Enhanced O-GlcNAc protein modification is associated with insulin resistance in GLUT1-overexpressing muscles

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Division of Endocrinology, Departments of 1Medicine, Diabetes, and Medical Genetics and 2Biochemistry/Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425; Division of Endocrinology and Metabolism, Departments of 3Pediatrics and 4Cell Biology and Physiology, School of Medicine, Washington University, St. Louis, Missouri 63110

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Buse, Maria G., Katherine A. Robinson, Bess A. Marshall, Richard C. Hresko, and Mike M. Mueckler. Enhanced O-GlcNAc protein modification is associated with insulin resistance in GLUT1-overexpressing muscles. Am J Physiol Endocrinol Metab 283: E241–E250, 2002. Published March 27, 2002; 10.1152/ajpendo.00060.2002.—O-linked glycosylation on Ser/Thr with single N-acetylglucosamine (O-GlcNAcylation) is a reversible modification of many cytosolic/nuclear proteins, regulated in part by UDP-GlcNAc levels. Transgenic (T) mice that overexpress GLUT1 in muscle show increased basal muscle glucose transport that is resistant to insulin stimulation. Muscle UDP-GlcNAc levels are increased. To assess whether GLUT4 is a substrate for O-GlcNAcylation, we translated GLUT4 mRNA (mutated at the N-glycosylation site) in rabbit reticulocyte lysates supplemented with [35S]methionine. O-GlcNAcylated proteins were galactosylated and separated by lectin affinity chromatography; >20% of the translated GLUT4 appeared to be O-GlcNAcylated. To assess whether GLUT4 or GLUT4-associated proteins were O-GlcNAcylated in muscles, muscle membranes were prepared from T and control (C) mice labeled with UDP-[3H]galactose and immunoprecipitated with anti-GLUT4 IgG (or nonimmune serum), and N-glycosyl side chains were removed enzymatically. Upon SDS-PAGE, several bands showed consistently two- to threefold increased labeling in T vs. C. Separating galactosylated products by lectin chromatography similarly revealed approximately threefold more -GlcNAc-modified proteins in T vs. C muscle membranes. RL-2 immunoblots confirmed these results. In conclusion, chronically increased glucose flux, which raises UDP-GlcNAc in muscle, results in enhanced O-GlcNAcylation of membrane proteins in vivo. These may include GLUT4 and/or GLUT4-associated proteins and may contribute to insulin resistance in this model.

Posttranslational O-GlcNAc modification of proteins (O-GlcNAcylation) is defined as the O-linked attachment of single β-N-acetylglucosamine moieties to specific Ser/Thr hydroxyl groups. It was first described in 1984 and differs in many respects from the “classical” forms of protein glycosylation, i.e., Asn-linked N-glycosylation or O-GalNAc-Ser-(Thr)-linked O-glycosylation. Whereas the latter processes occur in the endoplasmic reticulum and/or Golgi apparatus, the major sites of O-GlcNAcylation are the nucleus and the cytosol. While proteins bearing classical glycosyl side chains are typically cell surface or secreted proteins, O-GlcNAcylated proteins are ubiquitous and most abundant in the nucleus and the cytoplasm. Known O-GlcNAcylated proteins include nucleoporins, RNA polymerase II catalytic subunit, many transcription factors, and numerous cytoskeletal proteins. All O-GlcNAc-modified proteins identified to date are subject to phosphorylation, and in many instances the sites of O-phosphorylation and O-GlcNAcylation are identical or adjacent, suggesting a regulatory role in cell signaling (reviewed in Refs. 6 and 12). O-GlcNAcylation is a dynamic and reversible process, and enzymes that catalyze the addition (22, 27) and removal (9) of O-GlcNAc to and from polypeptides have been identified and cloned.

Insulin resistance is a hallmark of type 2 diabetes and is associated with uncontrolled type 1 diabetes. Sustained exposure to high glucose also causes insulin resistance, and “glucose toxicity” accounts for the insulin resistance in uncontrolled type 1 diabetes (39, 44). Insulin accelerates glucose utilization by responsive cells (skeletal muscle, heart muscle, and adipocytes) by stimulating glucose transport. The above cells express the insulin-responsive glucose transporter GLUT4, which is mainly segregated intracellularly in the basal state and translocates to the plasma membrane (PM) in response to insulin. GLUT1 is constitutively expressed at the PM and is mainly responsible for basal glucose transport (reviewed in Ref. 2).

Transgenic mice, which overexpress GLUT1 in skeletal muscle, exhibit mild fasting hypoglycemia, without significant changes in circulating insulin or glucose...
gon. Basal glucose transport and glycogen stores are markedly increased in GLUT1 transgenic muscles (28, 36). A remarkable characteristic of these muscles is that, in vitro, insulin fails to stimulate glucose transport, although GLUT4 expression is unchanged. Other stimuli, e.g., insulin-like growth factor-I, hypoxia, and contractile activity, which normally stimulate muscle glucose transport and GLUT4 translocation, also fail to stimulate glucose transport (10).

UDP-GlcNac is the major product of the hexosamine biosynthesis pathway (HBSP) and the obligatory substrate of polyepitide-O-GlcNac transferase (OGT), the cytosolic/nuclear enzyme, which catalyzes protein O-GlcNacylation (22, 27). Glucose entry into HBSP is regulated by glutamine-fructose-6-phosphate amidotransferase (GFAT). The products of the reaction are glucosamine 6-phosphate (GlcN-6-P) and glutamate (21). The concentration of UDP-N-acetylhexosamine(s) (UDP-GlcNac + UDP-GalNac, in an ~3:1 ratio) is increased two- to threefold in GLUT1-overexpressing muscles, and GFAT activity is increased ~60% (5).

Increased flux via HBSP has been implicated in glucose-induced insulin resistance (4, 5, 7, 13, 14, 16, 29, 37). In GLUT1-overexpressing muscles, glucose flux is chronically increased, and insulin-resistant glucose transport is associated with increased HBSP activity. We hypothesized that the increased intracellular concentrations of UDP-GlcNac may promote O-GlcNacylation of critical proteins involved in insulin-stimulated GLUT4 trafficking. In the experiments reported here, we investigated whether GLUT4 is a potential substrate of O-GlcNacylation and assessed whether or not increased O-GlcNacylation of GLUT4 or of proteins associated with GLUT4 are detectable in membrane preparations from GLUT1-overexpressing skeletal muscle.

**EXPERIMENTAL PROCEDURES**

**Materials.** Except where otherwise noted, all reagents were purchased from Sigma Chemical (St. Louis, MO) and were of the highest quality available. Site-specific, COOH-terminal polyclonal antibodies against GLUT1 and GLUT4 were raised in rabbits and purified before use by protein A affinity chromatography. Preimmune serum was purified identically. A monoclonal mouse antibody (RL-2) that recognizes O-GlcNacylated proteins (17, 40) was purchased from Affinity Bioreagents (Golden, CO), and the mouse monoclonal site-specific anti-GLUT4 antibody was from Genzyme (Cambridge, MA). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgGs were purchased from Jackson Immunoresearch Laboratories (West Grove, PA), and enhanced chemiluminescence (ECL) reagents were from Pierce (Rockford, IL).

**Animals.** The transgenic mouse line that overexpresses GLUT1 in skeletal muscle has been described previously (5, 10, 28, 36). Transcription of the human GLUT1 gene was controlled by the rat myosin light chain promoter (28). For experiments, mice expressing a single GLUT1 gene were mated with wild-type B6SJLF1/J mice (Jackson Laboratories, Bar Harbor, ME). The offspring of these matings consisted of a 50:50 mixture of heterozygous transgenic mice and wild-type controls (5). Male transgenic mice and controls from the same litter were used in experiments. At the time of study, mice were between 2 and 6 mo old.

Mice were housed in a facility equipped with a 12:12-h light-dark cycle and were fed ad libitum (Rodent Blox; Ralston Purina, St. Louis, MO) until food was removed 2 h before death. Mice were killed at 10:00 AM under halothane anesthesia. Hindlimb muscles, including calf, thigh, and hip muscles, were rapidly removed and frozen in liquid nitrogen.

**Preparation of total muscle membranes.** Frozen muscles (~2 g) were powdered in a mortar cooled with liquid nitrogen and homogenized immediately in 5 vol of ice-cold 10 mM Tris, pH 7.4, 1 mM EDTA, 250 mM sucrose, 10 μg/ml leupeptin and aprotinin, and 2 mM phenylmethylsulfonyl fluoride (PMSP). The homogenate was centrifuged for 5 min at 1,000 g, and the pellet was reextracted with another 5 vol of homogenization buffer and centrifuged for 5 min at 1,000 g. Pooled supernatants were centrifuged for 5 min at 10,000 g. The supernatant was adjusted to 0.8 M KCl, mixed for 30 min at 4°C, and centrifuged for 2 h at 174,000 g. The pellet was resuspended in 50 mM HEPES, pH 7.4, 150 mM NaCl, 1% 3-[3-cocoamidopropyl]dimethylamine-N, N-propanesulfonate (CHAPS), and 10 μg/ml leupeptin and aprotinin and solubilized for 1 h; the resultant sample was centrifuged for 5 min at 10,000 g to remove insoluble material. Protein content was assessed by the Bradford assay using Coomassie Protein Assay Reagent (Pierce). The protein concentration was adjusted to 2 mg/ml for the galactosylation procedure.

**Galactosylation of membrane proteins.** Terminally O-GlcNac-modified membrane proteins were identified by the galactosyltransferase probe method using purified UDP-GalNac (O-Galβ-1→4) galactosyltransferase (Gal-transferase) and UDP-[3H]galactose, as described by Roquemore et al. (38). Briefly, 0.5 or 1 mg of total crude membrane proteins in 50 mM HEPES, pH 7.4, 150 mM NaCl, and 0.5% CHAPS was incubated for 1 h at 37°C with the addition of 10 mM HEPES, pH 7.3, 10 mM galactose, 5 mM MnCl2, 1 mM diethiothreitol (DTT), 25 mM 5′-AMP, 20 μCi/ml UDP-[6-3H]galactose (specific activity 40 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO), and 1 U/ml galactosyltransferase (previously auto-galactosylated for 30 min at 37°C in 50 mM Tris, pH 7.3, 5 mM MgCl2, 1 mM mercaptoethanol, 0.4 mM UDP-galactose, and 0.1 mg/ml aprotinin). Enzyme reactions were terminated by the addition of 10 mM EDTA. Immunoprecipitation of galactosylated membranes is described below.

**Purification of galactosylated membrane proteins by ricianus communis lectin chromatography.** Galactosylation of 0.5 mg muscle membrane proteins was performed as described above except that the reactions were stopped by adding 10 mM EDTA and 1% SDS. Reaction products were separated from unincorporated label on a Sephadex G-50 column equilibrated with 50 mM ammonium formate and 0.1% SDS. The macromolecular peak was lyophilized to remove the ammonium formate, reconstituted in 50 mM HEPES, pH 7.4, 150 mM NaCl, and 10 mM EDTA, and the SDS concentration was reduced to 0.1% by spin filtration. The sample was reconstituted in the same buffer containing 1% Triton X-100. Average label incorporated over 0.5 mg protein was 1.6 × 106 disintegrations/min (dpm) for the control and 1.9 × 106 dpm for the transgenic mice. N-linked glycosyl side chains were removed by digesting the samples overnight at 37°C with 4 U/ml endoglycosidase F (Boehringer Mannheim, Indianapolis, IN). Ricinus communis agglutinin I agarose (Vector Laboratories, Burlingame, CA) chromatography was performed on a 0.7×18-cm (7 ml) column preequilibrated with 50 mM HEPES, pH 7.4, 150 mM NaCl, and 0.1% Triton X-100 (buffer A). Sixty 0.4-ml fractions were collected before the addition of 0.2 M lactose in buffer A, after which an additional 20
fractions were collected. The radioactivity in the fractions was monitored by scintillation counting of 40-μl aliquots and the protein content by ultraviolet absorption of the undiluted fractions at 280 nm. Peak 1, which eluted just after the void volume, and peak 2, which eluted after the addition of lactose, were concentrated and analyzed by 10% SDS-PAGE. The only visible proteins using silver stain or Coomassie blue stain were from peak 1. The majority of the protein-associated radioactivity, however, was in peak 2. Proteins were transferred to nitrocellulose, and 0.5-cm slices were digested in dimethyl sulfoxide (DMSO) before scintillation counting.

Immunoprecipitation of galactosylated membrane proteins with GLUT1 and GLUT4 antibodies and endoglycosidase F digestion. Membrane preparations galactosylated as described above were preabsorbed with Protein G-Sepharose for 2 h at 4°C. Beads were pelleted by centrifugation, and supernatants were incubated overnight at 4°C with rabbit polyclonal anti-GLUT1 IgG (10 μg/ml) and Protein G-Sepharose. The supernatants were incubated overnight at 4°C with rabbit polyclonal anti-GLUT4 IgG or mouse monoclonal anti-GLUT4 IgM or Protein G-Sepharose beads. Beads were washed three times with 50 mM HEPES, pH 7.4, 150 mM NaCl, and 0.1% CHAPS, and bound proteins were eluted with 10 mM Tris, pH 8.3, 1% SDS, 10 mM DTT, and 25 mM iodoacetic acid at room temperature for 40 min. The elution buffer was exchanged by spin filtration with 50 mM HEPES, pH 7.4, 150 mM NaCl, and 10 mM EDTA. The detergent concentration was adjusted to 1% Triton X-100, and 10 μg/ml leupeptin and aprotinin were added. Samples were incubated overnight at 37°C with or without 4 U/ml endoglycosidase F. Digestions were terminated by the addition of Laemmli sample buffer, and proteins were separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane, and sample lanes were cut into 0.5-cm slices, which were dissolved in DMSO. Radioactivity was quantified by scintillation counting.

Preparation of muscle extracts for Western blotting with RL-2 antibody. Frozen, powdered hindlimb muscle was homogenized in 10 vol of 50 mM HEPES, pH 7.4, 1 mM EDTA, 10% glycerol, 150 mM sodium chloride, 1 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.2 mM sodium vanadate, 100 μg/ml aprotinin, 10 μg/ml leupeptin, 1.5 mg/ml benzamidine, and 1 mM PMSF (homogenization buffer). Samples were centrifuged at 2,000 g for 2 min, and supernatants were saved. Pellets were reextracted in 8 vol of homogenization buffer and centrifuged as before. Pooled supernatants were solubilized for 1 h in 1% Igepal CA-630 (Sigma) at 4°C. After centrifugation at 10,000 g for 10 min, supernatant protein concentrations were normalized, samples were diluted with Laemml sample buffer, and 40 μg of protein were analyzed by 7% SDS-PAGE. Proteins were transferred to nitrocellulose membranes, incubated overnight with RL-2 antibody (1:1,000), and developed by the ECL procedure.

In vitro expression and galactosylation of GLUT4. GLUT4(Thr 57) was transcribed, translated, and O-GlcNAcylation by incubation for 90 min at 37°C using the Promega TNT rabbit reticulocyte lysate coupled transcription/translation system (Promega, Madison, WI) in the presence of canine microsomal membranes, in a methionine-free amino acid mixture supplemented with [35S]methionine (2 μCi/ml; ICN, Costa Mesa, CA) in a final volume of 50 μl. Asn 57 is the N-glycosylation site in GLUT4. Mutated aglyco-GLUT4 was translated to avoid galactosylating terminal GlcNAc moieties of the N-glycosyl side chain. The template was the rat GLUT4 cDNA in the oocyte expression vector pSP64T (24). Site-directed mutagenesis was carried out using the Clontech Transformer site-directed mutagenesis kit (Clontech Laboratories, Palo Alto, CA). Pancreatic microsomes were prepared as described (42). After 90 min of translation at 37°C, samples were pelleted by centrifugation at 10,000 g for 30 min at 4°C. Pellets were rinsed with five reaction volumes of 10 mM Na2HPO4, pH 7.4, and 150 mM NaCl (buffer B) and resuspended in 1 volume of 1% SDS in buffer B. Samples were diluted with 2.5 vol of 3% Nonidet P-40 (NP-40) in buffer B and galactosylated as described for total membrane proteins from muscle, except that UDP-galactose (0.3 mM) was not labeled. Galactosylated products were diluted to 1 ml with 0.2% NP-40 in buffer B (buffer C) and mixed at room temperature for 30 min with 400 μl of ricinus communis agglutinin I agarose prewashed with 30 ml of buffer B. The lectin beads were transferred to a column for washing and elution. The flow through and a 1-ml wash were collected, and the beads were washed with an additional 20 ml of buffer C. Beads were mixed with 1 ml of 0.5 M GleanAc in buffer C (buffer D) for 30 min, the eluate was collected, and the column was washed with 2 ml of buffer D and 2 ml of buffer C. The beads were mixed for 30 min with 1 ml of 0.5 M galactose in buffer C (buffer E), the eluate was collected, and the beads were washed with two 1-ml aliquots of buffer E. All fractions were precipitated with TCA or immunoprecipitated with GLUT4 antibody and Protein G-Sepharose as described for membrane proteins from muscle, except that the immunoprecipitates were washed with buffer C. Washed TCA precipitates and immunoprecipitates were solubilized in Laemml sample buffer, separated by 10% SDS-PAGE, and analyzed by fluorography using En3Hance reagent (NEN Life Science, Boston, MA).

RESULTS

To assess whether or not GLUT4 is a potential substrate for O-GlcNAcylation, we analyzed the products generated by the rabbit reticulocyte lysate system during translation of GLUT4 mRNA, mutated at the N-glycosylation site Asn 57 to Thr 57. The commercially available rabbit reticulocyte lysate preparation is known to contain sufficient substrate (UDP-GlcNAc) and OGT enzyme activity for efficient O-GlcNAcylation of susceptible proteins (38). Indeed, translation in this system has been recommended for the identification of low-abundance proteins, which are subject to O-GlcNAcylation (38). After galactosylation, O-GlcNAc-bearing proteins can be separated from complex mixtures by lectin affinity chromatography on a ricin column. Because Gal-transferase does not distinguish between O-GlcNAc attached to Ser/Thr residues and terminal GlcNAc moieties in glycosyl side chains, mutated GLUT4 mRNA, which is not subject to N-glycosylation, was translated (Fig. 1).

The data in Fig. 1 are consistent with the concept that GLUT4 is a substrate for O-GlcNAcylation. In the presence of GLUT4 mRNA, the major labeled translation product was an ~50-kDa protein, consistent with GLUT4 (Fig. 1A, lanes 2, 5, and 7). In samples that were not treated with galactosyltransferase, essentially all of the translated GLUT4 was recovered in the flow-through fraction of the ricin column (Fig. 1A, lane 2), and elution of the column with 0.5 M GleanAc (to test for nonspecific binding,) or with 0.5 M galactose (specific binding) yielded essentially no labeled products (Fig. 1A, lanes 3 and 4). In the Gal-transferase-treated
GLUT4 translated in the rabbit reticulocyte lysate system appears to be a substrate for O-linked glycosylation on Ser/Thr with single N-acetylglucosamine (O-GlcNAcylation). Aglyco-GLUT4 was translated in the presence of [35S]methionine by the Promega TNT rabbit reticulocyte lysate coupled transcription/translation system. The products were galactosylated with 0.3 mM UDP-galactose in the presence of Gal-transferase and adsorbed to and eluted from a ricinus communis agglutinin I agarose column. Eluted fractions were precipitated with TCA (A) or immunoprecipitated with mouse or rabbit anti-GLUT4 antibodies (B). Washed TCA precipitates or immunoprecipitates were analyzed by 10% SDS-PAGE followed by fluorography using En3Hance reagent. Negative controls shown in A are translated in the absence of GLUT4 mRNA (lane 1) or sham galactosylated (without Gal-transferase, lanes 1–4). FT, flow through (not bound to the lectin column); GlcNAc, eluted with 0.5 M GlcNAc (nonspecific binding); Gal, eluted with 0.5 M galactose (specific binding). FT and Gal in B were prepared under the same conditions as lanes 5 and 7 in A. The data shown are representative of 4 or more similar experiments, carried out independently in 2 laboratories.

samples (Fig. 1A, lanes 5–7), a major portion of translated GLUT4 was recovered in the flow-through fraction of the ricin column (Fig. 1A, lane 5). Eluting the column with GlcNAc yielded essentially no GLUT4 (lane 6), but, upon elution with 0.5 M galactose, a prominent GLUT4 band appeared (Fig. 1A, lane 7). In the experiments shown in Fig. 1A, the labeled proteins were precipitated with TCA before SDS-PAGE. Essentially identical results were obtained when the column fractions (corresponding to lanes 5 and 7 in Fig. 1A) were immunoprecipitated with mouse or rabbit anti-GLUT4 antibodies before SDS-PAGE (Fig. 1B), confirming the identity of the labeled bands. Quantitative analysis of GLUT4 recovery in the different fractions indicated that at least 20% of the translated GLUT4 was galactosylated, presumably on O-GlcNAc. This calculation may underestimate the extent of O-GlcNAcylation in view of the known inefficiency of the galactosyltransferase reaction, which was likely incomplete (38), and because proteins bearing only one O-GlcNAc may be retarded but not firmly bound to the lectin column (15). Qualitatively similar results were also obtained when wild-type GLUT4 was translated in the reticulocyte lysate system, adsorbed to a wheat germ agarose column, which after extensive washing was eluted with 0.5 M GlcNAc, and the eluate was digested with endoglycosidase F (data not shown). However, the yield of O-GlcNAcylated GLUT4 was greater using the method shown in Fig. 1.

Having shown that GLUT4 is susceptible to O-GlcNAcylation, we addressed the question whether GLUT4 or proteins associated with it are subject to the O-GlcNAc modification in skeletal muscles, in vivo, and whether the process is augmented in GLUT1-overexpressing, insulin-resistant muscles. The graphs in Fig. 2A represent [3H]galactose incorporation into total membrane preparations from muscles of heterozygote transgenic mice overexpressing GLUT1 in skeletal muscle and their wild-type littermates. The galactosylation reaction serves as a probe for the presence of protein-associated terminal GlcNAc moieties. The transgenic mice express 10-fold more GLUT1 in muscle than the controls but have equal amounts of GLUT4 (28). To enrich the preparation in the proteins of interest and minimize nonspecific background radioactivity, the galactosylated muscle membranes were submitted to two-step immunoprecipitation, first with an anti-GLUT1 antibody followed by immunoprecipitation of the supernatant with either anti-GLUT4 or nonimmune γ-globulin. [3H]galactose associated with the anti-GLUT4 immunoprecipitates from 0.5-mg muscle membranes prepared from control rats was very low and similar to the radioactivity associated with membranes from control or transgenic muscles that were treated with nonimmune γ-globulin. However, there was significantly more radioactivity associated with membrane proteins of GLUT1-overexpressing muscles that had been immunoprecipitated with anti-GLUT4 IgG. As assessed by their migration on SDS-PAGE, there were three major peaks between ~100 and ~40 kDa and three minor peaks ranging from 23 to 35 kDa. Comparing the heights of the major peaks, the [3H]galactose associated with these fractions in anti-GLUT4 immunoprecipitates prepared from transgenic muscles was consistently two to three times higher than in identical preparations from control rats.

To assess whether the [3H]galactose was incorporated in O-GlcNAc-modified sites rather than into N-linked glycosyl side chains, a parallel sample of the immunoprecipitates was digested with endoglycosidase F before SDS-PAGE (Fig. 2B). The results are essentially identical to those shown in Fig. 2A. In each experiment, an aliquot of the immunoprecipitated membranes was saved for immunoblotting before and after endoglycosidase F digestion (Fig. 3). The change in the apparent molecular mass of GLUT4 after digestion indicates that the N-glycosyl side chain was removed by the endoglycosidase. Therefore, the en-
hanced galactosylation of membrane proteins from the transgenic mice likely represents enhanced O-GlcNAcylation of these proteins. It is noteworthy that, after treatment with endoglycosidase F, GLUT4 still migrated as a broad band, albeit with increased mobility, suggesting that GLUT4 heterogeneity on SDS-PAGE is not determined solely by the heterogeneity of the N-glycosyl side chain and that additional posttranslational modifications are also likely involved.

In Fig. 2, A and B, membrane proteins were immunoprecipitated with a polyclonal anti-GLUT4 antibody raised in rabbits. To ensure that the immunoprecipitation of O-GlcNAcylated proteins from transgenic mice was not an artifact peculiar to the antibody used, we repeated the experiments using a monoclonal mouse anti-GLUT4 antibody for immunoprecipitation (Fig. 2C). The data shown represent the distribution of [3H]galactose among the immunoprecipitated proteins after endoglycosidase F digestion. Although the overall recovery of labeled proteins was less with the mouse antibody than with the polyclonal rabbit antibody, the pattern of protein recovery and distribution was similar.

For statistical analyses of the data in Fig. 2, the areas under the curves were integrated using National Institutes of Health Image software. Data from three independent experiments were analyzed for each parameter shown in Fig. 2, A-C. The graphic analysis eliminates variations in labeling between experiments but allows for comparison of controls and transgenics in each experiment because they were always processed in parallel. Data were analyzed as uncorrected and corrected (specific) anti-GLUT4 immunoprecipitates. The latter represent the area under the immunoprecipitate curve minus the area under the curve generated from a corresponding sample treated with non-immune γ-globulin. The significance of differences between means was analyzed by two-tailed Student’s t-test or by paired Student’s t-test where indicated in the text. Values represent means (in arbitrary units) ± SE. For Fig. 2A, uncorrected mean values were 7,061 ± 793 for controls and 11,441 ± 332 for transgenics (n = 3, P < 0.01), and corrected values were 2,394 ± 651 for controls and 5,896 ± 1,196 for transgenics (P < 0.07). The mean ratio of specific immunoprecipitates (transgenic/control) was 2.66 ± 0.4 (P < 0.025).

In Fig. 2B, means for uncorrected immunoprecipitates were 7,969 ± 1,280 and 10,980 ± 892 for controls and transgenics, respectively (n = 3, P < 0.03, paired Student’s t-test), and the corrected values were 2,141 ± 614 for controls and 4,960 ± 940 for transgenics (n = 3, P < 0.03, paired Student’s t-test). The mean ratio of specific immunoprecipitates (transgenic/control) was 2.53 ± 0.398 (P < 0.03). In Fig. 2C, the corresponding values were 7,710 ± 1,199 for controls and 14,687 ± 1,375 for transgenics (uncorrected immunoprecipitates, n = 3, P < 0.02), and the specific immunopre-
precipitates were 700 ± 296 for controls and 7,049 ± 967 for transgenics (P < 0.005, n = 3).

In the course of the experiments shown in Fig. 2, we also routinely assessed the recovery of [3H]galactosylated proteins in the immunoprecipitates generated with the anti-GLUT1 rabbit antibody, after separating the immunoprecipitated proteins by SDS-PAGE. We were unable to detect a consistent pattern or reproducible differences between the anti-GLUT1 immunoprecipitates prepared from muscle membranes of control and transgenic mice (data not shown).

In the experiments described above, the yield of O-GlcNacylated labeled proteins was too low to attempt identification. In an effort to improve the yield, we performed lectin affinity chromatography of the galactosylated membrane proteins from muscles of control and transgenic mice. After labeling with [3H]galactose, the membranes were digested with endoglycosidase F before affinity chromatography on a ricin column. Most of the radiolabeled proteins were retained on the column and were recovered upon elution with 0.2 M lactose. The distribution of the radioactivity eluted after the addition of lactose. The labeled, lactose-eluted fractions were pooled, concentrated, and analyzed by 10% SDS-PAGE. The distribution of radioactivity on the gel was quantified as described in the legend for Fig. 2. Data are representative of 3 similar experiments.

The integrated areas under the curves were determined by graphic analysis of four independent experiments, as described in Fig. 2. Means (arbitrary units ± SE) were 2,913 ± 378 and 6,275 ± 663 for controls and transgenics, respectively (n = 4, P < 0.005). Unfortunately, the protein concentrations in the concentrated lactose eluates were insufficient for detection after SDS-PAGE by silver or by Coomassie blue staining. We also attempted to ascertain whether GLUT4 was raised against nuclear pore proteins, which are recognized numerous proteins, and the signal was marked increases in muscle membranes of transgenic mice.

Fig. 5. Immunoblot of muscle extracts from GLUT1-overexpressing muscles and controls with monoclonal mouse (RL-2) antibody. Postnuclear, detergent-solubilized muscle extracts were prepared from homogenates as described in EXPERIMENTAL PROCEDURES. Proteins (40 μg/lane) were separated by 7% SDS-PAGE, transferred to nitrocellulose membranes, and incubated overnight with RL-2 antibody (1:1,000) in the presence or absence of 0.1 M GlcNAc. The blots were developed using the enhanced chemiluminescence procedure. Data are representative of 3 similar experiments.

The RL-2 mouse monoclonal antibody was originally raised against nuclear pore proteins, which are O-GlcNAcylated (17, 40); it recognizes the O-GlcNAc modification on numerous proteins. To confirm our finding that O-GlcNAcylation was increased in muscles of mice that overexpress GLUT1, proteins separated by SDS-PAGE from postnuclear muscle extracts of transgenic and control mice were immunoblotted with the RL-2 antibody (Fig. 5). As expected, the antibody recognized numerous proteins, and the signal was markedly increased in muscles from transgenic mice vs. the controls. Inclusion of 0.1 M N-acetylglucosamine with

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eFig. 4. Lectin affinity chromatography of galactosylated membrane proteins from transgenic and control muscles. Total muscle membranes were prepared and galactosylated with UDP-[3H]galactose and Gal-transferase, as described in the legend of Fig. 2. Labeled proteins were separated from unincorporated label by Sephadex G-50 chromatography, and the protein fraction was lyophilized, reconstituted in buffer containing 1% Triton X-100, and digested with endoglycosidase F. The samples were adsorbed to a ricin communis agglutinin I agarose column (0.7 cm), which after extensive washing (60 0.4-ml fractions were collected) was eluted with 0.2 M lactose (20 0.4-ml fractions were collected). Most of the radioactivity eluted after the addition of lactose. The labeled, lactose-eluted fractions were pooled, concentrated, and analyzed by 10% SDS-PAGE. The distribution of radioactivity on the gel was quantified as described in the legend for Fig. 2. Data are representative of 4 similar experiments.
the RL-2 antibody, as a competitive inhibitor of specific binding, diminished the signal in both controls and transgenics, although it did not abolish it. GlcNAc competes relatively poorly with protein-bound O-GlcNAc for binding to the antibody.

DISCUSSION

In recent years, there has been increasing awareness of the potential role of protein O-GlcNAcylation in cellular regulation. Studies from several laboratories suggest potential roles in 1) signaling (as an alternative to or modulator of phosphorylation); 2) protein-protein interactions; 3) modulation of protein degradation; and 4) regulation of DNA transcription and mRNA translation, as well as other processes (6, 12, 38). Despite this interest, there are very few studies addressing the metabolic regulation of protein O-GlcNAcylation in vivo. Yki-Jarvinen et al. (45) reported increased O-GlcNAcylation of proteins in rat skeletal muscles after 6 h of infusion of insulin and glucosamine (GlcN). GlcN infusions caused insulin resistance, impaired activation of early insulin-signaling intermediates in muscle, and, based on RL-2 reactivity, enhanced the O-GlcNAcylation of insulin receptor substrate-1 (32). Using in vivo metabolic radiolabeling, Hawkins and colleagues (13, 14) reported markedly enhanced glycosylation of skeletal muscle proteins associated with GLUT4-containing vesicles (GCV) in rats rendered insulin resistant by 7 h of infusion of GlcN plus insulin. However, >90% of the label was incorporated into N-linked glycosyl side chains (13). Thirty minutes of sustained hyperglycemia markedly increased protein O-GlcNAcylation in the islets of Langhans, based on RL-2 immunocytochemistry. Pancreatic islets appear to be exceptionally rich in O-GT (26).

GlcN-6-P is the product of the transamidase reaction catalyzed by GFAT, the first and rate-limiting step in HBSP. The entry of extracellular GlcN into cells is facilitated by glucose transporters (in the case of muscle, GLUT1 and GLUT4). GlcN is phosphorylated to GlcN-6-P by hexokinase and enters the HBSP, bypassing GFAT. GFAT activity is subject to allosteric, negative feedback regulation by the major product of the HBSP, UDP-GlcNAc (21, 29). The circulating concentration of GlcN is normally very low (13). Increasing the ambient GlcN concentration can cause metabolic changes in the intact animal (41) and in cell culture systems (18, 30, 31), which do not mimic the effects of increased glucose flux via HBSP. Thus, although both GlcN and sustained hyperglycemia cause insulin resistance, the cellular mechanisms may not always be identical (18, 30, 31, 41).

Skeletal muscle is a major site of insulin resistance in diabetic patients and in animal models of type 1 and type 2 diabetes. The major defect is impaired insulin stimulation of glucose transport (reviewed in Ref. 33). The transgenic mouse overexpressing GLUT1 in skeletal muscle is an interesting model, because the only underlying abnormality is chronically increased glucose flux into muscle. Circulating levels of nutrients, e.g., glucose and free fatty acids, and hormones, e.g., insulin and glucagon, are essentially normal. GLUT4 expression is similar to that of controls, and GLUT4 translocates to the PM in response to insulin (11). However, glucose transport is not stimulated by insulin (10, 11, 28, 36), suggesting that either the translocated GLUT4 is inappropriately inserted in the PM or GLUT4 or proteins associated with GLUT4 are modified in a manner that impairs the intrinsic activity of GLUT4 (11). The chronically increased glucose flux into muscle cells increases flux via HBSP, resulting in increased concentrations of UDP-GlcNAc (5). The data presented here demonstrate concomitant marked increases in the O-GlcNAcylation of muscle membrane proteins (Fig. 4), a subset of membrane proteins that coimmunoprecipitate with GLUT4 (Fig. 2), and numerous proteins in postnuclear total muscle extracts (Fig. 5). To our knowledge, this is the first demonstration of increased protein O-GlcNAcylation without high glucose or GlcN in the extracellular milieu, indicating that increased glucose flux via HBSP, resulting in increased UDP-GlcNAc, is sufficient to promote the O-GlcNAcylation of certain proteins in vivo. The insulin resistance that develops in rats infused with lipid emulsions, resulting in increased plasma free fatty acids, is also associated with increased UDP-GlcNAc in skeletal muscle, presumably reflecting impaired glycolytic flux distal to fructose-6-phosphate (F-6-P), resulting in increased F-6-P flux via HBSP (14). Because increased circulating free fatty acids and their enhanced utilization by muscle are clearly associated with the insulin resistance of obesity and type 2 diabetes, the question arises whether these conditions also promote O-GlcNAcylation of certain proteins in skeletal muscle. Relatively modest elevations in UDP-GlcNAc may promote protein O-GlcNAcylation in part by mass action and by positively regulating OGT activity (23). The concentration of UDP-GlcNAc appears to regulate not only the velocity of the enzyme, but also its Michaelis constant (Km) for different peptide substrates, i.e., substrate selectivity. Furthermore, OGT itself is subject to posttranslational modification by tyrosine phosphorylation and O-GlcNAcylation (23).

Our data obtained in the reticulocyte lysate system (Fig. 1) support the concept that GLUT4 can serve as a substrate for OGT. Although the majority of O-GlcNAc-modified proteins are nuclear or cytosolic, several membrane-associated O-GlcNAcylated proteins have been identified (6, 12, 32). As reported for other O-GlcNAc-modified proteins, GLUT4 is subject to reversible O-phosphorylation (25). The COOH-terminal portion of GLUT4 contains two sequences, which may represent potential O-GlcNAcylation sites, i.e., Val and/or Pro in close proximity to Ser/Thr (38): Thr-Pro-Ser-Leu-Leu-Glu-Gln-Glu-Val-Lys-Pro-Ser-Thr (amino acids 486–498) and Val-Pro-Glu-Thr-Ser (amino acids 469–472). Ser488 is an identified phosphorylation site (25). However, we were not able to conclusively determine that GLUT4 was O-GlcNAcylated in muscle in vivo. Clearly, the muscle membranes, which were pre-

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cipated with anti-GLUT4 antibody, yielded GLUT4 in the immunoprecipitates (Fig. 3), and the labeled protein peaks, which represent the O-GlcNAcylated membrane proteins prepared from GLUT1-overexpressing muscles, may contain the O-GlcNAcylated GLUT4 (consisting of 509 amino acids, ~51 kDa without the NH₂-linked glycosyl side chain; see, e.g., peak 3 in Fig. 2B). Unfortunately, the low yield precluded the characterization and identification of O-GlcNAcylated proteins. We chose the Gal-transferase method to probe for O-GlcNAcylated proteins because it has been extensively used and validated (38). Provided that N-glycosyl side chains are removed, the probe is specific for proteins modified by O-GlcNAc. However, galactosylation is not stoichiometric, and the yield is low. This reflects, in part, the lability of the O-GlcNAc modification, which may be lost during sample preparation, the relatively high Km of Gal-transferase for UDP-galactose, and the accessibility of the peptide substrate to the enzyme (23, 38).

The proteins that are highly O-GlcNAcylated in GLUT1-overexpressing muscle membranes and immunoprecipitate with GLUT4 (Fig. 2) have not been identified. The question arises whether they represent proteins and/or protein complexes, which interact with GLUT4 or nonspecific coinmunoprecipitation of O-GlcNAcylated proteins, which are more abundant in membrane preparations from transgenic muscles. The following support the former interpretation. 1) The O-GlcNAcylated protein peaks were not observed in “sham immunoprecipitates” treated with nonimmune γ-globulin. 2) There was no difference in the distribution or abundance of O-GlcNAcylated proteins between membranes prepared from control and transgenic muscles after immunoprecipitation with anti-GLUT1 γ-globulin (GLUT1 and GLUT4 are largely segregated in separate vesicles; reviewed in Ref. 34). 3) Similar results were obtained using two different anti-GLUT4 antibodies (rabbit polyclonal and mouse monoclonal). The source of the putative GLUT4-associated O-GlcNAcylated membrane proteins is not clear. GLUT4 is associated with the endoplasmic reticulum and the Golgi apparatus; it is stored in GCV, which translocates to the PM. The GCV interact with cytoskeletal components in the process of translocation, with the docking/fusion machinery at the PM, and GLUT4 recycles in endocytotic vesicles (34). The COOH-terminal portion of GLUT4 appears to be associated with unidentified proteins in skeletal muscle, which mask the antibody recognition site, an association that is released by insulin (43).

To galactosylate the O-GlcNAcylated membrane proteins, membranes had to be resuspended in detergent (1% CHAPS, a zwitterionic detergent)-containing buffer. It seems highly unlikely that this treatment preserved the integrity of GCV. However, GCV share many characteristics with small synaptic vesicles (SSV) in neurons (34, 35). Solubilization of SSV membranes in CHAPS preserved multimeric protein complexes (including SNARE proteins), but not solubilization in Triton X-100 or octylglucoside (3), and CHAPS-stabilized complexes were resistant to treatment with high salt or DTT, suggesting lysophilic interactions (3). Numerous cytoskeletal and bridging proteins, which are subject to O-GlcNAcylation, have been identified, including the microtubule-associated proteins-1, -2, and -4, tau, vinculin, talin, clathrin assembly protein-3 (AP-3) and synapsin 1, which is thought to anchor synaptic vesicles to the cytoskeleton and mediate their release upon phosphorylation (reviewed in Refs. 6 and 12). O-GlcNAcylation may modulate protein-protein interactions involved in numerous cellular processes (6), which may include GLUT4 trafficking, its appropriate docking/fusion at the PM, and/or its intrinsic activity.

The development of glucose-induced insulin resistance is time dependent. It requires several hours of hyperglycemia in animal models and in cell culture systems or, as in the model presented here, chronically increased glucose flux without hyperglycemia. Numerous O-GlcNAcylated proteins are transcription factors. Altered regulation of gene expression may contribute to the insulin resistance associated with chronically increased glucose flux into muscle and the resulting increase in the UDP-GlcNAc pool. Work from several laboratories links the deleterious effects of chronic hyperglycemia on the vascular system to increased HBSP activity. Chronic exposure to high glucose of cultured vascular smooth muscle cells increases OGT protein expression and activity and induces qualitative and quantitative changes in nuclear O-GlcNAcylated proteins (1). In cultured mesangial cells, the high glucose induction of transforming growth factor (TGF)-β1 (20) and of plasminogen inhibitor-1 (19) is mediated at least in part by products of HBSP. The transcription factor Sp-1 has several O-GlcNAcylation sites, and the O-GlcNAc modification competes with Ser/Thr phosphorylation (reviewed in Refs. 6, 8, and 12). In bovine aortic endothelial cells, culture in high glucose induces TGF-β1 and PAI-1 expression, which is dependent on GFAT activity. O-GlcNAcylation of Sp-1 is enhanced in high glucose, and mutation of Sp-1 binding sites on the PAI-1 promoter prevents its induction by glucose. Thus induction of genes known to be involved in the vascular complications of diabetes reflect, at least in part, the enhanced O-GlcNAcylation of Sp-1 (8).

In conclusion, our data indicate that chronically increased glucose flux into skeletal muscle is associated with insulin resistance, increased intracellular UDP-GlcNAc, and enhanced O-GlcNAc modification of membrane proteins in vivo. They support the notion that the UDP-GlcNAc concentration in muscle may serve as an indicator of and a contributor to the development of insulin resistance (5, 13) by promoting the O-GlcNAcylation of critical proteins. These may include GLUT4 and/or GLUT4-associated proteins.

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