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Distinct Regulation of IRS Proteins in Adipose Tissue from Obese Aged and Dexamethasone-Treated Rats

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In the present study, we investigated the protein levels and phosphorylation status of the insulin receptor and insulin receptor substrates (IRS-1, IRS-2, and IRS-3) as well as their association with PI(3)-kinase in the rat adipose tissue of two models of insulin resistance: dexamethasone treatment and aging. AKT and atypical PKC phosphorylation detection were also performed. Both models showed decreased insulin-induced IRS-1 and IRS-2 tyrosine phosphorylation, accompanied by reduced protein levels of IRS-1 and IRS-2. Nevertheless, IRS-3 protein level was unchanged in aging but increased in dexamethasone-treated rats. PI(3)-kinase association with IRS-1 was reduced in aged rats, whereas dexamethasone-treated rats showed a reduced IRS-2/PI(3)-kinase association. However, IRS-3 association with PI(3)-kinase was reduced in both models, as well as insulin-induced AKT and PKC phosphorylation. The alterations described in the present study show that the action of insulin is differently impaired depending on the origin of insulin resistance. These differences might be directly linked to the singular metabolic features of the models we tested.

Key Words: Adipose tissue; insulin receptor substrates; dexamethasone; aging; insulin resistance.

Introduction

There are some key hormones, in particular insulin and catecholamines, that act together in the storage and utilization of energy in the triglyceride depots *(1)*. The capacity of the adipose cell to respond to insulin with a significant increase in glucose uptake is critical to its function *(2)*. Insulin resistance occurs in muscle and fat, leading to decreased glucose uptake and utilization, and in liver, leading to increased glucose production *(3)*. Insulin resistance in adipose cells may be an important component of the overall regulation of glycemia because of the relationship between free fatty acid (FFA) and glucose production, glucose uptake, and insulin release *(4)*. Insulin resistance is a common feature of obesity and predisposes the affected individuals to a variety of diseases, including hypertension, dyslipidemias, cardiovascular problems, and type 2 diabetes mellitus *(5)*.

Aging *(6–10)* and glucocorticoid excess *(11–17)* are both classically associated with insulin resistance. Impaired glucose uptake in adipocytes from aged rats may be attributed to impaired function and depletion of GLUT-4 *(18)*. Buren et al. (2002) described that dexamethasone-impaired insulin-induced glucose uptake of adipocytes in vitro *(19)*.

Insulin receptor substrate (IRS) proteins play a central role in insulin signaling in many tissues as they are the main intracellular substrates for insulin receptor (IR). At least three IRS proteins (IRS-1, -2, and -3) have been identified in adipose tissue and share a similar overall structure *(20)*. All these IRS proteins contain a pleckstrin homology domain and a phosphotyrosine-binding domain at the N-terminus that are related to the kinase activity of the insulin receptor *(21–23)*. The C-terminal regions are less similar to IRS-1 and IRS-2 although they contain many conserved tyrosine phosphorylation motifs responsible for the interaction of IRS proteins with downstream SH2 (src homology 2) domain containing signaling molecules such as PI(3)-kinase *(24)*. These IRS proteins are likely to be redundant in function because they are involved in similar signaling pathways. However, the studies using knockout mice for each one of these IRS proteins demonstrated a particular function for each one of the IRS proteins rather than a redundant role *(25)*. IRS-3 protein is the main substrate for the insulin receptor tyrosine kinase in adipocytes from IRS-1 knockout mice *(26)*. Interestingly, the IRS-3 null mice have a quite normal phenotype with respect to glucose homeostasis and growth *(27)*. Overexpression of IRS-3 in rat adipocytes results in

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Table 1
Characteristics of the Rats Studied

Groups	Body weight (g)	Glycemia (mg%)	Insulin (μU/mL)	K_{itt} (%/min)
Control	248 ± 6[a]	128 ± 11[a]	29 ± 4[a]	3.3 ± 0.4[a]
DEXA	219 ± 10[b]	190 ± 16[b]	46 ± 4[b]	2.2 ± 0.3[b]
13MO	383 ± 11[c]	124 ± 11[a]	44 ± 5[b]	2.7 ± 0.7[b]

The data are represented as the mean ± SEM. Distinct letters mean statistical significance $p < 0.05$.

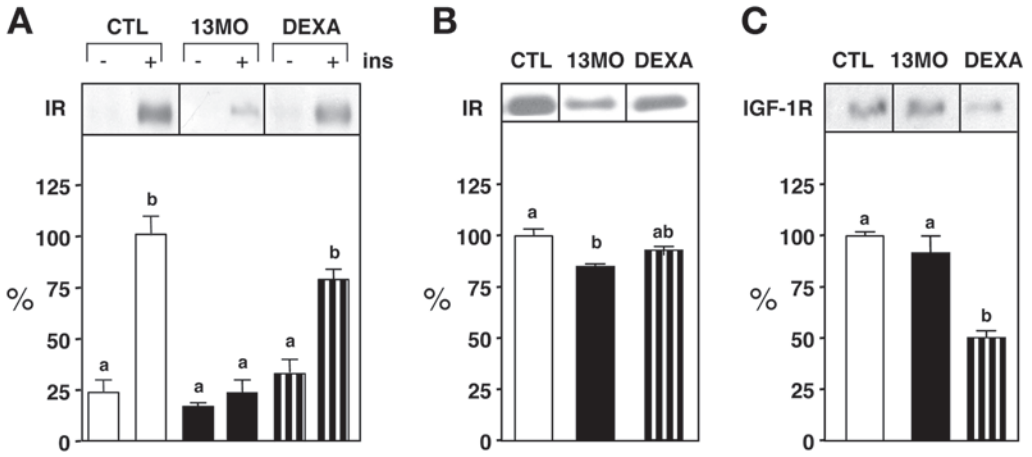

Fig. 1. Effect of aging and dexamethasone treatment in tyrosine phosphorylation degree of insulin receptor (IR) and protein level of IR and insulin-like growth factor-1 receptor (IGF-1R). Adipose tissue extracted from rats was used for immunoblotting analysis. Some tissue samples were submitted to immunoprecipitation with anti-IR antibody, before immunoblotting with antiphosphotyrosine antibody (**A**), with anti-IR (**B**), and with anti-IGF-1R (**C**). One representative blot of nine separate experiments is shown. The results of densitometry analysis are shown and expressed as percentage of the amount of signaling protein in the control situation (e.g., with insulin, +, control, 100%). Data are means ± SEM of nine separate experiments. Distinct letters mean statistical significance, $p < 0.05$.

increased translocation of glucose transporter (GLUT4) in absence of insulin, while the expression of a mutant IRS-3 unable to bind PI(3)-kinase leads to block insulin-induced translocation of GLUT4 *(28)*.

The modulation of IRSs and downstream signaling in adipose tissue of these animal models of insulin resistance still remain undescribed. This information might be helpful to elucidate the molecular basis of particular features observed in each model: for instance, aged rats display increased adipose tissue mass, while glucocorticoid excess decreased body weight *(29,30)*. For this reason, we have investigated the modulation of the insulin intracellular pathway cascade in adipose tissue of obese aged rats and dexamethasone treated rats.

Results

Animal Characteristics

Table 1 summarizes data on body weight, plasma glucose and serum insulin levels, and glucose disappearance rate during an intravenous insulin tolerance test (K_{itt}) in control (2-mo-old), Dexa-treated rats (Dexa) and obese 13-mo-old rats (13MO). Both dexamethasone administration and aging show fasting hyperinsulinemia and insulin resistance characterized by increased fasting insulin levels and a marked decrease in glucose disappearance rate. The dexamethasone treatment induced a 12% reduction in body weight, whereas 13-mo-old rats were obese compared to controls.

Impairment of the Insulin Signaling Cascade in White Adipose Tissue of 13-mo-old and Dexamethasone-Treated Rats

To investigate the tyrosine phosphorylation of insulin receptor β-subunit, IRS-1, IRS-2, and IRS-3 after stimulation by insulin, 2-mo-old (CTL), 13-mo-old (13MO) and dexamethasone-treated rats (Dexa) were injected with saline or insulin, and after 90 s, the epididymal fat pads were removed and homogenized as described in Materials and Methods. The soluble samples were used for immunoprecipitation with anti-insulin receptor antibody or anti-IRS-1, or anti-IRS-2, or anti-IRS-3, and immunoblotted with antiphosphotyrosine antibody. The nitrocellulose membranes were also stripped and reblotted with anti-PI(3)-kinase antibody, to assess the association of these proteins with the IRS-1, IRS-2, and IRS-3. Similarly, 75 μg protein samples were also subjected to SDS-PAGE and immunoblotted with anti-pAKT and anti-pPKCζ to analyze their degree of insulin-induced phosphorylation.

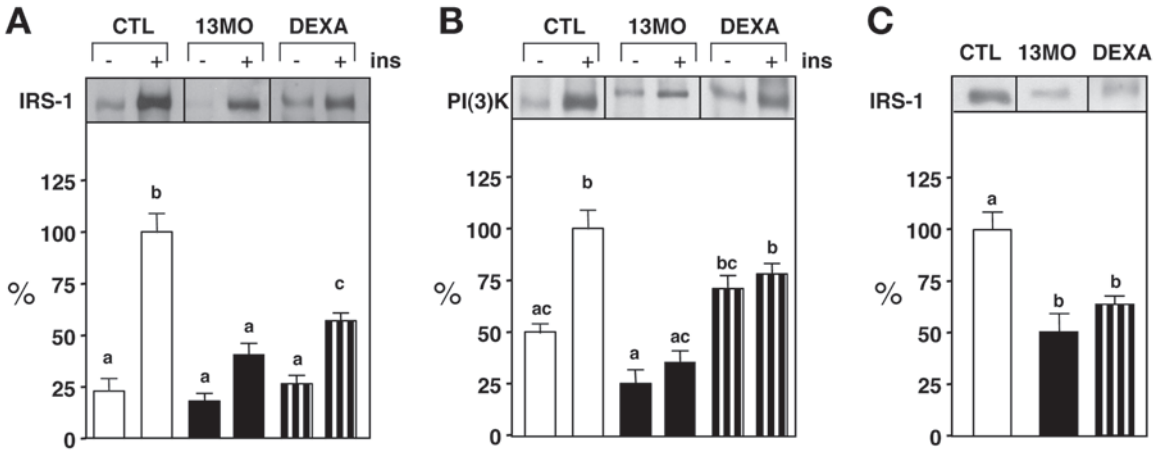

Fig. 2. Effect of aging and dexamethasone treatment in protein level and tyrosine phosphorylation degree of insulin receptor substrate 1 (IRS-1), and association of IRS-1 with PI(3)K. Adipose tissue extracted from rats was used for immunoblotting analysis. Some tissue samples were submitted to immunoprecipitation with anti-IRS-1 antibody before immunoblotting with antiphosphotyrosine antibody (**A**), with anti-PI(3)K (**B**), and with anti-IRS-1 (**C**). One representative blot of nine separate experiments is shown. The results of densitometry analysis are shown and expressed as percentage of the amount of signaling protein in the control situation (e.g., with insulin, +, control, 100%). Data are means ± SEM of nine separate experiments. Distinct letters mean statistical significance, $p < 0.05$.

After insulin stimulation, the extent of IR tyrosine phosphorylation was higher in control than in aging animals (CTL: 100 ± 9%; 13MO: 23 ± 6%; $p < 0.05$), with no difference in adipose tissue of Dexa-treated rats (Fig. 1A). The insulin receptor protein levels in 13-mo-old animals were decreased to 79 ± 1% ($p < 0.05$) of the value found for adipose tissue in control rats, as determined by immunoblotting with anti-IR antibody (Fig. 1B). No difference in the IR protein expression in the adipose tissue of Dexa-treated rats was observed (Fig. 1B).

The IR and IGF-1R are two closely related tyrosine kinases *(31)*. The IR plays a major role in metabolism control, whereas the IGF-1R is mainly involved in growth and differentiation. Contrasting with IR protein levels, IGF-1R values were similar in aging rats and decreased in Dexa-treated rats when compared to controls (CTL: 100 ± 1%; 13MO: 94 ± 12%; Dexa: 53 ± 4%; $p < 0.05$) (Fig. 1C).

A significant decrease in insulin-stimulated IRS-1 tyrosine phosphorylation was observed in adipose tissue of both aging and Dexa-treated rats when compared to controls (CTL: 100 ± 8%; Dexa: 63 ± 10%; 13MO: 37 ± 6%; $p < 0.05$) (Fig. 2A). The analysis of IRS-1 association to the p85 kDa subunit of the enzyme PI(3)K had a significant decrease in aging rats (CTL: 100 ± 11%; 13MO: 40 ± 4%; $p < 0.05$) while it was similar in Dexa-treated rats compared to control rats (Fig. 2B). The IRS-1 protein levels were similarly decreased to 45 ± 6% ($p < 0.05$) in aging rats and to 60 ± 7% ($p < 0.05$) in Dexa-treated rats compared to controls (Fig. 2C).

Insulin-induced IRS-2 tyrosine phosphorylation was reduced to 39 ± 4%, $p < 0.05$, in 13MO rats and to 42 ± 8% ($p < 0.05$; Fig. 3A) in Dexa-treated rats. However, the IRS-2/PI(3)K association induced by insulin infusion was maintained in aged rats and decreased in Dexa-treated rats to 47 ± 9% ($p < 0.05$), when compared to controls (Fig. 3B). The IRS-2 protein level was reduced in both models to similar extents (CTL: 100 ± 10%, 13MO: 63 ± 9%, Dexa: 64 ± 4%; $p < 0.05$; Fig. 3C).

A reduction in the insulin-induced IRS-3 tyrosine phosphorylation to 33 ± 7%, $p < 0.05$, was observed in 13MO rats when compared to control rats (Fig. 4A). In contrast to that detected for the former IRS proteins, the dexamethasone treatment had no impact in the tyrosyl phosphorylation content of IRS-3. However, the insulin-induced association of IRS-3 with PI(3)K was reduced in both animal models when compared to controls (CTL: 100 ± 10%; 13MO: 65 ± 1%; Dexa: 59 ± 4%; $p < 0.05$) (Fig. 4B). The IRS-3 protein level was similar to controls in the 13MO rats, but was increased to 128 ± 4% in the dexamethasone treated rats ($p < 0.05$, Fig. 4C).

The p85 subunit of PI(3)-kinase protein expression was reduced to 70 ± 8%, $p < 0.05$, in 13MO rats while it was similar in control and Dexa-treated rats (Fig. 5A).

It is well established that insulin-induced association/activation of PI(3)-kinase with IRS proteins are accompanied by an increase in AKT serine phosphorylation *(32,33)* and PKCζ/λ *(34,35)*. The 13MO rats and Dexa-treated rats showed a decreased in insulin-induced AKT serine phosphorylation when compared to control rats (CTL: 100 ± 8%, 13MO: 56 ± 8%, Dexa: 54 ± 7%; $p < 0.05$; Fig. 5B) and in insulin-induced rats the PKCζ phosphorylation (CTL: 100 ± 5%, 13MO: 57 ± 10%, Dexa: 61 ± 7%; $p < 0.05$; Fig. 5C).

Discussion

The family of IRS proteins is among the most studied insulin receptor substrates *(36)*. We analyzed the insulin-

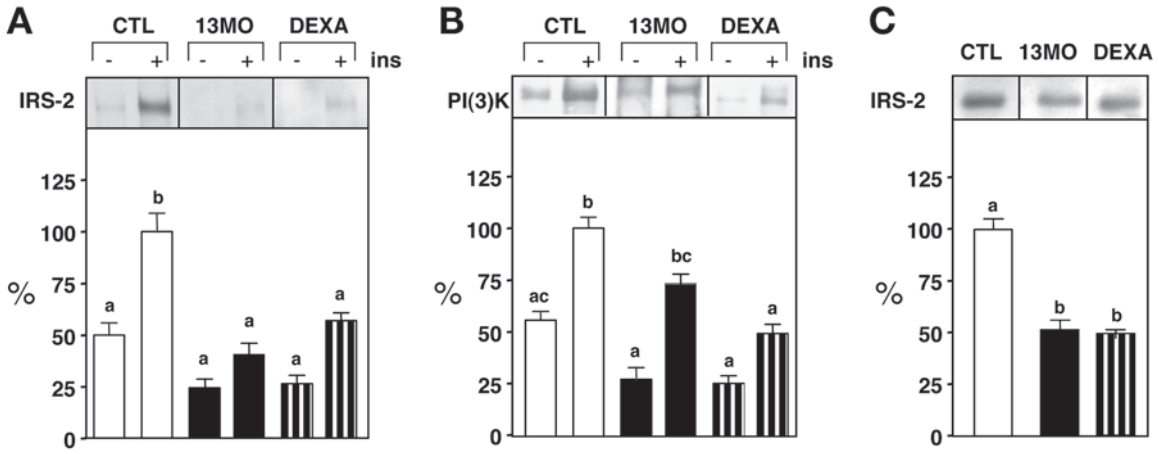

Fig. 3. Effect of aging and dexamethasone treatment in protein level and tyrosine phosphorylation degree of insulin receptor substrate 2 (IRS-2), and association of IRS-2 with PI(3)K. Adipose tissue extracted from rats was used for immunoblotting analysis. Some tissue samples were submitted to immunoprecipitation with anti-IRS-2 antibody before immunoblotting with antiphosphotyrosine antibody (**A**), with anti-PI(3)K (**B**), and with anti-IRS-2 (**C**). One representative blot of nine separate experiments is shown. The results of densitometry analysis are shown and expressed as percentage of the amount of signaling protein in the control situation (e.g., with insulin, +, control, 100%). Data are means ± SEM of nine separate experiments. Distinct letters mean statistical significance, $p < 0.05$.

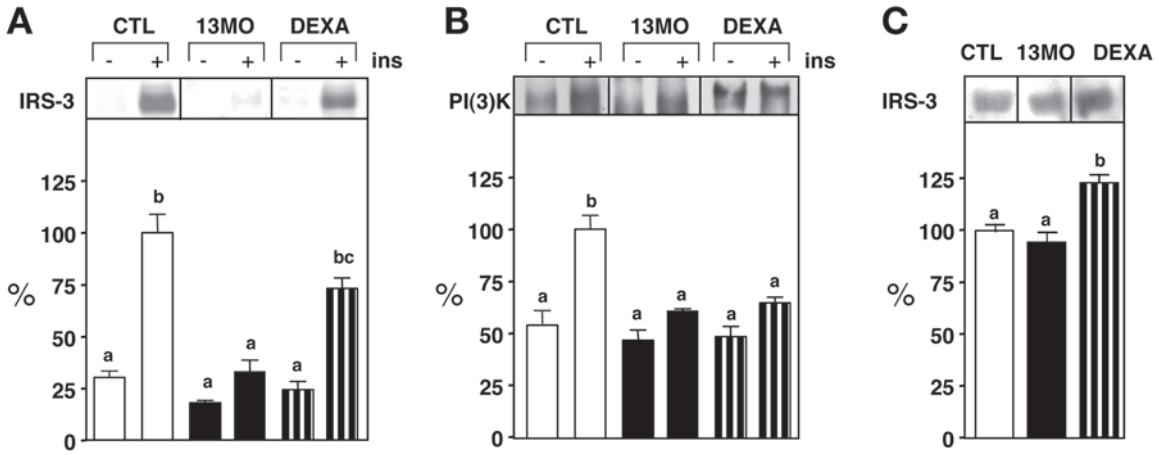

Fig. 4. Effect of aging and dexamethasone treatment in protein level and tyrosine phosphorylation degree of insulin receptor substrate 3 (IRS-3), and association of IRS-3 with PI(3)K. Adipose tissue extracted from rats was used for immunoblotting analysis. Some tissue samples were submitted to immunoprecipitation with anti-IRS-3 antibody before immunoblotting with antiphosphotyrosine antibody (**A**), with anti-PI(3)K (**B**), and with anti-IRS-3 (**C**). One representative blot of nine separate experiments is shown. The results of densitometry analysis are shown and expressed as percentage of the amount of signaling protein in the control situation (e.g., with insulin, +, control, 100%). Data are means ± SEM of nine separate experiments. Distinct letters mean statistical significance, $p < 0.05$.

induced phosphorylation of insulin receptor β chain, IRS-1, IRS-2, IRS-3, AKT, and atypical PKC in the adipose tissue of rats chronically treated with dexamethasone, a shorter model of pharmacological-induced insulin resistance, and in obese 13-mo-old aged rats, a physiological and chronic model of insulin resistance. Aging and dexamethasone treatment are classical models of insulin resistance and, in this work, insulin resistance was demonstrated by a reduced glucose disappearance rate. It is important to emphasize that both models of insulin resistance attend with hyperinsulinemia, and increased insulin levels in animals or cell cultures may change early events of insulin action *(37–39)*.

Hypercortisolism is associated with increased glucose production by the liver, decreased transport and utilization of peripheral glucose, decreased protein synthesis, increased protein degradation in muscle, and decreased lipogenesis in adipocytes of rats *(11,13,14,40)*. The insulin resistance that develops along aging in rats is associated with increased glucose intolerance, decreased inhibition of hepatic glucose production *(7)*, decrease of glycogen synthesis in muscle *(9)*, with current evidence suggesting that there is no clear defect in insulin secretion in aging *(41,42)* and the effects of insulin on whole-body amino-acid and protein metabolism do not seem to be impaired in the aged *(41)*.

Studies of mice with target disruption of IRS genes lend some support for the physiological roles of IRS proteins. Studies with IRS-1 knockout mice suggest that IRS-1 plays a key role in relaying the growth-stimulating effects of insulin and insulin-like growth factor *(43,44)*. The absence of

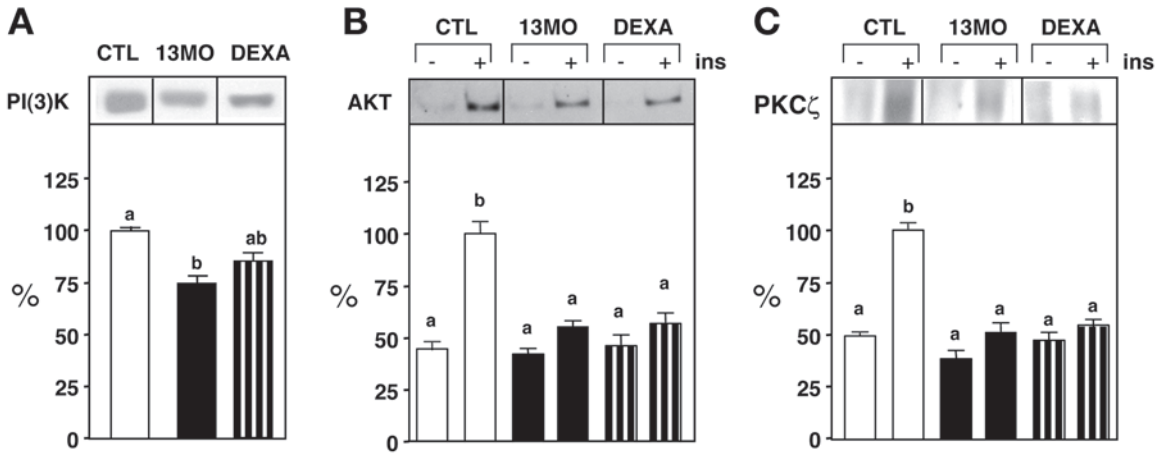

Fig. 5. Effect of aging and dexamethasone treatment in protein level of PI(3)K and phosphorylation degree of AKT and atypical PKCζ. Adipose tissue extracted from rats was used for immunoblotting analysis. Some aliquots containing 75 μg total protein were immunoblotted with anti-PI(3)K (**A**), or with phosphoserine-AKT (pAKT, **B**), or with anti-phospho-atypical PKC (pPKCζ, **C**) antibodies. One representative blot of nine separate experiments is shown. The results of densitometry analysis are shown and expressed as percentage of the amount of signaling protein in the control situation (e.g., with insulin, +, control, 100%). Data are means ± SEM of nine separate experiments. Distinct letters mean statistical significance, $p < 0.05$.

IRS-2 also produces insulin resistance, and in addition, a defect in β-cell proliferation, leading to diabetes at a young age *(45)*. In cultured brown adipocytes, loss of IRS-1 results in a failure of differentiation and lipid accumulation, whereas loss of IRS-2 results in a defect in insulin-stimulated glucose transport, despite normal differentiation *(46,47)*. These data indicate that both IRS-1 and IRS-2 play significant and nonredundant roles in growth and regulation of glucose homeostasis.

The models investigated in the present study showed specific patterns of IRS proteins level, phosphorylation, and association with PI(3)-kinase. While aging rats present decreased insulin-induced tyrosine phosphorylation of IRS-1 and IRS-3 and their association with PI(3)-kinase, the treatment with dexamethasone reduced insulin-induced IRS-2 phosphorylation and association with PI(3)-kinase. The decreased protein level of IRS-1 and IRS-2 detected in both animal models could be due to at least two mechanisms: increased degradation and/or reduced synthesis. However, there is evidence in the literature indicating that proteasome-mediated degradation of IRS-1 and IRS-2 are associated to obesity, aging *(48,49)*, and hyperinsulinemic states *(50)*. On the other hand, incubation of 3T3-L1 adipocytes with dexamethasone induces reduction of IRS-1 mRNA synthesis *(51)*. Therefore, at least in part the decreased IRS-1 and IRS-2 protein level in adipose tissue of aging and dexamethasone-treated rats might be mediated by proteasome activation.

Although IRS-3 is abundant in adipocytes and its mRNA is also detected in liver, heart, lung, and kidney *(52)*, it seems to be less important or perhaps redundant in terms of insulin effects on growth, development, and glucose homeostasis *(27)*. However, mice with combined deficiency of IRS-1 and IRS-3 result in development of early-onset lipoatrophy associated with marked hyperglycemia, hyperinsulinemia, insulin resistance, glucose intolerance, and islet hyperplasia *(25)*. Thus, IRS-3 has a physiological and complementary function in metabolism and development of white adipose tissue (adipogenesis) that is not clearly understood.

IRS-3 and IGF-1R showed different patterns of expression in the models herein. In the aged rats the IRS-3 and IGF-1R protein levels were similar to control young rats. On the other hand, dexamethasone treatment promotes higher protein level of IRS-3 and lower protein level of IGF-1R. These differences regarding IRS-3 and IGF-1R protein level could have an interesting association with the weight variation between these animal models: weight loss in the dexamethasone treatment and increased adipose mass in the aged rat. Considering that the heterozygous knockout mice for IGF-1R develop a modest growth deficit *(53)*, we suggest that the reduced IGF-1R protein level detected in adipose tissue of the dexametasone-treated rats may counter regulate the potential expected adipogenic effects of the increased IRS-3 protein level. Additionally, IRS-3 might contribute to insulin resistance by negatively regulating the tyrosine phosphorylation from other IRS proteins *(54)*.

Despite the absence of evidence in the literature describing a mechanism for IRS-3 degradation, the increased IRS-3 protein content observed in dexamethasone-treated rats could be consequence of increased IRS-3 gene transcription or even decreased protein degradation. However, further experiments are necessary to confirm such suggestions.

PhoshatidylinositoI (3)-kinase is likely to be a major mediator of AKT activation, which is important for many of the physiological responses to insulin *(32,33,55,56)*. PI(3)-kinase signaling diverges, at least, to activate atypical PKCζ, which contributes to GLUT4 translocation *(34,35)*. Our results suggest that in the adipose tissue of aged rats the

insulin resistance is related to reduction in both IRS-1/PI(3)K and IRS-3/PI(3)K pathway, while in the insulin resistance induced by dexametahose treatment the reduced IRS-2/PI(3)K pathway may mediate this effect.

Summarizing, we showed that insulin resistance in adipose tissue of dexamethasone-treated and aged rats may be linked to reduced insulin-induced AKT and atypical PKC phosphorylation. Aging displays reduced insulin-induced tyrosine phosphorylation of IRS-1 and IRS-3 as well as reduced IRS-1/PI(3)-kinase and IRS-3/PI(3)-kinase association, while dexamethasone treatment induces reduced IRS-2 phosphorylation and IRS-2/PI(3)-kinase association. Additionally, we showed that IRS-3 is differently regulated by dexamethasone treatment and aging. These differences and similarities might be helpful for the better understanding of the genesis and mechanisms underlying insulin resistance in different situations.

Materials and Methods

Materials

The reagents and apparatus for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoprecipitation, and immunoblotting were from Bio-Rad (Richmond, CA, USA). Tris [hydroxymethyl]amino-methane (Tris), phenylmethylsulfonylfluoride (PMSF), aprotinin, dithiothreitol (DTT), Triton X-100, Tween-20, and glycerol were obtained from Sigma Chemical Co. (St. Louis, MO). Human insulin was from Biobrás (Minas Gerais, Brazil). Enhanced chemiluminescence (ECL) detection system, protein A Sepharose 6MB, and nitrocellulose membrane (0.45 mm) were from Amersham Pharmacia Biotech (Uppsala, Sweden). Monoclonal anti-IRS-3 and anti-PI(3)-kinase (p85; phosphatidylinositol 3-kinase) antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-phosphoserine-AKT (protein kinase B) was obtained from Cell Signaling (Beverly, MA). Anti-IR, anti-IRS-1, anti-IRS-2, anti-pPKCζ (protein kinase C zeta), anti-IGF-1R and antiphosphotyrosine antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals

Two-mo-old (CTL) and obese 13-mo-old (13MO) male Wistar rats, obtained from the ICB/USP Central Animal Breeding Center (São Paulo SP, Brazil), were provided with standard rodent chow and water *ad libitum*. Food was withdrawn 12–14 h before the adipose tissue extraction experiments. To examine the influence of chronic hypercortisolism 2-mo-old rats received dexamethasone for 5 consecutive days (1 mg/kg of body weight each day, ip) and the experiments were performed on the morning of the sixth day. All studies were performed in parallel using control and dexamethasone-treated (Dexa-treated) rats or control and aged rats. The procedures with animals were conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Epididymal Fat Extracts

Rats were anesthetized with sodium thiopental (25 mg/kg, ip) and used 10–15 minutes later, as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the portal vein was exposed and 0.5 mL saline (0.9% NaCl) with or without 10^{-5} *M* insulin was injected. We previously detected that the maximum rate of insulin-induced tyrosine phosphorylation of insulin receptor β-subunit in adipose tissue of rats was at 90 s after administration of insulin into the portal vein, as well as to its substrates IRS-1, -2, and -3. In this regard, the epididymal fat pads were removed 90 s after the infusion and homogenized immediately in an ice-cold extraction buffer [1% Triton-X 100, 100 m*M* Tris (pH 7.4), 10 m*M* EDTA, 100 m*M* sodium pyrophosphate, 100 m*M* sodium fluoride, 10 m*M* sodium vanadate, 2 m*M* phenylmethylsulfonylfluoride, and 0.1 mg aprotinin/mL], maintained at 4°C, with a Polytron PTA 20S generator (model PT 10/35, Brinkmann Instruments, Inc., Westbury, NY) operated at maximum speed for 30 s.

The extracts were centrifuged at 15,000*g* at 4°C for 20 min to remove insoluble material. The supernatant of these tissues, containing 3 mg of protein, was used for immunoprecipitation with anti-IR, anti-IRS-1, anti-IRS-2, anti-IRS-3, and protein A-Sepharose 6MB before Laemmli sample buffer *(57)* containing 100 m*M* dithiothreitol treatment. Similar size samples (75 μg) with Laemmli sample buffer were used for total extracts. Samples were heated in a boiling water bath for 5 min, after which they were subjected to SDS-PAGE (6.5% *bis*-acrylamide).

Protein Analysis by Immunoblotting

Electrotransfer of proteins from gel to nitrocellulose was performed for 90 min at 120 V (constant) as described by Towbin et al. *(58)* except for the addition of 0.02% SDS to the transfer buffer to enhance the elution of high-molecular-mass proteins. Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter overnight at 4°C in blocking buffer (5% nonfat dry milk, 10 mm Tris, 150 mm NaCl, and 0.02% Tween-20). The nitrocellulose blot was incubated with antiphosphotyrosine (1:400), anti-IR (1:400), anti-IRS-1 (1:400), anti-IRS-2 (1:400), anti-IRS-3 (1:2000), the p85 subunit of PI(3)-kinase (1:2000), anti-pPKCζ (1:400), phosphoserine-AKT (1:1000), and anti-IGF-1R (1:400) antibody diluted in blocking buffer (3% BSA instead of nonfat dry milk) overnight at 4°C; and then washed for 30 min with blocking buffer without milk.

Bound antibodies were detected with horseradish peroxidase-conjugated anti-IgG (1:10000) and visualized with ECL detection system. HRP-conjugated anti-IgG bound to the antibodies was detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak Co., Rochester, NY). Band intensities were quantitated from the developed autoradiographs using Scion Image program (www.scioncorp.org).

Other

Insulin was determined by a standard radioimmunoassay. Some animals in the three groups also underwent an intravenous insulin tolerance test. Twenty microliters of blood were collected from the tail of anesthetized rats at 0 (basal), 4, 8, 12, and 16 min after 10^{-5} *M* insulin infusion into the portal vein. The blood glucose level was measured by glucose oxidase using a commercial kit from Labtest (Guarulhos, Brazil). The K_{itt} (glucose disappearance rate) was calculated from the formula $0.693/t_{1/2}$. The glucose $t_{1/2}$ was calculated from the slope of the least square analysis of blood glucose concentration during linear phase of decline *(59)*.

Statistical Analysis

Experiments were always performed using samples from aged (13MO) or dexamethasone-treated (Dexa) animals in parallel with a control group (CTL). Comparisons were made using one way ANOVA, with Tukey post-test, and two-way ANOVA, with Bonferroni post-test. The level of significance was set at $p < 0.05$.

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