Regulation of insulin-stimulated tyrosine phosphorylation of Shc and IRS-1 in the muscle of rats: effect of growth hormone and epinephrine

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Abstract Insulin receptor substrate-1 (IRS-1) and Shc protein have the same binding site at the insulin receptor and compete in their association with the phosphorylated receptor. The present study demonstrates that a decrease in the level of muscle insulin receptor phosphorylation induced by chronic growth hormone (GH) treatment or acute epinephrine infusion is accompanied by a reduction in the level of IRS-1 phosphorylation and in the association with phosphatidylinositol 3-kinase. In contrast, no change is observed in insulin-stimulated Shc tyrosine phosphorylation, or in the association of this substrate with Grb2. These data suggest that a reduction in insulin receptor phosphorylation may affect post-receptor processes differentially by preserving the phosphorylation of some substrates and pathways, but not of others.

Key words: Insulin signal transduction; Growth hormone; Epinephrine

1. Introduction

The insulin receptor is the principal mediator of insulin action in cellular mitogenic and metabolic processes. The insulin receptor β-subunit, which contains an intrinsic tyrosine kinase, undergoes tyrosyl autophosphorylation and is activated in response to insulin binding to the extracellular α-subunit [1,2]. This interaction further enhances the tyrosine kinase activity of the receptor towards other intermediate molecules, including insulin receptor substrate-1 (IRS-1), IRS-2 and Shc [3–9]. These molecules, rather than the insulin receptor itself, then couple to a downstream signaling pathway by serving as binding sites for SRO homology 2 (SH2) domain-containing signaling molecules [10]. IRS-1 binds to the 85 kDa subunit of phosphatidylinositol 3-kinase (PI 3-kinase), Grb2 and other SH2-containing proteins [11]. Shc protein has been shown to directly induce the association with Grb2 [12]. Recent studies indicate that Shc and IRS-1 have the same binding site at the insulin receptor, a finding consistent with the suggestion that both Shc and IRS-1 compete in their association with the phosphorylated receptor. As yet, there has been no comparison of insulin-induced IRS-1 and Shc tyrosine phosphorylation in situations of insulin resistance accompanied by a reduction in insulin receptor phosphorylation.

We have demonstrated elsewhere that there are common molecular events in the muscle of rats treated chronically with growth hormone (GH) [13] or acutely with epinephrine [14], including a decrease in insulin-induced insulin receptor and IRS-1 tyrosine phosphorylation and in the association of the latter with PI 3-kinase. These reductions correlate with the insulin resistance and reduced glucose uptake described in the muscle of these animals [15,16]. In this study, we investigated Shc and IRS-1 tyrosine phosphorylation in the muscle of the above two animal models of insulin resistance, both of which show reduced insulin-induced insulin receptor autophosphorylation.

2. Materials and methods

2.1. Materials

The reagents and apparatus for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were from Bio-Rad (Richmond, CA, USA). Tris[hydroxymethyl]amino-methane (Tris), phenylmethylsulfonyl fluoride (PMSF), aprotinin, silicone, and dithiothreitol (DTT) were from Sigma Chemical Co. (St. Louis, MO, USA). Sodium amobarbital and human recombinant insulin (Humulin R) were from Lilly. Human biosynthetic GH (Norditropin) was purchased from Novo Nordisk (Bagsvaerd, Denmark). [^{125}I]Protein A was from Amersham (Amersham, UK) and protein A Sepharose 6 MB from Pharmacia (Uppsala, Sweden). Nitrocellulose (BA85, 0.2 μm) was from Schleicher&Schuell. Male Wistar rats were from the UNICAMP Central Animal Breeding Center. Monoclonal antiphosphotyrosine antibody (1 μg/ml), polyclonal anti-Shc, polyclonal anti-Grb2 and polyclonal anti-PI 3-kinase (p85) antibody were from Upstate Biotechnology Incorporated (UBI, Lake Placid, NY, USA). Anti-IRS-1 antibodies were raised in rabbits using a synthetic peptide (Pep 80) derived from the amino acid sequence (YIPGATMGTSPALTGDEAA) corresponding to residues 489–507 of the rat protein and purified by affinity chromatography on a column prepared by coupling the synthetic peptide to Affi-Gel 10 (Bio-Rad Laboratories) as previously described [17]. Anti-insulin receptor antibody was raised in rabbits using a synthetic peptide derived from the amino acid sequence (KKNGRILPRSNPS) corresponding to the C-terminus of the rat protein [17].

2.2. Animals

Male Wistar rats, 6 weeks old, were divided into two groups and the studies were performed in parallel using the control and treated rats. All groups received standard rodent chow and water ad libitum. An excess of GH was induced by the s.c. injection of human GH at a dose of 1 mg twice a day for 5 days. The control group received the equivalent amount of normal saline (0.9% NaCl). The experiments were performed on the morning of the fifth day. In another series of experiments, anesthetized rats were injected i.p. with epinephrine (25 μg/100 g body weight) or an equal volume of saline (control group) and the animals used 5 min later. In all groups, the rats were fasted for 12 h before being used as described below.

2.3. Methods

Rats were anesthetized with sodium amobarbital (15 mg/kg body weight, i.p.), and were used 10–15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the vena cava exposed, and 6 μg of insulin diluted in normal saline (0.9% NaCl) was injected. Ninety seconds later, the hindlimb muscle was removed, minced coarsely and homogenized immediately in extraction buffer (1% Triton X-100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and

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0.1 mg of aprotinin/ml) at 4°C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 30 s. For immunoprecipitation with anti-Shc, hindlimb muscle was removed 5 min after the injection of insulin.

The extracts were centrifuged at 15 000 rpm and 4°C in a Beckman 70.1 Ti rotor (Palo Alto, CA, USA) for 45 min to remove insoluble material, and the resulting supernatant was used for immunoprecipitation with anti-IRS-1, anti-Shc or anti-IR (insulin receptor) antibody and protein A Sepharose 6 MB.

2.4. Protein analysis by immunoblotting

Proteins were denatured by boiling in Laemmli [18] sample buffer containing 100 mM DTT, run on SDS-PAGE and transferred to nitrocellulose membranes in Towbin [19] buffer containing 0.02% SDS and 20% methanol. The membranes were blocked, probed, and developed as described previously [20]. Blots were exposed to preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at −80°C for 12–48 h. Band intensities were quantitated by optical densitometry (Hoefer Scientific Instruments, San Francisco, CA; model GS 300) of the developed autoradiographs that were used at exposures in the linear range.

2.5. Statistical analysis

The experiments were performed by studying all groups of animals in parallel. For comparisons, Student's unpaired *t*-test was used as appropriate. The level of significance employed was $P < 0.05$.

3. Results

3.1. Animal characteristics

Seven animals from each group of rats underwent an insulin tolerance test. The glucose disappearance rate (K_{itt}) of the control group was 4.40 ± 0.39%/min. Chronic GH treatment and acute epinephrine treatment induced similar levels of insulin resistance, as reflected in their glucose disappearance rates which were 1.94 ± 0.81%/min and 1.89 ± 0.63%/min, respectively. Both of these values were significantly ($P < 0.05$) lower that the control K_{itt}.

3.2. Characteristics of insulin-stimulated insulin receptor, IRS-1 and Shc phosphorylation in the hindlimb muscle of GH-treated rats

There was no change in the insulin receptor level of muscle from rats treated with GH, as determined by immunoblotting with an antibody to the COOH-terminus of the insulin receptor (Fig. 1A). In muscle samples previously immunoprecipitated with anti-insulin receptor antibody and immunoblotted with antiphosphotyrosine antibody, there was a decrease to 43 ± 11% ($P < 0.05$, $n = 6$) in the insulin-stimulated phosphorylation of the 95 kDa β-subunit of the insulin receptor in GH-treated rats when compared to the controls (Fig. 1B).

Using a specific anti-peptide antibody against IRS-1, the level of this protein was found to be unchanged in the muscle of rats treated chronically with GH (Fig. 2A), although there were changes in the level of phosphorylation of this protein (Fig. 2B). Following the administration of insulin, the intensity of this band increased in both groups of rats. However, comparison of the bands stimulated by insulin revealed that the extent of IRS-1 phosphorylation was reduced to 38 ± 11% ($P < 0.05$, $n = 6$) in GH-treated rats compared to the controls. Previous studies [6,10,21–24] have suggested that there is a relatively stable, high affinity interaction between IRS-1 and the 85 kDa subunit of PI 3-kinase such that both proteins can be co-precipitated by antibodies to either protein. In muscle samples previously immunoprecipitated with anti-IRS-1 antibody and immunoblotted for the 85 kDa subunit of PI 3-kinase (Fig. 2C), there was little basal PI 3-kinase immunoreactivity in the control and GH-treated rats. After stimulation with insulin, a band with the expected molecular mass (85 kDa) of the PI 3-kinase regulatory subunit was observed in anti-IRS-1 antibody immunoprecipitates of muscle from rats in both groups. This finding is consistent with a stable association between IRS-1 and PI 3-kinase. However, the amount of PI 3-kinase associated with IRS-1 was reduced to 45 ± 7% ($P < 0.05$, $n = 4$) in GH-treated rats, thus suggesting a diminished association between IRS-1 and PI 3-kinase. This reduction was probably a consequence of reduced IRS-1 tyrosyl phosphorylation since the level of PI 3-kinase did not change after GH treatment.

Chronic GH treatment did not significantly change the level of Shc protein (Fig. 2D). To define better the extent of Shc phosphorylation, we performed a Western blot analysis of

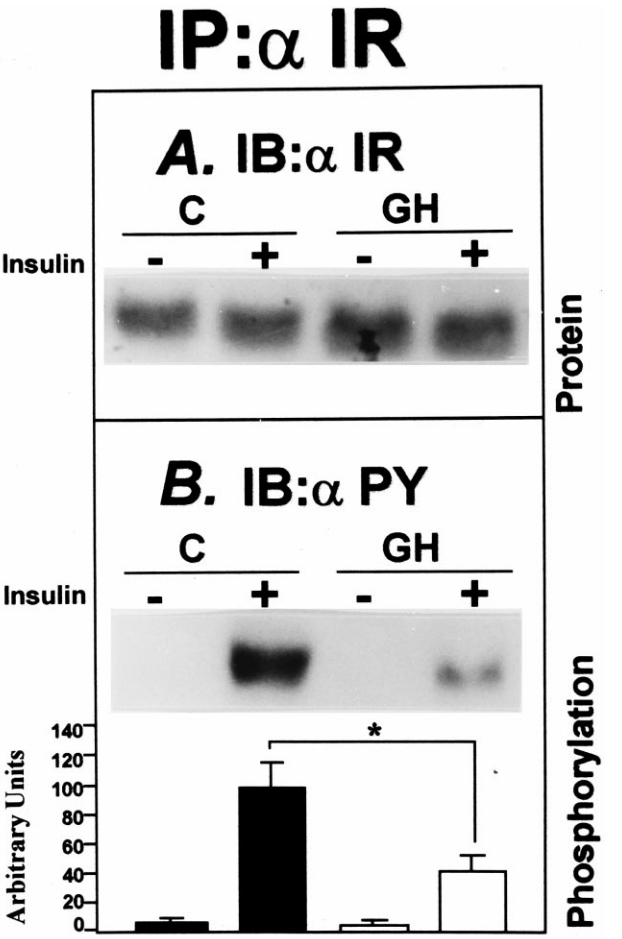

Fig. 1. Insulin-stimulated tyrosine phosphorylation of the insulin receptor in muscle from GH-treated rats. Saline (0.9%, lanes 1 and 3 or insulin 6 μg, lanes 2 and 4) was administered into the portal vein as a bolus injection and 90 s later the muscle was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-insulin receptor antibody and immunoblotted with the same antibody (A). The same samples were immunoprecipitated with anti-insulin receptor antibody and immunblotted with antiphosphotyrosine antibody. (B) Scanning densitometry of autoradiograms was performed in six experiments. The black bars represent the control group and the white bars represent the GH-treated group. The data are expressed as the mean ± S.E.M. *$P < 0.05$.

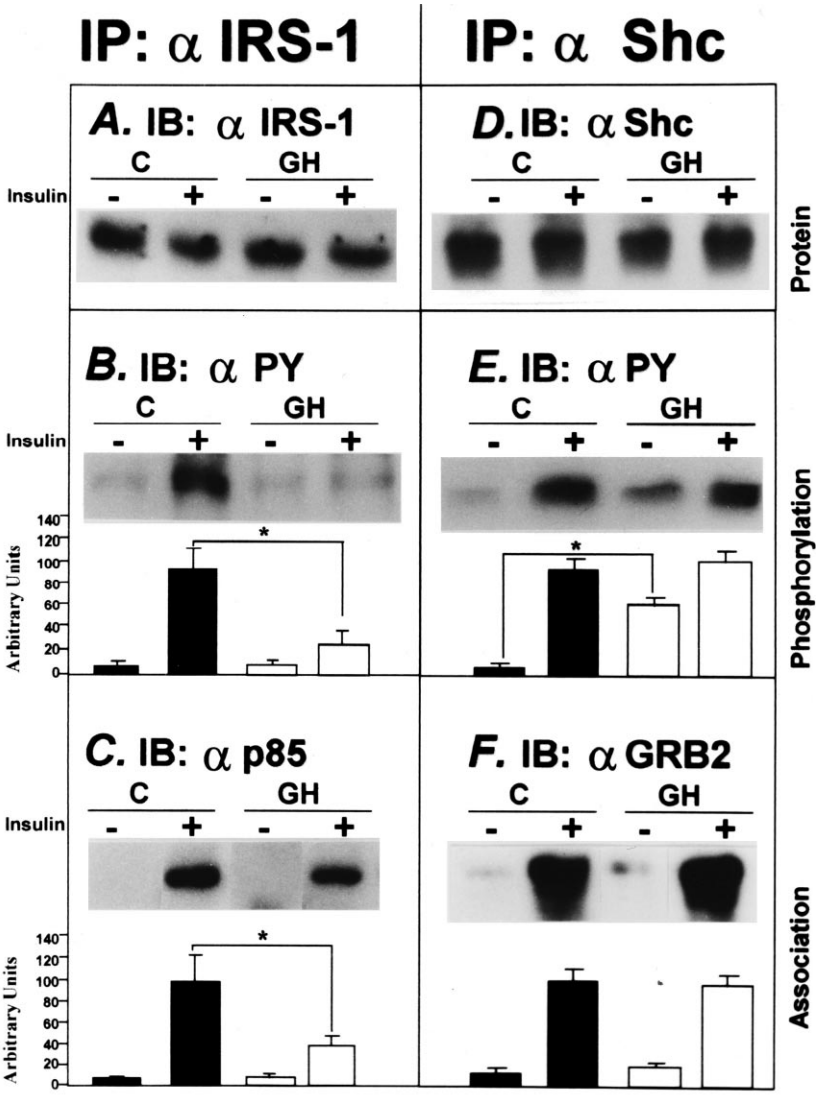

Fig. 2. Insulin-stimulated tyrosine phosphorylation of IRS-1 and Shc in intact muscle from GH-treated rats. Saline (0.9%, lanes 1 and 3 or insulin 6 μg, lanes 2 and 4) was administered into the portal vein as a bolus injection and 90 s later the muscle was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-IRS-1 and immunoblotted with the same antibody (A). The same samples were immunoprecipitated with anti-IRS-1 antibody and immunoblotted with antiphosphotyrosine antibody (B). C: The immunoblotting results of PI 3-kinase in anti-IRS-1 immunoprecipitates from the muscle of control and GH-treated rats. Five minutes after the bolus injection of saline or insulin (6 μg), the muscle was excised and aliquots immunoprecipitated with anti-Shc antibody and blotted with the same antibody (D). The same samples were immunoprecipitated with anti-Shc antibody and blotted with antiphosphotyrosine antibody (E). F: The immunoblotting results of Grb2 in anti-Shc immunoprecipitates from the muscle of control and GH-treated rats. Scanning densitometry of autoradiograms was performed in six experiments. The black bars represent the control group and the white bars represent the GH-treated group. The data are expressed as the mean ± S.E.M. $*P < 0.05$.

tyrosyl-phosphorylated proteins in anti-Shc immunoprecipitates before and after stimulation with insulin in both groups of animals. Basal Shc phosphorylation was higher in GH-treated rats (138 ± 7%, $P < 0.05$, $n = 4$) than in the control group. After stimulation with insulin, the intensity of this band increased in both groups of animals, although the extent of Shc phosphorylation in GH-treated rats was not significantly different from that in the controls (Fig. 2E). Since Shc can associate with Grb2, after insulin stimulation, blots with samples which had been previously immunoprecipitated with anti-Shc were incubated with anti-Grb2 and no change was observed in this association (Fig. 2F).

3.3. *Characteristics of insulin-stimulated insulin receptor, IRS-1 and Shc phosphorylation in the muscle of epinephrine-treated rats*

As in the rats treated with GH, acute epinephrine treatment did not significantly change the insulin receptor protein level (Fig. 3A). However, following stimulation with insulin, phosphorylation of the insulin receptor was reduced to 47 ± 4% ($P < 0.001$, $n = 6$) in epinephrine-treated rats compared to the controls (Fig. 3B).

Similarly, no significant change occurred in the level of IRS-1 protein in the muscle of rats treated with epinephrine when compared to the controls (Fig. 4A). To define better the

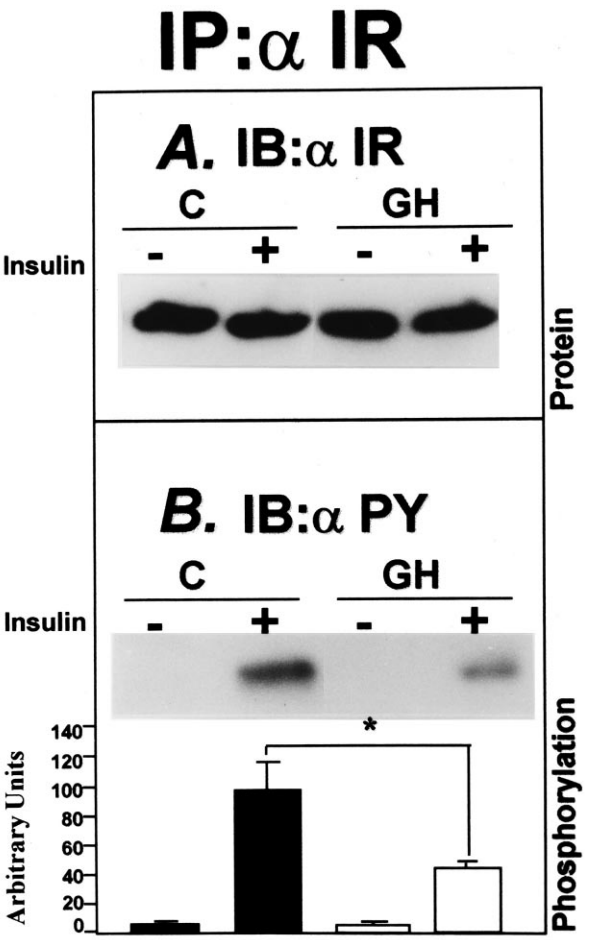

Fig. 3. Insulin-stimulated tyrosine phosphorylation of insulin receptor in intact muscle from epinephrine-treated rats. Saline (0.9%, lanes 1 and 3 or insulin 6 μg, lanes 2 and 4) was administered into the portal vein as a bolus injection and 90 s later the muscle was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-insulin receptor antibody and immunoblotted with the same antibody (A). The same samples were immunoprecipitated with anti-insulin receptor antibody and immunblotted with anti-phosphotyrosine antibody (B). Scanning densitometry of autoradiograms was performed in six experiments. The black bars represent the control group and the white bars represent the epinephrine-treated group. The data are expressed as the mean ± S.E.M. $^*P < 0.05$.

level of IRS-1 phosphorylation, we performed a Western blot analysis of the tyrosyl-phosphorylated proteins in anti-IRS-1 immunoprecipitates before and after stimulation with insulin in both groups of animals. Fig. 4B shows that there was a marked reduction to 53 ± 10% ($P < 0.001$, $n = 9$) in insulin-stimulated IRS-1 phosphorylation in the muscle of animals pretreated with epinephrine. To examine the association of the 85 kDa subunit of PI 3-kinase with IRS-1, blots of samples which had been previously immunoprecipitated with anti-IRS-1 antibodies were incubated with anti-PI 3-kinase antibody. As expected, in both groups an 85 kDa band was present in the IRS-1 immunoprecipitates after exposure to insulin and there was a decrease to 41 ± 8% ($P < 0.001$, $n = 4$) in the intensity of this band in epinephrine-treated rats (Fig. 4C).

Acute epinephrine treatment had no effect on the Shc protein level in muscle as determined by immunoblotting of cell lysates with anti-Shc antibody (Fig. 4D). In samples from muscle previously immunoprecipitated with anti-Shc antibody and immunoblotted with anti-phosphotyrosine antibody, there was no change in insulin-stimulated Shc tyrosine phosphorylation in epinephrine-treated rats when compared to the controls (Fig. 4E). Similarly, no change was observed in the association between Shc/Grb2 when rats treated with epinephrine were compared with controls (Fig. 4F).

4. Discussion

Following insulin stimulation, several proteins undergo tyrosine phosphorylation, including the β chain of the insulin receptor, IRS-1, IRS-2 and Shc proteins [25]. We analyzed the insulin-induced phosphorylation of these signaling intermediates in the muscle of rats treated chronically with GH and in rats receiving an acute infusion of epinephrine. Insulin resistance in these animals was demonstrated by a decrease in the glucose disappearance rate after insulin infusion [13]. We observed a reduction in insulin receptor and IRS-1 tyrosine phosphorylation and also in the PI 3-kinase association with IRS-1 in the muscle of both treated groups. These alterations in the three early steps of insulin action may explain some aspects of the insulin resistance observed in both of these models since these steps seems to have an important role in glucose homeostasis. Previous studies demonstrated that mice homozygous for target disruption of the IRS-1 gene were resistant to the glucose-lowering effects of insulin [5,6], and this correlated with a marked reduction in insulin-stimulated glucose transport in isolated adipocytes [6]. Distinct experimental approaches have also demonstrated a correlation between PI 3-kinase activity and glucose transport [26–28] and PI 3-kinase activity and glycogen metabolism [28]. Thus, it seems reasonable to speculate that the IRS-1/PI 3-kinase pathway may be linked to the activation of glucose transport and to glycogen synthesis in the muscle, and that a reduction in these associations in both models may have a role in the resulting insulin resistance.

Despite the reduction in insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1, no change was observed in the tyrosine phosphorylation of Shc. After insulin-induced Shc phosphorylation, Shc associates with the SH2 domain of the adapter protein Grb2 [29]. This association induces Grb2 to bind to the nucleotide exchange factor SOS, which in turn associates with and activates the GTP binding protein p21Ras. This pathway has been implicated in the mitogenic effects of insulin [12,30]. Although Ras is an upstream activator of the mitogen-associated protein kinase (MAPK) cascade, recent evidence has shown that stimulation of the MAPK pathway by insulin is not required for many of the metabolic activities of the hormone in cultured fat and muscle cells such as glucose uptake and glycogen synthesis [31]. These results are in accordance with our findings since in both animal models of insulin resistance we observed a marked decrease in the glucose disappearance rate that paralleled a decrease in IRS-1 phosphorylation and association with PI 3-kinase, but not Shc tyrosine phosphorylation. It is interesting that the 60% reduction in IRS-1/PI 3-kinase association in both models was quite similar to the 60% reduction in glucose disappearance rate observed in the two cases.

The dissociation of insulin-induced IRS-1 and Shc tyrosine phosphorylation has been described in cell lines with mutations at two tyrosine phosphorylation sites (Y1158, Y1162F,

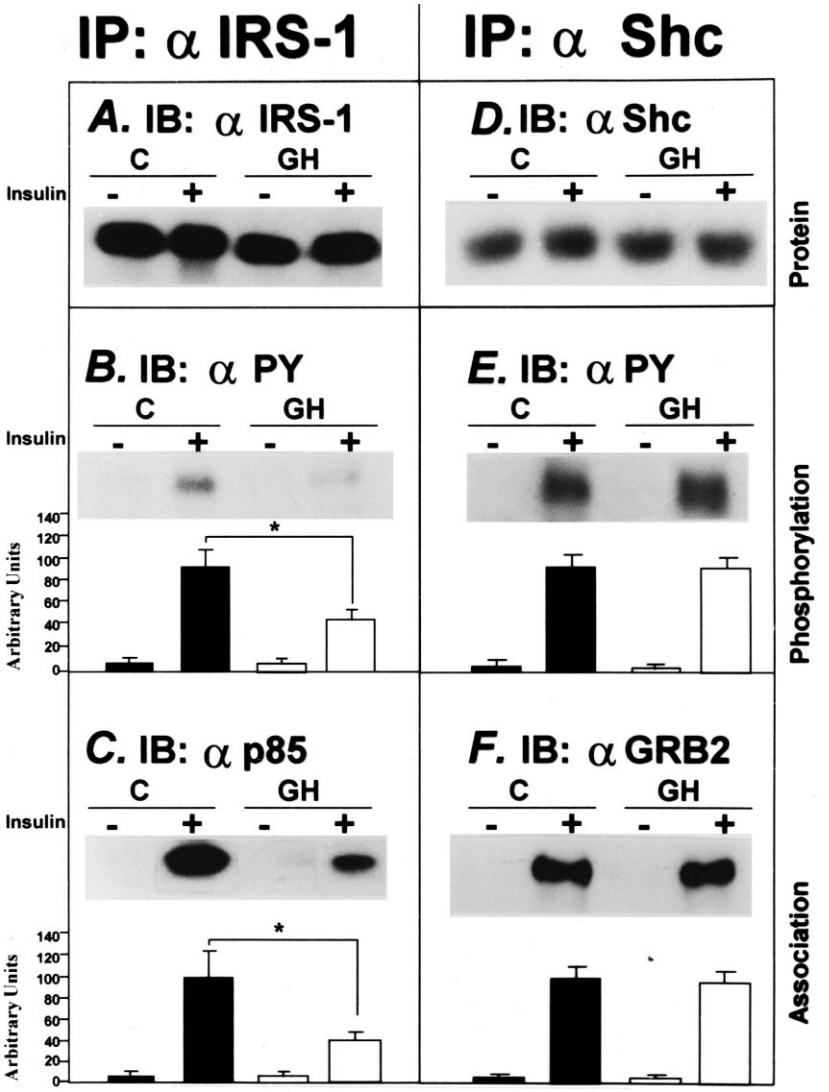

Fig. 4. Insulin-stimulated tyrosine phosphorylation of insulin receptor and Shc in intact muscle from epinephrine-treated rats. Saline (0.9%, lanes 1 and 3 or insulin 6 μg, lanes 2 and 4) was administered into the portal vein as a bolus injection and 90 s later the muscle was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-insulin IRS-1 and immunoblotted with the same antibody (A). The same samples were immunoprecipitated with anti-IRS-1 antibody and immunoblotted with antiphosphotyrosine antibody (B). C: The immunoblotting results of PI 3-kinase in anti-IRS-1 immunoprecipitates from the muscle of control and GH-treated rats. Five minutes after the bolus injection of saline or insulin (6 μg), the muscle was excised and aliquots were immunoprecipitated with anti-Shc antibody and blotted with the same antibody (D). The same samples were immunoprecipitated with anti-Shc antibody and blotted with antiphosphotyrosine antibody (E). F: The immunoblotting results of GRB2 in anti-Shc immunoprecipitates from the muscle of control and epinephrine-treated rats. Scanning densitometry of autoradiograms was performed in six experiments. The black bars represent the control group and the white bars represent the GH-treated group. The data are expressed as the mean ± S.E.M. $^*P < 0.05$.

Y1163F-YFF) on the insulin receptor [32]. Such cell lines showed insulin-induced tyrosine phosphorylation of Shc, Shc-Grb2 complex formation, and p21Ras-GTP formation, but had a reduced tyrosine phosphorylation of IRS-1 as well as a reduced IRS-1 association with Grb2 and PI 3-kinase [32]. These results, together with our own, suggest that a reduction in insulin receptor phosphorylation may differentially induce post-receptor processes by preserving the phosphorylation of some substrates and pathways, but not that of others. A dissociation between IRS-1 and Shc tyrosine phosphorylation without any change in insulin receptor level has also been observed after treatment with dexamethasone [33] or wortmannin [34].

The mechanism by which a reduction in insulin receptor phosphorylation differentially regulates its own substrates is unknown. Shc can directly associate with the insulin receptor, by a binding site to phosphorylated Tyr-960 in the receptor's juxtamembrane region [35]. This is the same binding site for IRS-1 [36], and is consistent with the suggestion that both Shc and IRS-1 compete for this tyrosine residue in their association with the phosphorylated receptor [37]. Although IRS-1 is best known as a substrate for tyrosine phosphorylation, it is mainly a phosphoserine-containing protein. IRS-1 contains over 30 potential serine/threonine phosphorylation sites in motifs recognized by various kinases such as PKC, MAP kinases and cyclic AMP- and cyclic GMP-dependent protein kinases [25,38,39]. Serine phosphorylation also plays a role in the regulation of IRS-1 signalling. Okadaic acid, a serine phosphatase inhibitor, increases serine phosphorylation of IRS-1 in 3T3-L1 adipocytes and this appears to inhibit insulin-

stimulated tyrosine phosphorylation [40]. Thus, it is possible that serine phosphorylation of IRS-1 (induced by chronic GH treatment and/or hyperinsulinemia in one model and by an increase in intracellular cAMP levels following exposure to epinephrine in another) may reduce this substrate's affinity for the insulin receptor, allowing Shc to be more competitive. In support of this hypothesis, Li and Goldstein [34] have recently shown that reducing IRS-1 serine phosphorylation results in increased insulin-induced IRS-1 tyrosine phosphorylation and decreased Shc tyrosine phosphorylation.

Accelerated dephosphorylation of the insulin receptor and IRS-1 by tyrosine phosphatases cannot be excluded in these models of insulin resistance. In this regard, Wilson and Kaczmarek [41] have demonstrated that an increase in cellular cAMP through the activation of PKA increases the activity of endogenous PTPase, thereby leading to a sequence of dephosphorylation.

A further interesting finding in GH-treated rats was the increase in basal Shc tyrosine phosphorylation without any change in basal IRS-1 tyrosine phosphorylation. This increase in phosphorylation may be related to the hormonal milieu of these animals. There is evidence that GH can induce Shc tyrosine phosphorylation in cultured cells [42] and in animal tissues [43]. It is therefore possible that GH, hyperinsulinemia and/or high serum levels of IGF-1 in the animals we studied could preferentially induce Shc tyrosine phosphorylation to the detriment of IRS-1 tyrosine phosphorylation for the same reasons as those discussed above.

We have shown that chronic GH or acute epinephrine treatment can specifically decrease insulin-induced IRS-1 tyrosine phosphorylation and association with PI 3-kinase. This may help to explain the insulin resistance of these animals. In muscle, both chronic GH treatment and the acute administration of epinephrine preserve insulin-induced Shc tyrosine phosphorylation. Further investigation of the hormonal regulation of the distinct signaling pathways activated by insulin receptors will contribute to our understanding of the mechanisms involved in this phenomenon.

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