Prolonged Incubation in PUGNAc Results in Increased Protein O-Linked Glycosylation and Insulin Resistance in Rat Skeletal Muscle

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Increased flux through the hexosamine biosynthetic pathway and increased O-linked glycosylation (*N*-acetylglucosamine [O-GlcNAc]) of proteins have been implicated in insulin resistance. Previous research in 3T3-L1 adipocytes indicated that insulin-stimulated glucose uptake and phosphorylation of Akt were reduced after incubation with O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc; 100 μmol/l), an inhibitor of the O-GlcNAcase that catalyzes removal of O-GlcNAc from proteins. Therefore, in this study, we tested the effects of PUGNAc on skeletal muscle. Incubation of rat epitrochlearis muscles for 19 h with 100 μmol/l PUGNAc resulted in a marked increase in O-GlcNAcylation of multiple proteins. Incubation with PUGNAc reduced glucose transport with a physiologic insulin concentration without affecting glucose transport without insulin or with supraphysiologic insulin. PUGNAc did not significantly alter insulin-stimulated phosphorylation of Akt (serine and threonine) or its substrates glycogen synthase kinase (GSK)3α and GSK3β. Insulin stimulated a dose-dependent (12.0 > 0.6 > 0 nmol/l) increase in the phosphorylation of a 160-kDa protein detected using an antibody against an Akt substrate phosphomotif. PUGNAc treatment did not alter phosphorylation of this protein. These results indicate that PUGNAc is an effective inhibitor of O-GlcNAcase in skeletal muscle and suggest that O-GlcNAc modification of proteins can induce insulin resistance in skeletal muscle independent of attenuated phosphorylation of Akt, GSK3α, GSK3β, and a 160-kDa protein with an Akt phosphomotif. *Diabetes* 53: 921–930, 2004

Insulin resistance, a subnormal biological response to insulin, is important in a number of diseases, most notably for type 2 diabetes. Study of humans and animals with spontaneous or experimentally induced insulin resistance has revealed much about the multiple pathways that can lead to insulin resistance. However, the primary events that trigger insulin resistance remain incompletely understood.

Experimental induction of insulin resistance in isolated cells or tissues can be a useful approach for the identification of early events because it provides considerable experimental control. Studying cultured rat adipocytes, Marshall and colleagues (1,2) demonstrated that glutamine can participate in glucose-induced insulin resistance. In an elegant series of experiments, they discovered an apparent link between the hexosamine biosynthetic pathway (HBP) and the development of insulin resistance (3). In the first and rate-limiting step of the HBP, the amide group from glutamine is used to convert fructose-6-phosphate to glucosamine-6-phosphate via glutamine/fructose-6-phosphate amidotransferase (GFAT) and is ultimately converted to uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc), a terminal metabolite of the pathway and donor substrate of O-GlcNAcylation. Marshall and colleagues hypothesized that flux through the HBP served as a glucose sensor in adipocytes, and potentially other insulin target cells, and by some unknown negative feedback loop, regulated glucose transport.

Because skeletal muscle accounts for 75% of whole-body, insulin-mediated glucose uptake (4) and insulin resistance in this tissue is an early defect in type 2 diabetes (5), it was important to determine if the HBP was also linked to muscle insulin resistance. Robinson et al. (6) first demonstrated an association between the HBP and insulin resistance in isolated rat skeletal muscle. They found that incubating rat hemidiaphragms in glucosamine or high glucose significantly decreased glucose uptake in a dose- and time-dependent manner. Subsequently, using euglycemic-hyperinsulinemic clamps, Rossetti et al. (7) demonstrated that glucosamine infusion raised muscle UDP-GlcNAc levels four- to fivefold and induced insulin resistance in normoglycemic rats. Other studies using a variety of models have also supported a relationship between the HBP and peripheral insulin resistance. For example, transgenic mice overexpressing the first and rate-limiting enzyme of the HBP, GFAT (8), have exhibited greater GFAT activity, increased muscle UDP-GlcNAc levels, and insulin resistance. On the other hand, Gazdag et al. (9) described a calorie restriction model that resulted in decreased muscle UDP-GlcNAc levels and improved insulin sensitivity.

These studies demonstrated an inverse relationship between cell UDP-GlcNAc levels and insulin action, but did not reveal a specific mechanism for the association.

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3-MG, 3-*O*-methyl-D-glucose; DMEM, Dulbecco's modified Eagle's medium; GFAT, glutamine/fructose-6-phosphate amidotransferase; GSK, glycogen synthase kinase; HBP, hexosamine biosynthetic pathway; IRS, insulin receptor substrate; PI, phophatidylinositol.

UDP-GlcNAc is a substrate for O-GlcNAc transferase (uridine diphospho-*N*-acetylglucosamine/polypeptide-*N*-acetylglucosaminyltransferase, EC 2.4.1) that transfers single *N*-acetylglucosamine moieties to the hydroxyl group of serine and threonine residues on cellular target proteins in a dynamic and reversible process known as O-GlcNAcylation (10). The extent of cellular O-GlcNAcylation appears to be regulated, at least in part, by the cellular concentration of the UDP-GlcNAc substrate (11). McClain et al. (12) recently found direct evidence that O-GlcNAc transferase plays an important role in insulin action by overexpressing O-GlcNAc transferase in skeletal muscle and fat in transgenic mice and inducing insulin resistance.

O-GlcNAcase (β-*N*-acetylglucosaminidase, EC 3.2.1.52) is responsible for specific removal of single O-GlcNAc modifications from cellular proteins. Both O-GlcNAc transferase and O-GlcNAcase exhibit nuclear and cytoplasmic localization and are highly conserved (13). Hart and colleagues (10,14) have hypothesized that O-GlcNAcylation of proteins is a type of posttranslational modification that may be analogous to kinase and phosphatase regulation of phosphorylation and thus compete for similar or adjacent phosphorylation sites in a reciprocal manner. It seems possible that, in addition to flux through the HBP, regulation of either O-GlcNAc transferase (12) or O-GlcNAcase (14) may influence insulin action.

O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc) is a potent and nontoxic inhibitor of O-GlcNAcase in various cell lines (15). Recently, Vosseller et al. (14) used PUGNAc to alter the extent of O-GlcNAcylation in 3T3-L1 adipocytes and study the effect on insulin action. Following 19 h of incubation with PUGNAc, O-GlcNAcylation of cellular proteins was increased and insulin-stimulated glucose uptake was reduced, concomitant with reduced insulin-stimulated phosphorylation of Akt on threonine 308 and glycogen synthase kinase (GSK)3β on serine 9.

Recently, a 160-kDa GTPase-activating protein, designated Akt substrate 160 (AS160), has been described in 3T3-L1 adipocytes by Kane et al. (16) as a downstream substrate of Akt action, and Sano et al. (17) demonstrated that Akt-dependent AS160 phosphorylation is apparently required for GLUT4 translocation to the cell surface. The effect of insulin on AS160 phosphorylation has not been reported in skeletal muscle.

The effects of PUGNAc on skeletal muscle are also unknown. Therefore, our primary goals in this study were to determine whether prolonged exposure of isolated skeletal muscle to PUGNAc would *1*) increase O-GlcNAcylation of proteins, *2*) induce insulin resistance for glucose uptake, *3*) influence insulin-stimulated Akt(T308), *4*) influence Akt(S473) phosphorylation, and/or *5*) influence GSK3α or GSK3β phosphorylation. We also assessed the influence of insulin and PUGNAc on the phosphorylation of an Akt substrate, AS160, which was recently implicated as important for insulin-stimulated glucose transport in 3T3-L1 adipocytes. We found that incubation with PUGNAc for 19 h resulted in an increase in O-GlcNAcylation of proteins and reduced glucose uptake using a physiological concentration of insulin. This insulin resistance was not attributable to reduced phosphorylation of Akt(T308), Akt(S473), GSK3β(Ser9), or GSK3α(Ser21). Furthermore, we demonstrated, for the first time in skeletal muscle, that insulin leads to increased phosphorylation of a 160-kDa protein on an Akt phosphomotif and that PUGNAc did not alter phosphorylation of this protein.

RESEARCH DESIGN AND METHODS

Unless otherwise noted, all chemicals were purchased from Sigma Chemical (St. Louis, MO). Sodium pentobarbital was obtained from Abbott Laboratories (North Chicago, IL). Dulbecco's modified Eagle's medium (DMEM; cat. no. 31600) was purchased from Gibco Invitrogen (Grand Island, NY). PUGNAc was purchased from CarboGen Labs (Aarau, Switzerland). Human recombinant insulin (Humulin R) was purchased from Eli Lilly (Indianapolis, IN). [^{3}H]-3-*O*-methylglucose and [^{14}C]mannitol were purchased from NEN (Downers Grove, IL). Teflon syringe filters were purchased from Osmonics (Rockford, IL). Reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA). Anti–O-GlcNAc (CTD110.6) antibody was purchased from Covance (Princeton, NJ). Anti–phospho-Akt(T308) (cat. no. 06-678) was purchased from Upstate Biotechnology (Lake Placid, NY). Anti–phospho-(Ser/Thr)Akt substrate (cat. no. 9611), anti–phospho-Akt(S473) (cat. no. 9271), and anti–phospho-GSK3α/β (cat. no. 9331) were obtained from Cell Signaling Technology (Beverly, MA). Serine-phosphorylated GSK3α and GSK3β are recognized by the same antibody and distinguished on the basis of molecular weights (51 and 46 kDa, respectively). Anti-Akt1 (cat. no. 1618) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Total protein concentrations were performed using the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL). Enhanced chemiluminescence kits were purchased from Amersham Pharmacia Biotechnology (Piscataway, NJ).

Treatment of rats. Male Wistar rats were obtained from Harlan (Indianapolis, IN) and provided with Purina rat chow and water ad libitum. Upon arrival at the animal facility, the animals were acclimated to their surroundings for 2–3 days and fasted at 1800 on the night before each experiment. The procedures were approved by the Animal Care Committee of the University of Wisconsin–Madison.

On the experimental day between 1200 and 1400, the animals (~160 g body wt) were anesthetized with an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body wt). Upon loss of pedal reflexes, epitrochlearis muscles were quickly excised and transferred into the tissue incubation media.

Muscle incubation and glucose transport. Figure 1 summarizes the protocols used in the incubation steps in the experiments. Following dissection, muscles were incubated for 18 h in flasks containing 1.5 ml low-glucose (5.5 mmol/l) DMEM supplemented to contain 2.54 mmol/l $CaCl_2$, 25 mmol/l $NaHCO_3$, 0.6 nmol/l insulin, and 0.1% BSA. Insulin was included for the initial 18 h of incubation because previous research demonstrated that this approach prevented the increase in basal glucose transport found with prolonged incubation of isolated skeletal muscle (18). Flasks were placed in a shaking water bath at 35°C and continuously gassed with 95% O_2/5% CO_2 throughout each experiment. The media was replaced with fresh DMEM every 6 h during the initial 18 h incubation. To minimize bacterial contamination during prolonged incubations, 100 μU/ml penicillin and 100 μg/ml streptomycin were added to the DMEM, and Teflon air filters were used to minimize aerosol contaminants.

Following the 18 h incubation in supplemented DMEM, muscles were rinsed in Krebs Henseleit buffer supplemented with 0.1% BSA, 100 μU/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l sodium pyruvate, and 5 mmol/l mannitol for two 30-min periods at 30°C to rinse away glucose and DMEM. During the rinse steps, the Krebs Henseleit buffer was supplemented with 0, 0.6, or 12 nmol/l insulin. Muscles treated with PUGNAc in the initial incubation steps continued to be exposed to 100 μmol/l PUGNAc during the subsequent rinse steps. Thus PUGNAc-treated muscles were exposed to PUGNAc for a total duration of 19 h before incubation with 3-*O*-methyl-D-glucose (3-MG).

Finally, muscles were incubated at 30°C for 15 min in 1.5 ml Krebs Henseleit buffer containing 8 mmol/l 3-MG (0.5 mCi/mmol) and 2 mmol/l mannitol (0.2 mCi/mmol). Insulin concentrations remained the same as in the preceding rinse steps (0, 0.6, or 12 nmol/l insulin). Muscles were then rapidly blotted, trimmed, clamp frozen with aluminum tongs cooled to the temperature of liquid N_2, and stored at −80°C until further processing.

Muscle preparation. Frozen muscles were weighed, transferred to prechilled glass tissue grinding tubes (Kontes, Vineland, NJ), and homogenized in ice-cold lysis buffer (1 ml/muscle) containing 20 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1% NP-40 (vol/vol), 2 mmol/l EDTA, 2 mmol/l EGTA, 1 mmol/l dithiothreitol, 2.5 mmol/l sodium pyrophosphate, 10 mmol/l sodium fluoride, 2 mmol/l sodium vanadate, 20 mmol/l β-glycerophosphate, 2 μmol/l PUGNAc, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 2 mmol/l phenylmethylsulfonyl fluoride. Homogenates were transferred to microfuge tubes, rotated for 1–2 h at 4°C, and then centrifuged at 12,000*g* for 15 min to remove

insoluble material. Following homogenization, processed muscle samples were used for determination of glucose transport as well as for immunoprecipitations and Western blotting. Supernatants were transferred to fresh microfuge tubes and duplicate 200-μl aliquots were used for scintillation counting. [^{3}H]-3-MG uptake was determined as previously described (19). Protein concentrations of the same supernatants were determined by the bicinchoninic acid method (20).

Immunoblotting. Aliquots of supernatants (20–30 mg protein) were solubilized in SDS sample buffer, boiled for 3 min, and subjected to 10% SDS-PAGE. Resolved proteins were transferred to nitrocellulose paper overnight at a constant current of 150 mA/transfer apparatus in electrotransfer buffer (20 mmol/l Tris, pH 8.0, 150 mmol/l glycine, 0.025% SDS, and 20% methanol). Nitrocellulose blots were blocked in TBST-HT (3% Tween-20 in Tris-buffered saline, pH 7.5) for 1 h at room temperature and transferred to fresh TBST-HT with anti-GlcNAc (CTD110.6; 1:1,000) for 2 h at room temperature or overnight at 4°C. Blots were then washed and incubated in goat anti-mouse IgM-horseradish peroxidase (1:5,000) for 1 h at room temperature. Blots were washed of excess antibody, subjected to enhanced chemiluminescence, and immunoreactive protein was quantified by densitometry. Western blots probed with anti–phospho-Akt(T308), anti–phospho-Akt(S473), anti–phospho-(Ser/Thr)Akt substrate, and anti–phospho-GSK3α and -GSK3β were performed following the manufacturer's protocol. Blots probed with anti–phospho-Akt(T308) and -Akt(S473) were reprobed with anti-Akt1 following a heat/detergent-stripping protocol. Briefly, blots were incubated in stripping buffer (100 mmol/l 2-mercaptoethanol, 2% wt/vol SDS, and 62.5 mmol/l Tris-HCl, pH 6.7) with gentle shaking for 30 min at 50°C, washed for 10 min in TBST, and subjected to enhanced chemiluminescence to confirm removal of secondary antibody.

Statistical analysis. All data are expressed as mean ± SE. Differences between two groups were determined by paired t test using SigmaSTAT (SPSS, Chicago, IL). Two-way ANOVA was used to evaluate the effect of PUGNAc and insulin on Akt substrate–phosphorylation-(Ser/Thr) (SigmaSTAT). $P < 0.05$ was considered statistically significant.

RESULTS

For control (no PUGNAc) muscles incubated for 19 h, there was a clear dose-dependent effect of insulin on glucose transport ($0 < 0.6 < 12$ nmol/l) (Fig. 2). PUGNAc incubation for 19 h did not alter glucose transport measured with no insulin or 12 nmol/l insulin. However, PUGNAc incubation for 19 h resulted in a 34% decrease ($P = 0.014$) in glucose transport for muscles incubated with submaximally effective insulin (0.6 nmol/l).

The same homogenates used for glucose transport were also used in Western blotting to assess PUGNAc-induced O-GlcNAcation of muscle proteins. There was no evidence that differing insulin concentrations during the final 75 min of incubation affected O-GlcNAcation, with or without PUGNAc (data not shown). In every muscle pair, Western blotting using an anti-GlcNAc antibody showed that muscle proteins exhibited greater O-GlcNAcation when muscles were exposed to 100 μmol/l PUGNAc (Fig. 3*A*). Several protein bands (apparent molecular weights of 58, 67, 74, 117, 129, and 147 kDa) were consistently observed to be approximately two- to eightfold greater ($P < 0.01$) in PUGNAc-treated versus no PUGNAc controls (Fig. 3*B*).

Akt(T308) phosphorylation was not detectable in muscles incubated without insulin during the final 75 min, regardless of PUGNAc exposure, and a stepwise increase was induced with 0.6 and 12 nmol/l insulin (Fig. 4*A*). PUGNAc did not affect Akt(T308) phosphorylation in samples measured with 0.6 nmol/l insulin (Fig. 4*B*) for 3-MG uptake. However, muscles stimulated with 12 nmol/l insulin following incubation with PUGNAc had a tendency ($P = 0.052$) for an 18% decrease in Akt(T308) phosphorylation (Fig. 4*C*). Insulin (0.6 and 12 nmol/l) resulted in the expected increase above basal (without insulin) phosphorylation of Akt(S473) (data not shown). PUGNAc did not

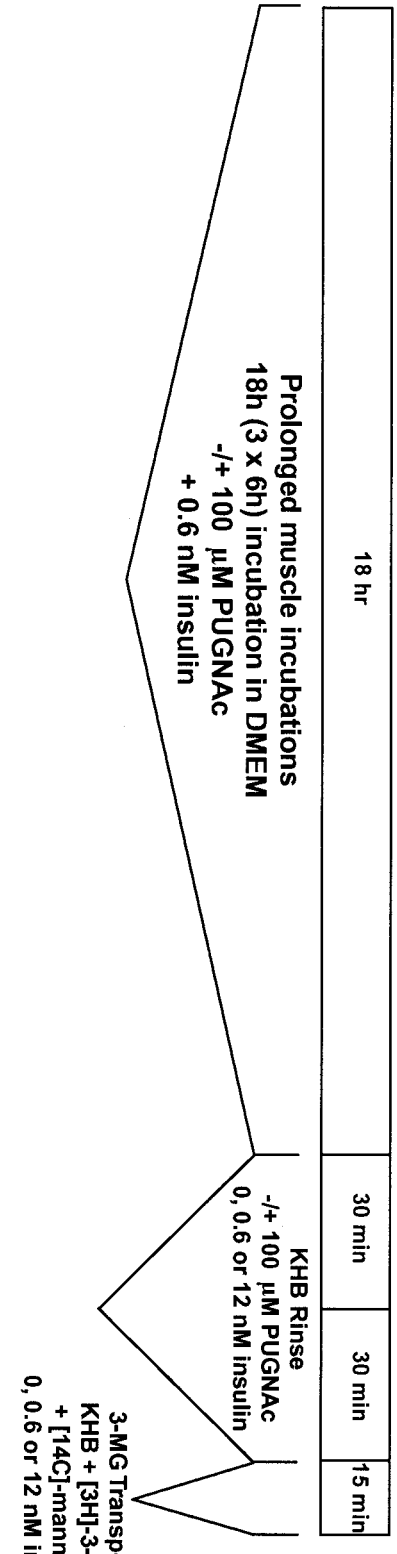

FIG. 1. Schematic diagram of muscle incubation protocol. KHB, Krebs Henseleit buffer.

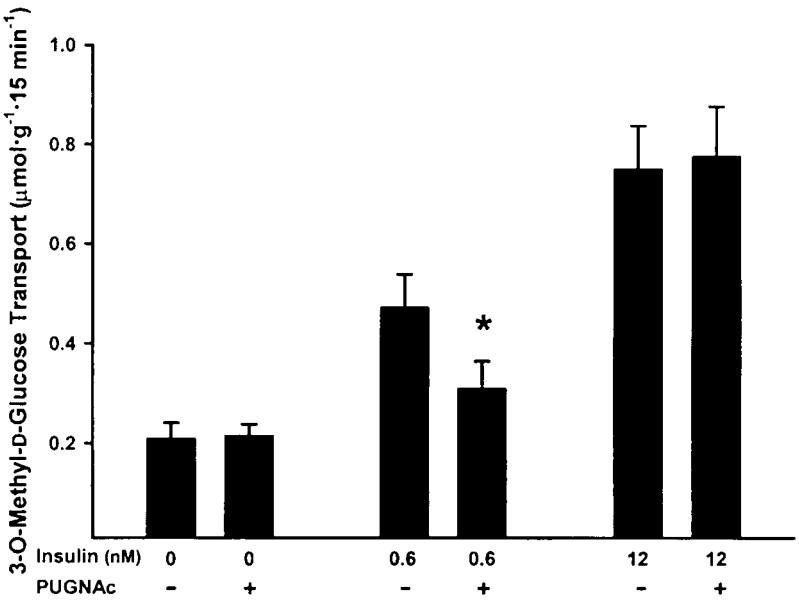

FIG. 2. Rate of 3-MG transport in paired muscles incubated for 19 h in the presence or absence of 100 μmol/l PUGNAc. During the final 75 min of incubation, muscles were exposed to 0, 0.6, or 12 nmol/l insulin. Data are mean ± SE (n = 12 per group). *Significant difference for with PUGNAc versus without PUGNAc for paired muscles incubated with 0.6 nmol/l insulin ($P < 0.05$).

significantly affect Akt(S473) phosphorylation in muscles measured without insulin (data not shown) or with insulin (0.6 or 12 nmol/l) (Fig. 5*A* and *B*). Reprobing these blots with anti-Akt1 demonstrated no difference in Akt1 abundance following 19 h incubation with or without 100 μmol/l PUGNAc (data not shown).

Phosphorylation of a protein at ~160 kDa, presumably Akt substrate protein, AS160, was increased by insulin in a dose-dependent manner (Fig. 6*A*). Phosphorylation of the 160-kDa protein was unaffected by prior PUGNAc incubation, regardless of insulin concentration (Figs. 6*B* and C).

As expected, insulin led to phosphorylation of GSK3α(S21) and GSK3β(S9) (data not shown). GSK3β(S9) phosphorylation was unaffected by prolonged exposure to PUGNAc in muscles stimulated with 0.6 or 12 nmol/l insulin during the final 75 min of incubation (Figs. 7*A* and *B*). GSK3α(S21) phosphorylation was also unaffected by PUGNAc (data not shown).

DISCUSSION

The most important, new results of this study were that *1*) we have described a protocol for prolonged (up to 19 h) in vitro incubation of rat skeletal muscle with which the muscle retains the insulin dose-dependent stimulation of glucose transport; *2*) in skeletal muscle, insulin stimulates increased phosphorylation on an Akt phosphomotif of a 160-kDa protein; *3*) the O-GlcNAcase inhibitor PUGNAc increased the O-GlcNAcylation of multiple proteins in skeletal muscle; *4*) PUGNAc also induced insulin resistance for glucose transport with a physiologic insulin dose; and *5*) this insulin resistance was not attributable to attenuated Akt1 abundance or perturbed phosphorylation of Akt(T308), Akt(S473), GSK3-α, GSK3-β, or the 160-kDa protein with an Akt phosphomotif.

Gulve et al. (18) described the experimental conditions for incubating isolated rat epitrochlearis muscles for up to 9 h before measurement of glucose transport. Subsequently, Ojuka and colleagues (21,22) modified the incubation conditions (including antibiotics, using sterile media, and gassing through Teflon filters) and extended incubations up to 18 h. However, they did not assess glucose transport of the muscles. Therefore, it was important that, using similar conditions, we found that rat epitrochlearis muscles can be incubated for up to 19 h and retain an insulin dose response for glucose transport, such that a physiologic insulin concentration (0.6 nmol/l) induced an approximately half-maximal increase in glucose transport, and glucose transport with a supraphysiologic insulin dose (12 nmol/l) was ~3.8-fold above basal.

We used PUGNAc, a nonmetabolizable analog of glucosamine and potent inhibitor of O-GlcNAcase, and found increased O-GlcNAcylation of multiple cellular proteins in rat epitrochlearis muscle. PUGNAc incubation has previously been shown to cause a 1.5- to 2-fold elevation of O-GlcNAcylation of multiple proteins in various cultured cell lines, including HT 29, NIH3T3, and CV1 cells; however, PUGNAc had relatively little effect on the O-GlcNAcylation of proteins in HeLa cells (15). Vosseller et al. (14) recently demonstrated that 19 h of PUGNAc (100 μmol/l) exposure induced a two- to sixfold increase in O-GlcNAc modification of multiple proteins. In this context, it was essential to first test the efficacy of PUGNAc for increasing O-GlcNAcylation of proteins in isolated rat skeletal muscle. We found a striking approximately two- to eightfold increase in the intensity of multiple O-GlcNAc–modified proteins bands (apparent molecular weights of 58, 67, 74, 117, 129, and 147 kDa) in muscles incubated with PUGNAc, indicating that the compound could be used to probe O-GlcNAc effects in isolated skeletal muscle.

Vosseller et al. (14) further reported that PUGNAc-induced GlcNAcylation of proteins in 3T3-L1 adipocytes is accompanied by insulin resistance for glucose uptake over a range (0.1–10 nmol/l) of insulin concentrations. Basal (no insulin) glucose uptake of muscle was unaffected by PUGNAc, which is consistent with results for 3T3-L1 adipocytes (14). Following 19 h incubation in 100 μmol/l PUGNAc, glucose transport was decreased significantly in muscle stimulated with a physiological insulin concentration (0.6 nmol/l). The 34% decrement compares with the 23–39% decrement reported for 3T3-L1 adipocytes with various insulin concentrations. The insulin-stimulated glu-

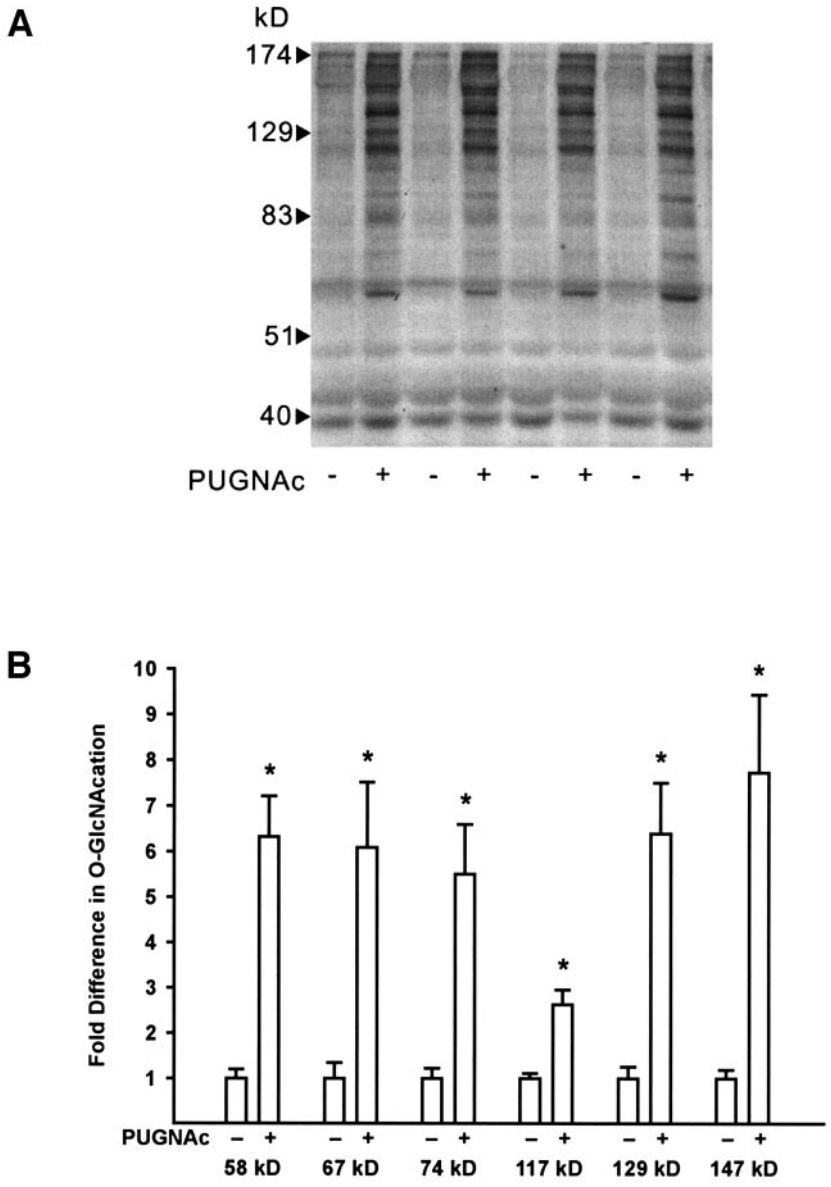

FIG. 3. O-linked glycosylation of muscle proteins following 19 h of incubation of paired skeletal muscles with or without 100 μmol/l PUGNAc. *A*: Muscle homogenates were subjected to 8% SDS-PAGE. Subsequent protein blots were probed with anti–O-GlcNAc and visualized following enhanced chemiluminescence. Relative molecular weight standards are denoted. *B*: Protein bands were quantitated by densitometry, and values for PUGNAc-treated samples are expressed relative to the mean value for the corresponding protein band in muscles incubated without PUGNAc (mean ± SE; $n = 6$ per group). *Significant difference for paired muscles with PUGNAc versus without PUGNAc ($P < 0.005$).

cose transport, calculated by subtracting the basal glucose transport rate, was reduced by 66% in muscle. In contrast to results with 3T3-L1 adipocytes, PUGNAc did not attenuate glucose transport with a supraphysiologic insulin (12 nmol/l) concentration.

We next evaluated the effects of PUGNAc on Akt phosphorylation at T308 and S473. Although we did not find an effect of PUGNAc on S473 phosphorylation of Akt regardless of insulin concentration, there was a trend for lesser Akt phosphorylation at T308 with 12 nmol/l insulin in PUGNAc-treated muscles, and this result corresponds to the PUGNAc-induced decrement in threonine phosphorylation of Akt in 3T3-L1 adipocytes stimulated with 10 nmol/l insulin (14). However, we found no insulin resistance for glucose uptake with 12 nmol/l insulin. Furthermore, with a physiologic insulin concentration at which glucose uptake was diminished by PUGNAc, we found no PUGNAc effect on Akt phosphorylation of T308 or S473 in skeletal muscle. Vosseller et al. (14) reported PUGNAc effects on Akt phosphorylation of 3T3-L1 adipocytes only with 10 nmol/l insulin, which based on their glucose uptake data were at least 10-fold higher than that needed for maximal glucose uptake, so it is uncertain if the ~50% reduction of Akt phosphorylation found at 10 nmol/l insulin played a role in the insulin resistance in adipocytes. Regardless, our findings clearly uncouple the effect of PUGNAc on glucose transport in skeletal muscle from altered Akt T308 and S473 phosphorylation.

Lienhard et al. (16) recently demonstrated in 3T3-L1 adipocytes that insulin stimulates the phosphorylation of a 160-kDa protein that was identified as a substrate for Akt and designated AS160 (Akt substrate 160). They subsequently reported that mutation of AS160 on sites that are motifs for phosphorylation by Akt resulted in marked inhibition of insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (17). These results provide strong evidence that AS160 is an essential mediator of Akt-activated GLUT4 vesicle trafficking. Kane et al. (16) also demonstrated that AS160 is expressed by skeletal muscle, but they did not evaluate the ability of insulin to stimulate phosphorylation of this protein. Therefore, we next eval-

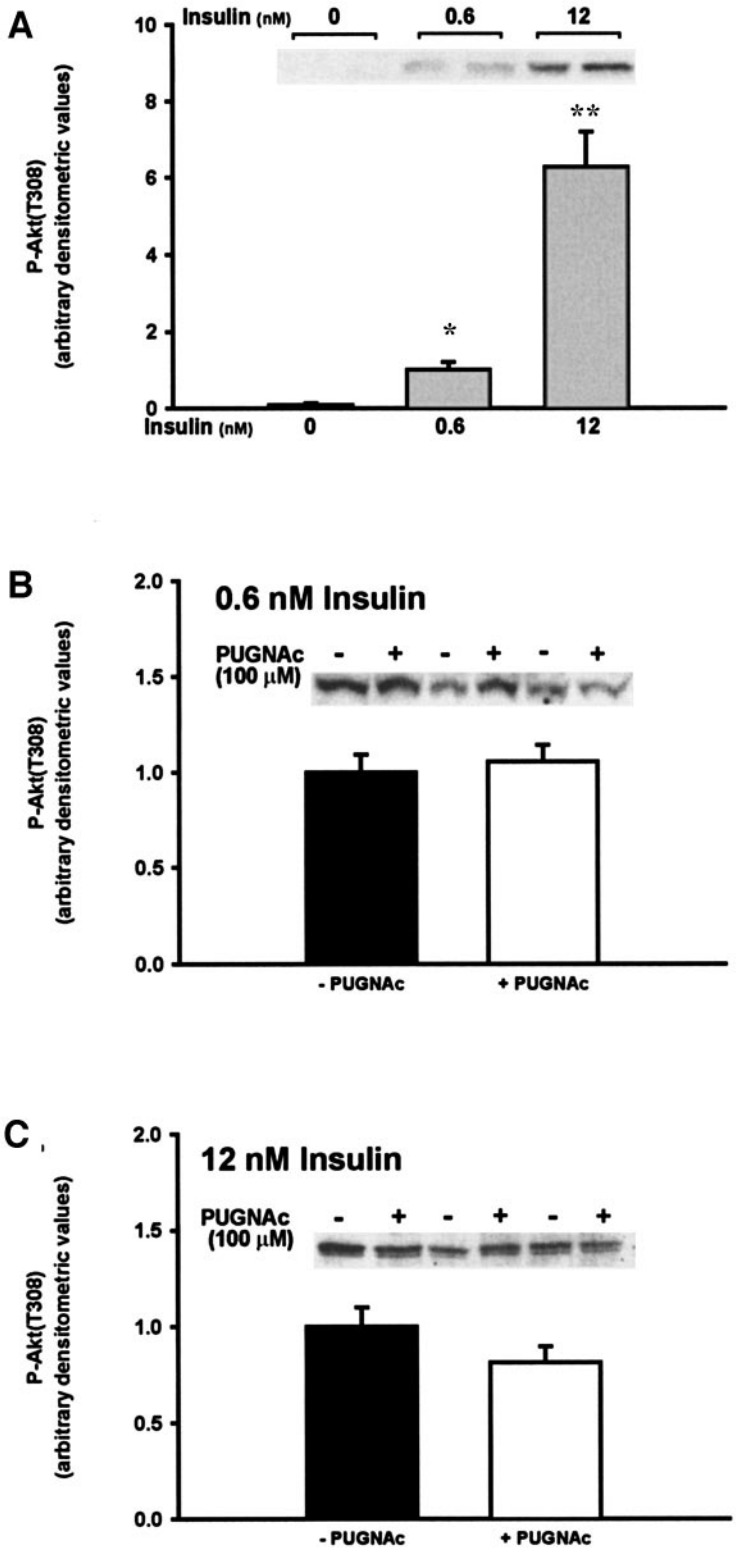

FIG. 4. Phosphorylation status of Akt(T308) following 19 h incubation of muscle with or without 100 μmol/l PUGNAc. *A*: Dose response for insulin in muscles incubated without PUGNAc at basal (no insulin), 0.6, or 12 nmol/l insulin during the final 75 min of incubation (n = 4 per group). Paired muscles incubated with or without PUGNAc for 19 h and with 0.6 nmol/l insulin (*B*) during the final 75 min of incubation (n = 11 per group) or 12 nmol/l insulin (*C*) during the final 75 min of incubation (n = 12 per group). Bands corresponding to P-Akt(T308) were quantitated by densitometry and expressed as the mean ± SE. *Basal versus 0.6 nmol/l insulin (P < 0.005); **basal versus 12 nmol/l insulin (P < 0.001) and 0.6 vs. 12 nmol/l insulin (P < 0.001).

uated the effects of insulin and PUGNAc in skeletal muscle on phosphomotifs of Akt substrates using the same antibody as used by Lienhard and coworkers (16,17). Our results provide the first evidence that, in skeletal muscle, insulin stimulates a dose-dependent phosphorylation of an Akt phosphomotif on a 160-kDa protein. It is especially notable that we found a significant increase in phosphorylation with a physiologic insulin concentration and a further increase with a supraphysiologic insulin level, particularly because Kane et al. (16) had only reported results using a single, very high insulin concentration (160 nmol/l). It is tempting to speculate that the 160-kDa protein in muscle that became phosphorylated with insulin stimulation is AS160. Regardless, PUGNAc has no significant effect on phosphorylation of this protein at any insulin concentration studied. It is clear that the insulin

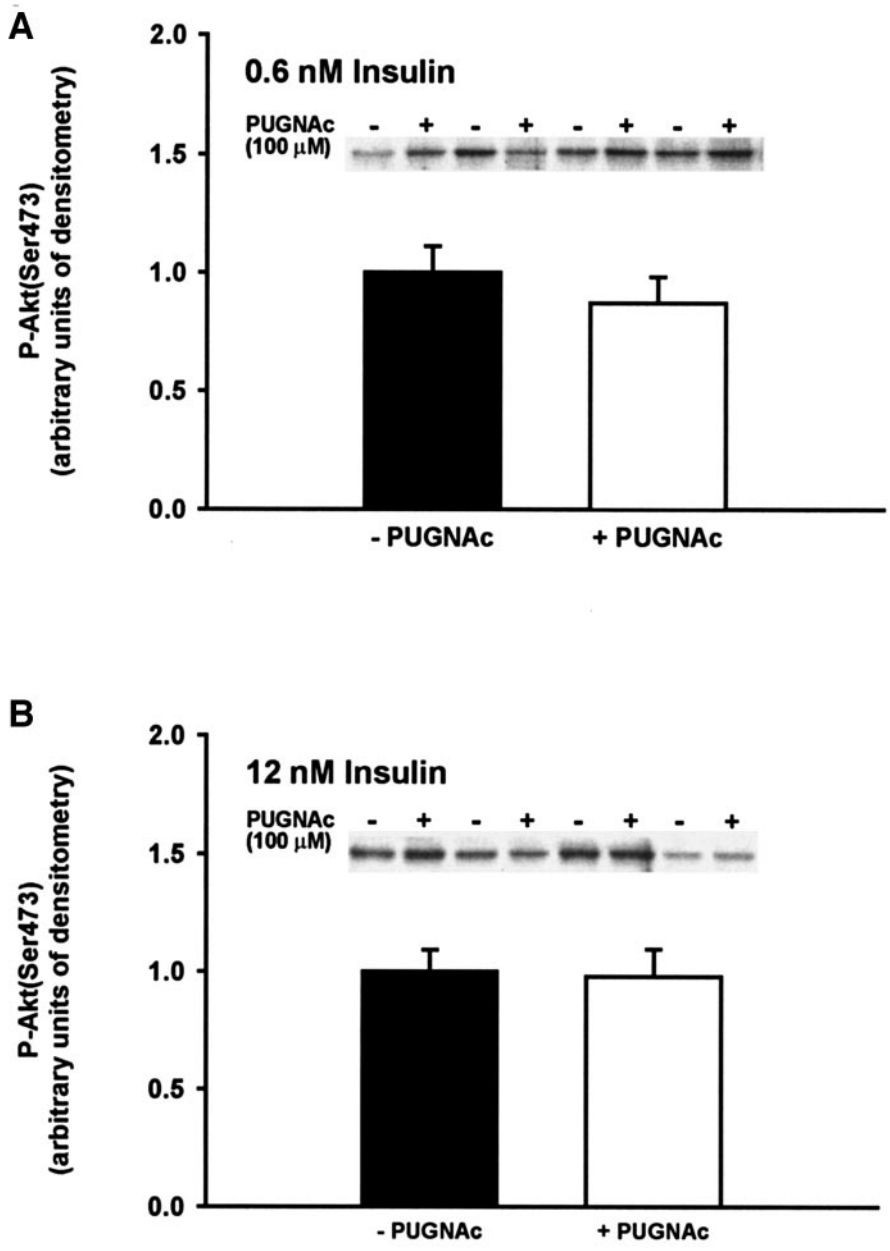

FIG. 5. Phosphorylation status of Akt(S473) following 19 h of incubation of muscle with or without 100 μmol/l PUGNAc. Paired muscles incubated with or without PUGNAc for 19 h and with 0.6 nmol/l insulin (*A*) during the final 75 min of incubation (n = 7 per group) or 12 nmol/l insulin (*B*) during the final 75 min of incubation (n = 8 per group). Bands corresponding to P-Akt(S473) were quantitated by densitometry and expressed as the mean ± SE.

resistance with 0.6 nmol/l insulin is not attributable to attenuated phosphorylation of the 160-kDa protein.

GSK3α and GSK3β are well-known Akt substrates, and their insulin-stimulated phosphorylation attenuates their activity for phosphorylation and inhibition of glycogen synthase, thereby promoting the insulin-mediated increase in glycogen synthesis. Vosseller found a PUGNAc-induced decrease in GSK3β(S9) phosphorylation in insulin-stimulated 3T3-L1 adipocytes. Consistent with the lack of an effect of PUGNAc on Akt phosphorylation in skeletal muscle, we did not detect an effect of PUGNAc on GSK3β(S9) phosphorylation following either physiologic or supraphysiologic insulin stimulation. In addition, we found no affect of PUGNAc on the phosphorylation of GSK3α(S21), regardless of insulin concentration.

Several previous studies (23–25) have demonstrated that infusion of rats with high concentrations of glucosamine can lead to insulin resistance for glucose uptake by skeletal muscle concomitant with impairment of insulin receptor substrate (IRS)-1–associated phophatidylinositol (PI) 3-kinase. In two of these studies, the insulin resistance and impaired IRS-1/PI 3-kinase were not accompanied by attenuated activation of Akt (23,25). These results suggest that processes that are IRS-1/PI 3-kinase–dependent but Akt independent are crucial for the glucosamine-induced insulin resistance. We have focused on Akt-related signaling events in this study because of the evidence that, in 3T3-L1 adipocytes, PUGNAc inhibits the activation of Akt and downstream signaling processes (14). It will be valuable to also assess the influence of PUGNAc on IRS-1/PI3-kinase as well as other downstream signaling events. Regardless, the current results demonstrate that PUGNAc-induced insulin resistance is independent of altered Akt phosphorylation and phosphorylation of several downstream signaling proteins.

It seems reasonable to suspect that the mechanism

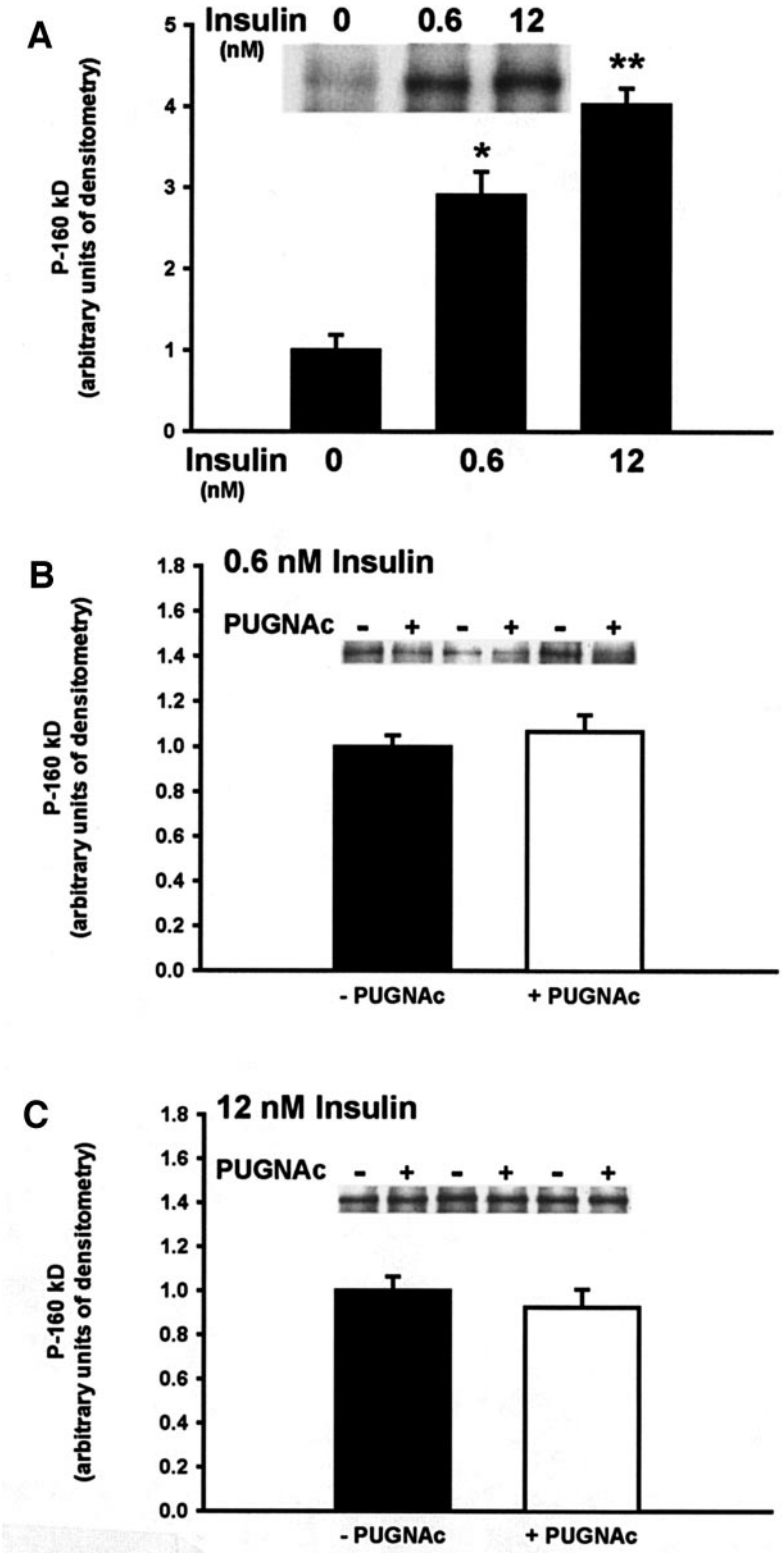

FIG. 6. Effect of insulin and PUGNAc on phosphorylation of the 160-kDa protein on the Akt phosphomotif. *A*: Dose response for insulin in muscles incubated without PUGNAc at basal (no insulin), 0.6, or 12 nmol/l insulin during the final 75 min of incubation (n = 4 per group). Paired muscles incubated with or without PUGNAc for 19 h and with 0.6 nmol/l insulin (*B*) during the final 75 min of incubation (n = 11 per group) or 12 nmol/l insulin (*C*) during the final 75 min of incubation (n = 12 per group). Protein blots were probed with anti–phospho-(Ser/Thr)Akt substrate. Bands corresponding to the phosphorylated 160-kDa protein were quantitated by densitometry and expressed as the mean ± SE. *Basal vs. 0.6 nmol/l insulin (P < 0.001); **basal vs. 12 nmol/l insulin (P < 0.001) and 0.6 vs. 12 nmol/l insulin (P < 0.05).

whereby PUGNAc induces insulin resistance involves O-GlcNAc modification of specific proteins. Vosseller et al. (14) found that in 3T3-L1 adipocytes, PUGNAc led to elevated O-GlcNAcylation of IRS-1 and β-catenin, concomitant with no detectable O-GlcNAc modification of Akt, p85 subunit of PI3-kinase, or GSK3β, with or without PUGNAc treatment. In rats made insulin resistant by infusing high levels of glucosamine, Patti et al. (25) found increased O-GlcNAc modification of IRS-1, IRS-2, and p85–PI 3-kinase in skeletal muscle. In L6 myotubes incubated for 18 h with 10 nmol/l insulin and 5 or 25 mmol/l glucose, Walgren et al. (26) found a high glucose–related increase in O-GlcNAcylated Sp1, α-tubulin, and HSP70. Sp1 is one of several transcription factors known to undergo O-GlcNAc modification, raising the possibility that PUGNAc might act by altering transcription.

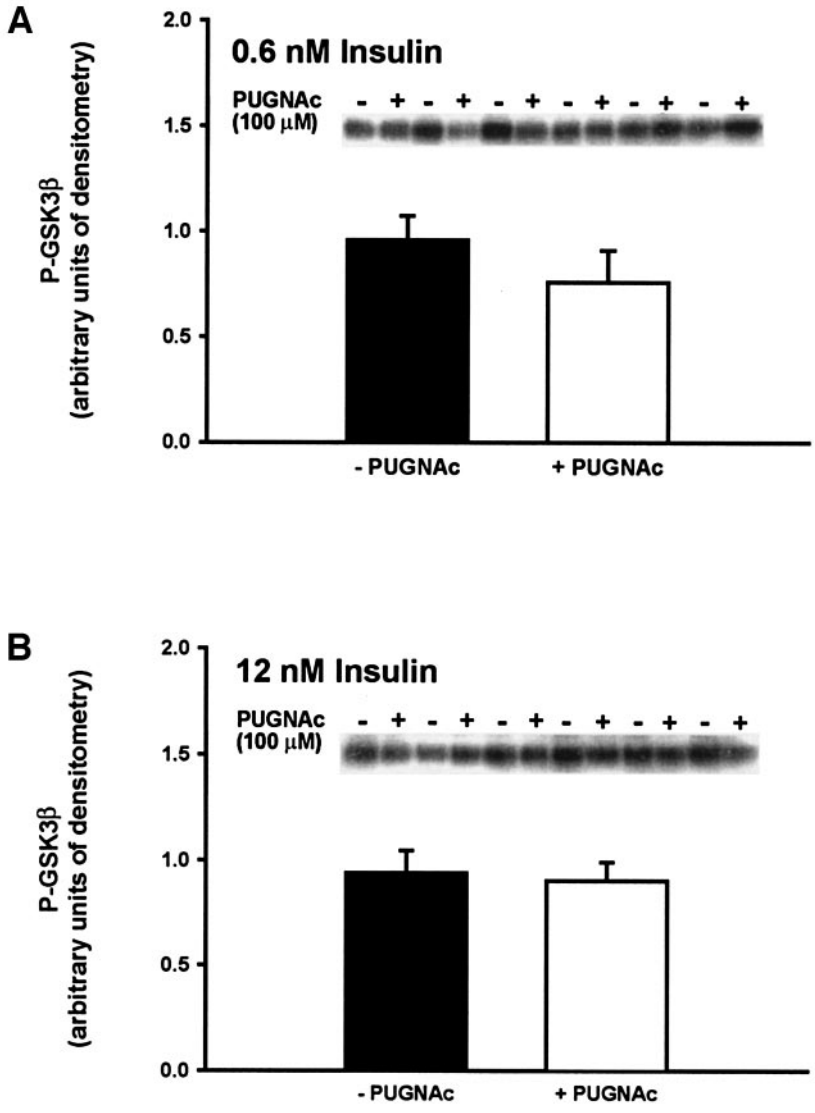

FIG. 7. Phosphorylation status of GSK3β phosphorylation following 19 h of incubation of muscle with or without PUGNAc and with 0.6 nmol/l insulin (*A*) (*n* = 7 per group) or 12 nmol/l insulin (*B*) (*n* = 11 per group). Protein blots were probed with anti–phospho-GSK3α/β. Bands corresponding to P-GSK3β (molecular weight of 46 kDa) were quantitated by densitometry and expressed as the mean ± SE.

In conclusion, we have found that PUGNAc can be effectively used to induce O-GlcNAc modification of multiple proteins in isolated rat skeletal muscle. Furthermore, PUGNAc significantly reduced glucose transport when measured with a physiologic insulin concentration, and the insulin resistance could be overcome with a supraphysiologic insulin concentration. The reduced glucose transport with physiologic insulin was not attributable to attenuated threonine or serine phosphorylation of Akt, serine phosphorylation of GSK3α or GSK3β, or phosphorylation of an insulin-responsive, putative Akt substrate with an apparent molecular weight of 160 kDa. The lack of a PUGNAc-induced change in the phosphorylation of several proteins with Akt phosphomotifs provides strong evidence that the insulin resistance in skeletal muscle was not the result of impaired Akt function. Our results are consistent with the idea that O-GlcNAc modification of proteins can induce insulin resistance in skeletal muscle. Further research will be needed to determine whether the PUGNAc effect on insulin resistance in muscle is a direct effect involving the O-GlcNAcylation of proteins required for insulin signaling and/or GLUT4 translocation or an indirect effect, e.g., mediated by increasing O-GlcNAcylation of transcription factors, leading to altered gene expression.

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