Endocrine (1995) 3, 755–759

Modulation of early steps in insulin action in the liver and muscle of epinephrine treated rats

Mario J.A. Saad, Luiz G.C. Hartmann, Daniela S. de Carvalho, Cesar A.O. Galoro, Sigisfredo L. Brenelli & Carla R.O. Carvalho

Department of Internal Medicine, FCM, UNICAMP, Campinas, SP, 13081-970, Brazil

Epinephrine is known to produce insulin resistance, but the exact molecular mechanism involved is unknown. In the present study we have examined the levels and phosphorylation state of the insulin receptor and of insulin receptor substrate 1 (IRS-1), as well as the association between IRS-1 and phosphatidylinositol 3-kinase (PI 3-kinase) in the liver and muscle of rats treated with epinephrine. The results demonstrate a decrease in insulin-stimulated receptor and IRS-1 phosphorylation levels which was accompanied by a reduction in the association of IRS-1 with PI 3-kinase *in vivo* in liver and muscle of epinephrine treated rats. These data suggest that molecular post-receptor defects may explain some aspects of the insulin resistance induced by catecholamines.

Keywords: epinephrine; insulin resistance; insulin receptor; insulin receptor substrate; phosphatidylinositol 3-kinase

Introduction

Insulin initiates its metabolic and growth-promoting effects by binding to the α subunit of its tetrameric receptor, thereby activating the kinase in the β subunit (Kasuga *et al.*, 1982; Rosen, 1987). This interaction catalyzes the intramolecular autophosphorylation of specific tyrosine residues of the β subunit further enhancing the tyrosine kinase of the receptor toward other protein substrates (White *et al.*, 1985). In most cells, this primary event leads to the subsequent tyrosyl phosphorylation of a cytoplasmic protein with an apparent molecular weight between 165 kDa and 185 kDa, called insulin receptor substrate 1 (IRS-1) (White *et al.*, 1985; Rothenberg *et al.*, 1991; Sun *et al.*, 1991; Wang *et al.*, 1993). Considerable evidence indicates that insulin receptor tyrosine kinase and the associated IRS-1 phosphorylation are essential for many, if not all, of the biological effects of insulin (Ebina *et al.*, 1987; Rosen, 1987; Maegawa *et al.*, 1988). In cells in culture and *in vitro*, phosphorylated IRS-1 associates with the lipid metabolizing enzyme phosphatidylinositol 3-kinase (PI 3-kinase) thus activating the enzyme (Backer *et al.*, 1992; Folli *et al.*, 1992). Thus, the insulin receptor, IRS-1 and PI-3-kianse represent three of the earliest steps in insulin action, and each of these can be demonstrated in two of the main target tissues for the metabolic actions of insulin *in vivo*, namely liver and muscle (Folli *et al.*, 1992). Recently, we demonstrated alterations in these early steps in insulin action in liver and muscle of insulin resistant animals (Saad *et al.*, 1992, 1993; Folli *et al.*, 1993).

It has long been known that an excess of epinephrine causes insulin resistance (Chiasson *et al.*, 1981; Kirsch *et al.*, 1983; Pessin *et al.*, 1983; Wallberg-Henriksson, 1987; Bonen *et al.*, 1992). Catecholamines antagonize the action of insulin by stimulating gluconeogenesis, glycogenolysis and lipolysis and by inhibiting peripheral glucose use via a β-adrenergic mechanism that may also involve a decrease in cellular glucose transport (Pessin *et al.*, 1983). High intracellular cyclic AMP-levels seem to induce insulin resistance at both receptor and post-receptor levels (Kirsch *et al.*, 1983; Joost *et al.*, 1986; Kuroda *et al.*, 1987). Using purified receptors and artificial substrates *in vitro*, decreased insulin receptor phosphorylation and kinase activity have been observed as a consequence of increased cAMP and cAMP kinase (Stadmauer & Rosen, 1986; Roth & Beaudoin, 1987). The role of the phosphorylation of IRS-1 the endogenous substrate for the insulin receptor, and its association with PI 3-kinase in insulin resistance induced by catecholamines have not yet been examined. In the present study we have investigated the phosphorylation state of the insulin receptor and of IRS-1 as well as the association of the latter with PI 3-kinase in the liver and muscle of epinephrine-treated rats stimulated with insulin.

Correspondence: Mario J.A. Saad
Received 1 June 1995; accepted 24 July 1995

Results

The effect of epinephrine on insulin receptor and IRS-1 phosphorylation in rat muscle

Following *in vivo* stimulation with insulin, a phosphotyrosine band of 95 kDa, previously identified as the insulin receptor β-subunit, appeared and became prominently phosphorylated. The level of phosphorylation of this band was reduced to 47 ± 4% ($P<0.01$) in animals treated with epinephrine (Figure 1A). In the same anti-phosphotyrosine blots of whole tissue extracts, in addition to the 95 kDa band seen after insulin injection, a broad band migrating between 165 and 185 kDa was also detectable. This band is known as pp185 and contains IRS-1 as one of its components (Araki, 1994; Tamemoto, 1994). The phosphorylation of pp185 was reduced to 42 ± 3% ($P<0.001$) in animals treated with epinephrine (Figure 1A).

In samples from muscle previously immunoprecipitated with anti-IRS-1 antibody and immunoblotted with anti-phosphotyrosine antibody, there was a clear decrease in insulin stimulated IRS-1 phosphorylation to 35 ± 10% ($P<0.01$) in epinephrine-treated rats when compared to the controls (Figure 1B and 1D).

Acute epinephrine treatment had no effect on the insulin receptor and IRS-1 protein levels in muscle as determined by immunoblotting with an antibody to the C-terminus of the insulin receptor and an antibody to the C-terminus of IRS-1, respectively (Figure 1D).

Previous studies (Backer *et al.*, 1992; Folli *et al.*, 1992; Hadari *et al.*, 1992; Lavan *et al.*, 1992; Yonezawa *et al.*, 1992; Giorgetti *et al.*, 1993; Kelly & Ruderman, 1993; Kuhnne *et al.*, 1993; Skolnik *et al.*, 1993) have suggested that there is a relatively stable, high affinity interaction between IRS-1 and the 85 kDa subunit of the PI 3-kinase, such that both proteins are coprecipitated by antibodies to either protein. In samples from muscle previously immunoprecipitated with anti-IRS-1 antibody and immunoblotted with antibodies directed against the 85 kDa subunit of PI 3-kinase, there was little or no detectable PI 3-kinase immunoreactivity in the

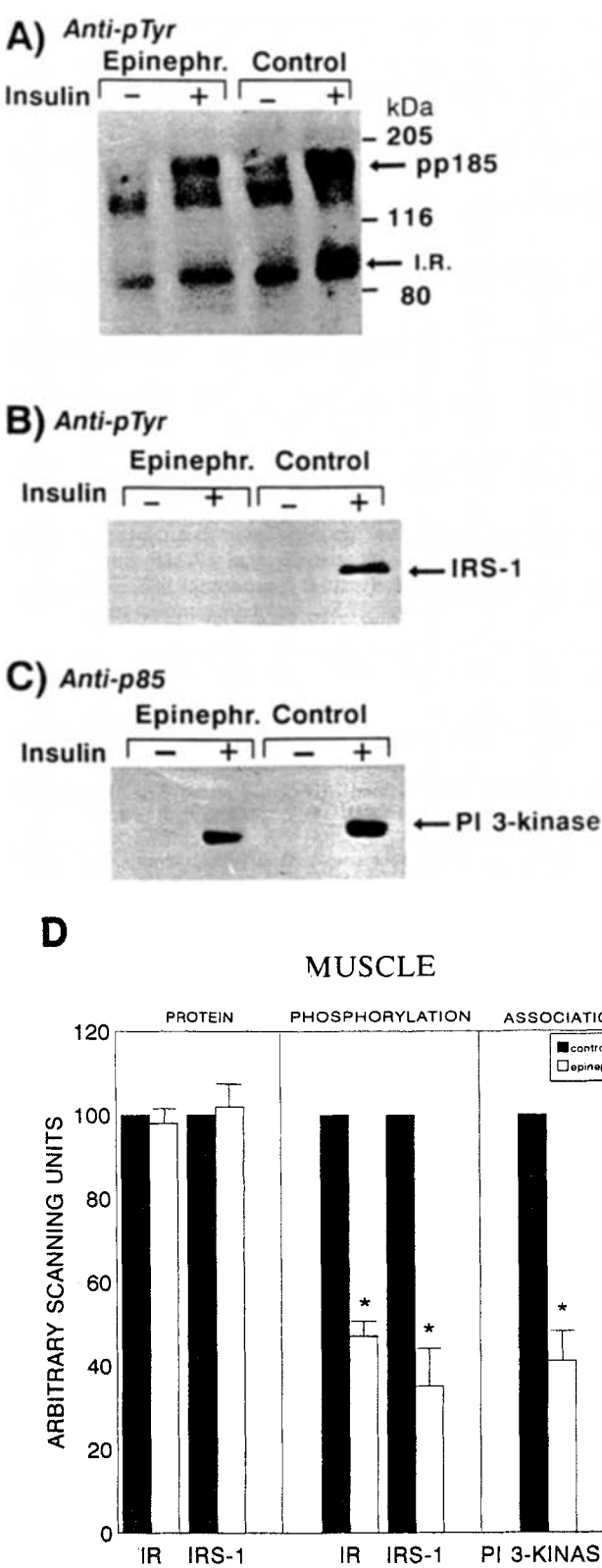

Figure 1 Insulin-stimulated tyrosine phosphorylation in the intact muscle from epinephrine-treated rats. Rats were anesthetized, and the abdominal wall incised to expose the viscera. Normal saline (lanes 1 and 3) or 10^{-5} M insulin (lanes 2 and 4) was infused into portal vein as a bolus injection and 90 s later muscle was excised and homogenized in extraction buffer A at 100°C as described in Methods. After centrifugation, aliquots containing the same amounts of protein were resolved on 6% SDS-polyacrylamide gel, transferred to nitrocellulose, and detected with anti-phosphotyrosine antibody (**A**), anti-IRS-1 antibody, or anti-insulin receptor antibody, and [^{125}I]protein A and subjected to autoradiography. (**B** and **C**) show the immunoblotting of tyrosyl phosphorylated proteins and PI 3-kinase in anti-IRS-1 immunoprecipitates from muscle of control and epinephrine-treated rats. The muscle proteins were extracted and processed in extraction buffer B as described in Methods and then incubated at 4°C with anti-IRS antibody and Protein A-sepharose 6MB. Immunoprecipitated proteins were analysed by immunoblotting with anti-phosphotyrosine (**B**) and anti-PI 3-kinase (85 kDa subunit) (**C**) antibodies and [^{125}I]protein A and subjected to autoradiography. The data shown are representative of several independent experiments. (**D**) Insulin receptor and IRS-1 protein levels and tyrosine phosphorylation in the muscle of control and epinephrine-treated rats. Scanning densitometry of autoradiograms was performed on seven experiments for insulin receptor and IRS-1 concentration, six experiments for tyrosine phosphorylation levels of both and four experiments for association of PI 3-kinase with IRS-1. The data are expressed as mean ± SEM and are normalized per protein. *Significant differences at least at $P<0.05$

basal state in either the normal or epinephrine-treated animals (Figure 1C). After insulin stimulation, a band with the expected molecular weight of the regulatory subunit of the PI 3-kinase (85 kDa) was present in anti-IRS-1 antibody immunoprecipitates of muscle from both groups of rats. This observation is consistent with a stable association of IRS-1 and PI 3-kinase. However, the amount of PI 3-kinase associated with IRS-1 was reduced to 41 ± 8% ($P<0.05$, $n=4$) in epinephrine-treated rats, thus suggesting a reduced association between IRS-1 and PI 3-kinase (Figure 1D).

The effect of epinephrine treatment on insulin receptor and IRS-1 phosphorylation in rat liver

Following insulin treatment, there were changes in the phosphorylation of the insulin receptor and pp185 in the liver of rats treated with epinephrine. After insulin, the extent of phosphorylation of insulin receptor was reduced by 57 ± 4% ($P<0.01$) in epinephrine-treated rats compared to the controls (Figure 2A and D). In agreement with this, subsequent to the administration of insulin the tyrosine phosphorylation of pp185 decreased to 40 ± 12% of the controls ($P<0.05$) in animals treated with an excess of catecholamine (Figure 2A). As determined by immunoblotting there was no change in the hepatic insulin receptor and IRS-1 protein levels of animals treated acutely with epinephrine (Figure 2D).

To better define the levels of IRS-1 phosphorylation, we performed Western blot analysis of tyrosyl-phosphorylated proteins in anti-IRS-1 immunoprecipitates before and after stimulation with insulin in both groups of animals. Figure 2B and D show that there is a marked reduction to 31 ± 9% ($P<0.01$) of the control values of insulin-stimulated IRS-1 phosphorylation in the liver of animals previously treated with epinephrine. To examine the association of the 85 kDa subunit of PI 3-kinase with IRS-1, the same blot was incubated with antibodies to this subunit. As expected, in both groups, a band of 85 kDa was present in the IRS-1 immunoprecipitates after exposure to insulin, although there was a 67 ± 8% ($P<0.01$, $n=4$) decrease in the intensity of this band in epinephrine-treated rats (Figure 2C and D). This result suggests that administration of epinephrine reduces the association between IRS-1 and PI 3-kinase.

Discussion

In the present study we investigated the importance of insulin receptor and IRS-1 phosphorylation and of the association of PI 3-kinase with IRS-1 in the liver and muscle of rats treated with epinephrine. It is known that epinephrine induces marked insulin resistance (Chiasson *et al.*, 1981; Kirsch *et al.*, 1983; Pessin *et al.*, 1983; Wallberg-Henriksson, 1987; Bonen *et al.*, 1992) and that this is accompanied by a reduced glucose uptake in peripheral tissues and an increase in hepatic glucose output. Our results indicate that epinephrine

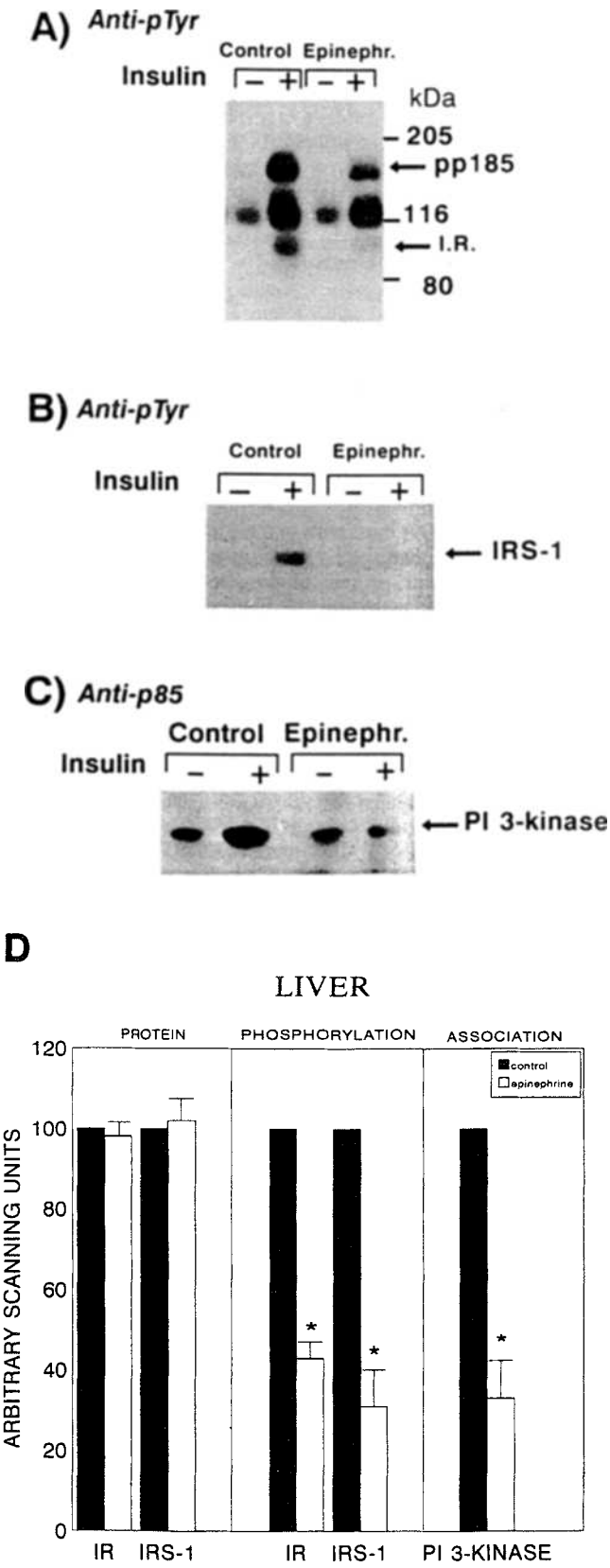

Figure 2 Insulin-stimulated tyrosine phosphorylation in the intact liver from epinephrine-treated rats. Rats were anesthetized, and the abdominal wall incised to expose the viscera. Normal saline (lanes 1 and 3) or 10^{-5} M insulin (lanes 2 and 4) was infused into portal vein as a bolus injection and 30 s later liver were excised and homogenized in extraction buffer A at 100°C as described in Methods. After centrifugation, aliquots containing the same amounts of protein were resolved on 6% SDS-polyacrylamide gel, transferred to nitrocellulose, and detected with anti-phosphotyrosine antibody (**A**), anti-IRS-1 antibody or anti-insulin receptor antibody, and [^{125}I]protein A and subjected to autoradiography. (**B** and **C**) show the immunoblotting of tyrosyl phosphorylated proteins and PI 3-kinase in anti-IRS-1 immunoprecipitates from liver of control and epinephrine-treated rats. The liver proteins were extracted and processed in extraction buffer B as described in Methods and then incubated at 4°C with anti-IRS antibody and Protein A-sepharose 6MB. Immunoprecipitated proteins were analysed by immunoblotting with anti-phosphotyrosine (**B**) and anti-PI 3-kinase (85 kDa subunit) (**C**) antibodies and [^{125}I]protein A and subjected to autoradiography. The data shown are representative of several independent experiments. (**D**) Insulin receptor and IRS-1 protein levels and tyrosine phosphorylation in the liver of control and epinephrine-treated rats. Scanning densitometry of autoradiograms was performed on six experiments for insulin receptor and IRS-1 concentration, five experiments for tyrosine phosphorylation levels of both and four experiments for PI 3-kinase associated with IRS-1. The data are expressed as mean ± SEM and are normalized per protein. *Significant differences at least at $P<0.05$

has no significant effect on the levels of the insulin receptor in rat liver and muscle. Despite the normal number of receptors, there was a 57% reduction in insulin receptor autophosphorylation in liver and a 53% reduction in muscle after stimulation with insulin. This observation is consistent with previous studies which demonstrated that increasing the cAMP content of cells alters the phosphorylation state and protein kinase activity of the insulin receptor (Stadmauer & Rosen, 1986; Roth & Beaudoin, 1987). Weber *et al.* (1991) demonstrated that isoproterenol markedly reduced insulin-stimulated [^{32}P] phosphate incorporation into the plasma membrane receptor β subunit, in parallel to a change in glucose transporter intrinsic activity (lowered maximal activity) and a decrease in insulin sensitivity.

The phosplorylation of IRS-1 (reflecting the kinase activity of the insulin receptor towards its endogenous substrate) was reduced by 69% in the liver and by 65% in the muscle of epinephrine-treated rats. Interestingly, PI 3-kinase associated with IRS-1 was also reduced in the liver and muscle of animals exposed to an excess of catecholamine. This decrease in the three early steps of insulin action after binding may explain some aspects of the insulin resistance observed in animals treated with catecholamines. Recently, it was demonstrated that mice homozygous for target disruption of the IRS-1 gene had resistance to the glucose-lowering effects of insulin (Araki *et al.*, 1994; Tamemoto *et al.*, 1994). This correlated with a marked reduction in insulin-stimulated glucose transport in isolated adipocytes (Araki *et al.*, 1994). Evidence from other sources, and using different approaches also have demonstrated a correlation between PI 3-kinase activity and glucose transport (Kanai *et al.*, 1993; Cheatham *et al.*, 1994; Quon *et al.*, 1994). Thus, it is reasonable to speculate that the IRS-1/PI 3-kinase pathway may be linked to the activation of glucose transport, and that a reduction in this association in rats treated with epinephrine may have a role in the reduced glucose uptake induced by catecholamines in muscle. In this regard, Vannucci *et al.* (1992) demonstrated in rat adipose cells that isoprenaline promoted a decrease in the accessibility of insulin stimulated cell surface GLUT 4, which directly correlated with the observed inhibition of glucose transport activity.

The mechanism(s) whereby epinephrine induces these alterations are unknown but at least two possibilities should be considered. First, it is known that agents which raise intracellular cAMP levels also increase phosphorylation of the insulin receptor at serine and threonine resides, reduce insulin-mediated receptor phosphorylation on tyrosine and inhibit the insulin-dependent tyrosine protein kinase activity of the receptor. Since insulin receptor kinase activity is reduced, a reduction in IRS-1 phosphorylation is also expected as is the association between IRS-1 and PI 3-kinase. The second possibility arises from recent data showing that

an increase in cellular cAMP through the activation of PKA increases the activity of endogenous PTPase, thereby leading to a sequence of dephosphorylation (Wilson & Kaczmarek, 1993).

In summary, this study has shown a reduced insulin receptor and IRS-1 phosphorylation and a reduced association of IRS-1 with PI 3-kinase in the liver and muscle of epinephrine-treated rats. These alterations may explain some aspects of insulin resistance in these animals.

Materials and methods

Materials

Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting were from Bio-Rad (Richmond, CA). N-2-Hydroxyethyl-piperazine-N-2 ethanesulfonic acid (HEPES), phenylmethylsulfonylfluoride (PMSF), aprotinin, dithiotreitol (DTT), Triton X-100, Tween 20, glycerol and bovine serum albumin (BSA, fraction V) were from Sigma Chemical Co. (St. Louis, MO). Protein A-sepharose 6MB was from Pharmacia (Uppsala, Sweden), [^{125}I]protein A was from ICN Biomedicals (Costa Mesa, CA) and nitrocellulose paper (BA85, 0.2 mm) was from Schleicher & Schuell. Sodium amobarbital (Amytal) and human recombinant insulin (Humulin R) were from Eli Lilly. Monoclonal antiphosphotyrosine antibody and anti-PI 3-kinase antiserum was from UBI (Lake Placid, NY). Anti-insulin receptor antibody and anti-IRS-1 antibodies were raised in rabbits using a synthetic peptide derived from the amino acid sequence to the C-terminus of the proteins, as previously described (Saad *et al.*, 1993), and were kindly provided by Drs C.R. Kahn and M.F. White.

Methods

Animals and tissue extracts Male rats (150–180 g) were allowed access to standard rodent chow and water *ad libitum*. The animals were studied after 12 h of fasting. The studies were performed in parallel for the control and treated rats in each pair.

The rats were anaesthetized with sodium amobarbital (15 mg/kg body weight intraperitoneally) and used as soon as anesthesia was assured by loss of the pedal and corneal reflexes. Initially, the animals were injected i.p. with epinephrine 25 μg/100 g body weight or an equal volume of saline (control group). Five minutes later, the abdominal cavity was opened, the portal vein exposed and 0.5 ml of normal saline (0.9% NaCl) with or without 10^{-5} M insulin was injected. Thirty seconds later, the livers were removed, minced coarsely and homogenized immediately in approximately 10 volumes of solubilization buffer A in a water bath maintained at 100°C using a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed (setting 10) for 30 s. The solubilization buffer A was composed of 1% SDS, 50 mM HEPES (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA and 10 mM sodium vanadate. The homogenate was heated further to boiling for 10 min and then cooled in an ice bath for 40 min.

Approximately 90 s after insulin injection, hindlimb muscles were excised and homogenized with a Polytron in 6 volumes of homogenization buffer A at 100°C. The extracts were centrifuged at 55 000 r.p.m. at 4°C in a Beckman 70.1 Ti rotor for 60 min to remove insoluble material and the supernatant was used as a sample. In some experiments, the tissues were extracted at 4°C with homogenization buffer B (same as buffer A except that 1% Triton-X 100 replaced 1% SDS and 2 mM PMSF and 0.1 mg/ml aprotinin were added) and after centrifugation the supernatant was used for immunoprecipitation with anti-IRS-1 antibody.

Protein analysis by immunoblotting The samples were treated with Laemmli sample buffer (Laemmli, 1970) containing 100 mM DTT and heated in a boiling water bath for 4 min. For total extracts, similar sized aliquots of sample (150 μg of protein) were subjected to SDS–PAGE (6% Tris acrylamide) in a Bio-Rad miniature slab gel apparatus. The electrotransfer of proteins from the gel to nitrocellulose was performed for 2 h at 120 V (constant) using a Bio-Rad miniature transfer apparatus (Mini-Protean) as described by Towbin *et al.* (1979) but with 0.02% SDS added to the transfer buffer to enhance the elution of high molecular mass proteins. Non-specific protein binding to the nitrocellulose was reduced by preincubating the filter overnight at 4°C in blocking buffer (3% BSA, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). Prestained molecular weight standards were myosin (205 kDa), β galactosidase (116 kDa), bovine serum albumin (80 kDa) and ovalbumin (49.5 kDa).

The nitrocellulose blot was incubated with antiphosphotyrosine antibodies, anti-insulin receptor antibodies, with anti-IRS-1 antibodies or anti-PI 3-kinase antibodies diluted in blocking buffer for 4 h at 22°C and washed for 60 min in blocking buffer without BSA. The blots were then incubated with 2 mCi of [^{125}I]protein A (30 mCi/mg) in 10 ml of blocking buffer for 1 h at 22°C and washed again as described above for 2 h. [^{125}I]Protein A bound to the antiphosphotyrosine and anti-IRS-1 antibodies were detected by autoradiography using preflashed Kodak XAR film with Cronex Lighting Plus intensifying screens at −70°C for 12–48 h. Band intensities were quantitated by optical densitometry (Molecular Dynamics) of the developed autoradiogram.

Other

Protein determination was performed by the Bradford dye method (Bradford, 1976) using the Bio-Rad reagent and BSA as the standard.

Statistics

Experiments were always performed by analysing samples from the epinephrine-treated animals in parallel with a control group. Comparisons were made using Student's paired and unpaired *t* test as appropriate. The level of significance selected was $P < 0.05$.

Acknowledgements
This work was supported by FAPESP grants.

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