Maternal protein restriction during early lactation induces GLUT4 translocation and mTOR/Akt activation in adipocytes of adult rats

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Garcia-Souza EP, da Silva SV, Félix GB, Rodrigues AL, de Freitas MS, Moura AS, Barja-Fidalgo C. Maternal protein restriction during early lactation induces GLUT4 translocation and mTOR/Akt activation in adipocytes of adult rats. *Am J Physiol Endocrinol Metab* 295: E626–E636, 2008. First published June 17, 2008; doi:10.1152/ajpendo.00439.2007.—Epidemiological and experimental studies have demonstrated that early postnatal nutrition has been associated with long-term effects on glucose homeostasis in adulthood. Recently, our group demonstrated that undernutrition during early lactation affects the expression and activation of key proteins of the insulin signaling cascade in rat skeletal muscle during postnatal development. To elucidate the molecular mechanisms by which undernutrition during early life leads to changes in insulin sensitivity in peripheral tissues, we investigated the insulin signaling in adipose tissue. Adipocytes were isolated from epididymal fat pads of adult male rats that were the offspring of dams fed either a normal or a protein-free diet during the first 10 days of lactation. The cells were incubated with 100 nM insulin before the assays for immunoblotting analysis, 2-deoxyglucose uptake, immunocytochemistry for GLUT4, and/or actin filaments. Following insulin stimulation, adipocytes isolated from undernourished rats presented reduced tyrosine phosphorylation of IR and IRS-1 and increased basal phosphorylation of IRS-2, Akt, and mTOR compared with controls. Basal glucose uptake was increased in adipocytes from the undernourished group, and the treatment with LY294002 induced only a partial inhibition both in basal and in insulin-stimulated glucose uptake, suggesting an involvement of phosphoinositide 3-kinase activity. These alterations were accompanied by higher GLUT4 content in the plasma membrane and alterations in the actin cytoskeleton dynamics. These data suggest that early postnatal undernutrition impairs insulin sensitivity in adulthood by promoting changes in critical steps of insulin signaling in adipose tissue, which may contribute to permanent changes in glucose homeostasis.

undernutrition; glucose transporter 4; insulin signaling

MATERNAL AND CHILD UNDERNUTRITION still represent an important problem in public health, persisting as pervasive and damaging challenges in low and middle-income countries (5, 65). Epidemiological studies in humans suggest that undernutrition during various phases of gestation and/or lactation can predispose some individuals to become obese and insulin resistant (13, 17, 21, 38, 49, 53, 61).

There is a high correlation between undernutrition and insulin functions. During the past decade, many experimental studies have investigated the impact of a varying maternal, and hence fetal, nutrition on patterns of postnatal growth, insulin secretion, glucose tolerance, and the insulin sensitivity of postnatal tissues such as skeletal muscle, adipose tissue, and liver (46–48). Although the biochemical basis of these deleterious consequences are not yet well defined, it has been suggested that poor nutrition during gestation and/or lactation results in metabolic changes in the offspring that can lead to permanent alteration in the structure and/or function of organs and tissues (33, 34). Undernutrition during early life is also associated with alterations in the insulin secretion mechanism, resulting in reduced insulin sensitivity in adulthood that may be the start of type 2 diabetes (66, 69). A series of studies has also highlighted the possibility that alterations in maternal nutrition during critical windows of development may also alter the expression and activation of specific proteins involved in the insulin signaling cascade of the offspring (2, 45, 47).

Insulin is the primary hormone involved in glucose homeostasis and in the stimulation of glucose transport. Insulin action is characterized by several effects, including changes in vesicle trafficking, stimulation of protein kinases and phosphatases, control of cellular growth and differentiation, and activation or repression of transcription (55). At the molecular level, insulin signaling begins with the hormone binding and activation of the insulin receptor (IR), resulting in tyrosine phosphorylation of several substrates, including a family of the IR substrate (IRS). Phosphorylated IRS generates docking sites for several SH2-containing proteins that function as effector molecules. Tyrosine-phosphorylated IRS proteins can recruit and activate phosphoinositide 3-kinase (PI3K), which generates phosphatidylinositol 3,4,5-triphosphate (PIP3), using inositol-containing phospholipids as substrates (58). One downstream target of PI3K is the serine/threonine kinase Akt (also known as protein kinase B). The activity of Akt is markedly stimulated in a PI3K-dependent manner (30). This phenomenon predominantly relies on the phosphorylation of Akt on two of its amino acid residues: threonine308 by phosphoinositide-dependent kinase-1 (PDK1) and serine473 by mammalian target of rapamycin (mTOR)/Rictor complex (23). The PI3K/Akt pathway has an important role in the metabolic effects of insulin (30, 42). This signaling cascade culminates in the translocation of the glucose transporter-4 (GLUT4) from intracellular compartments to the plasma membrane, which in turn allows glucose uptake by the cell (50, 56). Moreover, several studies have shown that the actin cytoskeleton is required for insulin-dependent GLUT4 translocation, playing a
role in organization of the insulin signaling complex (14, 63) or in the movement of vesicles to the plasma membrane (29, 44).

Glucose transport is the rate-limiting step of glucose metabolism in insulin-sensitive tissues such as muscle and fat, under most physiological conditions (67). Various cellular defects resulting in insulin-resistant glucose transport have been proposed, including alterations in insulin receptor function, depletion of GLUT4 pool, and alterations in the post-receptor signaling pathway (26, 68, 71).

Using an experimental model with rats, we recently established an association between maternal protein deficiency during lactation and changes in the glucose homeostasis of the offspring in adulthood. In short, these animals present reduced insulin secretion and, as an adaptation mechanism, there is an increase in insulin sensitivity (8, 39). We have shown that neonatal nutrient restriction causes increased insulin secretion and GLUT2 expression by pancreatic β-cells in neonates (33) and increased glucocorticoid secretion, reduced plasma insulin, and alterations in innate inflammatory response (3), as well as changes in feeding behavior associated with insulin and leptin concentrations in adult rats (41). Furthermore, to investigate the point in development at which the improvement in insulin response occurred in undernourished adult rats, we also evaluated the expression and activation of key proteins of insulin signaling cascade in skeletal muscle from the suckling period until adulthood. Our results showed that undernutrition during early lactation leads to a permanent upregulation of PI3K and an increase in GLUT4 translocation in skeletal muscle of adult rats, explaining why the malnourished rats are able to maintain normal glucose tolerance, despite reduced insulin secretion (11).

It has been well established that adipose tissue is an important insulin target, and it regulates glucose metabolism. The aim of our study was to investigate the effects of protein restriction during early lactation on insulin-induced glucose uptake and in the expression of key proteins of the insulin signaling cascade in isolated adipocytes from rats at 90 days of age.

**MATERIAL AND METHODS**

**Animals and diets.** Wistar rats were housed in rooms with controlled temperature at 23–25°C. Virgin female rats were mated, and pregnant dams, housed in individual cages, were fed a normal diet containing 22% protein during gestation. Following delivery, each pregnant dam was kept with six male pups, and one group of dams received a protein-free diet during the first 10 days of lactation, whereas the other group received a normal diet (control group; C). Both groups were fed ad libitum, and one group of dams was killed, and blood was collected to evaluate biochemical parameters. Plasma glucose concentration was determined by glucose oxidase method (Glucose PP; Gold Analisqa Diagnostica, Belo Horizonte, MG, Brazil). Plasma levels of triglycerides, total cholesterol, LDL cholesterol, and HDL cholesterol were determined by enzymatic colorimetric methods (Gold Analisqa Diagnostica).

**Adipocyte isolation.** Adipocytes were isolated from epidydimal fat pads as described previously (54) by collagenase digestion. Briefly, fat pads were minced in Krebs-Ringer-HEPES buffer, pH 7.4, containing 2% BSA (Sigma-Aldrich, St. Louis, MO) and 0.7 mg/ml collagenase (Sigma-Aldrich) and incubated at 37°C for 40 min with shaking at 120 rpm. After digestion, the cell suspension was passed through a filter, and the floating cells were washed three times with fresh Krebs-Ringer-HEPES buffer containing BSA. After the final wash, cells were diluted to a 20% suspension and submitted to the treatments described in the legends. Rapamycin was purchased from Sigma-Aldrich.

**Preparation of cell extracts.** To obtain the total cell extracts, isolated adipocytes were lysed in 50 mM HEPES, pH 6.4, 1 mM MgCl2, 10 mM EDTA, 1% SDS, 1 µg/ml DNase, and 0.5 µg/ml RNase and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 1 µM leupeptin, and 1 µM soybean trypsin inhibitor (Sigma-Aldrich).

**Immunoprecipitation.** Isolated adipocytes were lysed in 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na3VO4, 1% NP-40, 0.1% SDS, 10% aprotinin, 10 µg/µl leupeptin, 2 µg/µl pepstatin, and 1 mM PMSF. Lysates (2 µg/µl) were incubated for 2 h at 4°C under rotation with either polyclonal rabbit anti-IRS-1 or polyclonal goat anti-IRS-2 antibodies (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). Then, protein A/G agarose (20 µg/mg; Santa Cruz Biotechnology) was added, and samples were incubated at 4°C overnight. The content of IRS-1, IRS-2, and phosphotyrosine was analyzed by Western blotting as described below.

**Western blotting analysis.** The total protein content in the cell extracts was determined by the Bradford method. Cell lysates were denatured in sample buffer (50 mM Tris–HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and heated in a boiling bath for 3 min. Samples (50 µg of total protein) were subjected to 10 or 12% SDS-PAGE, transferred to polyvinylidene difluoride filters (PVDF Hybond-P, Amersham Pharmacia Biotech), and blocked with Tween-PBS (0.01% Tween-20) containing 1% BSA. Primary antibodies used in Western analysis were as follows: anti-insulin receptor β-subunit (1:1,000; Santa Cruz Biotechnology), anti-phosphotyrosine (1:500, Santa Cruz Biotechnology), anti-IRS-1 (1:1,000; Santa Cruz Biotechnology), anti-IRS-2 (1:1,000; Santa Cruz Biotechnology), anti-Akt 1/2 (1:1,000; Santa Cruz Biotechnology), anti-pAktSer473 (1:2,000; Promega, Madison, WI), anti-mTOR (1:1,000; Cell Signaling Technology), and anti-phospho-mTORSer2448 (1:1,000; Cell Signaling Technology). The PVDF filters were then incubated with appropriate secondary antibodies conjugated to biotin (1:1,000, Santa Cruz Biotechnology), followed by 1-h incubation with horseradish peroxidase-conjugated streptavidin (1:100; Caltag Laboratories, Burlingame, CA). Immunoreactive proteins were visualized by 3,3′-diaminobenzidine staining (Sigma). The bands were quantified by densitometry, using Image J software (Wayne Rasband, National Institutes of Health).

**Glucose uptake.** The glucose transport in adipocytes was assayed as described previously (18). Briefly, isolated adipocytes were incubated in Krebs-Ringer-HEPES buffer with or without LY294002 (Calbiochem, San Diego, CA) at 37°C for 10 min before the addition of insulin (100 nM) for 20 min. Then, samples were incubated for 10 min in the presence of 50 µM 2-deoxy-D-[14C]glucose (1 µCi/ml; Amersham Biosciences). The reaction was stopped by washing the cells three times with ice-cold PBS. The cells were then solubilized in 1% Triton X-100 at room temperature for 30 min. Radioactivity was determined by liquid scintillation counting (Beta Counter; Beckman Instruments, Fullerton, CA). Protein concentration was determined by the Bradford method.

**Immunolocalization of GLUT4.** The immunolocalization of GLUT4 in adipocytes was assayed as reported earlier (36). The isolated
adipocytes were resuspended in 4% paraformaldehyde in PBS for 1 h at room temperature. After being washed three times with PBS containing 10 mg/ml of BSA, fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, and nonspecific binding sites were blocked with PBS containing 1% BSA for 30 min at room temperature. The cells were washed three times with PBS and incubated overnight with anti-GLUT4 antibody (1:50; Santa Cruz Biotechnology) at 4°C. Next, the cells were incubated for 1 h with donkey anti-rabbit IgG conjugated with biotin (1:50; Santa Cruz Biotechnology) before incubation with streptavidin conjugated with FITC (1:50; Santa Cruz Biotechnology) for 1 h at room temperature. Finally, the cells were washed with PBS, and coverslips were mounted on the slides using a solution of 20 mM N-propylgallate and 20% glycerol in PBS. Microscopic analysis of fluorescence images was done using an epifluorescence microscope (Olympus BX-40F4; Tokyo, Japan) equipped with appropriate filters and objectives. Image capture was performed with a CCD camera (Photometrics, Tucson, AZ). Images from 50 cells were obtained from each group, and quantitative analysis of fluorescent images was performed by Image-Pro Plus 4 software (Media Cybernetics), measuring GLUT4 fluorescence signal at the peripheral rim of individual cells, reported as integrated optical density. All images were captured using identical camera settings, such as time of exposure, brightness, contrast, and sharpness, and an appropriate white balance set according to the fluorescence filter. Subcellular fractionation. Subcellular fractionation of adipocytes was carried out as previously described (37). Following experimental treatment, all subsequent steps were carried out at 4°C. Cell pellets were resuspended in buffer I (250 mM sucrose, 1 mM EDTA, 1 mM Na3VO4, 200 μM PMSF, 1 μM leupeptin, 1 μM pepstatin A, and 20 mM HEPES pH 7.4) and then homogenized, using a Polytron, three times for 20 s. The homogenate was centrifuged at 760 g for 5 min to remove nuclei and unbroken cells. The supernatant was then centrifuged at 31,000 g for 60 min to pellet crude plasma membranes (CPM). The light microsomes (LM) were collected from the 31,000 g supernatant by centrifugation at 190,000 g for 60 min. Both CPM and LM pellets were suspended in buffer I and frozen at −20°C. Protein concentration was assayed by the Bradford method. Equal amounts of protein from both the LM and CPM fractions were analyzed by immunoblotting using anti-GLUT4 antibody.

Immunocytochemistry for actin filaments and GLUT4. Double labeling was performed to visualize actin filaments and GLUT4. The isolated adipocytes were fixed and washed in PBS as described above. The cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, and nonspecific binding sites were blocked with PBS containing 1% BSA for 30 min at room temperature. The cells were incubated with TRITC-phalloidin (1:1,000; Sigma) for 1.5 h at room temperature. The cells were washed three times with PBS and incubated overnight with anti-GLUT4 antibody (1:50; Santa Cruz Biotechnology) at 4°C. Next, the cells were incubated with donkey anti-rabbit IgG conjugated with biotin (1:50; Santa Cruz Biotechnology) for 1 h before incubation with FITC-conjugated streptavidin (1:50; Santa Cruz Biotechnology) for 1 h at room temperature. Finally, the cells were washed with PBS, and slides were mounted using a solution of 20 mM N-propylgallate and 20% glycerol in PBS. Microscopic analysis of fluorescent images was done using an epifluorescence microscope (Olympus BX-40F4) equipped with appropriate filters and ×40 objectives. Image capturing was performed with a CCD camera (Photometrics), and all images were captured using identical camera settings, such as time of exposure, brightness, contrast, and sharpness, and an appropriate white balance set according to the fluorescence filter. Images from 25 cells were captured from each group and were processed by Image-Pro Plus 4 software (Media Cybernetics).

Statistical analysis. Data are shown as means ± SD. Statistical significance of the results was determined by one-way ANOVA followed by Bonferroni’s t-test. A P value <0.05 was considered significant.

RESULTS

Body weight, epididymal fat weight, and metabolic parameters. Table 1 shows, as previously reported by our group (11), that the body weight of rats from the UN group is lower (by ~20%) compared with that of controls. Maternal protein restriction during the first 10 days of lactation also affected the offspring epididymal fat pad weights, which were also significantly reduced (by ~35%) compared with the C group. No differences in fasting blood glucose, total cholesterol, LDL cholesterol, and triacylglycerol levels were observed between the C and UN groups.

Expression and tyrosine phosphorylation of IR, IRS-1, and IRS-2. Adipocytes of UN rats did not show significant differences in IR immunocomponent compared with controls (Fig. 1A).

Table 1. Body weight, epididymal fat weight, and metabolic parameters in adult rats from control and undernourished groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C</th>
<th>UN</th>
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<tbody>
<tr>
<td>Body wt, g</td>
<td>292 ± 22.6</td>
<td>260 ± 16.0*</td>
</tr>
<tr>
<td>Epididymal fat wt, g</td>
<td>3.5 ± 0.44</td>
<td>2.25 ± 0.27*</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>9.17 ± 1.43</td>
<td>9.93 ± 1.03</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>1.51 ± 0.21</td>
<td>1.62 ± 0.21</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>0.6 ± 0.22</td>
<td>0.88 ± 0.06</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>0.69 ± 0.05</td>
<td>0.58 ± 0.07</td>
</tr>
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</table>

* P < 0.05 compared with C group. ** P < 0.05 compared with UN group.
However, after insulin stimulation, tyrosine phosphorylation was significantly reduced in insulin-stimulated adipocytes of UN rats, whereas IR in adipocytes of C rats showed a significant increase in phosphotyrosine content (Fig. 1B).

Expression and phosphorylation of IRS-1 and IRS-2 were also analyzed by Western Blotting in adipocytes from rats of both groups following insulin stimulation (Fig. 2). No differences in IRS-1 and IRS-2 expression were observed in C or UN rats (Fig. 2, A and C). Maternal undernutrition during early lactation induced significant changes in insulin-stimulated phosphorylation of IRS-1, which was expressively decreased in adipocytes from the UN group (Fig. 2B). On the other hand, nonstimulated adipocytes of UN animals presented highly phosphorylated IRS-2 compared with controls. As expected, insulin induced an increase in tyrosine phosphorylation of IRS-2 in adipocytes of the C group. However, it failed to increase phosphorylation of IRS-2 in UN rats (Fig. 2D).

**Effect of insulin and LY294002 on glucose uptake.** Basal glucose uptake (i.e., the rate of glucose transported without acute insulin stimulation) was increased in adipocytes from the UN group, as shown in Fig. 3. Insulin stimulation increased glucose uptake by adipocytes of C rats, reaching a fourfold increase over the basal values. In contrast, insulin was unable to increase glucose uptake by adipocytes from UN rats.

It is well established that activation of PI3K is downstream from IRS-1 and IRS-2 activation (58). To evaluate the involvement of PI3K in the responses of UN rat adipocytes, glucose uptake was assayed in the presence of LY294002, a specific inhibitor of this enzyme. The treatment with LY294002 (10 and 30 μM) inhibited, in a concentration-dependent manner, the insulin-stimulated glucose uptake in adipocytes of control rats (Fig. 3). In contrast, in adipocytes of UN rats, the treatment with LY294002 at the highest concentration (30 μM) induced only a partial inhibition (35%) both in basal and in insulin-stimulated glucose uptake, suggesting an involvement of PI3K activity (Fig. 3).

**Effect of insulin treatment on Akt activation.** Akt is the main target of PI3K. Therefore, Akt phosphorylation was evaluated in adipocytes of C and UN rats that had been stimulated, or not, with insulin. Figure 4 shows that total Akt content did not differ between both groups. Nevertheless, in the basal state, Akt was highly phosphorylated in adipocytes of UN rats, reaching the same levels of activation observed for control cells stimulated with insulin. The treatment with insulin failed to increase serine phosphorylation of Akt in adipocytes of the UN group (Fig. 4).

**Immunolocalization and subcellular distribution of GLUT4.** The translocation of GLUT4 to the plasma membrane following insulin stimulation was investigated in adipose tissue from UN and C groups (Fig. 5). As previously reported (44), in nonstimulated control adipocytes, GLUT4 is found as a few punctuated spots distributed throughout the cytoplasm, as detected by fluorescence immunocytochemistry (Fig. 5, A and C). On insulin stimulation, GLUT4 is rapidly translocated to adipocyte membrane, presenting an increased fluorescence on the cell surface (Fig. 5A). In contrast, adipocytes from UN rats showed, already in basal state, a significant increase of GLUT4 immunofluorescence on the cell surface (Fig. 5A) that was not altered following insulin stimulation (Fig. 5A). The GLUT4 content was also analyzed by immunoblotting in CPM and LM

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**Fig. 2.** IR substrate-1 (IRS-1) and IRS-2 expression and phosphorylation in isolated adipocytes from rats given either normal or protein-free diet. Isolated adipocytes in Krebs-Ringer-HEPES buffer from C and maternal UN groups were stimulated with (+) or without (−) 100 nM insulin for 10 min. Immunoprecipitation was performed with anti-IRS-1 and anti-IRS-2 antibodies, and Western blotting analysis was probed with anti-IRS-1 (A), anti-IRS-2 (C), and anti-Py (B and D) antibodies. IRS-1 and IRS-2 content and phosphorylation levels were quantified by scanning densitometry of the bands. Results are means ± SD; n = 6. *P < 0.05 compared with C group. **P < 0.05 compared with UN group.
fracture fractions of adipocytes from C and UN rats. As expected, Fig. 5B shows that the subcellular fractions of adipocytes from the UN group present a higher content of GLUT4, indicating an increased translocation to membranes.

Effect of latrunculin B treatment on the subcellular localization of GLUT4 and actin cytoskeleton. Insulin-stimulated translocation of GLUT4 is a dynamic process that requires a functional actin network. To determine the involvement of actin cytoskeleton dynamics in the upregulation of GLUT4 in adipocytes from the UN group, we performed experiments using latrunculin B, an actin-disrupting agent especially effective in disassembling actin filaments in adipocytes. Actin dynamics were determined using rhodamine-labeled phalloidin. As shown in Fig. 6, treatment of control adipocytes with insulin increased polymerized cortical actin along with GLUT4 translocation. Merged images suggest that both phenomena are associated (Fig. 6B). When control cells were treated with 60 μM latrunculin B (Fig. 6, C and D) or 100 μM latrunculin B (Fig. 6, E and F) for 1 h, we observed a concentration-dependent loss of actin filaments accompanied by a significant reduction in GLUT4 immunofluorescence, followed by a decrease in the co-localization of spots in merged images. As previously reported (24), treatment with latrunculin B also resulted in a complete inhibition of insulin-stimulated actin rearrangement (Fig. 6, D and F). Accordingly, nonstimulated adipocytes of UN rats presented a strong fluorescence for polymerized actin and GLUT4, with co-localized labeling (Fig. 6G). Furthermore, insulin stimulation did not alter GLUT4 translocation or actin polymerization levels in adipocytes of UN rats (Fig. 6H). The treatment of UN adipocytes with 20 or 60 μM latrunculin B had no effect on the actin filament assembly or on GLUT4 surface distribution. Analysis of merged images showed that the co-localized spots remained following this treatment (Fig. 6, I and J). Actin cytoskeleton disassembly and the inhibition of GLUT4 translocation were only observed in adipocytes from UN rats following treatment with 100 μM latrunculin B (Fig. 6, K and L), although this inhibition was not complete (Fig. 6, E, F, K, and L).

Effect of latrunculin B treatment on the basal and insulin-stimulated glucose uptake was measured as described in MATERIALS AND METHODS. When control adipocytes were treated with 60 μM latrunculin B (Fig. 6, I and J), the ratio of phosphorylated Akt to total Akt content was determined. Figure 7A shows that, in control cells, the treatment with latrunculin B decreased Akt phosphorylation induced by insulin in a concentration-dependent manner. However, in UN animals, phosphorylation of Akt in serine residues was inhibited only partially, even with the highest concentration of latrunculin B (100 μM), both in stimulated and nonstimulated adipocytes (Fig. 7B).

Effect of rapamycin treatment on the insulin-stimulated activation of mTOR. mTOR functions as a nutrient sensor and mediates cross talk between amino acids and insulin signaling. To evaluate mTOR expression and activation, cells were treated with 20 nM rapamycin before insulin stimulation, and the phosphorylated mTOR and total mTOR contents were determined. Figure 8 shows that mTOR expression did not differ between both groups. Nevertheless, in the basal state, mTOR was highly phosphorylated in adipocytes of UN rats,
reaching higher levels of activation, comparable with those observed for control cells stimulated with insulin. The treatment with insulin failed to increase activation of mTOR in adipocytes of the UN group (Fig. 8). The treatment with rapamycin decreased mTOR phosphorylation induced by insulin in both C and UN groups.

**DISCUSSION**

Clinical studies have shown that malnutrition during critical developmental periods of neonatal life is associated with later metabolic disorders, including insulin resistance and glucose intolerance (21, 53, 61). Additionally, several studies using animal models have demonstrated that undernutrition in the first few days of life leads to long-term effects on glucose homeostasis and on the endocrine system (39, 40, 45, 46, 59).

In this work, we demonstrated that maternal protein restriction during early lactation leads to alterations in glucose uptake and in insulin signaling response in adipocytes of adult rats. We found that, at 3 mo of age, these animals presented lower body weight and lower epididymal fat pad weight compared with controls, despite presenting higher membrane GLUT4 expression. Epididymal adipocytes from the undernourished group had an elevated basal glucose uptake compared with controls. Although the mechanistic basis for this elevated basal glucose uptake is not known, a number of studies have shown that the offspring of mothers fed a low-protein diet have a higher glucose tolerance than controls in young adult life (8, 17, 45, 48). This improved insulin sensitivity in young adults...
maldnourished as pups is often associated with an increased glucose uptake into the muscle (2, 48) and in the adipocytes (45, 47). Furthermore, insulin has been shown to be an important player in the maintenance of key adipocyte functions. Despite the fact that glucose uptake by adipocytes accounts for only a small fraction of total body glucose utilization, adipocytes are thought to have a crucial role in the pathogenesis of insulin resistance (14, 27). Adipose tissue can indirectly influence insulin action in other tissues through multiple mechanisms, including release of fatty acids, cytokines, and hormones, some with insulin-sensitizing properties, such as leptin and adiponectin, and others that antagonize insulin action, such as resistin and tumor necrosis factor-α (22).

Analyzing the insulin signaling in adipocytes of both groups, we demonstrated that IR, IRS-1, and IRS-2 expressions were studied and obtained by fluorescence microscopy of representative cells from control group (A–F) and maternal UN group (G–L). Panels at right represent the merged images. These fields are representative of 3 independent experiments. Magnification, ×400.

**Fig. 6.** Effect of latrunculin B treatment on the subcellular localization of GLUT4 and actin cytoskeleton in isolated adipocytes from rats given either normal or protein-free diet. After incubation without (A, B, G, H) or with 60 μM (C, D, I, J) or 100 μM (E, F, K, L) latrunculin B for 1 h at 37°C, adipocytes in Krebs-Ringer-HEPES buffer were incubated for an additional 20 min with (B, D, F, H, J, L) or without (A, C, E, I, G, K) 100 nM insulin. Cells were then labeled with TRITC-labeled phalloidin and anti-GLUT4 antibody, as described in MATERIALS AND METHODS. Images were obtained by fluorescence microscopy of representative cells from control group (A–F) and maternal UN group (G–L). Panels at right represent the merged images.
hepatic glyconeogenesis would be reduced and/or lipid oxidation pathway increased, which would favor a greater breakdown of fat deposits. Furthermore, it is well known that insulin has anti-lipolytic and lipogenic action in adipose tissue. As demonstrated in our previous works, these animals present lower insulin levels. It is conceivable then to suggest that the hormone-sensitive lipase, which under normal conditions is inhibited by insulin to block lipolysis, would be more activated in undernourished adipocytes, increasing lipolysis in the adipose tissue, contributing to lower body weight and fat deposits.

In corroboration with this hypothesis, Ozanne et al. (45) showed that low-protein offspring adipocytes are resistant to both the anti-lipolytic action of insulin and the action of insulin to stimulate glucose uptake. Additionally, the adipocytokine, adiponectin, can enhance insulin sensitivity through an increase in fatty acid oxidation and inhibition of hepatic glucose production (32). Since undernourished rats present lower body fat weight, and adiponectin has negative correlation with measures of adiposity, we can suggest that adipocytes from undernourished rats may secrete higher levels of adiponectin, which would contribute to the adaptive mechanisms of skeletal muscle adipose tissue and liver to maintain glucose homeostasis. Moreover, an increase in the thermogenesis in undernourished rats, as described for other undernourished rat models (62, 64), could not be excluded as a possible explanation for the lean profile of these animals.

Many reports have linked mTOR with diet nutrient sensing and its ability to be widely regulated by amino acids (16). Rictor is an essential component of mTOR complex-2 (mTORC2), a kinase complex that phosphorylates Akt at Ser173 on activation of PI3K (49). Akt is known to mediate some insulin actions, including the stimulation of the glucose uptake in adipocytes (70). We observed that adipocytes of undernourished rats, in contrast to controls, present higher levels of phosphorylated Akt and mTOR in the basal state, which can contribute to an increase in the basal uptake of glucose. Nevertheless, LY294002 was able to affect the increased basal uptake only partially, even at high doses, suggesting a PI3K upregulation in this system. Our data corroborate the study by Ozanne et al. (45), who demonstrated in an experimental model of protein restriction an increase of glucose uptake in the basal state with resistance to the effect of insulin in epididymal adipocytes of older rats. Moreover, other works showed that inhibitors of PI3K, like LY294002 and wortmannin, can directly inhibit mTOR activity (7, 18), which strengthens the hypothesis that mTOR signaling may be partly responsible for the elevation in basal glucose uptake. In contrast, adipocytes of the undernourished group failed to increase serine phosphorylation of Akt following insulin stimulation, suggesting resistance to the actions of the hormone. An increase in activation in the IRS-2/PI3K/Akt pathway in white adipose tissue was demonstrated in monoglutamate-resistant rats (20) and in rats given a high-fat diet (52).

In adipocytes, a substantial amount of cellular GLUT4 is located in a specific highly insulin-responsive storage pool, termed GLUT4 storage vesicle (51). It is likely that the actin...
cytoskeleton is also crucial for insulin-stimulated GLUT4 translocation (6). Insulin causes remodeling of cortical actin filaments just below the plasma membrane and induces membrane ruffling. In this study, we evaluated the insulin-stimulated translocation of GLUT4 and the involvement of the actin cytoskeleton dynamics in this process. Adipocytes from undernourished rats showed in the basal state an increase in GLUT4 translocation to the cell surface as well as alterations in the actin cortical polymerization, which was not altered after insulin stimulation. The treatment with latrunculin B was effective in disassembling actin filaments and GLUT4 translocation induced by insulin in adipocytes of control rats, as previously reported (24, 44). In contrast, adipocytes of undernourished rats were resistant to latrunculin B, which inhibited partially GLUT4 translocation, actin polymerization, and Akt phosphorylation only after treatment with the higher concentration (100 μM). Previous studies showed that constitutive targeting of Akt1/PKBα in the membrane (myr-Akt) signals in transfected cells for GLUT4 translocation in the absence of insulin and presence of wortmannin (13, 22). In accordance with our data, a recent study showed that 3T3-L1 adipocytes that targeted Akt1/PKBα in the membrane (myr-Akt) retained the ability to signal GLUT4 translocation in latrunculin B-treated cells, even in the absence of an intact actin cytoskeleton, suggesting a role for actin in the organization of the insulin signaling complex before Akt/PKB activation (14). Other studies support the idea that actin filaments may be regulated in vivo. Barreto et al. (4) concluded that hyporesponsiveness of mast cells observed in alloxan-evoked diabetes may be due to a reduction in actin filament plasticity. Furthermore, glucocorticoids are shown to induce the reorganization and stabilization of the actin cytoskeleton in the pituitary cell as well as in other cell types (10). Since our animal model was already shown to exhibit constitutively low blood levels of insulin in parallel with high plasma glucocorticoid, (3), it is conceivable to assume that alterations observed could be, at least in part, related to these events.

In summary, we provide evidence that maternal protein restriction during a short period in early lactation may affect the expression and activation of key proteins of the insulin signaling cascade in adipocytes of adult rats, which could result in metabolic alterations in glucose homeostasis. The alterations in activation of key proteins of the insulin signaling cascade observed in muscle (11) and adipose tissue are clear evidences that explain why these animals are able to maintain glucose tolerance, despite reduced insulin secretion. The adipose tissue presents an increase in IRS-2/PI3K/Akt activation, mTOR phosphorylation, actin polymerization and GLUT4 translocation, and glucose uptake but also a resistance to actions of insulin, which might predispose the animal to the development of diseases later in life. In conclusion, the data demonstrate that undernutrition during lactation promotes alterations in the insulin response in peripheral tissues in the adult offspring. Our findings suggest that adaptive mechanisms imposed by nutritional conditions during a critical phase of the development may program significant alterations in the cellular and molecular mechanisms regulated by insulin in peripheral tissues.

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