Low expression of insulin signaling molecules impairs glucose uptake in adipocytes after early overnutrition

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Abstract

Experimental and clinical studies have demonstrated that early postnatal overnutrition represents a risk factor for later obesity and associated metabolic and cardiovascular disturbance. In the present study, we assessed the levels of glucose transporter 4 (GLUT-4), GLUT-1, insulin receptor (IR), IR substrate 1 (IRS-1), phosphatidylinositol 3-kinase (PI3K) and Akt expression, as well as insulin-stimulated glucose transport and Akt activity in adipocytes from adult rats previously raised in small litters (SL). The normal litter (NL) served as control group. We also investigated glycemia, insulinemia, plasma lipid levels, and glucose tolerance. Our data demonstrated that early postnatal overfeeding induced a persistent hyperphagia accompanied by a significant increase in body weight until 90 days of age. The SL group also presented a significant increase (~42%) in epididymal fat weight. Blood glucose,

plasma insulin, and lipid levels were similar among the animals from the SL and NL groups. While insulin-stimulated glucose uptake was approximately twofold higher in adipocytes from the NL group, no stimulatory effect was observed in the SL group. The impaired insulin-stimulated glucose transport in adipose cells from the SL rats was associated with a significant decrease in GLUT-4, IRS-1 and PI3K expression, and Akt activity. In contrast, IR and Akt expression in adipocytes was not different between the SL and NL groups. Despite these alterations, our results showed no differences in glucose tolerance test in rats raised under different feeding conditions. Our findings reinforce a potent and long-term effect of neonatal overfeeding, which can program major changes in the metabolic regulatory mechanisms.

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Introduction

Obesity in childhood represents an important problem in public health, showing increasing incidence in the Western countries (Troiano *et al.* 1995, Friedman 2000). Besides genetic factors, epigenetic environmental factors such as dietary intake can contribute to obesity development. It has been shown that early feeding conditions, the quantity and quality of food during gestation, or the postnatal development period may exert lifelong effects on body weight regulation (Basset & Craig 1988, Plagemann *et al.* 1992, Routh *et al.* 1993, Jones *et al.* 1995, Kozak *et al.* 2000).

Experimental and clinical studies have demonstrated that early postnatal overnutrition represents a risk factor for later obesity and associated metabolic and cardiovascular disturbance (Plagemann *et al.* 1992, Kramer *et al.* 1998). Reduction of pre-weaning litter size in rats is an appropriate experimental model to study immediate and long-term consequences of overnutrition during the critical perinatal period (Faust *et al.* 1980, Basset & Craig 1988). Rats from small litters (SL) can develop persistent hyperphagia, obesity, elevated triacylglycerols, increased systolic blood pressure, hyperleptinemia, hyperinsulinemia, and impaired glucose

tolerance (Cryer & Jones 1980, You *et al.* 1990, Plagemann *et al.* 1992, 1999a,b).

There is a high correlation between obesity and insulin functions. Insulin acts as a satiety signal, stimulating the central nervous regulatory system responsible for food intake and body weight control (McGowan *et al.* 1990). At the cellular level, 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. The insulin receptor (IR) belongs to the large family of growth factor receptors with intrinsic tyrosine kinase activity. Following insulin binding, IR undergoes autophosphorylation in multiple tyrosine residues. It results in the receptor kinase activation and tyrosine phosphorylation of a family of IR substrate (IRS) proteins (Patti & Kahn 1998, White 1998). The tyrosine phosphorylation of IRS generates docking sites for several SH2-containing proteins. Among these, the predominant partner seems to be the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K; Myers *et al.* 1992). The PI3K activation may transmit multiple signals. PI3K catalyzes the phosphatidylinositol (PtdIns) phosphorylation in the 3 position, generating PtdIns (3,4,5)P₃, which markedly

stimulates the serine kinase Akt (also known as protein kinase B; Lawlor & Alessi 2001). This phenomenon predominantly relies on the phosphorylation of Akt at Thr308 and Ser473 by phosphoinositide-dependent kinase 1 and mammalian target of rapamycin/rapamycin intensive companion of mTOR (MTOR/RICTOR) complex respectively (Alessi *et al.* 1997, Hresko & Mueckler 2005).

Akt activation mediates glucose transporter 4 (GLUT-4) translocation from the intracellular sites to the plasma membrane. This in turn allows glucose uptake by the cell (Pessin *et al.* 1999).

Obesity may cause insulin resistance and predisposition to type 2 diabetes, demonstrating that adipose tissue is crucial in regulating metabolism beyond its ability to take up glucose. Previous studies have demonstrated that alterations of insulin signaling in fat tissue are associated with impaired glucose tolerance, apparently due to insulin resistance in obesity. It has been observed that insulin resistance in adipocytes from obese patients was due to depletion of GLUT-4, which was determined by the level of encoding mRNA (Garvey *et al.* 1991). Along this line, Pedersen *et al.* (1991) showed that impaired insulin-stimulated glucose transport in adipose cells from high-fat-fed rats occurs in the presence of a dramatic decrease in GLUT-4 expression concomitant with reduction of glucose transporter GLUT-1 expression. In addition to the association between glucose transporter and insulin resistance, Bjornholm *et al.* (2002) demonstrated that IRS-1 signaling was a site of insulin resistance in adipocytes from obese women.

We have shown that neonatal nutrient restriction affects insulin sensitivity by increasing GLUT-4 translocation in skeletal muscle from adult rats (De Freitas *et al.* 2003). Furthermore, alterations in glucocorticoid secretions and innate inflammatory response were also observed in this model (Barja-Fidalgo *et al.* 2003). The aim of our study was to investigate the effects of early postnatal overnutrition on insulin-induced glucose uptake and expression of key proteins of the insulin signaling cascade in isolated adipocytes from rats at 90 days of age. We also evaluated blood glucose, plasma insulin, total cholesterol, lipoproteins (VLDL, LDL, and HDL), and triacylglycerols levels, as well as glucose tolerance in this experimental model.

Materials and Methods

Animal model

Wistar rats were housed in temperature-controlled rooms at 23–25 °C with a 12 h light: 12 h darkness cycle. Virgin female rats were time mated with normal males at the age of 3 months. During pregnancy and lactation, mother rats were housed in individual cages and had *ad libitum* water and standard pellet diet (commercial control diets for rats). To induce early postnatal overnutrition, the litter size was adjusted at 3 days of age to four male rats in each litter (SL, overnutrition; Plagemann *et al.* 1992, Velkoska *et al.* 2005).

The litter containing ten pups per mother served as control (normal litter (NL), normonutrition). At weaning (day 21), the four male pups were separated from dams and received *ad libitum* commercial diet. In the control group, only four male pups from each litter were analyzed. The body weight gain was measured throughout life. From days 30 to 90 of age, the daily mean food intake of the animals was measured by the determination of 24-h consumption of standard pellets. The animals were starved overnight before starting the experimental procedures. Only male rats were used and all experiments occurred at 90 days of age. The animal procedures were carried out in accordance with the National Institute of Health animal care guidelines and were approved by our Institutional Ethics Committee.

Metabolic parameters

Blood was obtained from deeply anesthetized rats (50 mg/kg ketamine, 20 mg/kg xylazine, i.p.) by cardiac puncture. The blood was centrifuged (302 g, 24 °C, 30 min) and the separated plasma stored at –70 °C for subsequent determination of plasma insulin, total cholesterol, lipoproteins (VLDL, LDL, and HDL), and triacylglycerols. Plasma insulin concentrations were determined using RIA kit (ICN Pharmaceuticals Inc., Orangeburg, NY, USA) and plasma total cholesterol, lipoproteins, and triacylglycerols were measured colorimetrically using commercial kits (Bioclin, Belo Horizonte, MG, Brazil). The glucose levels were measured by Advantage II Glucose Blood Monitor (Roche Diagnosis Co).

I.p. glucose tolerance test

Rats were fasted overnight and then injected intraperitoneally with 2 g/kg D-glucose (35% stock solution in saline). Blood samples were taken by tail venesection before and 30, 60, and 120 min after the glucose load and glucose levels were measured by Advantage II Glucose Blood Monitor.

Adipocytes isolation

Adipocytes were isolated from the epididymal fat, as described previously, by collagenase digestion (Nadler *et al.* 2001). Briefly, fat pads were mixed in Krebs–Ringer–HEPES buffer (pH 7.4), containing 2% BSA and 0.7 mg/ml collagenase (Sigma–Aldrich), and incubated at 37 °C for 1 h with shaking at 45 g. After digestion, the cell suspension was passed through a filter, and the floating cells were washed thrice with fresh Krebs–Ringer–HEPES buffer plus BSA. After the final wash, cells were diluted to a 20% suspension. To analyze Akt activity, isolated adipocytes were incubated in Krebs–Ringer–HEPES buffer at 37 °C for 10 min in the absence or presence of insulin (100 nM; Biohulin, Biobrás, Brazil).

Glucose uptake

The glucose transport in adipocytes was assayed as described earlier (Kanzaki & Pessin 2001). Briefly, isolated adipocytes were incubated in Krebs–Ringer–HEPES buffer at 37 °C for 20 min in the absence (basal state) or presence of insulin (100 nM). 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 thrice 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, USA). Protein concentration was determined by the Bradford method (Bradford 1976).

Immunocytochemistry assay

The immunolocalization of GLUT-4 in epididymal adipocytes was assayed as reported earlier (Malide & Cushman 1997). The isolated adipocytes incubated for 10 min at 37 °C in the absence (basal state) or presence of insulin (100 nM) were fixed in suspension with 4% paraformaldehyde in PBS for 1 h at room temperature. Cells were permeabilized in PBS containing 0.1% Triton X-100 for 5 min, non-specific binding sites blocked with PBS containing 1% BSA for 30 min at room temperature, and incubated with anti-GLUT-4 antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Subsequently, cells were incubated with donkey anti-rabbit IgG conjugated with biotin (1:50; Santa Cruz Biotechnology) for 1 h at room temperature and then incubated with streptavidin fluorescein isothiocyanate (FITC)-conjugated (1:50; Santa Cruz Biotechnology) for 1 h at room temperature. Coverslips were mounted on the slides using a solution of 20 mM *N*-propyl gallate and 20% glycerol in PBS. Microscopic analysis of fluorescent images was done using an epifluorescence microscope (Olympus BX40; Tokyo, Japan) equipped with appropriate filters and using 40× objectives. Image capturing was performed with a CCD camera (Photometrics, Tucson, AR, USA), and all images were captured using identical camera settings, as time of exposure, brightness, contrast and sharpness, and an appropriated white balance set according to the fluorescence filter. Gray images were taken using Adobe Photoshop software.

Preparation of cell extracts

To obtain the whole cell extracts, cells 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 containing the following protease inhibitors: 10 µg/µl aprotinin, 10 µg/µl leupeptin, 2 µg/µl pepstatin, and 1 mM phenylmethylsulphonyl fluoride (Sigma–Aldrich). The content of IR, IRS-1, PI3K, Akt, p-Akt, GLUT-4, and GLUT-1 was analyzed by western blotting as described below.

Western blotting analysis

The total protein content in the cell extracts was determined (Bradford 1976). 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 at 90 °C for 3 min. Samples (50 µg total protein) were subjected to 8, 10, or 12% SDS–PAGE and transferred to polyvinylidene filters (PVDF Hybond-P; Amersham Pharmacia Biotech). Rainbow markers (Amersham Biosciences) were run in parallel to estimate molecular weights. Membranes were blocked with Tween–TBS (20 mM Tris–HCl (pH 7.5), 500 mM NaCl, 0.1% Tween 20) containing 1% BSA. Primary antibodies (Santa Cruz Biotechnology) such as anti-IR β-subunit (1:1000), anti-IRS-1 (1:500), anti-PI3K p85 subunit (1:500), anti-Akt1/2 (1:1000), anti-p-Akt^{ser473} (1:500), anti-GLUT-4 (1:1000), anti-GLUT-1 (1:500), and anti-actin (1:500) were used. The PVDF filters were then washed thrice with Tween–TBS, followed by 1-h incubation with appropriate secondary antibody conjugated to biotin (1:1000; Santa Cruz Biotechnology). Then, the filters were incubated with streptavidin-conjugated horseradish peroxidase (1:1000; Caltag Laboratories, Burlingame, CA, USA). Immunoreactive proteins were visualized by 3,3'-diaminobenzidine (Sigma–Aldrich) staining. The bands were quantified by densitometry using Image J 1.34s software (Wayne Rasband National Institute of Health, Bethesda, MD, USA).

Statistical analysis

The data are represented as means ± s.d. One-way ANOVA and Newman–Keuls multiple comparison tests were used to analyze glucose uptake and Akt activity. The other experimental data were analyzed by Student's *t*-test with the significance level set at *P* < 0.05.

Results

Effect of early postnatal overnutrition on body weight, weight gain rate, and mean food intake during development

Early postnatal overnutrition affected the offspring body weight during the suckling period (from birth until 21 days of age). The SL group had a significant increase in body weight since 7 days of age, in comparison with the NL group. The highest difference (50%) in body weight between the groups was observed at 21 days of age (Fig. 1A). This difference decreased at 30, 60, and 90 days of age, but a significant increase still remains until 90 days of age (Fig. 1B). The weight gain rate in rats from the SL and NL groups during the postnatal development was also measured (Fig. 2A). The early postnatal overnutrition conditions resulted in a significant weight gain during the suckling period when compared with the control group. However, no differences in the rate of weight gain were observed after weaning between both the groups. In addition, daily food intake was measured after weaning period until 90 days of age. In the SL group, a significant increase in daily food consumption was observed

Figure 1 Effect of early postnatal overnutrition on body weight during development. Body weight was evaluated in NL (●; $n=12$) and SL (○; $n=12$) groups during suckling period (A) and after weaning (B). Results are expressed as mean $\pm$ s.d. * $P<0.05$, $^{\dagger}P<0.01$, $^{\ddagger}P<0.0001$, compared with NL group.

during this period when compared with the NL group (Fig. 2B), suggesting that the SL group developed a persistent hyperphagia.

Epididymal fat weight and metabolic parameters in the NL and SL groups in adulthood

Epididymal fat weight and metabolic parameters were measured in the SL and NL groups at 90 days of age (Table 1). The SL group presented a statistically significant increase ($\sim 42\%$) in epididymal fat weight. Fasting blood glucose and plasma insulin levels were quite similar in rats from the SL and NL groups. Table 1 also shows no differences in plasma total cholesterol, VLDL cholesterol, LDL cholesterol, HDL cholesterol, and triacylglycerol levels between the SL and NL groups.

Early postnatal overnutrition induces decrease in glucose uptake in adipocytes

Figure 3 shows that, when *in vitro* basal glucose uptake (i.e., the rate in the absence of acute insulin stimulation) was evaluated, similar values were obtained in isolated

Figure 2 Effect of early postnatal overnutrition on rate of weight gain and mean food intake during development. (A) Rate of weight gain was evaluated in NL (●; $n=12$) and SL (○; $n=12$) groups during development. (B) Mean food intake was evaluated in NL (●; $n=10$) and SL (○; $n=10$) groups during development. Results are expressed as mean $\pm$ s.d. * $P<0.05$, $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$, compared with NL group.

adipocytes from the SL and NL groups at 90 days of age. Insulin produced a twofold stimulatory effect on glucose uptake compared with basal values in the NL group, and had no stimulatory effect on adipocytes from the SL group (Fig. 3).

Early postnatal overnutrition induces decrease in insulin-stimulated GLUT-4 translocation and in GLUT-4 and GLUT-1 expression in adipocytes

We analyzed *in vitro* translocation of GLUT-4 to the plasma membrane in adipocytes from the SL and NL groups at 90 days of age after insulin stimulation, by immunofluorescence technique. Figure 4A shows that, in basal conditions, a slightly GLUT-4 immunolabeling is detected in the plasma membrane of adipocytes from both the groups. However, upon insulin stimulation, an increased fluorescence on cell surface was visualized in adipocytes from the NL group (Fig. 4A). In contrast, adipocytes from the SL group presented no changes in GLUT-4 immunofluorescence after acute insulin stimulation when compared with the basal conditions (Fig. 4A). We also evaluated the glucose transporters

Table 1 Epididymal fat weight and metabolic parameters in the normal litter (NL) and small litter (SL) groups in adulthood (90 days of age)

	NL	SL
Epididymal fat weight (g)	3.65 ± 0.40 (n=14)	6.08 ± 1.23 (n=14)*
Glucose (mmol/l)	5.55 ± 0.35 (n=9)	5.24 ± 0.39 (n=11)
Insulin (pmol/l)	400.78 ± 106.07 (n=9)	404.57 ± 122.93 (n=11)
Total cholesterol (mmol/l)	1.74 ± 0.10 (n=8)	1.62 ± 0.15 (n=8)
VLDL cholesterol (mmol/l)	0.68 ± 0.04 (n=8)	0.65 ± 0.05 (n=8)
LDL cholesterol (mmol/l)	0.69 ± 0.05 (n=8)	0.75 ± 0.05 (n=8)
HDL cholesterol (mmol/l)	0.37 ± 0.07 (n=8)	0.32 ± 0.05 (n=8)
Triacylglycerols (mmol/l)	0.81 ± 0.15 (n=8)	0.69 ± 0.11 (n=8)

Results are expressed as mean ± s.d. n, number of animals used. * $P < 0.0001$ comparing SL group with NL group.

expression in isolated adipocytes from the SL and NL groups. Figure 4B shows that early postnatal overnutrition induced a significant decrease in both GLUT-4 and GLUT-1 (40% and 59% respectively) expression in adipocytes from the SL group when compared with the NL group.

Effect of early postnatal overnutrition on IR, IRS-1, PI3K, and Akt expression in adipocytes

IR, IRS-1, PI3K, and Akt protein content was analyzed by western blotting in isolated adipocytes from the SL and NL groups at 90 days of age. Figure 5A shows that IR expression was not affected by early postnatal overnutrition. In contrast, a significant decrease in IRS-1 expression (2.8-fold) was observed in adipocytes from the SL group when compared with the NL group (Fig. 5B). Furthermore, early postnatal overnutrition also induced a decrease (~25%) in PI3K expression (Fig. 5C). However, both the groups presented similar Akt protein content (Fig. 6).

Figure 3 Early postnatal overnutrition induces decrease in glucose uptake in adipocytes. Isolated adipocytes from NL and SL groups at 90 days of age were incubated in the absence (basal state) or presence of insulin (100 nM) before addition of 50 μ M 2-deoxy-D-[14 C] glucose. Results are expressed as mean ± s.d. from independent experiments. * $P < 0.001$, comparing insulin-stimulated group with basal group. $n = 8$ animals from each group.

Effect of early postnatal overnutrition on insulin-stimulated Akt activity in adipocytes

Considering that our results showed that overnutrition condition induces a decreased expression of upstream proteins (IRS-1 and PI3K) in Akt signaling, we decided to analyze Akt activity in these cells. In the basal state, serine phosphorylation of Akt protein was similar in both the groups. As expected, insulin induced an increase in Akt activity (60%) in adipocytes from the NL group (Fig. 6). However, Fig. 6 shows that no differences in serine phosphorylation of Akt were observed between unstimulated and stimulated adipocytes from animals raised in SL.

Glucose tolerance test in the NL and SL groups in adulthood

Glucose tolerance was evaluated after an i.p. glucose load in the SL and NL groups during different periods of time. Similar to the control animals, the overfeeding animals presented elevated glucose circulating levels after 30 min of glucose load (Fig. 7). Blood glucose levels were reduced after 60 min reaching fasting glycemia values after 120 min in both the groups.

Discussion

Clinical studies have shown that overweight during critical developmental periods of neonatal life is associated with adult obesity (Kramer *et al.* 1998, Ong *et al.* 2000, Stettler *et al.* 2005). Additionally, several studies using animal models have demonstrated that overfeeding in the first few days of life leads to long-term obesity, perhaps by promoting alterations in the developing brain and/or in the endocrine system (Plagemann *et al.* 1992, 1999a,b, Velkoska *et al.* 2005). In the present study, early postnatal overnutrition induced by SL size resulted in the increased body weight and development of persisting hyperphagia. Our data demonstrated that early overfeeding induced a dramatic increase in body weight during suckling period. Despite the decrease in the differences between both the groups from 30 to 90 days of age, a significant overweight was observed in SL animals during this period of life. This same profile of weight gain was also found by Zippel *et al.*

Figure 4 Early postnatal overnutrition induces decrease in insulin-stimulated GLUT-4 translocation and in GLUT-4 and GLUT-1 expression in adipocytes. (A) Isolated adipocytes from SL and NL groups were incubated for 10 min in the absence (basal state) or presence of insulin (100 nM). The cells were immunolabeled with anti-GLUT-4 antibody. Immunolocalization of GLUT-4 was visualized by fluorescence microscopy, and images are shown as representative fields from four independent experiments. (B) Whole cellular extracts of isolated adipocytes from SL and NL groups were obtained and GLUT-4 and GLUT-1 detection was performed by western blotting. GLUT-4 and GLUT-1 content was quantified by scanning densitometry of the bands (AU: arbitrary units). Actin content was used as control loading. Results are expressed as mean $\pm$ s.d. * $P < 0.05$, compared with NL group. A representative experiment is shown from three independent experiments.

Figure 5 Effect of early postnatal overnutrition on IR, IRS-1, and PI3K expression in adipocytes. Whole cellular extracts of isolated adipocytes from SL and NL groups were obtained and IR (A; $n=4$), IRS-1 (B; $n=4$), and PI3K (C; $n=6$) detections were performed by western blotting. IR, IRS-1, and PI3K content were quantified by scanning densitometry of the bands (AU: arbitrary units). Actin content was used as control loading (data not shown). Results are expressed as mean $\pm$ s.d. * $P<0.01$, compared with NL group. A representative experiment is shown from three independent experiments.

Figure 6 Effect of early postnatal overnutrition on insulin-stimulated Akt activity in adipocytes. Isolated adipocytes from SL and NL groups were incubated for 10 min in the absence or presence of insulin (100 nM). Total Akt and p-Akt content ($n=6$) were performed by western blotting and then quantified by scanning densitometry of the bands (AU: arbitrary units). Results are expressed as mean $\pm$ s.d. from the ratio between p-Akt and total Akt content. * $P<0.01$, compared with NL group. A representative experiment is shown from two independent experiments.

(2003). However, other studies have demonstrated a significant difference in body weight between the SL and NL groups from weaning until later adulthood (Basset & Craig 1988, Wiedmer *et al.* 2002, Velkoska *et al.* 2005). Although body weight of both the groups was quite similar at 90 days of age, epididymal fat weight from the SL animals was significantly higher (42%) than the NL group, suggesting that early postnatal overnutrition may lead to alterations in body fat composition as well as induce an increased adiposity.

Figure 7 Glucose tolerance test in NL and SL groups in adulthood. Blood glucose levels were evaluated at fasting period and 30, 60, and 120 min after i.p. glucose loading in NL (●; $n=6$) and SL (○; $n=8$) groups. Results are expressed as mean $\pm$ s.d.

Similar to the earlier studies by other investigators using the same experimental model presented here, our results revealed clearly persisting hyperphagia (Plagemann *et al.* 1992, 1999a,b, Velkoska *et al.* 2005). Plagemann *et al.* (1999a,b) have shown that persistent hyperphagia is a consequence of hypothalamic malformation. This alteration may be a consequence, at least in part, of hyperinsulinemia observed during the development period, once insulin becomes an essential organizer of the developing brain, especially of the hypothalamus (Plagemann *et al.* 1999c). It has also been demonstrated that early postnatal overnutrition leads to neonatal hyperinsulinemia (Plagemann *et al.* 1999a). It can be assumed that hyperinsulinemia gives rise to permanent hypoplasia and hypotrophy of the glucoregulatory hypothalamic ventromedial nucleus (VMH), which is considered as a satiety centre, and consequently its hypoactivity can lead to hyperphagia (Dorner *et al.* 1988). On the contrary, several studies have suggested that the adipocyte-derived hormone leptin, in addition to regulating neuronal activity and neuropeptide release and expression, also affects neuronal plasticity in the hypothalamic neurons that are critical to the regulation of food intake and body weight (Bouret *et al.* 2004, Pinto *et al.* 2004). These studies emphasize that this activity is restricted to a neonatal critical period that precedes leptin regulation of food intake in adults (Bouret *et al.* 2004). According to this, previous findings have shown that neonatal hyperleptinemia in rats promotes hypothalamic leptin resistance, overweight, and higher food intake in adulthood (De Oliveira Cravo *et al.* 2002, Toste *et al.* 2006).

Early postnatal overnutrition results in the development of hyperinsulinemia and an increased insulin/glucose ratio in early and later life (Cryer & Jones 1980, You *et al.* 1990, Plagemann *et al.* 1992, 1999a,b). These alterations may lead to peripheral insulin resistance. In the present study, we decided to investigate some metabolic parameters in early adulthood (age 90 days) SL rats. The results showed that blood glucose and plasma insulin levels, as well as plasma lipid levels, are similar between the animals raised in different litter size. However, Boullu-Ciocca *et al.* (2005) described how adult animals (age 120 days) neonatally overfed show increased insulin and insulin/glucose ratio, whereas fasting glycemia was not changed. In agreement with this, it has been shown that neonatally overfed rats present significant metabolic disturbances in adulthood, such as increased systolic blood pressure and impaired glucose tolerance (Cryer & Jones 1980, You *et al.* 1990, Plagemann *et al.* 1992, Boullu-Ciocca *et al.* 2005).

Adipocytes express at least two glucose transporter isoforms, GLUT-1 and GLUT-4, but GLUT-4 is by far the most abundant isoform and appears to be responsible for the acute increase in glucose transport stimulated by insulin (Zorzano *et al.* 1989, Holman *et al.* 1990). In the present work, we found a direct correlation between insulin-stimulated glucose transport and GLUT-1 and GLUT-4 protein levels. Our findings demonstrated a significant decrease in insulin-induced glucose uptake into isolated adipocytes from SL animals. It has been documented that

insulin resistance in obesity and type 2 diabetes is manifested by decreased insulin-stimulated glucose transport and metabolism in adipocytes and skeletal muscle and by impaired suppression of hepatic glucose output (Reaven 1995). In a recent study, Prada *et al.* (2005) reported that early insulin resistance in muscle and hypothalamus, but not in adipose tissue, was observed in rats fed with the Western diet, and they also showed that the compensatory hyperinsulinemia was evident only when altered insulin signaling was installed in the liver. These functional defects may result, in part, from impaired insulin signaling in all three target tissues. It has been observed that downregulation of GLUT-4 is a major factor contributing to the impaired insulin-stimulated glucose transport in adipocytes in all forms of obesity and diabetes (Shepherd & Kahn 1999). In the present study, the impairment in insulin-activated glucose transport could be due to a reduced GLUT-4 translocation and/or expression.

Despite the fact that insulin stimulates GLUT-1 translocation to the cell surface only one to twofold compared with tenfold for GLUT-4 (Kahn & Flier 1990), some studies have shown a correlation between impairment in insulin-activated glucose uptake and reduction in both GLUT-4 and GLUT-1 expression. Here, we show that early overnutrition promoted a low expression of both glucose transporters in adipocytes. According to our results, Pedersen *et al.* (1991) demonstrated that fat feeding caused reduction in insulin-stimulated glucose transport, which was associated with reduction in GLUT-4 and GLUT-1 expression in adipocytes. These authors also reported that acute diabetes in obese rats causes a profound downregulation of glucose uptake and a concomitant reduction of GLUT-1 and GLUT-4 protein levels (Pedersen *et al.* 1992).

A significant low IRS-1 and PI3K expression was also observed in adipocytes from the SL rats. Low IRS-1 expression has been observed to be associated with low GLUT-4 expression and a marked impairment in maximally insulin-stimulated glucose transport in adipocytes from non-obese subjects with a genetic predisposition for type 2 diabetes (Carvalho *et al.* 2001). Changes in the expression of insulin signal transduction components, including PI3K, associated with altered glucose metabolism has also been described in the fat of obese insulin-resistant diabetic mice (Bonini *et al.* 1995). In addition, depletion of GLUT-4 transporters and suppression of encoding mRNA were observed in adipocytes from obese subjects accompanied by a decrease in maximally stimulated glucose transport rate (Garvey *et al.* 1991). These alterations have also been demonstrated in adipose cells from high-fat-fed rats (Pedersen *et al.* 1991). It appears that obese subjects also exhibit a low IRS-1 expression in adipose tissue (Bjornholm *et al.* 2002). These data suggest a possible coordinate regulation of IRS-1, PI3K, and GLUT-4, probably at the level of gene transcription.

Among the effector molecules downstream of PI3K, Akt is strongly implicated in the metabolic action of insulin including glucose uptake and glycogen synthesis (Kohn *et al.* 1996,

Cong *et al.* 1997). Upon insulin treatment, Akt is known to translocate from the cytosol to the plasma membrane where it is primarily activated by phosphorylation at Thr308 and Ser473 (Lawlor & Alessi 2001). Some changes in insulin-stimulated Akt activity can be explained by altered expression, but others are most likely due to altered allosteric activation. In the present work, no alterations were observed in Akt expression in adipocytes from our experimental model. However, the insulin-stimulated activation of Akt was significantly affected. This altered insulin responsiveness is possibly due to low IRS-1 and PI3K expression caused by early overnutrition. It has been reported that, in obese Zucker rats, insulin-stimulated Akt activity was found altered in insulin target tissues (Kim *et al.* 2000). Other studies have also demonstrated a decrease in IRS1/PI3K/Akt pathway in white adipose tissue from the rats submitted to a high-fat diet (Pedersen *et al.* 1991, Prada *et al.* 2005).

Considering the decreased glucose uptake associated with the low IRS-1, PI3K, GLUT-4 and GLUT-1 expression, and impaired Akt activity in adipocytes from the SL group, we investigated the glucose tolerance in these animals. Despite these alterations, our results showed no differences in glucose tolerance test in rats raised under different feeding conditions. However, Plagemann *et al.* (1999b) and Boullu-Ciocca *et al.* (2005) have demonstrated alterations in glucose tolerance test in animals raised in SL at 120 and 250 days of age respectively. These data suggest that early postnatal overfeeding may induce insulin resistance in later life. Concerning our findings, we suggest that adaptive mechanisms involving skeletal muscle and/or hepatic tissue may occur to maintain glucose homeostasis in young animals. For example, the glucose uptake would be increased in muscle tissue and/or hepatic gluconeogenesis would be reduced. According to this, we previously demonstrated that changes in early nutritional status promoted an increased glucose uptake by skeletal muscle, in young rats (60 days of age), associated with an increased GLUT-4 trafficking (De Freitas *et al.* 2003).

In summary, in the present work, we demonstrated that early overnutrition environment leads to a low responsiveness of insulin-stimulated glucose uptake by adipocytes from adult rats. This hyporesponsiveness is a consequence of insulin signaling downregulation caused by a significant decrease in IRS-1, PI3K and GLUT-4 expression, and altered Akt activity. Our findings reinforce a potent and long-term effect of neonatal overfeeding, which can program major changes in the metabolic regulatory mechanisms.

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