Rats That Are Made Insulin Resistant by Glucosamine Treatment Have Impaired Skeletal Muscle Insulin Receptor Phosphorylation

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The current study sought to verify whether glucosamine (GlcN)-induced insulin resistance is associated with impaired insulin receptor (IR) autophosphorylation. Rats were given either saline or primed continuous GlcN infusion (5 μmol · kg^{-1} · min^{-1}) 10 minutes prior to and during euglycemic hyperinsulinemic clamp (primed continuous infusion of 20 mU · kg^{-1} · min^{-1} insulin for 2 hours). IR autophosphorylation was measured in skeletal muscle after in vivo insulin stimulation (ie, during clamp) by Western blot and then retested after subsequent in vitro 0.1 to 100 nmol/L insulin stimulation (by enzyme-linked immunosorbent assay [ELISA]). Tissue PC-1 enzymatic activity was also measured. In vivo, insulin/GlcN rats had decreased ($P < .01$) whole body glucose uptake (37.7 ± 2.1 *v* 49.7 ± 2.7 mg · kg^{-1} · min^{-1} in respect to insulin/saline), receptor autophosphorylation (37 ± 5 *v* 82 ± .0 arbitrary units/mg protein), and insulin receptor substrate-1 (IRS-1) phosphorylation (112% ± 15% *v* 198% ± 23% of saline infusion rats). Receptor autophosphorylation was correlated with whole body glucose uptake ($r = 0.62$, $P < .05$). Skeletal muscle PC-1 activity (58.8 ± 10.7 *v* 55.7 ± 5.8 nmol · mg^{-1} · min^{-1}) was not different in the 2 groups. Our data show that GlcN-induced insulin resistance is mediated, at least in part, by impaired skeletal muscle IR autophosphorylation.

GLUCOSAMINE (GlcN) treatment of rats causes insulin resistance,[1] but the early molecular events of GlcN-induced insulin resistance are still poorly understood. In normal rats GlcN infusion inhibits skeletal muscle phosphatidylinositol 3-kinase (PI3K) activity, a key step for insulin-stimulated GLUT 4 translocation and glucose transport.[2-4] However, the role of PI3K activity impairment as the unique molecular defect in GlcN-induced insulin resistance has recently been questioned.[4] Some[5] but not all[2,3] studies reported impaired insulin receptor (IR) tyrosine-kinase (TK) activity on GlcN-induced insulin resistance. These discrepancies may be due to several causes, including different models (in vivo *v* cultured cell lines[2,3,5]) and different tissues examined (ie, skeletal muscle tissue[2,3] *v* adipose cell line[5]). In vivo studies on skeletal muscle[2,3] may be more valuable than in vitro studies on cultured adipose cells.[5] However, previous in vivo studies[2,3] may have been affected by methodological problems obscuring, at least in part, the inhibiting effect of GlcN on on insulin receptor (IR) tyrosine-kinase (TK) activity. In one study,[2] GlcN-pretreated rats were administered intravenous (IV) insulin at 10 U/kg body weight, a very high pharmacological dose that might have overridden some GlcN inhibitory effect. In the other study,[3] GlcN was coinfused with insulin during the euglycemic clamp, thus allowing an initial IRTK activity stimulation before the inhibitory effect of GlcN was established.

The present study was therefore aimed at verifying whether at usual, and lower (ie, 20 mU/kg/min/body weight), insulin concentrations skeletal muscle IRTK stimulation is impaired and contributes to insulin resistance in GlcN-pretreated rats.

MATERIALS AND METHODS

Animal Preparation

Male Sprague-Dawley rats (Charles River Laboratories, Lecco, Italy; body weight, ~250 g) were maintained as described[1] and randomly assigned to the insulin/glucosamine (Ins/GlcN, n = 6), insulin/saline (Ins/Sal, n = 5), or saline control (Sal, n = 4) group.

Methods

In Vivo Studies

Based on previous experience,[1] a priming bolus (180 μmol · kg^{-1}) followed by a constant (5 μmol · kg^{-1} · min^{-1}) GlcN infusion was started 10 minutes prior to the clamp (primed continuous infusion of 20 mU · kg^{-1} · min^{-1} of insulin plus approximately 0.4 μCi/min of [3-^{3}H]-glucose (Du Pont-New England Nuclear, Milan, Italy) and continued throughout the 2-hour study,[1] when rats were injected pentobarbital (60 mg/kg body weight, IV), and the hindlimb muscle tissues (selected because of their mixed composition of fiber types) were freeze-clamped in situ with aluminium tongs precooled in liquid nitrogen. Whole body glucose uptake was calculated on [^{3}H]-glucose specific activities during steady-state.[1] All tissue samples were kept frozen at −80°C until analyzed.

IR Phosphorylation and Binding Measurements

IR phosphorylation in vivo. Rat muscle IR phosphorylation in vivo stimulated by insulin infusion during clamp were measured by Western blot analysis and by enzyme-linked immunosorbent assay (ELISA) (see below). For Western blot analysis, tissues were homogenized and solubilized in Hepes buffer (50 mmol/L Hepes pH 7.6, 150 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 20 mmol/L sodium pyrophosphates, 1 mmol/L PMSF, 1 mmol/L sodium vanadate, 20 mmol/L sodium fluoride, 1 mg/mL bacitracin).[6] Muscle extracts (1.0 mg protein) were immunoprecipitated (2 hours at 4°C) with 4 μg specific anti-IR monoclonal antibody αCT-1 conjugated with rabbit antimouse

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IgG prebound to protein A-Sepharose and subjected to 7.5% polyacrylamide gel electrophoresis (PAGE). Proteins were transferred (3 hours at 4°C) to nitrocellulose membranes and IR phosphorylation detected by an antiphosphotyrosine (PY) antibody (UBI, Lake Placid, NY). After 16 hours at 4°C, membranes were incubated with a rabbit antimouse serum conjugated with horseradish peroxidase (1 hour at 22°C) and the reaction developed according to an enhanced chemiluminescence (ECL) detection system and the specific signaling revealed by autoradiography.

IR phosphorylation in vitro. IR phosphorylation in response to increased insulin concentration was measured by a specific ELISA.[7] In detail, rat (Sal, Ins/Sal, and Ins/GlcN) muscle extracts were solubilized in Hepes buffer (see above) at 4°C for 60 minutes. After centrifugation at 12,000 × *g* for 15 minutes at 4°C, IRs (50 μg of protein) were immunocaptured on plastic wells precoated with a monoclonal antibody specific to the IR (αCT-1). IR extracts were incubated with 5 mmol/L $MnCl_2$, 24 mmol/L $MgCl_2$ in the absence or presence of 0.1 to 100 nmol/L insulin. After 15 minutes, adenosine triphosphate (ATP) (10 μmol/L) was added for 1 hour, and then wells washed 5 times and a biotinylated anti-PY antibody added for 2 hours at 22°C. Bound antibody was revealed by the peroxidase-conjugated streptavidin method. Since IR phosphorylation is measured on purified IR extracts in presence of exogenous ATP, the measured activity is independent of any (possible) decrease in cellular ATP production.

Specific ^{125}I-insulin binding in solubilized tissues was also studied as previously described.[7]

Insulin Receptor Substrate-1 Phosphorylation

For insulin receptor substrate-1 (IRS-1) phosphorylation measurement, muscle tissues, stimulated in vivo during clamp, were solubilized in Hepes buffer (see above). The muscle extracts were immunoprecipitated by an anti–IRS-1 monoclonal antibody (4 μg/mL) (UBI, Lake Placid, NY) conjugated with protein A-Sepharose (16 hours at 4°C). Proteins were then separated by sodium dodecyl sulfate (SDS)-PAGE (7.5% polyacrylamide), transferred to nitrocellulose membranes, incubated with anti-PY antibody (1 μg/mL), and quantified by rabbit antimouse antiserum conjugated with horseradish peroxidase and ECL system.

PC-1 Activity in Skeletal Muscle

PC-1 phosphodiesterase activity was measured by hydrolysis of thymidine 5′monophosphate *p*-nitrophenyl ester (PNTP) as previously described.[6]

Statistical Analysis

Differences between groups were tested by Student's *t* test and 1-way analysis of variance (ANOVA). Correlations between variables were analyzed by linear regression.

RESULTS

In Vivo Studies

Whole body glucose uptake was significantly decreased in Ins/GlcN rats (37.7 ± 2.1 mg · kg^{-1} · min^{-1}) as compared to Ins/Sal animals (49.7 ± 2.7, $P < .01$). As already reported,[1] decreased glucose uptake was observed from the very beginning of glucose infusion, and was due to a reduction in both glycogen synthesis and glycolysis (data not shown).

IR autophosphorylation, measured by Western blot (Fig 1A), was significantly ($P < .01$) reduced in skeletal muscle of Ins/GlcN rats (37 ± 5 arbitrary densitometric units/mg protein, mean ± SE of 5 different experiments) as compared to Ins/Sal animals (82 ± 3.0, mean ± SE). In control (Sal) rats, IR autophosphorylation was barely detectable and significantly ($P < .01$) lower than in the other 2 groups (Fig 1A). When data from Ins/GlcN-infused animals were considered, individual IR autophosphorylation values were significantly correlated with individual whole body glucose uptake at euglycemic clamp ($r = 0.81$, $P = .05$); this correlation remain significant when all data, from animals infused both with Ins/Sal and Ins/GlcN, were considered together ($r = 0.62$, $P < .05$) (Fig 1B).

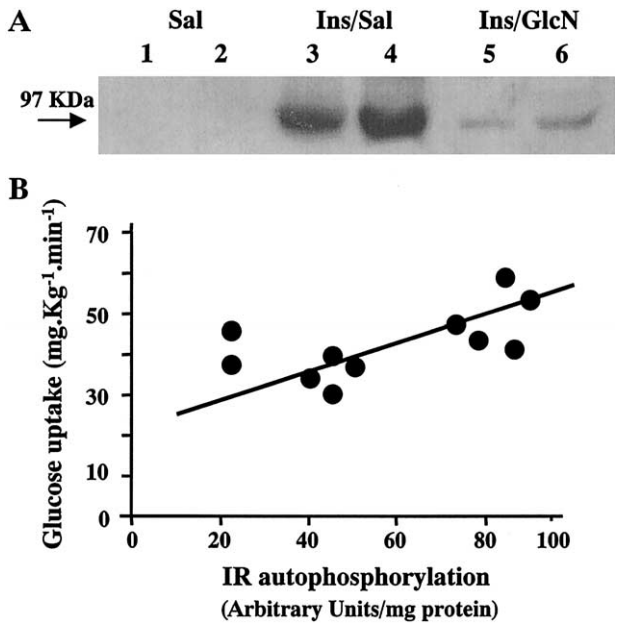

Fig 1. (A) IR autophosphorylation in rat skeletal muscle in response to in vivo insulin stimulation (during euglycemic hyperinsulinemic clamp. Western blots were performed by immunoprecipitating muscle extracts with an anti-IR (αCT-1) antibody and immunoblotting the electrophoresed protein with an anti-PY antibody. A representative experiment is shown in 2 animals infused with saline (Sal), 2 with insulin and saline (Ins/Sal), and 2 with insulin and glucosamine (Ins/GlcN), as described in the Methods. (B) Correlation between muscle IR autophosphorylation measured by Western blot and glucose uptake during euglycemic clamp in 11 rats is shown.

In these solubilized muscle tissues, a significant reduction of IR autophosphorylation was observed in Ins/GlcN as compared to Ins/Sal group also by ELISA (446 ± 60 *v* 968 ± 111 optical density/50 μg of protein, $P < .0001$). Differences reflected those observed by Western blot analysis. In fact, a significant correlation between ELISA and Western blot data was observed in tissues from the same animals ($r = 0.83$, $P < .01$). Also, receptor phosphorylation measurements by ELISA significantly correlated with whole body glucose uptake at euglycemic clamp ($r = 0.76$, $P < .03$).

To examine if GlcN has any significant effect on the other component of the insulin signaling cascade, we measured the IRS-1 phosphorylation by Western blot. In the solubilized muscle tissue of GlcN-infused (Ins/GlcN) rats, a significant ($P < .02$) reduction of IRS-1 phosphorylation was observed in respect to insulin (Ins/Sal)-infused rats (Fig 2).

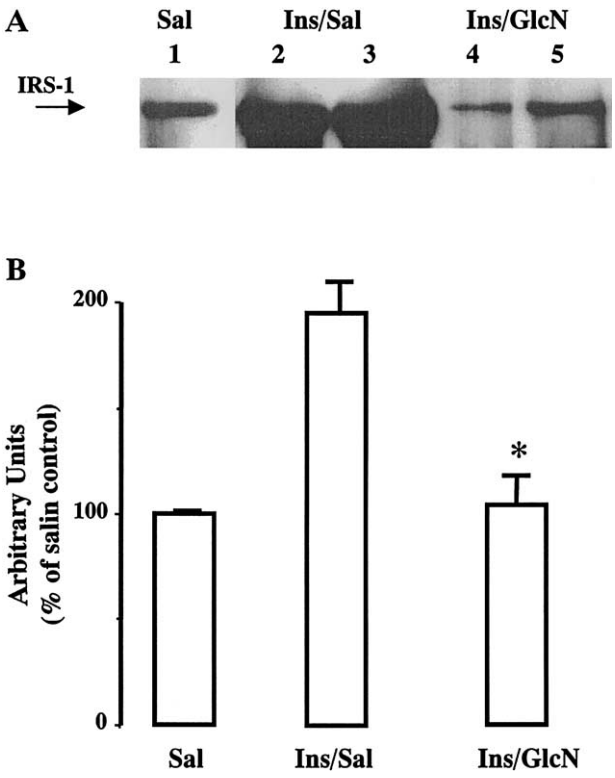

Fig 2. In vivo insulin-stimulated IRS-1 phosphorylation is reduced by GlcN infusion. (A) A representative Western blot experiment is shown. Muscle extract, obtained from animals infused only with saline (Sal, line 1), with insulin and saline (Ins/Sal, lines 2 and 3), and with insulin and glucosamine (Ins/GlcN, lines 4 and 5) were immunoprecipitated with an anti–IRS-1 antibody. Proteins were subjected to electrophoresis and immunoblotted with an anti-PY antibody. (B) Quantitative results from 3 different experiments are shown. Data are expressed as percent of value of saline-infused rats (*$P < .02$ *v* Ins/Sal).

In Vitro Studies

In contrast to in vivo studies, when solubilized muscle tissues were incubated in vitro with 0.1 to 100 nmol/L insulin, a similar IR autophosphorylation was observed in Ins/GlcN as compared to Ins/Sal and Sal rats (Fig 3A). Differences between Ins/Sal and Ins/GlcN rats in receptor phosphorylation in the absence (insulin 0) of in vitro insulin stimulation are due to the above reported different receptor stimulation by in vivo insulin infusion (Fig 3B). This is also suggested by the lower level of IR autophosphorylation in the absence of in vitro insulin stimulation in tissues from animals (Sal) that did not undergo euglycemic hyperinsulinemic clamp (Fig 3B).

No difference in specific ^{125}I-Insulin binding was observed in the solubilized tissues from from Sal, Ins/Sal, and Ins/GlcN rats (2.2 ± 0.75 *v* 2.6 ± 0.29 *v* 3.3 ± 0.61 ng/mL of insulin, respectively).

PC-1 Activity in Skeletal Muscle

PC-1 activity, as measured by its phosphodiesterase activity, was not different (54.2 ± 3.9 *v* 55.7 ± 5.8 *v* 58.8 ± 10.7 nmol · mg^{-1} · min^{-1}, P = not significant) in solubilized skeletal muscle from Sal, Ins/Sal, and Ins/GlcN rats.

DISCUSSION

Our data indicate that GlcN-induced insulin resistance in the rat is mediated, at least in part, by impairment of skeletal muscle IR phosphorylation and IRS-1 phosphorylation. The impairment of IR phosphorylation in presence of high GlcN concentration is in agreement with previous studies in cultured adipose cells,[5] but not with previous animal studies.[2,3] As already mentioned, several methodological differences might explain the discrepancies between the present and previous studies.[2,3] In the study of Patti et al,[3] no GlcN pretreatment was used and, as a consequence, the effects of the increased hexosamine metabolism on glucose uptake become evident only when insulin stimulation already occurred, ie, after 2 hours. Although GlcN treatment is a valid model for increasing hexosamine metabolism (as it occurs in glucose and lipo-toxicity) GlcN pretreatment is mandatory to mimic insulin resistance

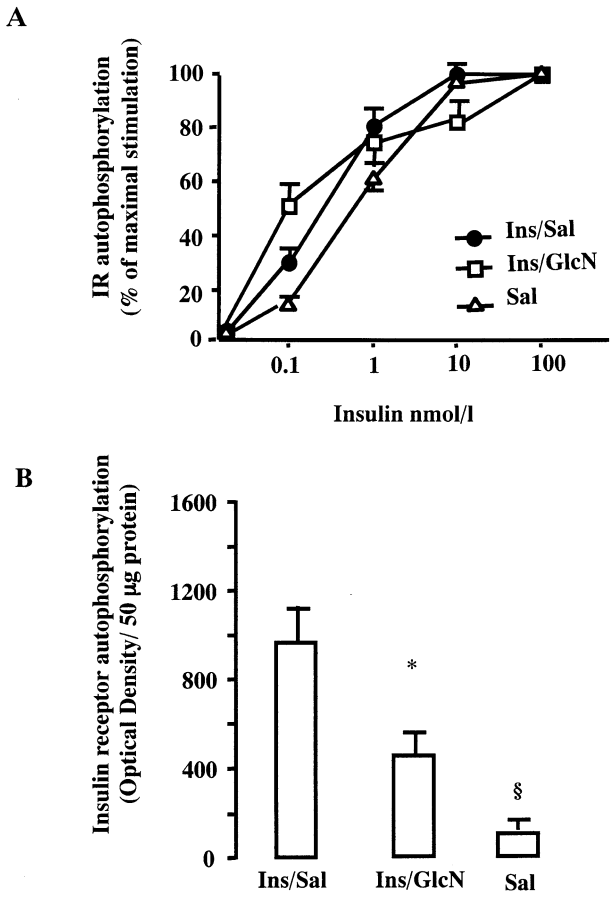

Fig 3. IR autophosphorylation measured in vitro by ELISA in skeletal muscle from animals infused only with insulin and saline (Ins/Sal), with insulin and glucosamine (Ins/GlcN), and with saline (Sal) (see Methods). IR phosphorylation was measured in the presence or absence of increasing insulin concentrations. (A) Data are expressed as percent of maximal activity of the IR autophosphorylation. (B) IR phosphorylation measured in the absence of insulin (*$P < .001$ and §$P < .05$ *v* Ins/Sal).

that occurs in these conditions. In contrast, in our GlcN-pretreated rats a reduced glucose uptake was observed from the very beginning of the clamp study (data not shown). In the study of Kim et al,[2] animals were preinfused with GlcN for 2 hours before the 10-U/kg body weight insulin bolus. However, the use of a pharmacological insulin dose (ie, 10^3-fold the highest effective dose that can override some GlcN inhibitory effects) leaves open the possibility that impaired tyrosine phosphorylation may be observed when a lower dose of insulin is used.

In contrast to in vivo studies, when purified insulin receptors were stimulated in vitro with insulin, no effect of the previous in vivo infusion with GlcN was observed. One can speculate that the GlcN effect can be overridden by higher insulin concentrations; otherwise we cannot exclude that differences between in vivo versus in vitro insulin stimulation of partially purified IR can explain this apparent discrepancy. In fact, it is possible that phosphorylation response of immunopurified IR to in vitro insulin stimulation cannot perfectly reflect in vivo IR phosphorylation response. In addition, although it is difficult to image that 2 hours of GlcN exposure may significantly alter IR and IRS-1 expression, we cannot exclude this possibility. Studies using tissue sample not previously exposed to insulin in vivo deserve further and deeper investigation.

The overexpression of several negative modulators may negatively affect IRTK activity and, eventually, cause insulin resistance. Among these inhibitors is PC-1 a membrane class II glycoprotein with phosphodiesterase and pyrophosphatase enzymatic activity.[6-8] When PC-1 cDNA is transfected and PC-1 overexpressed in cells, IRTK activity and subsequent insulin signaling are decreased, suggesting that PC-1 is an inhibitor of IR signalling.[9] Also, in insulin target tissues (including muscle and adipose tissues), PC-1 overexpression is associated with whole body insulin resistance and reduced IRTK activity.[6-9] It has been suggested that PC-1 inhibits IR signaling through protein-protein interaction with the alpha subunit of IR.[10] Our data demonstrate that PC-1 is not overexpressed after skeletal muscle exposure to high GlcN. PC-1, therefore, is unlikely to play a role in GlcN-induced IR phosphorylation impairment and insulin resistance.

The present data, together with previous data from other studies,[2-5] indicate that GlcN-induced insulin resistance in rat skeletal muscle is also due to a defective IRTK activity. This, of course, does not imply at all that GlcN reduces insulin sensitivity only by IRTK activity modulation. In fact, other mechanisms for GlcN inhibition of insulin sensitivity have been clearly reported.[2-4] Previous reports[2-5] and the present findings strongly support the idea that impairments at more than one site in the insulin signaling cascade are likely to be responsible for GlcN-induced insulin resistance.

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