Endocrine, *vol. 21, no. 2, 115–133, July 2003* 0969–711X/03/21:115–122/$25.00

Regulation of Insulin Receptor Substrate-2 Tyrosine Phosphorylation in Animal Models of Insulin Resistance

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Insulin induces a wide variety of growth and metabolic responses in many cell types. These actions are initiated by insulin binding to its receptor and involve a series of alternative and complementary pathways created by the multiple substrates of the insulin receptor (insulin receptor substrates [IRSs]). We investigated IRS-1 and IRS-2 tyrosine phosphorylation; their association with phosphatidylinositol-3-OH kinase (PI3-K); and the phosphorylation of Akt, a serine-threonine kinase situated downstream of PI3-K, in liver and muscle of two animal models of insulin resistance: epinephrine- or dexamethasone-treated rats. We used in vivo insulin infusion followed by tissue extraction, immunoprecipitation, and immunoblotting. IRS-1 and IRS-2 protein expression did not change in liver and muscle of the epinephrine-treated rats, but in dexamethasone-treated rats IRS-1 presented an increase in liver and a decrease in muscle tissue. PI3-K and Akt protein expression did not change in liver or muscle of the two animal models of insulin resistance. There was a downregulation in insulin-induced IRS-1 and IRS-2 tyrosine phosphorylation and association with PI3-K in both models of insulin resistance. In parallel, insulin-induced Akt phosphorylation was reduced in both tissues of epinephrine-treated rats, and in liver but not in muscle of dexamethasone-treated rats. The reduction in insulin-induced Akt phosphorylation may help to explain the insulin resistance in liver and muscle of epinephrine-treated rats and in the liver of dexamethasone-treated rats.

Key Words: Insulin receptor substrate-2; dexamethasone; epinephrine; liver; muscle.

Introduction

The primary role of insulin is to control plasma glucose concentration by stimulating glucose transport into muscle and adipose cells as by reducing glucose output from the liver. These actions of insulin are initiated by insulin binding to its receptor. On binding insulin, the intrinsic tyrosine kinase activity of the β-subunit of the receptor increases, allowing it to phosphorylate itself as well as intracellular substrates *(1)*. Numerous studies have shown that insulin receptor has various substrates, including insulin receptor substrate-1 (IRS-1), IRS-2, IRS-3, IRS-4, Shc, and possibly others *(2–9)*. This cellular response to insulin, phosphorylation of several cytosolic docking proteins (IRS proteins), couples the insulin receptor to various effector molecules, including phosphatidylinositol-3-OH kinase (PI3-K), SHP2, Grb2/SOS, NCK, and CRK *(10)*. PI3-K may be involved in mediating several insulin-regulated metabolic pathways, including glucose uptake *(11)*, antilipolysis *(12)*, glycogen synthesis *(13)*, the suppression of hepatic gluconeogenesis through the regulation of phosphoenolpyruvate carboxykinase (PEPCK) expression *(14)*, and abnormalities in this protein association could explain defective glucose homeostasis. Different approaches have demonstrated that Akt, a serine-threonine kinase with a pleckstrin homology domain, is functionally located downstream of PI3-K *(15–19)* and is important for insulin action in metabolic pathways *(20)*.

Alterations in the early steps of insulin signaling have been recognized as an important component of many insulin-resistant states. The decrease in insulin binding, receptor kinase activity, IRS-1 protein phosphorylation, and IRS-1-associated PI3-K activity have been demonstrated in different animal models of insulin resistance *(21–25)*. Interestingly, in mice made IRS-1 deficient by homologous recombinant gene targeting, IRS-2 serves as an alternative substrate and allows for significant residual insulin signaling *(25)*. In this regard, the investigation of the regulation of IRS-2 and downstream signaling may add new information in the molecular mechanisms of insulin resistance.

Previous studies showed that epinephrine-induced insulin resistance is accompanied by a reduction in IRS-1 tyrosine phosphorylation *(25)*, but dexamethasone induced a tissue-specific regulation of IRS-1 protein expression *(23)*, showing that these hormones may have different characteristics that may reveal more about mechanisms of impaired signaling. In this regard, the effect of these hormones on the regulation of IRS-2 and downstream signaling was not yet studied.

In the present study, we investigated the importance of IRS-2 phosphorylation and its association with PI3-K, and Akt phosphorylation after insulin stimulation, in two animal models of insulin resistance: epinephrine- or dexamethasone-treated rats.

Received August 29, 2002; Revised December 18, 2003; Accepted January 15, 2003.

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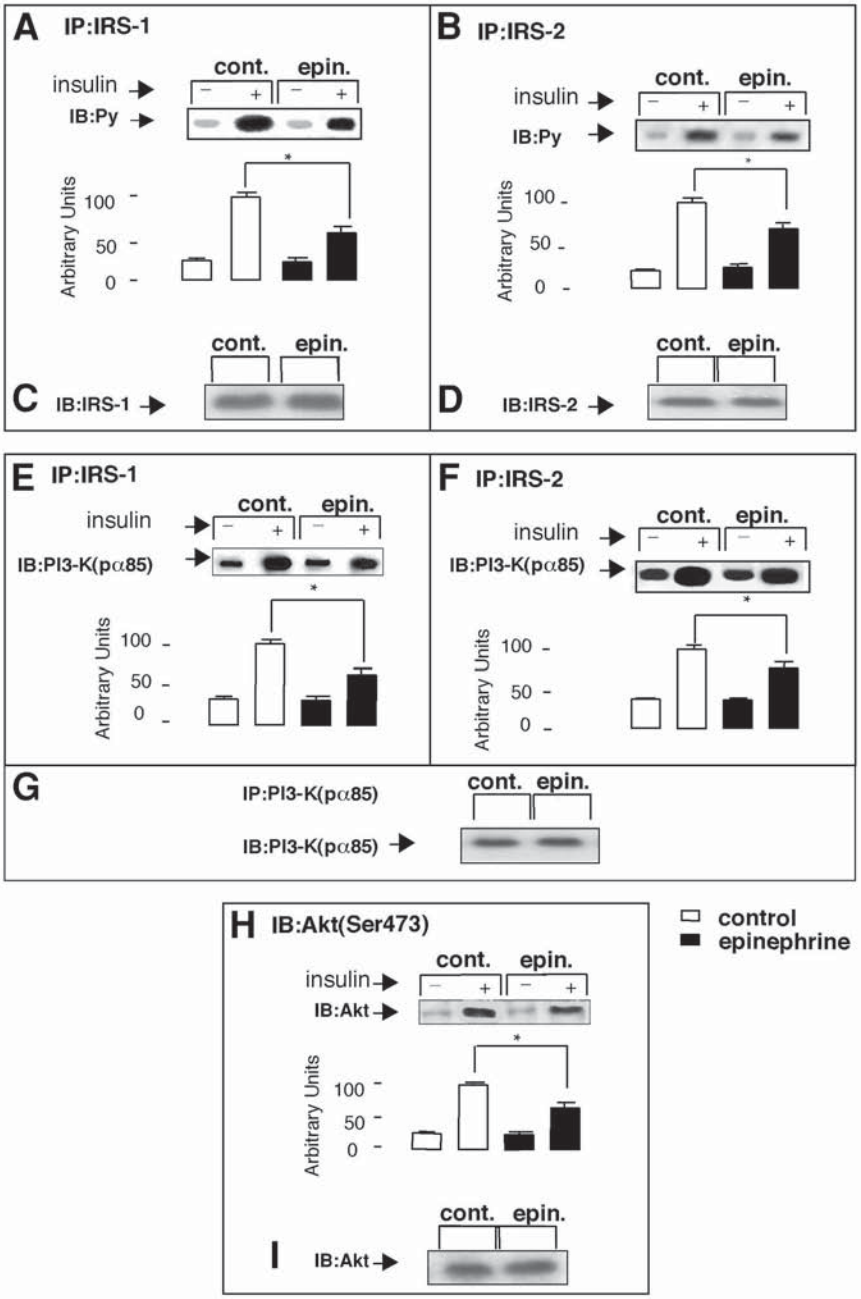

Fig. 1. Early steps in insulin action in intact liver from epinephrine-treated rats ($n = 6$). Saline (0.9%) (–) or insulin (6 μg) (+) was administered into portal vein as a bolus injection, and 30 s later the liver was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-IRS-1 and anti-IRS-2 and immunoblotted with antiphosphotyrosine (**A–D**) and anti-PI3-K(pα85) (**E–G**). Aliquots of samples were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and immunoblotted with anti-pAkt(Ser473) (**H**). Data are expressed as the mean ± SEM. *$p < 0.05$.

Results

Effect of Epinephrine on Insulin-Induced IRS-1 and IRS-2 Tyrosine Phosphorylation, Association of PI3-K with These Substrates, and Akt Phosphorylation in Liver of Rats

Insulin-induced IRS-1 and IRS-2 tyrosine phosphorylation levels in liver of epinephrine-treated rats were significantly decreased to 62 ± 8 and 63 ± 10%, respectively, when compared with control rats ($p < 0.05$) (Fig. 1A,B). Comparison of the bands in Fig. 1 showed that the amounts of IRS-1 or IRS-2 protein did not change after epinephrine treatment (Fig. 1C,D)

Previous studies have suggested that there is a relatively stable, high-affinity interaction between IRSs/PI3-K, such that both proteins are coprecipitated by antibodies to either protein *(26,27)*. In liver and muscle samples of all of the present experiments, previously immunoprecipitated with anti-IRS-

1 and IRS-2 antibodies and then immunoblotted with anti-PI3-K (p85α) antibody, a band corresponding to the latter protein was seen in both epinephrine and control rats, but in the basal state it was only a weak band. After stimulation with insulin, the intensity of this band increased in both groups of rats, in agreement with the formation of a stable association between IRS-1 and IRS-2 and PI3-K. Comparison of the bands corresponding to stimulation by insulin showed that the amount of PI3-K associated IRS-1 and with IRS-2 decreased to 63 ± 8 and 73 ± 8% ($p < 0.05$), respectively, in the liver of treated rats (Fig. 1E,F). There was no change in PI3-K protein levels in liver of epinephrine-treated rats (Fig. 1G). The Akt phosphorylation induced by insulin was significantly decreased to 60 ± 5% ($p < 0.05$) in liver of epinephrine-treated rats (Fig. 1H), whereas there was no change in the expression of this protein (Fig. 1I).

Effect of Epinephrine on Insulin-Induced IRS-1 and IRS-2 Tyrosine Phosphorylation, Association of PI3-K with These Substrates, and Akt Phosphorylation in Muscle of Rat

Insulin-induced IRS-1 and IRS-2 tyrosine phosphorylation levels in muscle of epinephrine-treated rats were significantly decreased to 67 ± 8 and 74 ± 4%, respectively, when compared with control rats ($p < 0.05$) (Fig. 2A,B). IRS-1 or IRS-2 protein expression did not change after epinephrine treatment (Fig. 2C,D).

Comparison of the bands in Fig. 2 corresponding to stimulation by insulin showed that the amount of PI3-K associated with IRS-1 and IRS-2 decreased to 45 ± 6 and 55 ± 11% ($p < 0.05$), respectively, in the muscle of treated rats (Fig. 2E,F), without changes in PI3-K protein levels (Fig. 2G). The Akt phosphorylation induced by insulin was significantly decreased to 70 ± 3% ($p < 0.05$) in muscle of epinephrine-treated rats (Fig. 2H), whereas there was no change in the expression of this protein (Fig. 2I).

Effect of Dexamethasone on Insulin-Induced IRS-1 and IRS-2 Tyrosine Phosphorylation, Association of PI3-K with These Substrates, and Akt Phosphorylation in Liver of Rats

Insulin-induced IRS-1 and IRS-2 tyrosine phosphorylation levels in liver of dexamethasone-treated rats were significantly decreased to 45 ± 8 and 50 ± 6%, respectively, when compared with control rats ($p < 0.05$) (Fig. 3A,B). IRS-1 protein expression increased to 173 ± 35% whereas IRS-2 tissue levels did not change after dexamethasone treatment (Fig. 3C,D). When the data are expressed as a function of the amount of IRS-1 molecules, there was an approx 75% decrease in the stoichiometry of the IRS-1 phosphorylation in the liver of dexamethasone-treated rats.

Analysis of insulin-induced IRS-1/PI3-K and IRS-2/PI3-K association showed a significant reduction to 82 ± 3 and 80 ± 10%, respectively ($p < 0.05$), in liver of dexamethasone-treated rats (Fig. 3E,F), but the expression of PI3-K did not change (Fig. 3G). The Akt phosphorylation induced by insulin in liver of dexamethasone-treated rats was significantly decreased to 70 ± 17% ($p < 0.05$) (Fig. 3H), whereas there was no change in the expression of this protein (Fig. 3I).

Effect of Dexamethasone on Insulin-Induced IRS-1 and IRS-2 Tyrosine Phosphorylation, Association of PI3-K with These Substrates, and Akt Phosphorylation in Muscle of Rats

Insulin-induced IRS-1 and IRS-2 tyrosine phosphorylation levels in muscle of dexamethasone-treated rats were significantly decreased to 41 ± 3 and 63 ± 1%, respectively, when compared with control rats ($p < 0.05$) (Fig. 4A,B). Comparison of the bands in Fig. 4 showed that the amounts of IRS-1 decreased to 65 ± 5% whereas the IRS-2 protein did not change in dexamethasone treatment (Fig. 4C,D). When the data are expressed as a function of the amount of protein for IRS-1 molecules, there was an approx 30% decrease in the stoichiometry of the IRS-1 phosphorylation in the muscle of dexamethasone-treated rats.

Analysis of insulin-induced IRS-1/PI3-K and IRS-2/PI3-K association showed a significant reduction to 47 ± 4 and to 84 ± 8%, respectively ($p < 0.05$), in muscle of dexamethasone-treated rats (Fig. 4E,F), but the expression of PI3-K did not change (Fig. 4G). In muscle, insulin-induced Akt phosphorylation and also Akt protein expression were similar in dexamethasone-treated rats and in controls.

Discussion

Insulin resistance is defined as a subnormal biologic response to a given concentration of insulin. Although the effects of insulin are pleiotropic, insulin resistance typically refers to the action of insulin on glucose homeostasis. In animal models of insulin resistance, there is evidence that regulation of IRS-1 may have a role in the modulation of glucose homeostasis *(20)*. In the present study, we investigated the regulation of insulin-induced IRS-2 tyrosine phosphorylation and the association with PI3-K and Akt phosphorylation in liver and muscle of epinephrine- or dexamethasone-treated rats.

It has long been known that an excess of epinephrine causes insulin resistance *(22,25)*. Catecholamines antagonized the action of insulin by stimulating gluconeogenesis, glycogenolysis, and lipolysis and by inhibiting peripheral use of glucose. High intracellular cyclic adenosine monophosphate (cAMP) levels have been shown to induce insulin resistance at both receptor and postreceptor levels *(25)*. Using purified receptors and artificial substrates in vitro, decreased insulin receptor phosphorylation and kinase activity has been observed as a consequence of increased cAMP and cAMP kinase *(28)*. It was previously demonstrated that in liver and muscle of epinephrine-treated rats there is a decrease in insulin-induced insulin receptor, and IRS-1 tyrosine phosphorylation levels *(25)*. The results of the present study extend this information, showing that there is also a decrease

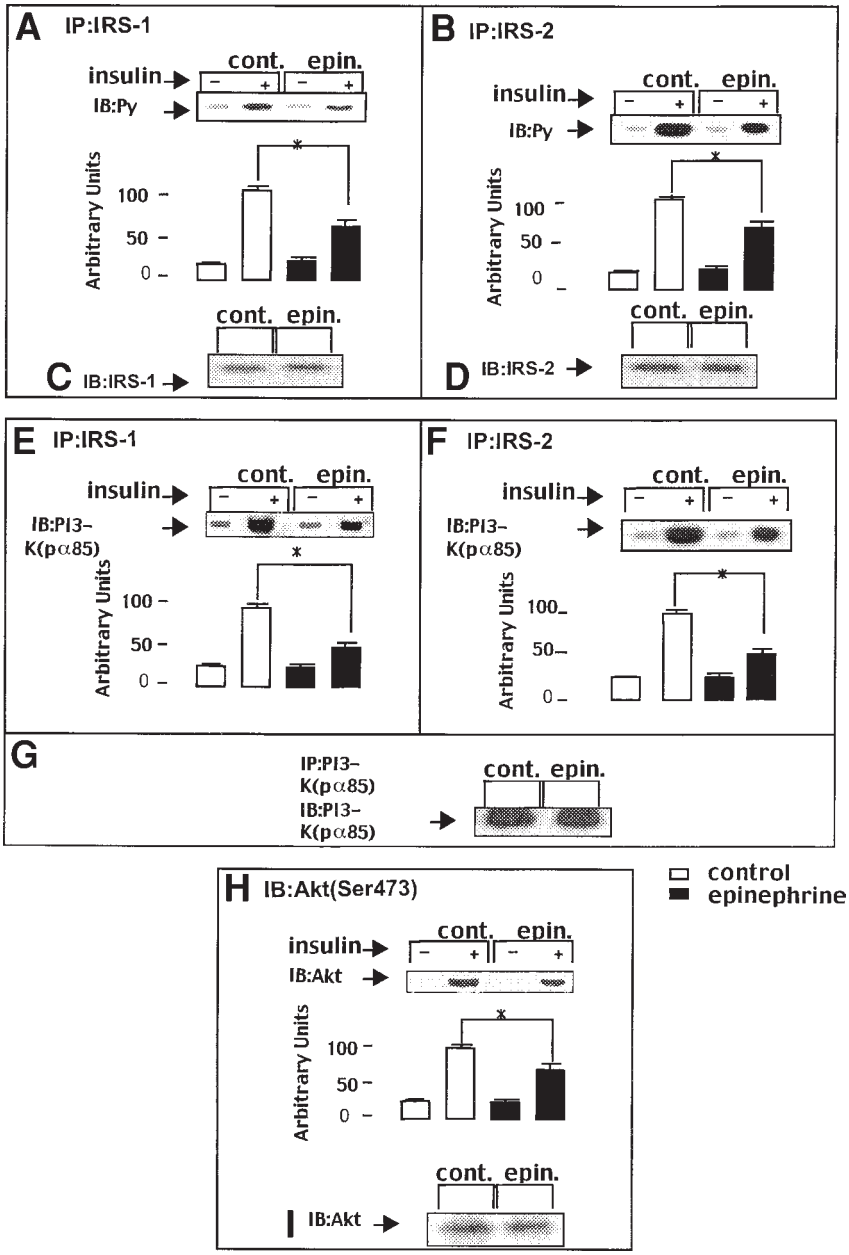

Fig. 2. Early steps in insulin action in intact muscle from epinephrine-treated rats ($n = 6$). Saline (0.9%) (–) or insulin (6 μg) (+) was administered into portal vein as a bolus injection, and 90 s later the muscle was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-IRS-1 and anti-IRS-2 and immunoblotted with antiphosphotyrosine (**A–D**) and anti-PI3-K(p85α) (**E–G**). Aliquots of samples were resolved on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-pAkt(Ser473) (**H**). Data are expressed as the mean ± SEM. *$p < 0.05$.

in IRS-2 tyrosine phosphorylation and in IRS-2/PI3-K association in liver and muscle of epinephrine-treated rats. These alterations may be secondary to an increased activity of protein kinase A (PKA), which can phosphorylate insulin receptor and probably IRS-1 and IRS-2 in serine, thereby reducing the kinase activity and autophosphorylation of the insulin receptors as well as the tyrosine phosphorylation of IRS-1 and IRS-2. Another possibility arises from data showing that an increase in cellular cAMP through activating PKA increases the activity of endogenous phosphotyrosine phosphatase, thus leading to a sequence of dephosphorylation *(28)*.

The serine/threonine kinase Akt (also termed *kinase B*) is stimulated by receptor tyrosine kinases and is a downstream target of PI3-K *(29–33)*, presumably through activation of the phosphatidylinositol 3,4,5-trisphosphate-dependent protein kinase *(34)*. Furthermore, expression of a constitutively active Akt is sufficient to promote GLUT-4 translocation and to increase glucose transport in 3T3-L1 adipocytes *(17,35)*, although this is not uniformly observed. Interest-

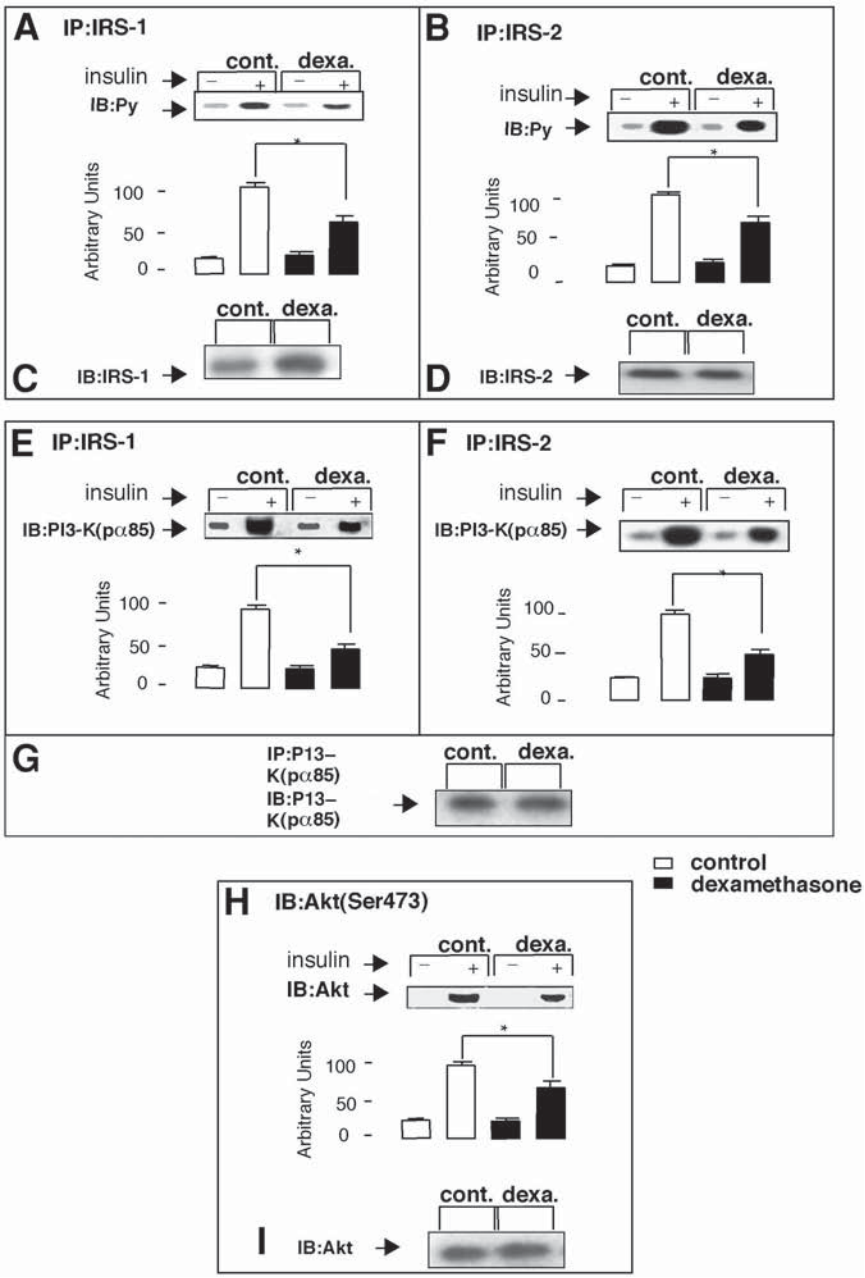

Fig. 3. Early steps in insulin action in intact liver from dexamethasone-treated rats ($n = 6$). Saline (0.9%) (–) or insulin (6 μg) (+) was administered into portal vein as a bolus injection, and 30 s later the liver was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-IRS-1 and anti-IRS-2 and immunoblotted with antiphosphotyrosine (**A–D**) and anti-PI3-K(p85α) (**E–G**). Aliquots of samples were resolved on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-pAkt(Ser473) (**H**). Data are expressed as the mean ± SEM. *$p < 0.05$.

ingly insulin-induced pAKT(Ser473) phosphorylation was attenuated in epinephrine-treated rats *(20)*. Recently, Akt activity has been reported to be essential for the activation of glycogen synthase by insulin *(19)*. Since Akt phosphorylation correlates with Akt activity, we can suggest that a decrease in insulin-induced Akt phosphorylation may contribute to the insulin resistance observed in epinephrine-treated rats. In this regard, reduced Akt phosphorylation may have a role in increased gluconeogenesis in liver and reduced glycogen synthesis in liver and muscle of epinephrine-treated rats.

An excess of glucocorticoids is a well-known situation of insulin resistance *(36)*. Hypercortisolemia is associated with increased glucose production by the liver, decreased transport and utilization of glucose in peripheral tissue, decreased protein synthesis, and increased protein degradation in muscle *(36–38)*.

Under conditions of hypercortisolemia similar to those used in the present study, dexamethasone has been reported to reduce insulin-induced insulin receptor autophosphorylation in the liver and muscle of rats in vivo and also IRS-1

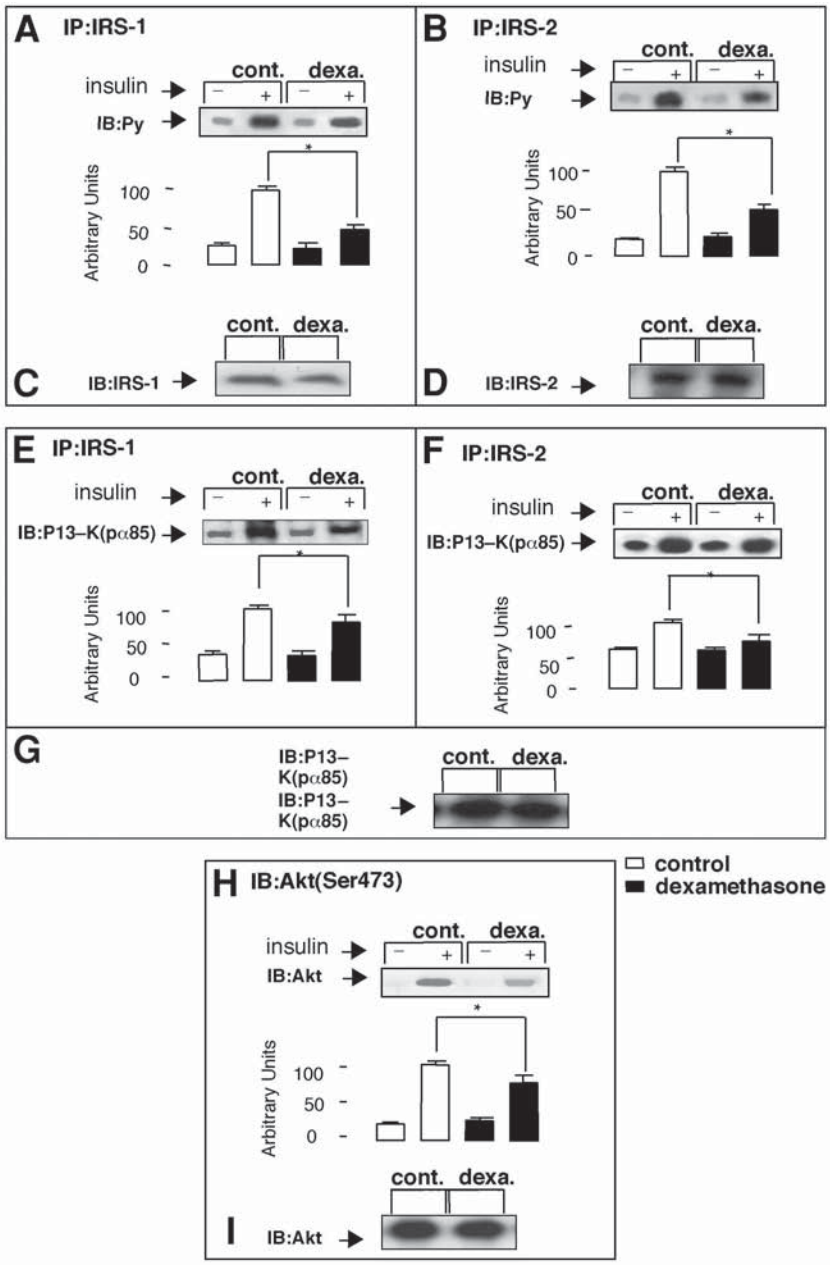

Fig. 4. Early steps in insulin action in intact muscle from dexamethasone-treated rats ($n = 6$). Saline (0.9%) (–) or insulin (6 μg) (+) was administered into portal vein as a bolus injection, and 90 s later the muscle was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-IRS-1 and anti-IRS-2 and immunoblotted with antiphosphotyrosine (**A–D**) and anti-PI3-K(p85α) (**E–G**). Aliquots of samples were resolved on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-pAkt(Ser473) (**H**). Data are expressed as the mean ± SEM. *$p < 0.05$.

activation of PI3-K in these tissues *(39)*. The results of the present study add new information in the molecular mechanism of insulin resistance in dexamethasone-treated rats, showing that insulin-induced IRS-2 tyrosine phosphorylation and association with PI3-K were also reduced in liver and muscle of these animals.

In liver, but not in muscle, of dexamethasone-treated rats there was also a decrease in insulin-induced Akt phosphorylation/activation. The alterations in early steps of insulin action may contribute to the explanation of the insulin resistance and the increased hepatic gluconeogenesis in the liver of these animals. PEPCK, a rate-controlling enzyme of gluconeogenesis, is regulated by insulin *(40)*. Transcription of PEPCK is not known to be subject to allosteric regulation, and the inhibition of gluconeogenesis by insulin in vivo is probably owing to an insulin-induced decrease in the amount of PEPCK protein. Moreover, observations that PEPCK gene expression in the liver is increased in animal models of diabetes *(41)* and that transgenic animals overexpressing the PEPCK gene develop a diabetic phenotype *(42,43)* also indi-

cate the importance of this enzyme in glucose homeostasis in vivo. Recently, it was demonstrated that the activation of Akt alone is sufficient to repress the glucocorticoid- and cAMP-stimulated increase in PEPCK gene transcription *(44)*. Our results showing that in dexamethasone-treated rats there is a decrease in insulin-induced pAkt(Ser473) phosphorylation may suggest a molecular mechanism for insulin resistance and increased gluconeogenesis observed in these animals.

In muscle of dexamethasone-treated rats, there was an important decrease in IRS-1/PI3-K association and a modest decrease in IRS-2/PI3-K association, and no alteration in pAkt (Ser473) phosphorylation. It is interesting that Yamauchi et al. *(45)* demonstrated that IRS-1 plays a central role in insulin-induced glucose transport in muscle. Recently, it was demonstrated that activation of Akt is not essential for glucose transport in muscle *(19)*. Taking together these findings, we can suggest that the marked reduction in insulin-induced IRS-1 tyrosine phosphorylation and association with PI3-K may have a role in the reduced insulin-stimulated glucose transport in muscle of dexamethasone-treated rats.

In summary, the results of the present study demonstrated that alterations in early steps of insulin action may have a role in the insulin resistance observed in epinephrine- or dexamethasone-treated rats. The reduction in insulin-induced pAkt(Ser473) phosphorylation may help to explain the insulin resistance in liver and muscle of epinephrine-treated rats and in the liver of dexamethasone-treated rats.

Materials and Methods

Reagents, Chemicals, and Antibodies

The reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA). Tris, phenylmethylsulfonyl fluoride (PSMF), aprotinin, silicone, and dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO). Sodium amobarbital and human recombinant insulin (Humulin R) were purchased from Eli Lilly (Indianapolis, IN). Protein A-Sepharose 6MB was purchased from Pharmacia (Uppsala, Sweden). [^{125}I] Protein A was obtained from Amersham (Aylesbury, UK), and nitrocellulose (BA85; 0.2 μm) was obtained from Schleicher & Schuell (Keene, NH). Male Wistar rats were from the UNICAMP Central Animal Breeding Center. Monoclonal antiphosphotyrosine antibodies and antiphospho Akt(Ser 473) and anti-PI3-K(p85α) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-IRS-1 and anti-IRS-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals

Six-week-old male wistar rats (mean body wt of 131 ± 6 g) were fed standard rodent chow and water ad libitum. Food was withdrawn 12–14 h before the experiments for the epinephrine- and dexamethasone-treated rats and their respective controls. Chronic hypercortisolism was induced with dexamethasone administered for 5 d (1 mg/[kg·d], intraperitoneally). In experiments with epinephrine, the rats were injected with 25 μg/100 g of body wt 5 min before the abdominal cavity was opened. All experiments were approved by the Ethics Committee of the State University of Campinas (UNICAMP). Each experiment was performed with its own control group.

Sampling of Liver and Muscle

The rats were anesthetized with sodium amobarbital (15 mg/kg of body wt, intraperitoneally) and used 10–15 min later, as soon as the anesthesia was ensured by the loss of foot and corneal reflexes. The abdominal cavity was opened, the portal vein was exposed, and 6 μg of insulin was injected. The liver and muscle were removed 30 and 90 s after the infusion of insulin, respectively; minced coarsely; and homogenized immediately in approx 10 vol of solubilization buffer A at 4ºC, using a Polytron PT 10/35 homogenizer fitted with a PTA 20S blade (Brinkmann) operated at maximum speed for 30 s. Buffer A (for solubilization) consisted of 1% Triton X-100, 50 m*M* HEPES (pH 7.4), 100 m*M* sodium pyrophosphate, 100 m*M* sodium fluoride, 10 m*M* EDTA, 10 m*M* sodium vanadate, 2 m*M* PMSF, and 0.1 mg of aprotinin/mL.

The liver and muscle were extracted in an identical fashion in each model studied. The extracts were centrifuged at 30,000*g* in a Beckman 70.1 Ti rotor at 4ºC for 20 min to remove insoluble material, and the resulting supernatant was used for immunoprecipitation with 13 μL of polyclonal anti-IRS-2 and IRS-1 antibodies. Equal amounts of protein were immunoprecipitated in all cases. We used 3 mg of protein for the liver extracts and 1 mg for muscle. In the same way, equal amounts of protein were loaded on the gels for Akt phosphorylation (200 μg of protein/lane). The immune complexes were precipitated with protein A-Sepharose 6MB and washed three times with 50 m*M* Tris (pH 7.4), 2 m*M* sodium vanadate, and 0.1% Triton X-100.

Protein Analysis by Immunoblotting

After washing, the pellet was resuspended in Laemmli sample buffer *(46)* with 100 m*M* DTT and heated in a boiling water bath for 5 min. The samples were subjected to SDS-PAGE (8.5% Tris-acrylamide) in a Bio-Rad miniature slab gel apparatus. Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described by Towbin et al. *(47)*. Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter overnight at 4ºC in blocking buffer (3% bovine serum albumin [BSA], 10 m*M* Tris, 150 m*M* NaCl, and 0.02% Tween-20). The prestained molecular mass standards used were myosin (206 kDa), β-galactosidase (120 kDa), BSA (85 kDa), and ovalbumin (47 kDa). The nitrocellulose filter thus treated was then incubated for 4 h at 22ºC with anti–phosphotyrosine antibody and anti-PI3-K (p85α) antibody (0.5 μg/mL, diluted in blocking buffer) and then washed

for 30 min in blocking buffer without BSA. Some samples were incubated with anti–phosphoAkt1(Ser473) antibody in blocking buffer. The blots were then incubated with ^{125}I-Protein A (30 μCi/μg) in 10 mL of blocking buffer for 1 h at 22ºC and washed again. ^{125}I-Protein A bound to the antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at −70ºC for 12–48 h. Band intensities were quantified by optical densitometry (Molecular Dynamics) of the developed autoradiogram and the data expressed as mean ± SEM in arbitrary units.

Statistical Analyses

The experiments were always performed by studying the physiologic or pathologic group of rats in parallel with a control group. The data in control vs epinephrine-treated and control vs dexamethasone-treated rats were analyzed using unpaired student's *t*-test. The level of significance employed was $p < 0.05$.

Acknowledgments

We wish to thank Fundação de Amparo à Pesquisa do Estado de São Paulo for financial support.

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