

Activation of the Hexosamine Pathway by Glucosamine in Vivo Induces Insulin Resistance of Early Postreceptor Insulin Signaling Events in Skeletal Muscle

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To explore potential cellular mechanisms by which activation of the hexosamine pathway induces insulin resistance, we have evaluated insulin signaling in conscious fasted rats infused for 2–6 h with saline, insulin ($18 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), or insulin and glucosamine ($30 \text{ } \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) under euglycemic conditions. Glucosamine infusion increased muscle UDP-*N*-acetylglucosamine concentrations 3.9- and 4.3-fold over saline- or insulin-infused animals, respectively ($P < 0.001$). Glucosamine induced significant insulin resistance to glucose uptake both at the level of the whole body and in rectus abdominis muscle, and it blunted the insulin-induced increase in muscle glycogen content. At a cellular level, these metabolic effects were paralleled by inhibition of postreceptor insulin signaling critical for glucose transport and glycogen storage, including a 45% reduction in insulin-stimulated insulin receptor substrate (IRS)-1 tyrosine phosphorylation ($P = 0.02$), a 44% decrease in IRS-1 association with the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase ($P = 0.03$), a 34% reduction in IRS-1-associated PI 3-kinase activity ($P = 0.03$), and a 51% reduction in insulin-stimulated glycogen synthase activity ($P = 0.03$). These alterations in postreceptor insulin signaling were time-dependent and paralleled closely the progressive inhibition of systemic glucose disposal from 2 to 6 h of glucosamine infusion. We also demonstrated that glucosamine infusion results in *O*-linked *N*-acetylglucosamine modification of IRS-1 and IRS-2. These data indicate that activation of the hexosamine pathway may directly modulate early postreceptor insulin signal transduction, perhaps via posttranslational mod-

ification of IRS proteins, and thus contribute to the insulin resistance induced by chronic hyperglycemia. *Diabetes* 48:1562–1571, 1999

Studies performed in patients with type 1 diabetes (1) and in rats in vivo (2) have established that chronic hyperglycemia is an independent cause of insulin resistance. Overactivity of the hexosamine pathway has recently been suggested to be one of the mechanisms mediating glucose-induced insulin resistance or “glucose toxicity” (3). Initial studies by Marshall and colleagues (42,43) demonstrated that prolonged incubation of adipocytes with insulin, glucose, and glutamine resulted in desensitization of glucose transport. The requirement for glutamine ultimately led to the identification of an important role for glutamine: fructose-6-phosphate amidotransferase (GFA), the rate-limiting enzyme of hexosamine biosynthesis, in mediating these effects. Overexpression of GFA in skeletal muscle of transgenic mice (4) and in Rat-1 fibroblasts (5) results in severe insulin resistance. Infusion of glucosamine, which directly activates the hexosamine pathway by bypassing the reaction catalyzed by GFA, mimics this insulin resistance in skeletal muscle of normal rats (6–8). In partially pancreatectomized diabetic rats, infusion of glucosamine has no effect on insulin-stimulated glucose uptake, suggesting that insulin resistance in this setting is due to preexisting activation of the hexosamine pathway (7).

The mechanisms that link activation of the hexosamine pathway to insulin resistance are presently poorly understood. Sustained increases in circulating glucosamine concentrations impair both whole-body and muscle glucose uptake, glycogen synthesis, and glycolysis without increasing intracellular glucose-6-phosphate (G-6-P) concentrations, suggesting that glucosamine exerts its effects at an early step in glucose transport (7). Therefore, we sought to investigate whether the insulin resistance induced by activation of the hexosamine pathway is mediated via downregulation of early steps in insulin signal transduction critical for glucose transport and metabolism.

RESEARCH DESIGN AND METHODS

Materials. Reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA). [γ - ^{32}P]ATP was supplied by Du Pont-NEN (Boston, MA). ^{125}I -labeled protein A was supplied by ICN Radiochemicals (Lisle, IL). All other chemical reagents were from Sigma (St. Louis, MO), except as noted.

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[^{14}C]DOG, 2-[1- ^{14}C]deoxyglucose; [^{14}C]DOG-6-P, 2-[1- ^{14}C]deoxyglucose-6-phosphate; CT, COOH-terminal; G-6-P, glucose-6-phosphate; GFA, glutamine:fructose-6-phosphate amidotransferase; GLCN, insulin and glucosamine; GlcNAc, *N*-acetylglucosamine; GlcN-6-P, glucosamine-6-phosphate; HPLC, high-performance liquid chromatography; INS, insulin and saline; IR, insulin receptor; IRS, insulin receptor substrate; NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases; PI, phosphatidylinositol; PKI, protein kinase inhibitor; PMSF, phenylmethylsulfonyl fluoride; R_g , glucose uptake; SAL, saline; UDP-Gal, UDP-galactose; UDP-GalNAc, UDP-*N*-acetylgalactosamine; UDP-Glc, UDP-glucose; UDP-GlcNAc, UDP-*N*-acetylglucosamine.

α -Insulin receptor substrate (IRS)-1-COOH-terminal (CT) antibodies were raised in rabbits using a synthetic peptide derived from the rat CT sequence (TYASINFQKQPEDRQ). α -Insulin receptor (IR)-CT antibodies were raised in rabbits using a synthetic peptide corresponding to the human CT 15 amino acids (KKNRILLTLPRSNPS). Polyclonal antibodies to the murine p85 α -subunit of phosphatidylinositol (PI) 3-kinase (0.5 mg/ml), IRS-2, and the monoclonal antiphosphotyrosine antibody 4G10 (0.2 mg/ml) were provided by Dr. Morris White. Other antibodies included antiphosphotyrosine (pY20; Transduction Labs, Lexington, KY), polyclonal anti-p85 (UBI, Lake Placid, NY), anti-human Akt, PH domain (UBI), and monoclonal anti-O-linked *N*-acetylglucosamine (GlcNAc) RL2 (Affinity Bioreagents, Golden, CO).

Preparation of animals. Male Wistar rats were purchased from the Helsinki University Experimental Animal Center. Animals were housed in a 12-h light-dark cycle and fed standard rodent food (Altromin 1323; Feedcon, Helsinki, Finland). At 6 days before the study, rats were anesthetized (pentobarbital, 60 mg/kg i.p.) and catheters were inserted into the aortic arch for blood sampling and into the right atrium for infusions, as previously described (9).

Study design. Three groups of rats were studied. The first received only saline (SAL, $n = 10$). Two other weight-matched groups were studied under normoglycemic-hyperinsulinemic conditions (insulin infusion rate of $18 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$). These rats received, in addition to glucose and insulin, intravenous infusions of either saline (INS, $n = 10$) or glucosamine (GLCN, $n = 10$) at a rate of $30 \text{ } \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, as detailed below.

Rats were studied after a 16-h fast. On the morning of the study, rats were weighed, connected to infusion and blood sampling lines, and placed in metabolic cages, where they were allowed to move freely. After 1 h, a primed-continuous infusion of insulin (INS and GLCN rats) or saline (SAL) was started (9–11). Plasma glucose was measured every 5 min (INS and GLCN rats) or 30 min (SAL) using the glucose oxidase method (Beckman Glucose Analyzer II, Fullerton, CA). In INS and GLCN rats, a variable rate infusion of 20% glucose was started at 4 min and adjusted to maintain the plasma glucose concentration at fasting concentrations. Serum insulin concentrations at 0, 30, 60, 120, 180, 240, 300, and 360 min were determined using radioimmunoassay (12).

At 315 min, a bolus injection of 2-[1- ^{14}C]deoxyglucose (^{14}C]DOG) (50 μCi) was given to determine tissue glucose uptake. Samples for determination of ^{14}C]DOG specific activity in plasma (dpm/ μmol glucose) were withdrawn at 317, 320, 325, 330, 335, 345, and 360 min. At the end of the study, rats were anesthetized with pentobarbital, and rectus abdominis muscle was freeze-clamped in situ with aluminum tongs precooled in liquid nitrogen for measurement of insulin signaling parameters and 2-[1- ^{14}C]deoxyglucose-6-phosphate (^{14}C]DOG-6-P) concentrations. Tissue samples were stored in liquid nitrogen until analysis. The experimental protocol was approved by the ethical committee of the Helsinki University Central Hospital.

Immunoprecipitation and immunoblotting. Rectus abdominis muscle samples frozen in liquid nitrogen were pulverized with a stainless steel mortar and pestle and then homogenized using a Polytron (Brinkmann Instruments, Westbury, NY) for 30 s at 4°C in buffer A (50 mmol/l HEPES, pH 7.4, 137 mmol/l NaCl, 1 mmol/l MgCl_2 , 1 mmol/l CaCl_2 , 2 mmol/l EDTA, 10 mmol/l sodium pyrophosphate, 10 mmol/l sodium fluoride, 2 mmol/l sodium orthovanadate, 2 mmol/l phenylmethylsulfonyl fluoride (PMSF), 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 mmol/l benzamidine, 10% glycerol, and 1% NP-40). Homogenates were allowed to solubilize at 4°C for 30 min and were then clarified by centrifugation at 15,000g for 30 min. Supernatants of tissue homogenates containing equal amounts of total protein (2–5 mg) were immunoprecipitated overnight with anti-IRS-1-CT, anti-pY (4G10), anti-p85, or anti-IR-CT antibodies. Immune complexes were collected with protein A Sepharose (Pharmacia, Uppsala, Sweden), washed extensively, and solubilized in Laemmli sample buffer. Proteins were separated using 7.5 or 10% SDS-PAGE, transferred onto nitrocellulose (Schleicher & Schuell, Keene, NH), and immunoblotted using the indicated antibodies and ^{125}I -protein A for detection (13). Quantitation was performed using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

PI 3-kinase activity. Tissues were homogenized immediately in buffer A, centrifuged, and immunoprecipitated with anti-IRS-1-CT antibodies; immune complexes were collected with protein A Sepharose and washed extensively, as described previously (13). In vitro kinase assays were performed using PI as a substrate. ^{32}P incorporation into PI 3-phosphate was quantified using a PhosphorImager. To convert PhosphorImager units to counts per minute equivalents, serial dilutions of the [γ - ^{32}P]ATP were spotted and imaged on the PhosphorImager in parallel with tissue immunoprecipitate samples. The dilution samples were then cut from the thin-layer chromatography plate and counted for ^{32}P . A linear standard curve was constructed by plotting PhosphorImager units against counts per minute for the diluted standard. This method permits normalization of tissue sample results to counts per minute equivalents and comparison of results from different experiments.

Akt immune complex kinase assay. Muscle homogenates were immunoprecipitated with an anti-Akt antibody (PH domain), washed twice with buffer A and

once with kinase buffer (50 mmol/l Tris, 10 mmol/l MgCl_2 , 1 mmol/l dithiothreitol, 1 mmol/l benzamidine, and 0.5 mmol/l PMSF), and resuspended in 30 μl of kinase buffer. Crosside and protein kinase inhibitor (PKI) were added to each tube; samples were prewarmed at 30°C for 5 min, followed by addition of hot ATP mix. Final concentrations in the 50- μl reaction volume were 1 $\mu\text{mol}/\text{l}$ PKI, 30 $\mu\text{mol}/\text{l}$ crosside substrate, 10 mmol/l MgCl_2 , 50 $\mu\text{mol}/\text{l}$ ATP, and 5 $\mu\text{Ci}/\text{tube}$ ^{32}P . Reactions were stopped by the addition of 1 mmol/l cold ATP to the final concentration; 40 μl of the reaction product was spotted onto p81 phosphocellulose paper, washed with 1% phosphoric acid, rinsed with acetone, and counted for incorporated radioactivity.

Glycogen synthase activity assay. Muscle homogenates were diluted 1:5 in glycogen synthase assay buffer; synthase activity was measured as described previously (14). Activity was expressed as fractional velocity of glycogen synthase, defined as the ratio of activity at 0.1 mmol/l G-6-P to that at 10 mmol/l G-6-P for each sample.

Muscle [^{14}C]DOG glucose uptake, glycogen, and ATP concentrations. Tissue concentration of [^{14}C]DOG-6-P (disintegrations per minute/milliliter) was measured using anion exchange chromatography, as previously described in detail (15,16). Tissue glucose uptake (R_g) was corrected for ^{14}C radioactivity incorporation into glycogen. Glycogen concentration was measured from freeze-dried tissue samples, which were dissected free of blood and connective tissue, and weighed and extracted with alkali as previously described (9,17). Glucose concentration was determined before and after amyloglucosidase treatment using a fluorometric assay (9). For the measurement of ATP concentrations, perchloric acid extract was neutralized with KHCO_3 . ATP concentrations were determined with a fluorometric enzymatic assay (18).

Muscle UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine, UDP-glycose, and UDP-galactose concentrations. The concentration of UDP-*N*-acetylglucosamine (GlcNAc), the end product of the hexosamine biosynthesis pathway, was measured in muscle tissue, as described by Robinson et al. (19) and Hebert et al. (4). Frozen abdominal muscle (~300 mg) was homogenized at 4°C in 4 vol of 0.3 mol/l perchloric acid. The precipitates were pelleted by centrifugation (10,000g for 15 min at 4°C). The supernatant was delipidated and neutralized using 2 vol of tri-*n*-octylamine:1,1,2-trichloro-trifluoroethane (1:4). Extracts were filtered (0.22 μm), and high-performance liquid chromatography (HPLC) was performed using two Spherisorb amino columns (25 cm \times 3 mm; Phase Separations, Norwalk, CT) connected in series. The columns were eluted with a concave gradient from 80 mmol/l potassium phosphate (pH 2.8), 35% acetonitrile, to 800 mmol/l potassium phosphate (pH 3.6) over 70 min at a flow rate of 0.3 ml/min at room temperature. Under these conditions, UDP-GlcNAc, UDP-*N*-acetylgalactosamine (UDP-GalNAc), UDP-glucose (UDP-Glc), and UDP-galactose (UDP-Gal) eluted as separate peaks between 40 and 45 min. Their concentrations were quantitated by UV absorption (254 nm) and by comparison to external standards. The standard curves were linear over a concentration range of 1–20 nmol/peak. The recovery of standards added to muscle homogenates was >95%.

Serum glucosamine concentrations. Serum glucosamine concentrations were assayed using the same sample preparation procedure and reverse-phase HPLC setup as that used for glucosamine-6-phosphate (GlcN-6-P) in the GFA assay described previously (20,21). The mobile phase consisted of a two-step gradient made of buffers identical to those used for the GlcN-6-P assay (20). Glucosamine eluted with a retention time of 20.6 min. The correlation coefficient between the concentration of glucosamine standards mixed in control serum and the area under the curve was 0.985.

Statistical analysis. All data are expressed as means \pm SE. For longitudinal comparisons between the study groups, data were analyzed using analysis of variance. For cellular analyses of tissue samples at either 2 or 6 h of infusion, data were compared using paired Student's *t* tests. Simple correlations between selected study variables were calculated using Pearson's correlation coefficient for variables that were not normally distributed.

RESULTS

Metabolic parameters. Fasting plasma glucose concentrations in the rats did not differ among the three experimental groups and were held steady at $5.6 \pm 0.1 \text{ mmol}/\text{l}$ during the hyperinsulinemic clamp with or without glucosamine (Table 1). Likewise, serum insulin concentrations did not differ during the clamp. However, plasma glucosamine concentrations were significantly elevated in the GLCN rats ($0.81 \pm 0.10 \text{ mmol}/\text{l}$) while being undetectable (<0.05 – $0.1 \text{ mmol}/\text{l}$) in the SAL and INS rats. The infusion of glucosamine and insulin for 360 min reduced whole-body glucose uptake by 29% as compared with insulin infusion alone ($P < 0.001$), as in previous studies (15). During the last hour of the study,

TABLE 1
Metabolic parameters in rats

	SAL	INS	GLCN
Fasting plasma glucose (mmol/l)	6.6 ± 0.3	6.6 ± 0.5	6.4 ± 0.3
Clamp plasma glucose (mmol/l)	5.7 ± 0.1	5.6 ± 0.1	5.6 ± 0.1
Clamp serum insulin (pmol/l)	—	1,284 ± 168	1,152 ± 177
Serum glucosamine (mmol/l)	ND	ND	0.81 ± 0.1
Glucose uptake (0–360 min) ($\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)	—	188 ± 8	133 ± 8*
Glucose uptake (300–360 min) ($\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)	—	201 ± 7	106 ± 12*
Rectus [^{14}C]DOG uptake (315–360 min) ($\text{nmol} \cdot \text{mg}^{-1} \text{protein} \cdot \text{min}^{-1}$)	—	2.6 ± 0.3	1.3 ± 0.2*
Muscle glycogen ($\mu\text{mol/g}$ dry wt)	138 ± 10	390 ± 25†	274 ± 27*
Muscle ATP (mmol/kg wet wt)	5.2 ± 0.2	5.6 ± 0.9	5.5 ± 0.3
UDP-GlcNAc (nmol/g wet wt)	25.4 ± 1.2	23.1 ± 0.9	99.2 ± 6.4‡
UDP-GalNAc (nmol/g wet wt)	10.6 ± 0.7	10.3 ± 1.0	19.5 ± 1.1‡
UDP-Glc (nmol/g wet wt)	18.3 ± 1.3	12.7 ± 1.7§	8.9 ± 0.6§
UDP-Gal (nmol/g wet wt)	7.6 ± 0.3	6.8 ± 1.3	3.0 ± 0.2*

Data are means ± SE. Rats were infused for 360 min with either saline (SAL), insulin and saline (INS), or insulin and glucosamine (GLCN). * $P < 0.001$ for GLCN vs. INS, † $P < 0.001$ for INS vs. SAL, ‡ $P < 0.001$ for GLCN vs. SAL and INS, § $P < 0.001$ for GLCN vs. SAL, $P < 0.01$ for INS vs. SAL. ND, not detectable (<0.05 – 0.1 mmol/l).

when muscle glucose uptake was measured, the rate of whole-body glucose uptake was 47% lower in the glucosamine-infused rats ($P < 0.001$).

To quantitate insulin-stimulated muscle-specific glucose uptake, the accumulation of [^{14}C]DOG-6-P during the last 45 min of the study was measured. The addition of glucosamine to insulin decreased [^{14}C]DOG uptake by 50% as compared with insulin alone ($P < 0.001$) in rectus abdominis muscle ($n = 6$ per group). The insulin-stimulated increase in muscle glycogen content was also significantly blunted in glucosamine-infused rats (46% reduction, $P < 0.001$ vs. insulin).

Recent data have demonstrated that incubation of 3T3-L1 cells with glucosamine reduces ATP content in parallel with inhibition of glucose transport (22). To exclude a similar effect of glucosamine in vivo, we measured ATP content in rectus abdominis muscle ($n = 8$ for SAL and INS groups, $n = 10$ for GLCN group). There were no differences in ATP content between the control, insulin-, or glucosamine-infused rats.

To confirm that glucosamine infusion increased key hexosamine pathway metabolites in skeletal muscle, we directly measured concentrations of UDP-GlcNAc, UDP-GalNAc, UDP-Glc, and UDP-Gal in rectus abdominis in seven animals from each group (Table 1). UDP-GlcNAc increased modestly after 2 h of glucosamine infusion (2-fold, NS, data not shown) but was significantly increased by 6 h (4.3-fold over INS, $P < 0.001$). UDP-GalNAc was also increased (1.9-fold vs. INS, $P < 0.001$) in 6-h glucosamine-treated rats, consistent with the ability of infused glucosamine to enter muscle cells and activate the hexosamine pathway. By contrast, glucosamine infusion decreased muscle concentrations of UDP-Glc at 2 h (44% reduction, $P < 0.05$) and at 6 h (30% reduction, $P < 0.001$); UDP-Gal was also reduced by 6 h (56% decrease vs. INS, $P < 0.001$). **Glucosamine infusion does not alter IR phosphorylation or expression.** Binding of insulin to the extracellular domain of its transmembrane receptor induces activation of the receptor tyrosine kinase, tyrosine phosphorylation of a variety of IRS proteins, and propagation of multiple signal trans-

duction cascades, which result in the biological effects of insulin. Because IR kinase activity and protein content are downregulated in some forms of insulin resistance (23–25) and alterations in IR phosphorylation could contribute to the observed inhibition of insulin-stimulated glucose disposal, we evaluated the effects of insulin and glucosamine infusion on IR tyrosine phosphorylation in skeletal muscle. Tissue homogenates were immunoprecipitated with anti-IR antibodies; immune complexes were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine antibodies. As seen in Fig. 1, insulin infusion resulted in a 3.6-fold stimulation of IR phosphorylation at 6 h ($P < 0.01$ vs. basal). However, this was not affected by the addition of glucosamine for either 2 or 6 h. In addition, there was no alteration in IR protein expression in either INS or GLCN animals (data not shown).

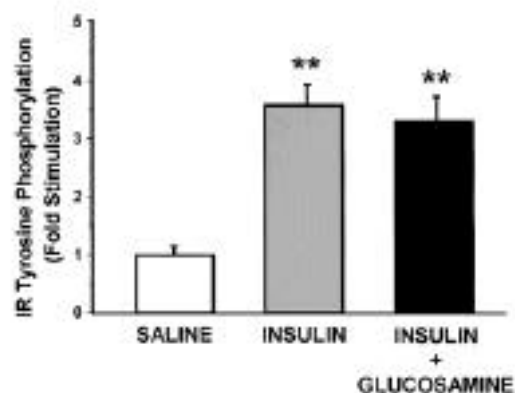


FIG. 1. Insulin-stimulated IR phosphorylation is unaltered by glucosamine infusion. Rectus abdominis muscle samples from rats infused with saline, insulin, or insulin plus glucosamine for 6 h were homogenized, immunoprecipitated with anti-IR-CT, and immunoblotted with antiphosphotyrosine antibody as described in METHODS. Data are summarized from 10 individual animals and are expressed as fold stimulation above control (saline-infused animals) (mean ± SE). ** $P < 0.01$ vs. saline.

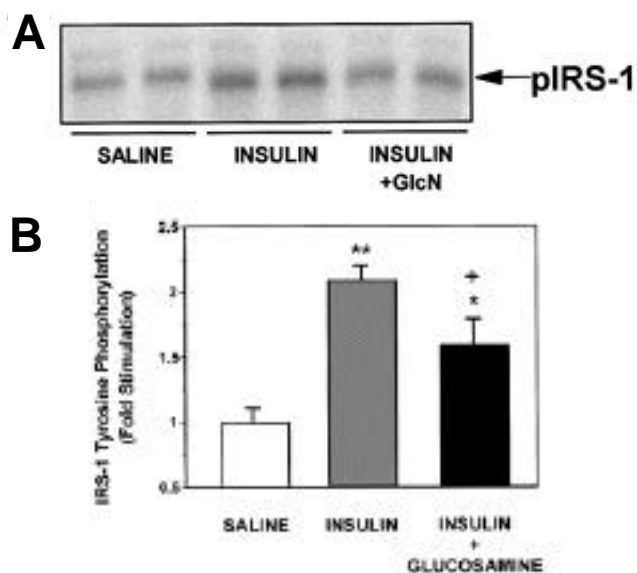


FIG. 2. Insulin-stimulated IRS-1 phosphorylation is decreased by glucosamine (GlcN) infusion. *A*: Representative antiphosphotyrosine immunoblot of anti-IRS-1 immunoprecipitates of rectus abdominis muscle from the three study groups. *B*: Quantitative results of IRS-1 phosphorylation from 10 rats per group. Data are expressed as fold stimulation (mean \pm SE) above saline-infused animals. * P < 0.05, ** P < 0.001 vs. saline, + P = 0.02 vs. insulin-stimulated animals.

Glucosamine infusion decreases insulin-stimulated IRS-1 tyrosine phosphorylation. Because insulin-stimulated IR phosphorylation was not altered by coinfusion of glucosamine, we next investigated whether the metabolic effects of glucosamine might be mediated via postreceptor pathways, including alterations in tyrosine phosphorylation of IRS proteins and activation of key enzymes upstream of glucose transport. As seen in Fig. 2*A*, 6 h of insulin infusion stimulated the tyrosine phosphorylation of IRS-1; this was reduced in animals infused with both insulin and glucosamine. Quantitative results are presented in Fig. 2*B*. Insulin stimulated IRS-1 tyrosine phosphorylation by 2.1-fold; the addition of glucosamine to insulin reduced the insulin-stimulated tyrosine phosphorylation by 45% (P = 0.02 vs. insulin alone). Although IRS-2 could be detected in anti-IRS-2 immunoblots of anti-IRS-2 immunoprecipitates, tyrosine phosphorylation of IRS-2 was not appreciably stimulated in either the insulin- or glucosamine-treated rats after 6 h of infusion.

IRS-1 and IRS-2 expression are not altered by glucosamine infusion. Alterations in IRS-1 protein expression have been described in a variety of insulin-resistant states, including chronic hyperinsulinemia, obesity, and dexamethasone treatment (26). Therefore, one potential mechanism for the reduction in IRS-1 phosphorylation induced by glucosamine infusion is a reduction in IRS-1 protein expression. To evaluate this possibility, anti-IRS-1 immunoblots of anti-IRS-1 immunoprecipitates were performed in parallel with the antiphosphotyrosine immunoblots. Quantification of protein expression revealed a nonsignificant decrease in IRS-1 protein expression in rats infused with insulin and glucosamine for 6 h (expressed relative to saline control: SAL 100 \pm 9%; INS 96 \pm 10%; GLCN 80 \pm 11%; NS). In parallel immunoprecipitations and Western blots, IRS-2 expression was not altered by glucosamine infusion (data not shown).

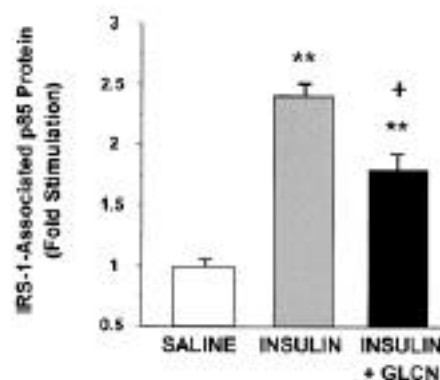


FIG. 3. Glucosamine infusion decreases insulin-stimulated association of IRS-1 with the p85 regulatory subunit of PI 3-kinase. Rectus muscle homogenates from each of the study groups were immunoprecipitated with anti-IRS-1 CT antibody and immunoblotted with anti-p85 polyclonal antibody. Data represent means \pm SE of p85 associating with IRS-1 from 10 animals. ** P < 0.001 vs. saline, + P = 0.03 vs. insulin-stimulated animals.

Glucosamine infusion decreases IRS-1 association with the p85 regulatory subunit of PI 3-kinase and its lipid kinase activity. Tyrosine phosphorylation is required for the association of SH2 domain-containing proteins, including the p85 regulatory subunit of the lipid and protein kinase PI 3-kinase, with IRS proteins. To determine whether the reduction in IRS-1 tyrosine phosphorylation induced by the addition of glucosamine to insulin was accompanied by decreased association with p85, anti-IRS-1 immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-p85 polyclonal antibody. Insulin infusion stimulated the association of p85 with IRS-1 by 2.4-fold; the addition of glucosamine to insulin reduced p85 association by 44% (Fig. 3). Although the anti-p85 antibody also detects other PI 3-kinase regulatory isoforms (27), the 85-kDa subunit was the dominant isoform associating with IRS-1 under these conditions; we did not detect any alteration in the relative binding of PI 3-kinase isoforms induced by glucosamine infusion.

The catalytic unit of PI 3-kinase is activated by binding of the p85 regulatory subunit to phosphotyrosine substrates. PI 3-kinase activation is critical for a variety of insulin-stimulated metabolic processes, including glucose transport (28–34) and glycogen synthesis (35). Because glucosamine infusion reduced p85 binding to IRS-1 protein, we assayed PI 3-kinase activity in skeletal muscle homogenates using an *in vitro* kinase assay, as previously described (36). After 6 h of infusion, insulin stimulated PI 3-kinase activity 3.8-fold, as indicated by increased incorporation of 32 P into PI 3-phosphate by anti-IRS-1 immunoprecipitates (representative chromatography [Fig. 4*A*] and quantification [Fig. 4*B*]). Insulin-stimulated PI 3-kinase activity was decreased by 35% in immunoprecipitates from glucosamine-infused animals (P = 0.03).

Akt, which is also known as protein kinase B or Rac, is a serine/threonine kinase localized downstream of PI 3-kinase that may play a role in glucose transport (37). To assess whether glucosamine modified the insulin-stimulated activation of this pathway, we performed both gel mobility shift and immune complex kinase assays. Muscle homogenates were separated by SDS-PAGE and immunoblots were performed using an anti-Akt antibody. In animals infused with insulin, an Akt mobility shift was clearly observed, indicating insulin-stimulated phosphorylation (Fig. 5*A*); this was not

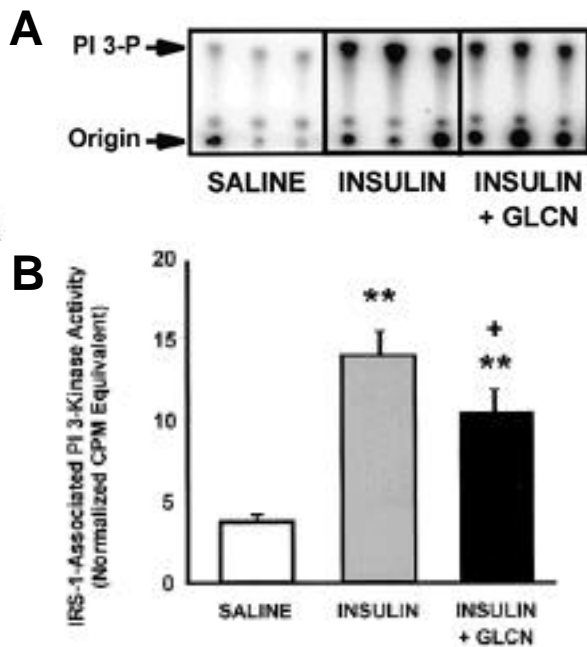


FIG. 4. Activation of IRS-1-associated PI 3-kinase by insulin is inhibited by concomitant glucosamine infusion. **A:** Representative chromatograph of *in vitro* PI 3-kinase assay. Kinase assays were performed on anti-IRS-1 immunoprecipitates using PI as an *in vitro* substrate. PI 3-P indicates the position of the 3-phosphorylated lipid. **B:** Quantitative data are presented from duplicate PI 3-kinase assays in rectus abdominis samples from 10 rats. Data are expressed as normalized counts per minute, using PhosphorImager units normalized to a counts per minute standard curve for each experiment, as described in METHODS. ** $P < 0.001$ vs. saline, + $P = 0.03$ vs. insulin-infused animals.

attenuated in animals also infused with glucosamine. Quantitative Akt immune complex kinase assays also revealed that insulin infusion stimulated Akt kinase activity by 3.5-fold at 2 h and 3.3-fold at 6 h, but again confirmed that glucosamine infusion did not modify Akt activation at either time point (Fig. 5B).

The glucosamine-induced decrease in insulin-stimulated IRS phosphorylation and PI 3-kinase activity in rectus muscle is time-dependent. As in previous studies, we have again demonstrated for the current experimental animal cohort that induction of significant resistance to insulin-stimulated glucose uptake requires 90–120 min of glucosamine infusion (Fig. 6A). To determine whether the reduction in insulin-stimulated IRS-1 phosphorylation and activation of PI 3-kinase paralleled this development of insulin resistance in the glucosamine-infused animals, we measured IR and IRS-1 phosphorylation and activation of PI 3-kinase in skeletal muscle from animals infused with saline, insulin alone, or insulin plus glucosamine for 2 h. IR phosphorylation was greater at 2 h of insulin infusion (6.5-fold) than at 6 h (3.6-fold); however, glucosamine was without effect on IR phosphorylation at either 2 or 6 h. Insulin stimulation of IRS-1 phosphorylation and IRS-1-associated PI 3-kinase activity was similar at 2 h and 6 h. However, the effect of glucosamine to decrease IRS-1 tyrosine phosphorylation and PI 3-kinase activity was evident only after 6 h of infusion (Fig. 6B and C).

Glycogen synthase enzyme activity is reduced by glucosamine infusion. To determine whether the inhibition of insulin signal transduction also extended to another primary

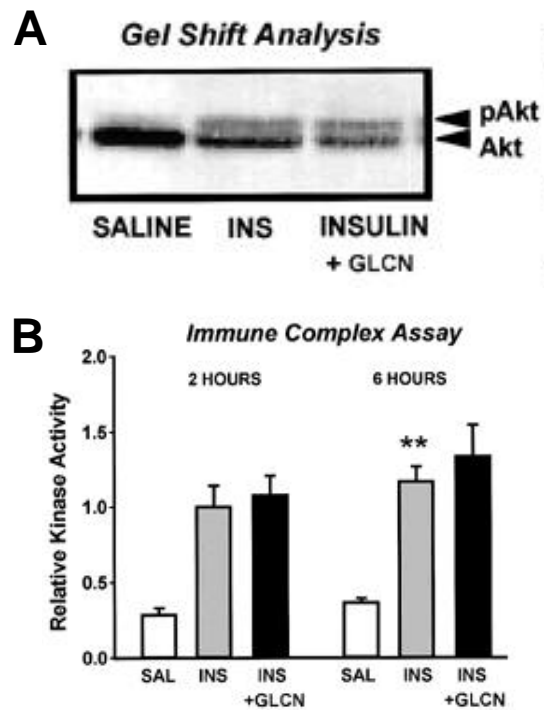


FIG. 5. Glucosamine (GlcN) infusion does not alter insulin-stimulated phosphorylation of Akt. **A:** Gel shift analysis: 75- μ g lysates of abdominal samples were separated by SDS-PAGE, immunoblotted with anti-Akt antibody, and visualized by enhanced chemiluminescence. The gel retardation of Akt induced by insulin in both insulin- and insulin-plus-glucosamine-treated animals is indicated by the arrows. Blot is representative of two independent experiments. **B:** Immune complex assay: Muscle homogenates were immunoprecipitated with anti-Akt antibody and assayed for phosphotransferase activity to the substrate crosside, as described in METHODS. Data are presented from two independent experiments, each performed in duplicate.

signaling and metabolic end point, namely glycogen synthesis, we evaluated the effects of glucosamine on both whole-body and muscle glycogen metabolism. Glucosamine infusion decreased whole-body glycogen synthesis as well as glycogen accumulation in skeletal muscle (Table 1). To determine whether glucosamine infusion directly altered the ability of insulin to stimulate glycogen synthase activity, we performed *in vitro* glycogen synthase assays on muscle homogenates. As seen in Fig. 7, insulin stimulated the activation of glycogen synthase fractional velocity by 2.2-fold at 2 h and by 1.6-fold at 6 h. Whereas the coinfusion of glucosamine had no effect at 2 h, there was a 51% reduction in insulin-stimulated activation of glycogen synthase at 6 h ($P = 0.03$).

Taken together, these data demonstrate that glucosamine infusion results in a time-dependent inhibition of insulin-stimulated glucose uptake and glycogen synthase activation and, in parallel, the IRS-1 and PI 3-kinase-dependent insulin signaling pathways required for stimulation of these metabolic end points.

Alterations in glycosylation of IRS proteins. *O*-linked GlcNAc modification of serine or threonine residues has been implicated as a dynamic regulated process common to many intracellular proteins. A potential role for this glycosylation would be to compete with phosphate for hydroxyl groups, and thus regulate available sites for phosphorylation (38,39). In fact, inhibition of the enzyme responsible for the removal of *O*-GlcNAc from proteins results in an increase in

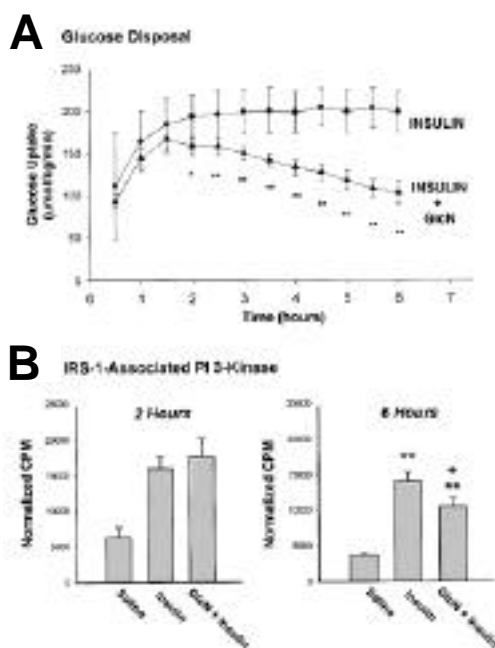


FIG. 6. The glucosamine-induced decrease in insulin-stimulated glucose uptake and IRS-1-associated PI 3-kinase are time-dependent. **A:** Glucose uptake data (means \pm SE) for the INS and GLCN groups in the present study (10 rats each) are indicated over the course of the 6-h infusion; * $P < 0.05$, ** $P < 0.01$ vs. insulin. **B:** IRS-1-associated PI 3-kinase was measured in rectus abdominis muscle from rats infused with saline, insulin, or insulin plus glucosamine (GLCN) for 2 h (2, 3, and 4 animals, respectively) or 6 h (10 rats per group). ** $P < 0.01$ vs. saline, + $P < 0.05$ vs. insulin.

glycosylation and a reduction in total phosphorylation of Sp1 (38). Because insulin-stimulated tyrosine phosphorylation of IRS-1 is reduced in animals infused with glucosamine, we explored whether the glycosylation of IRS proteins might be altered in this model. Muscle homogenates (2.5 mg) were immunoprecipitated with anti-IRS-1, anti-IRS-2, or anti-p85 antibodies, separated by SDS-PAGE, and immunoblotted with the anti-*O*-linked GlcNAc antibody RL2 (40). As seen in Fig. 8, IRS-1, IRS-2, or p85 immunoprecipitates from saline- or insulin-infused rats (*left and center lanes*) had minimal or no detectable immunoreactivity with the RL2 antibody. By

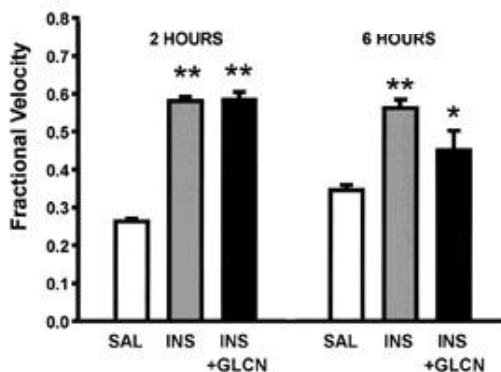


FIG. 7. Glucosamine infusion decreases glycogen synthase fractional velocity. Data are expressed as fractional velocity, namely, the ratio of synthase activity of muscle homogenates at an *in vitro* concentration of 0.1 mmol/l G-6-P to that at 10 mmol/l G-6-P and are the means \pm SE of two independent experiments, each in triplicate. * $P = 0.03$ vs. insulin, ** $P < 0.001$ vs. saline.

contrast, a distinct band of 170 kDa was detected by the RL2 antibody in IRS-1, IRS-2, and p85 immunoprecipitates from glucosamine-infused animals (*right lanes*). These data suggest that glucosamine infusion resulted in an increase in *O*-linked glycosylation on IRS proteins in skeletal muscle and supports a potential mechanistic role for increased glycosylation on critical IRS protein residues.

DISCUSSION

Chronic hyperglycemia is an independent cause of insulin resistance, which is clinically significant in the treatment of many patients with uncontrolled diabetes, both type 1 and type 2 (41). Initial studies to explore the mechanism of the insulin resistance induced by high levels of glucose *in vitro* were performed by Marshall and colleagues (42,43), who demonstrated that increasing concentrations of glucose downregulated insulin-stimulated glucose transport by decreasing translocation of glucose transporters in isolated adipocytes (42). This effect required the intracellular transport and metabolism of glucose as well as the presence of amino acids, particularly glutamine, and was more potently reproduced by glucosamine. These data led to the hypothesis that high levels of glucose are transported, phosphorylated, and metabolized to fructose-6-phosphate; a fraction of the fructose-6-phosphate then enters the hexosamine biosynthetic pathway, where the glutamine-requiring enzyme GFA generates GlcN-6-P. Elevated levels of glucosamine metabolites would then result in desensitization of glucose transport. Indeed, inactivation of GFA, the rate-limiting enzyme in hexosamine biosynthesis, prevents glucose-induced downregulation of glucose transport (43). Taken together, these early data pointed to a role for the hexosamine biosynthetic pathway in the induction of hyperglycemia-induced insulin resistance.

Additional studies have confirmed the importance of the hexosamine pathway in the modulation of several insulin-stimulated metabolic pathways, including glucose transport and glycogen synthesis. In isolated rat skeletal muscle, preincubation with glucosamine before insulin stimulation inhibits insulin-stimulated glucose transport in a dose- and time-dependent manner, by up to 70% (8). Infusion of glucosamine into healthy rats under euglycemic conditions

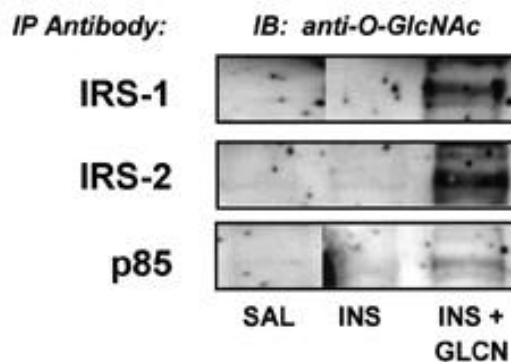


FIG. 8. Glucosamine infusion results in *O*-linked GlcNAc modification of IRS-1 and IRS-2. Muscle homogenates (2.5 mg) from saline- (SAL, *left*), insulin- (INS, *center*), or insulin-plus-glucosamine-infused (INS plus GLCN, *right*) rats were immunoprecipitated with the indicated antibody, separated by SDS-PAGE, and immunoblotted with the anti-*O*-linked GlcNAc antibody RL2 (40). The arrows indicate bands of ~170 kDa corresponding to IRS-1 (*upper panel*), IRS-2 (*middle panel*), or both IRS-1 and IRS-2 (*lower panel*, p85 precipitate).

decreases insulin- and IGF-1-stimulated glucose uptake by 22–49% (7,15,44,45). Importantly, the effect of glucosamine to induce peripheral insulin resistance is not seen in hyperglycemic animals but persists when hyperglycemia is corrected with phlorizin (7). These data support a common mechanism for both hyperglycemia- and glucosamine-induced insulin resistance, most probably via the hexosamine pathway. Increased activity of GFAT has been demonstrated in *ob/ob* mice (46) and in patients with type 2 diabetes (20). Transgenic mice overexpressing GFA in skeletal muscle and adipose tissue develop insulin resistance to glucose disposal (4). Recent studies have suggested that the hexosamine pathway may also play a wider role in nutrient sensing (47) and regulation of leptin gene expression (48).

One potential cellular mechanism for hexosamine-induced impairments in glucose disposal would be impaired translocation and/or activation of GLUT4 glucose transporters to the plasma membrane. Baron et al. (44) demonstrated that a 4-h glucosamine infusion in healthy rats resulted in a defect in translocation and/or trafficking of GLUT4. In another study, incubation of rat adipocytes *in vitro* with glucosamine for 4 h resulted in decreased glucose transport, without a concomitant effect on insulin-stimulated translocation of GLUT4 (49). Taken together, however, these data point to a potential upstream signaling defect induced by glucosamine that results in impaired glucose transport.

The effects of activation of the hexosamine pathway on glycogen metabolism are more complex. Stable overexpression of GFA in Rat-1 fibroblasts decreases insulin sensitivity for both glycogen synthase and net glycogen synthesis without a change in maximal synthase activity (50). At the same time, overexpression of GFA increases cell sensitivity to the effects of hyperglycemia to inhibit protein phosphatase-1 and glycogen synthase activity, consistent with increased flux through the hexosamine pathway in mediating the adverse effects of hyperglycemia (51). In HIR cells, preincubation with glucosamine inhibits insulin-stimulated glycogen synthesis by up to 80% (8) but slightly increases G-6-P-independent glycogen synthase activity. Incubation of isolated rat skeletal muscle with glucosamine increases fractional activity of glycogen synthase, but total insulin-stimulated glycogen synthesis is inhibited (8). In the heart, glucosamine infusion results in increased glycogen accumulation despite a concomitant decrease in glycogen synthase activation. This appears to be due to allosteric activation of glycogen synthase by high concentrations of GlcN-6-P (542% increase in synthase activity) (52). Taken together, these data demonstrate that hexosamine pathway activation decreases insulin-stimulated glycogen synthesis, despite concomitant increases in GlcN-6-P, suggesting modification of upstream insulin signal transduction cascades.

Thus, a variety of studies have demonstrated that activation of the hexosamine pathway induces insulin resistance to both glucose disposal and glycogen synthesis. We now demonstrate a potential cellular mechanism for these observations, namely, that activation of the hexosamine pathway by glucosamine infusion inhibits critical early steps in post-receptor insulin action in skeletal muscle, including decreased tyrosine phosphorylation of IRS-1 and binding of SH2 domain-containing proteins, such as the regulatory subunit of PI 3-kinase, and decreased PI 3-kinase activity, ultimately leading to decreased glucose transport and decreased activation of glycogen synthase enzyme activity. Although

Robinson et al. (8) noted increased half-life of insulin proreceptors, possibly reflecting alterations in glycosylation, in glucosamine-treated HIR cells, we did not detect any change in IR protein content or phosphorylation. The inhibition of insulin-stimulated IRS phosphorylation and PI 3-kinase activity in skeletal muscle may provide a mechanism for the observed changes in both whole-body and skeletal muscle glucose transport and glycogen synthesis, since PI 3-kinase-dependent pathways have been shown to play prominent roles in both of these insulin effects (28–35).

The importance of Akt in mediating insulin-stimulated glucose uptake remains controversial (37,53,54). It is interesting that, despite inhibition of PI 3-kinase activity and glycogen synthase activity by glucosamine in our experimental conditions, Akt kinase activation was not altered. Although Akt activation appears to be PI 3-kinase-dependent (55), normal Akt activation despite decreased PI 3-kinase activity has been demonstrated in other settings, including in skeletal muscle of patients with type 2 diabetes (56), rat skeletal muscle after long-term endotoxin treatment (57), and in hepatoma cells overexpressing dynamin (58). Although we cannot fully explain this dissociation between PI 3-kinase and Akt kinase activation in this context, potential mechanisms for this dissociation include the ability of small amounts of PI 3-kinase lipid products to fully activate Akt or activation of Akt by other pathways, including G-protein-coupled receptors (59).

Additional support for the mechanistic role for our findings comes from the *in vivo* time course data. The effect of glucosamine to downregulate glucose disposal begins at 2 h, but the magnitude increases dramatically from 2 to 6 h of infusion. In parallel, the effect of glucosamine to inhibit IRS-1 phosphorylation, activation of PI 3-kinase, and activation of glycogen synthase also requires several hours for its full manifestation. We cannot exclude a role for high concentrations of accumulated glucosamine metabolites, such as GlcN-6-P, to modestly inhibit glucose uptake directly by allosteric inhibition of hexokinase II. However, these effects appear to be modest in skeletal muscle. Virkamaki and Yki-Jarvinen (52) have demonstrated that the addition of GlcN-6-P (in concentrations approximating GlcN-6-P concentrations achieved in muscle *in vivo* during glucosamine infusion) to muscle extracts *in vitro* results in a 5% inhibition of hexokinase activity. Thus, the magnitude of the impairment in glucose disposal cannot be accounted for by hexokinase inhibition. In addition, our findings also demonstrate an independent and potentially additive signal transduction mechanism.

What are the mechanisms by which activation of the hexosamine pathway inhibits IRS-1 tyrosine phosphorylation and activation of downstream pathways? In this study, we demonstrate no reduction in IRS-1 protein expression after 6 h of glucosamine infusion. A minor reduction in IRS protein expression could not account for the observed 45% reduction in IRS-1 tyrosine phosphorylation, but a direct role for regulation of protein expression by hexosamine metabolites cannot be excluded.

Hresko et al. (22) recently reported similar insulin signal transduction defects induced by glucosamine in 3T3-L1 adipocytes, and they provide evidence for a role for depletion of intracellular ATP. In the present study, however, ATP levels were unchanged during glucosamine infusion. Thus, ATP depletion cannot explain the observed defects in either postreceptor signal transduction or insulin-stimulated glucose disposal.

Other potential mechanisms by which glucosamine may inhibit IRS tyrosine phosphorylation and activation of IRS-dependent downstream pathways would include activation of serine/threonine kinases that phosphorylate IRSs and diminish their suitability as substrates for the IR tyrosine kinase, as has been shown with tumor necrosis factor- α -induced insulin resistance (60), or activation of protein tyrosine phosphatases. Activation of protein kinase C has been reported in rat adipocytes developing insulin resistance to glucose transport during in vitro incubation with glucosamine (61); however, the effects on insulin signal transduction were not explored in these studies. Further studies will be required to investigate each of these possibilities.

Another intriguing possibility for the downregulation of IRS-1 tyrosine phosphorylation more specific to activation of the hexosamine pathway would be the direct posttranslational modification of key insulin signaling proteins, via *O*-linked glycosylation on serine and threonine residues with the GlcNAc moiety. This monosaccharide modification has been detected on many proteins in the cytoplasm, nuclear pore complex, and chromatin fraction, including transcription factors and nuclear receptors (40,62,63). In cell culture experiments, an increase in the ambient glucose concentration increases nuclear and cytoplasmic protein glycosylation, even in the absence of new protein synthesis, while inhibition of GFA inhibits the accumulation of these glycoproteins (64). Sayeski et al. (64) have also demonstrated that the effect of high glucose to stimulate transforming growth factor- α transcription requires its metabolism through GFA to glucosamine, suggesting a role for activation of glycosylation in mediating the effects of alterations in the metabolic milieu on gene transcription. In addition, the time course of *O*-GlcNAc turnover is very rapid relative to that of the proteins bearing this modification (65), suggesting a potential regulatory role for this glycosylation. In the case of insulin action, posttranslational glycosylation of a GLUT4-associated vesicular protein has been demonstrated in glucosamine-infused rats and may also play a role in the effect of glucosamine to impair GLUT4 translocation or activation (66). However, this glycosylation was sensitive to treatment with endoglycosidase F, suggesting that *N*-linked glycosylation was the more likely moiety identified in this protocol.

We now demonstrate that glucosamine infusion increases *O*-linked glycosylation on IRS-1 and IRS-2 proteins in skeletal muscle, as determined by immunoblotting with the RL2 antibody. This *O*-linked glycosylation may modulate early insulin signal transduction via alterations in global or site-specific phosphorylation of IRS proteins, resulting in decreased IRS protein tyrosine phosphorylation and induction of insulin resistance to glucose transport. These data are particularly intriguing in light of the hypothesis that *O*-linked glycosylation with GlcNAc may directly affect critical phosphorylation events in signal transduction cascades. *O*-GlcNAc transferases share substrate sequence similarities with known protein kinases, many of these sequences in a PV(S/T) motif (39). Because *O*-GlcNAc-modified proteins identified thus far are also phosphorylated, Hart and colleagues (67) have postulated that *O*-GlcNAc glycosylation may affect a variety of important cellular processes by inhibiting phosphorylation of proteins bearing these residues. For example, the nonphosphorylated I α form of RNA polymerase II is glycosylated on multiple residues, while the highly phosphory-

lated I β form does not contain any *O*-GlcNAc residues. Recent studies using an inhibitor of *N*-acetylglucosaminidase indicate that an increase in *O*-linked glycosylation of the transcription factor Sp1 results in a reciprocal decrease in its phosphorylation, further supporting this hypothesis (38). Further studies will be required to evaluate the potential role for *O*-GlcNAc modification on IRS and other key insulin-signaling proteins in the insulin resistance induced by activation of the hexosamine pathway.

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