)

Adipocytes were also incubated in a medium containing the tracers as above, but in the presence of 100 nmol/L insulin, and the net insulin effect was expressed by considering the values found in basals as zero (i.e., absence of insulin). As shown in Fig. 3a, the effect of insulin enhancing the incorporation of glucose into either fatty acids or glyceride glycerol was higher in adipocytes from 7-day pregnant rats than in those from either virgin, 14- or 20-day pregnant rats. When adipocytes were incubated in the presence of labeled glycerol and the medium did not contain glucose, insulin did not modify the incorporation of labeled glycerol into lipids (data not shown) as compared to those incubated in the absence of insulin. However, when adipocytes were incubated in the presence of labeled glycerol in medium containing 5 mmol/L glucose, insulin did not modify the incorporation of the tracer into fatty acids but decreased its incorporation into the glyceride glycerol fraction; this negative effect being greatest in those adipocytes coming from 7-day pregnant rats than from any of the other groups (Fig. 3b)

Protein levels of GLUT4 and aquoporin 7 are shown in Fig. 4. We did not observe any significant differences in total amount of GLUT4 protein between the experimental groups (Fig. 4a). Aquoporin 7 expression, however, was significantly higher in adipose tissue from 7-day pregnant rats as compared to non-pregnant and 20-day pregnant animals, being the values in this last group significantly lower than in any of the others (Fig. 4b).

Discussion

The present study demonstrates that adipocytes from early pregnant rats (7 days) have an enhanced glucose or glycerol incorporation into lipids as compared to those from virgin animals or from pregnant rats at

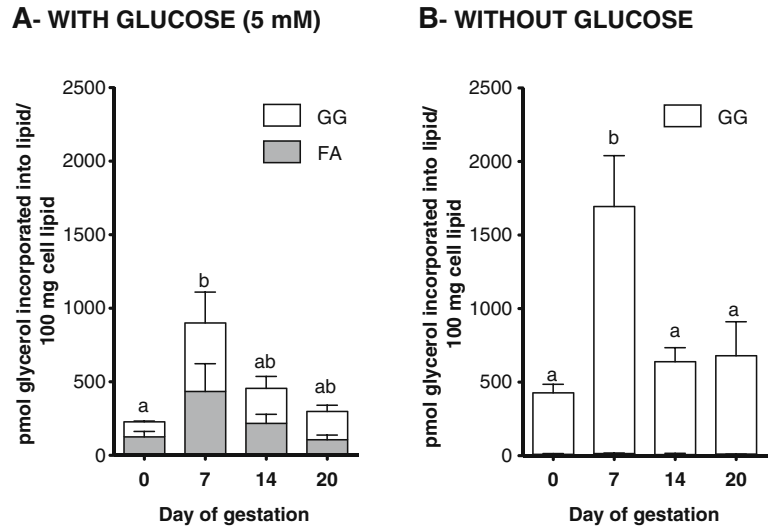

Fig. 2 Incorporation of glycerol into both fatty acids (FA) and glyceride glycerol (GG) in adipocytes isolated from virgin (0), 7-, 14-, and 20-day pregnant rats. Cells were incubated in vitro during 90 min in Krebs Ringer bicarbonate buffer containing BSA and D-[U-^{14}C]glycerol, in the presence (**a**) or the absence of 5 mM glucose (**b**). Statistical comparison between groups is shown by *letters* (*capital letters* fatty acids, *small case* letters to glyceride glycerol): *different letters* indicate statistical significant difference between the groups ($p<0.05$)

later stages of gestation (14 or 20 days). In addition, we also found that the responsiveness to insulin for the utilization of glucose for lipid synthesis is highly augmented in adipocytes from early pregnant rats. These findings give additional support to previous studies [37, 39] on the roles of both enhanced adipose tissue lipogenesis and insulin responsiveness on the fat deposition that normally take place during early

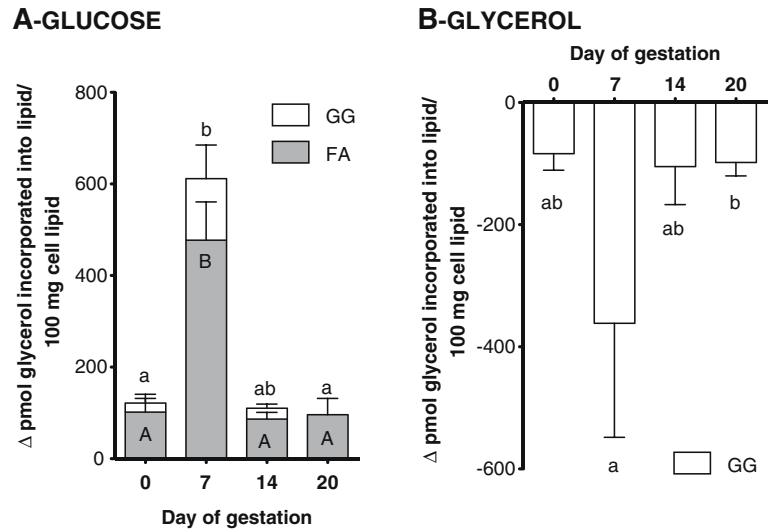

Fig. 3 Net effect of 100 nM insulin (i.e., change as related to the basals, incubated in the absence of insulin) on the incorporation of glucose (**a**) or glycerol (**b**) into both fatty acids (FA) and glyceride glycerol (GG) in adipocytes isolated from virgin (0), 7-, 14-, and 20-day pregnant rats. Cells were incubated in vitro in Krebs Ringer bicarbonate buffer containing BSA, 5 mM glucose and either D-[U-^{14}C]glucose (**a**) or D-[U-^{14}C]glycerol (**b**). Incubations were carried for 30 min in the presence of insulin, and then the corresponding labeled tracer was added and incubated for a further 90 min. Statistical comparison between groups is shown by *letters* (*capital letters* fatty acids, *small case letters* glyceride glycerol): *different letters* indicate statistical significant difference between the groups ($p<0.05$)

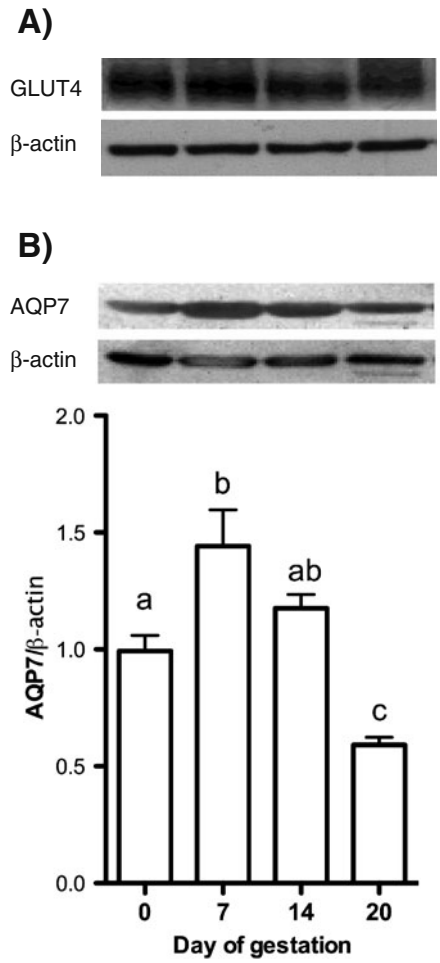

Fig. 4 Immunodetection of GLUT4 and aquoporin 7 (AQP7) in lumbar adipose tissue during pregnancy. Lumbar adipose tissue of virgin and 7-, 14-, and 20-day pregnant rats was homogenized in an ice-cold 30 mM HEPES buffer (pH 7.4), containing 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate and 2 mM protease inhibitor (Pefablock). Samples were subjected to SDS-PAGE and electrophoretically transferred to PVDF membranes that, subsequent to blocking, were incubated overnight at 4°C with the corresponding antibodies. **a** Representative immunoblots for GLUT4 and β-actin. **b** The graph shows the levels of AQP7 normalized to the total amount of β-actin in each sample (n=7); representative immunoblots are shown above the graph. Statistical comparison between the groups is shown by *letters*: *different letters* indicate statistical significant difference between the groups (p<0.05)

pregnancy. Moreover, although we agreed with previous reports [6, 44] that glycerol utilization by isolated adipocytes was very low under basal conditions, the increase in glycerol utilization observed in adipocytes from 7-day pregnant rats supports the recently proposed notion that, under conditions of enhanced insulin sensitivity [13, 46, 50], white adipose tissue contains sufficient glycerol kinase to play a role in triacylglycerol homeostasis in this tissue. Glycerol transport across the membrane of adipocytes is mediated by aquaporin 7 that acts as a glycerol channel [21]. In support of increased glycerol utilization by adipose tissue during early pregnancy, we found a significantly higher amount of aquoporin 7 in this group of rats as compared to virgin animals or pregnant rats at later stages of gestation.

Although the net utilization of labeled glycerol by isolated adipocytes could be considered negligible (in the order of pmoles/100 mg cell lipids) when compared to that of glucose (nmoles/100 mg cell lipids), such a value is actually much greater when the dilution of the tracer with the cold glycerol being continuously released into the incubation medium through lipolysis is taken into account, as previously described [17].

Recent studies addressed to determine the utilization of either glucose or glycerol for lipid synthesis by isolated adipocytes do not differentiate between their respective incorporation into fatty acids or glyceride glycerol [13, 50, 51]. However, we have observed here that the changes affect each of these lipid moieties differently, and since their intracellular physiological roles differ, they should be considered separately.

The effects of 7-day pregnancy enhancing glucose utilization for lipid synthesis under both basal or insulin-stimulated conditions corresponded to both fatty acids and glyceride glycerol, indicating an enhanced overall glucose utilization throughout glycolysis and lipogenesis. This finding extends our previous results, showing an enhanced response to insulin in the synthesis of fatty acids from glucose in adipocytes of 7-day pregnant rats [39]. Furthermore, it demonstrates that at this early stage of pregnancy, glucose contributes greatly to the production of glycerol-3-phosphate for fatty acid esterification, under both basal conditions and after its insulin stimulated metabolism. Through this pathway, the intracellular accumulation of free fatty acids and of their acyl-CoA derivatives is avoided. Consequently, the inhibitory action of these fatty acid derivatives on acetyl-CoA carboxylase, the key enzyme for lipogenesis [3], whose activity is enhanced by insulin [14] disappears. It is thus proposed that at this early stage of pregnancy, an enhanced glucose utilization for both fatty acid and

glycerol-3-phosphate synthesis and an augmented insulin responsiveness on these pathways actively contribute to the fat depot accumulation that takes place in the mother [31]. It was found here that the enhanced utilization of glucose by adipose tissue during early pregnancy occurs without modifying its total GLUT4 content, although a differentiated GLUT4 cell surface translocation cannot be discarded.

The enhanced glycerol utilization by isolated adipocytes found in adipocytes from 7-day pregnant rats incubated in either the presence or the absence of glucose in the medium agrees with the augmented glycerol kinase activity and glycerol incorporation into adipocyte lipids recently found under non-pregnant conditions after enhanced insulin sensitivity, such as the treatment with thiazolidinediones [13, 50]. These results also agree with the enhanced glycerol utilization found under conditions of obesity induction, such as that caused by rats treated with monosodium glutamate [6] as well as with the enhanced glycerol kinase activity and enhanced glycerol utilization consistently found in the adipose tissue of obese mice or rats [21, 22, 26, 29, 30, 48].

Since under in vivo conditions, adipose tissue does not take up a significant amount of circulating glycerol [37], the main source of glycerol for its phosphorylation by glycerol kinase, and its subsequent incorporation into acylglycerides would be that derived from intracellular lipolysis. Throughout this process, the tissue would reutilize intracellularly part of the lipolytic products, greatly contributing to the net accumulation of fat depots, as it was the case under obese conditions, and we propose here that it is also the case during early pregnancy.

Since glycerol kinase activity in adipose tissue from obese mice has been reported to be directly related to plasma insulin levels [29, 49], and its activity and/or its expression enhanced under conditions of insulin sensitisation by antidiabetic therapies [13, 46, 50], present findings of increased utilization of glycerol by adipocytes of 7-day pregnant rats may well be related to the enhanced insulin responsiveness seen in this condition [39]. This explanation is compatible with the lack of in vitro effect of insulin on glycerol utilization, when incubations were carried out in the absence of glucose. Aquaporin 7 acts as a glycerol channel and it is long term, but not short term, regulated by insulin at a transcriptional level [32]. Thus, the higher amount of aquoporin 7 found in adipose tissue from 7-day pregnant rats could be explained by the enhanced insulin sensitivity of the tissue during the first part of pregnancy [39]. Furthermore, the significant effect of insulin decreasing the synthesis of glyceride glycerol from glycerol, when incubations of adipocytes from 7-day pregnant rats were carried out in the presence of glucose, fits well with the enhanced insulin sensitivity of adipose tissue at this specific gestational age [39], since the augmented glucose utilization for glyceride glycerol synthesis would displace glycerol for its use by this same metabolic product.

In conclusion, the present results demonstrate an enhanced in vitro utilization of both glucose and glycerol by adipocytes of early pregnant rats and an augmented responsiveness of insulin to glucose utilization, although not to glycerol utilization, at this specific gestational age. The enhanced use of glycerol for glyceride glycerol synthesis in adipocytes in early pregnancy shown for the first time indicates that the induction of adipose tissue glycerol kinase activity contributes to the fat depot accumulation occurring at this anabolic stage of pregnancy. The fact that under normal conditions glycerol kinase activity is very low in adipose tissue, its induction when fat depots build up, as occurs during early pregnancy, may play a key role in warranting the appropriate availability of glycerol-3-phosphate for sustaining triacylglycerol synthesis. This action may contribute to maintain low intracellular FFA and their acyl-CoA derivates levels, allowing to increase lipogenesis, which is well known to be highly enhanced in the early stages of pregnancy in rat [37].

Acknowledgments This study was supported by grants from the Ministry of Science and Innovative Technology of Spain (SAF2007-64881 and SAF2008-04518) and from University CEU San Pablo (USP PC 12-09). We thank Brian Crilly for revising the manuscript.

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