

Increased Phosphorylation of Skeletal Muscle Glycogen Synthase at NH₂-Terminal Sites During Physiological Hyperinsulinemia in Type 2 Diabetes

Kurt Højlund,¹ Peter Stæhr,¹ Bo Falck Hansen,² Kevin A. Green,³ D. Grahame Hardie,³ Erik A. Richter,⁴ Henning Beck-Nielsen,¹ and Jørgen F.P. Wojtaszewski⁴

In type 2 diabetes, insulin activation of muscle glycogen synthase (GS) is impaired. This defect plays a major role for the development of insulin resistance and hyperglycemia. In animal muscle, insulin activates GS by reducing phosphorylation at both NH₂- and COOH-terminal sites, but the mechanism involved in human muscle and the defect in type 2 diabetes remain unclear. We studied the effect of insulin at physiological concentrations on glucose metabolism, insulin signaling and phosphorylation of GS in skeletal muscle from type 2 diabetic and well-matched control subjects during euglycemic-hyperinsulinemic clamps. Analysis using phospho-specific antibodies revealed that insulin decreases phosphorylation of sites 3a + 3b in human muscle, and this was accompanied by activation of Akt and inhibition of glycogen synthase kinase-3 α . In type 2 diabetic subjects these effects of insulin were fully intact. Despite that, insulin-mediated glucose disposal and storage were reduced and activation of GS was virtually absent in type 2 diabetic subjects. Insulin did not decrease phosphorylation of sites 2 + 2a in healthy human muscle, whereas in diabetic muscle insulin infusion in fact caused a marked increase in the phosphorylation of sites 2 + 2a. This phosphorylation abnormality likely caused the impaired GS activation and glucose storage, thereby contributing to skeletal muscle insulin resistance, and may therefore play a pathophysiological role in type 2 diabetes. *Diabetes* 52: 1393–1402, 2003

From the ¹Diabetes Research Centre, University of Southern Denmark and Department of Endocrinology, Odense University Hospital, Odense, Denmark; the ²Diabetes Biology, Novo Nordisk, Bagsvaerd, Denmark; the ³Wellcome Trust Biocentre, Division of Molecular Physiology, School of Life Sciences, Dundee University, Dundee, Scotland, U.K.; and the ⁴Copenhagen Muscle Research Centre, Institute of Exercise and Sport Sciences, Department of Human Physiology, University of Copenhagen, Copenhagen, Denmark.

Address correspondence and reprint requests to Kurt Højlund, MD, Department of Endocrinology, Odense University Hospital, Klovevænget 6, DK-5000, Odense C, Denmark. E-mail: k.hojlund@dadlnet.dk.

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AMPK, AMP-activated protein kinase; ECL, enhanced chemiluminescence; FFA, free fatty acid; G6P, glucose-6-phosphate; GDR, glucose disposal rate; GS, glycogen synthase; GSK-3, glycogen synthase kinase-3; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PP2A_C, catalytic subunit of protein phosphatase 2A; TBST, Tris-buffered saline with Tween.

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Insulin resistance in skeletal muscle, defined as reduced insulin-stimulated glucose disposal, is a hallmark feature of type 2 diabetes and is largely accounted for by reduced nonoxidative glucose metabolism (1–3), i.e., decreased rates of muscle glucose storage as glycogen (4). It is therefore not surprising that a defect in insulin activation of glycogen synthase (GS), a key enzyme in the regulation of glycogen synthesis, is one of the most consistent findings in muscle of patients with type 2 diabetes (1,2,5). This abnormality has also been demonstrated in skeletal muscle cells cultured from patients with type 2 diabetes (6,7) and in skeletal muscle of first-degree relatives of diabetic patients (8,9). This has led to the hypothesis that a primary defect of possible genetic origin is involved (1).

GS activity is controlled by multisite phosphorylation and by allosteric effectors (10). Phosphorylation leads to inactivation of GS, but full activity can be restored in the presence of the allosteric activator glucose-6-phosphate (G6P) (10,11). Of the nine serine residues that are phosphorylated in mammalian GS in vivo (12), the sites most important for enzyme activity are sites 2 and 2a in the NH₂-terminus and sites 3a and 3b in the COOH-terminus (12–19). In vivo, studies on rabbit skeletal muscle showed that most of the phosphate released from GS in response to insulin was removed from the tryptic peptide containing sites 3a and 3b (16). However, studies in cells expressing rabbit muscle GS and in rat skeletal muscle in vitro have demonstrated that supraphysiological concentrations of insulin (100–200 nmol/l) promote dephosphorylation of sites 2 and 2a as well (13,14,17–18). In vitro, glycogen synthase kinase-3 (GSK-3) is the most active kinase phosphorylating sites 3a and 3b, whereas site 2 could be phosphorylated by several protein kinases, including protein kinase A, protein kinase C (PKC), and AMP-activated protein kinase (AMPK) (12,19,20). CK1 is the only detectable site 2a kinase in skeletal muscle extracts (21).

It is currently believed that insulin signaling from the insulin receptor to GS involves activation of insulin receptor substrates (IRS)-1 and -2, phosphatidylinositol 3-kinase (PI 3-kinase), and Akt, which in turn leads to phosphorylation and inhibition of GSK-3 (10,12,22). Indeed, insulin has been shown to inhibit GSK-3 in skeletal muscle to a degree sufficient to account for the observed activation of GS (10,12,22–24). However, the final step, that GSK-3

TABLE 1
Clinical characteristics

	Control subjects	Diabetic subjects	<i>P</i>
<i>n</i>	10	10	
Duration of diabetes (years)		2.6 ± 0.5	
Age (years)	51.1 ± 2.0	50.4 ± 1.6	NS
BMI (kg/m ²)	29.6 ± 0.9	31.1 ± 1.1	NS
Fat-free mass (kg)	64.1 ± 2.3	63.6 ± 1.8	NS
HbA _{1c} (%)	5.1 ± 0.1	6.9 ± 0.5	<0.01
Fasting plasma triglycerides (mmol/l)	1.4 ± 0.2	3.4 ± 0.8	0.02
Fasting cholesterol (mmol/l)	5.6 ± 0.3	5.7 ± 0.2	NS
Fasting serum C-peptide (pmol/l)	657 ± 55	1051 ± 115	<0.01
Fasting serum insulin (pmol/l)	48 ± 7	74 ± 9	0.03
Fasting plasma glucose (mmol/l)	5.7 ± 0.1	10.7 ± 1.2	<0.01
Fasting serum FFA (mmol/l)	0.53 ± 0.07	0.51 ± 0.06	NS

Data are means ± SE. Clinical characteristics of type 2 diabetic and control subjects. NS, not significant.

inhibition leads to dephosphorylation of sites 3a and 3b and subsequent activation of GS in vivo has yet to be demonstrated. Moreover, the effect of insulin on phosphorylation of the sites believed to be essential for GS activity (sites 2, 2a, 3a, and 3b) and determination of the sites (if any) responsible for the impaired activation of GS in type 2 diabetes have not been addressed in human skeletal muscle.

The present study was undertaken to investigate the molecular mechanisms by which insulin activates GS in human skeletal muscle in vivo and to determine the mechanism(s) responsible for impaired GS activation and thereby insulin resistance in type 2 diabetes. The changes in glucose metabolism, insulin signaling, and phosphorylation of GS in skeletal muscle evoked by insulin in physiological concentrations (0.4 nmol/l) were studied in patients with type 2 diabetes and in well-matched healthy control subjects.

RESEARCH DESIGN AND METHODS

Subjects. Ten patients with type 2 diabetes and 10 healthy control male subjects, matched according to age and BMI participated in the study (Table 1). Patients with type 2 diabetes were either treated by diet alone or in combination with sulfonylurea or metformin, which were withdrawn 1 week before the study. The patients were all GAD65 antibody negative and without signs of diabetic retinopathy, nephropathy, neuropathy, or macrovascular complications. The control subjects had normal glucose tolerance and no family history of diabetes. All subjects had normal results on screening blood tests of hepatic and renal function. All subjects were instructed to refrain from strenuous physical activity for a period of 48 h before the experiment. Informed consent was obtained from all subjects before participation. The study was approved by the Local Ethics Committee and was performed in accordance with the Helsinki Declaration.

Study design. All study subjects were admitted to the Diabetes Research Center at Odense University Hospital. After an overnight fast the subjects underwent a euglycemic-hyperinsulinemic clamp (4-h insulin infusion, 40 mU/m² per min) as described in detail previously (25). In type 2 diabetic subjects, plasma glucose was allowed to decline to ~5.5 mmol/l before glucose infusion was initiated. Total glucose disposal rates (GDRs) were calculated using Steele's non-steady-state equations adapted for labeled glucose infusates (25). Distribution volume of glucose was taken as 200 ml/kg body wt and pool fraction as 0.65. The studies were combined with indirect calorimetry using the flow-through canopy gas analyzer system (Deltatrac; Datex, Helsinki, Finland). Rates of glucose oxidation were calculated from Frayn's equation (26). Nonoxidative glucose metabolism was calculated as the difference between GDR and glucose oxidation. Plasma glucose, serum insulin, C-peptide, and free fatty acids (FFAs) were measured as described previously (27). Muscle biopsies were obtained from the vastus lateralis muscle before and after the 4-h insulin infusion period using a modified Bergström needle with suction under local anesthesia. Muscle samples were

immediately blotted free of blood, fat, and connective tissue and frozen in liquid nitrogen within 20–30 s. The muscle samples were freeze-dried and dissected free of blood, fat, and connective tissue before analysis of muscle enzyme activity and phosphorylation, which were measured in muscle homogenates or lysates prepared as described previously (28).

Glycogen synthase activity and protein. GS activity was measured in homogenates by a modification of the method of Thomas (29) as described by Richter et al. (30). GS activity was determined in the presence of 0.02, 0.17, and 8 mmol/l G6P and given either as the percent of G6P-independent GS activity (% I-form activity) (100 × activity in the presence of 0.02 mmol/l G6P divided by the activity at 8 mmol/l G6P [saturated]) or as the percent of fractional velocity (% FV) (100 × activity in the presence of 0.17 mmol/l G6P divided by the activity at 8 mmol/l G6P). GS protein expression was examined in muscle lysates by SDS-PAGE (7.5% Criterion gels; Bio Rad) and Western Blotting using polyvinylidene fluoride membrane and semidry transfer. After membrane blocking (Tris-buffered saline with Tween [TBST] + 3% skim milk), GS protein was detected as a single band at ~90 kDa using a polyclonal GS antibody (TBST + 3% skim milk) (kindly provided by Prof. Oluf Pedersen), horse radish peroxidase-conjugated secondary antibody (in TBST + 3% skim milk) and enhanced chemiluminescence (ECL). The bands were visualized and analyzed using a Kodak Image Station E440CF.

IRS-1-associated PI 3-kinase activity. IRS-1-associated PI 3-kinase activity was measured in IRS-1 immunoprecipitated from muscle lysates using an anti-IRS-1 antibody (Upstate Biotechnology, Lake Placid, MA) and protein A agarose beads (Pierce, IN). Pellet was washed twice in PBS containing 1% NP-40 and 100 μmol/l Na₃VO₄, pH 7.5, twice in 10 mmol/l Tris buffer containing 100 mmol/l NaCl, 1 mmol/l EDTA, and 100 μmol/l Na₃VO₄, pH 7.5. The pellet was resuspended in 50 μl of the latter buffer, and the PI 3-kinase assay (34°C for 15 min) was performed as described before (31).

Akt phosphorylation. Phosphorylation of Akt at Ser473 and Thr308 was measured in muscle lysates by SDS-PAGE (7.5% Criterion gels; Bio Rad) and Western Blotting using PVDF membrane and semidry transfer. After membrane blocking (TBST + 1% skim milk), phosphorylated Akt protein was detected as a single band at ~60 kDa (in TBST + 1% skim milk) using phospho-specific antibodies (in TBST + 1% skim milk) (New England Biolabs, Beverly, MA, and Upstate Biotechnology, Lake Placid, MA, respectively), horse radish-peroxidase conjugated secondary antibody (in TBST + 1% skim milk), and ECL. The bands were visualized and analyzed using a Kodak Image Station E440CF.

GSK-3α activity. GSK-3α was immunoprecipitated from muscle lysates using an anti-GSK-3α antibody (Upstate Biotechnology) bound to G-Sepharose. A p81-filter paper assay, with the phospho-GS2-peptide (YRRAAVPPSPSLSRH-SSPHQSpEDEEE [Sp = phosphoserine]; Schafer-N) as substrate, was used to measure GSK-3α activity (24,28). The final concentration of the substrate used on the study muscle samples was 85 μmol/l. It has been suggested that the GSK-3 activity assay became more sensitive using lower concentrations of phospho-GS-peptide. These analyses were performed using human GSK-3β purified from 293 cells (32). However, in our hands, using immunopurified GSK-3α and GSK-3β from human muscle obtained under basal and hyperinsulinemic clamp conditions (~9 nmol/l), the same relative effect of insulin was detected when using lower peptide concentrations (42.5 and 21 μmol/l) in the *in vitro* kinase activity assay.

GS phosphorylation. Three phospho-specific polyclonal antibodies against GS were raised in sheep. The anti-site 2 (Ser7) and anti-site 2 + 2a (Ser7 and

Ser10) antibodies were raised against the peptides PLSRSLSpMSSLPGLLED and PLSRSLSpMSSLPGLLED (residues 1–16 of rat GS). The anti-site 3a + 3b (Ser640 and Ser644) antibody was raised against the peptide RYPRPASPVP-SpPSLSR (residues 634–50 of human GS). Peptides were synthesized with an additional cysteine at the NH₂-terminus, coupled to keyhole limpet hemocyanin, and used to immunize sheep as described previously (33). Serum was first passed through columns containing immobilized dephosphopeptides and then (in the case of antibodies against doubly phosphorylated peptides) through columns containing the singly phosphorylated peptides. Antibodies specific for phosphorylation state were then bound to columns containing immobilized peptides of the same phosphorylation state as the antigen used for immunization and eluted as described previously (33). Using these antibodies, phosphorylation of GS was measured in muscle lysates by SDS-PAGE (Bis-Tris 4–12% gel; Invitrogen) and Western Blotting using PVDF membrane and semidry transfer. After blocking (TBST + 5% skim milk), the membranes were incubated with the primary antibody (in TBST + 5% skim milk), followed by incubation with horse radish-peroxidase conjugated secondary antibody (in TBST + 5% skim milk). ECL was used as a detection system. Using a FUJIFILM Image Reader Las. 1,000, a single band at ~90 kDa corresponding to GS was detectable and quantified.

Protein preparations. GS, AMPK, and the catalytic subunit of protein phosphatase 2A (PP2A_C) was purified from rabbit muscle, rat liver, and bovine heart, respectively, as described previously (34–36). A bacterially expressed glutathione-S-transferase fusion of rat CK1δ (residues 1–294) and human recombinant GSK3β were a gift from the Division of Signal Transduction Therapy, University of Dundee.

Characterization of phospho-specific antibodies against GS using purified GS. Purified GS was exchanged into phosphorylation buffer (75 mmol/l Tris/HCl, pH 7.5, 1 mmol/l EDTA, 0.4 mmol/l EGTA, and 6 mmol/l Mg acetate) by dilution in the buffer and re-concentration using a Millipore Biomax centrifugal filter (this step was necessary to remove β-glycerophosphate, which inhibits PP2A, from the GS storage buffer). The GS was treated with PP2A_C (14 mU/ml) for 40 min at 30°C to dephosphorylate and inactivate the contaminating kinase, followed by okadaic acid (1 μmol/l) to inactivate the PP2A. It was then phosphorylated for 10 min at 30°C in phosphorylation buffer plus 200 μmol/l AMP, 200 μmol/l ATP, plus AMPK (2 units/ml) and/or CK1 (2 units/ml) as indicated in the text. The samples were analyzed by SDS-PAGE and immunoblotting, followed by detection of the primary antibodies with Alexa Fluor 680 donkey anti-sheep IgG (Molecular Probes). The second antibody was detected using a LI-COR Odyssey infrared imaging system at 700 nmol/l. Before SDS-PAGE and Western Blotting using the anti-site 3a + 3b antibody, the GS (0–5 μg) was phosphorylated by incubation at 24°C for 60 min in a buffer containing 8 mmol/l MOPS, 0.2 mmol/l EDTA, 10 mmol/l MgAc, 0.1% dithiothreitol, 0.03% Triton X100, and 100 μmol/l ATP either with or without GSK-3β (1 unit/ml).

Statistical analysis. Data calculation and statistical analysis were performed using the SSSP for Windows version 10.0 program. Data are presented as means ± SE. Differences between the groups or within groups were evaluated using the Student's *t* test for unpaired and paired data, respectively (two sided). Significance was accepted at the *P* < 0.05 level.

RESULTS

Metabolic characteristics. To assess the effects of insulin on glucose metabolism, we performed euglycemic-hyperinsulinemic clamps combined with indirect calorimetry in type 2 diabetic and healthy control subjects matched according to age, sex (all male), and BMI (Table 1). Fasting HbA_{1c}, plasma glucose, serum insulin, and C-peptide concentrations were significantly higher in the diabetic group, whereas fasting plasma FFA concentrations did not differ between the groups (Table 1). During the insulin-stimulated steady-state period (210- to 240-min period), euglycemia at a plasma glucose concentration of ~5.5 mmol/l and physiological hyperinsulinemia at a serum insulin concentration of ~400 pmol/l were obtained in both groups (Fig. 1A and B). In this period, plasma FFA concentrations were significantly higher in the diabetic versus the control group (0.08 ± 0.01 vs. 0.04 ± 0.00 mmol/l; *P* < 0.05).

In the basal state GDR was 21% higher (*P* < 0.01), whereas in the insulin-stimulated state GDR was 38%

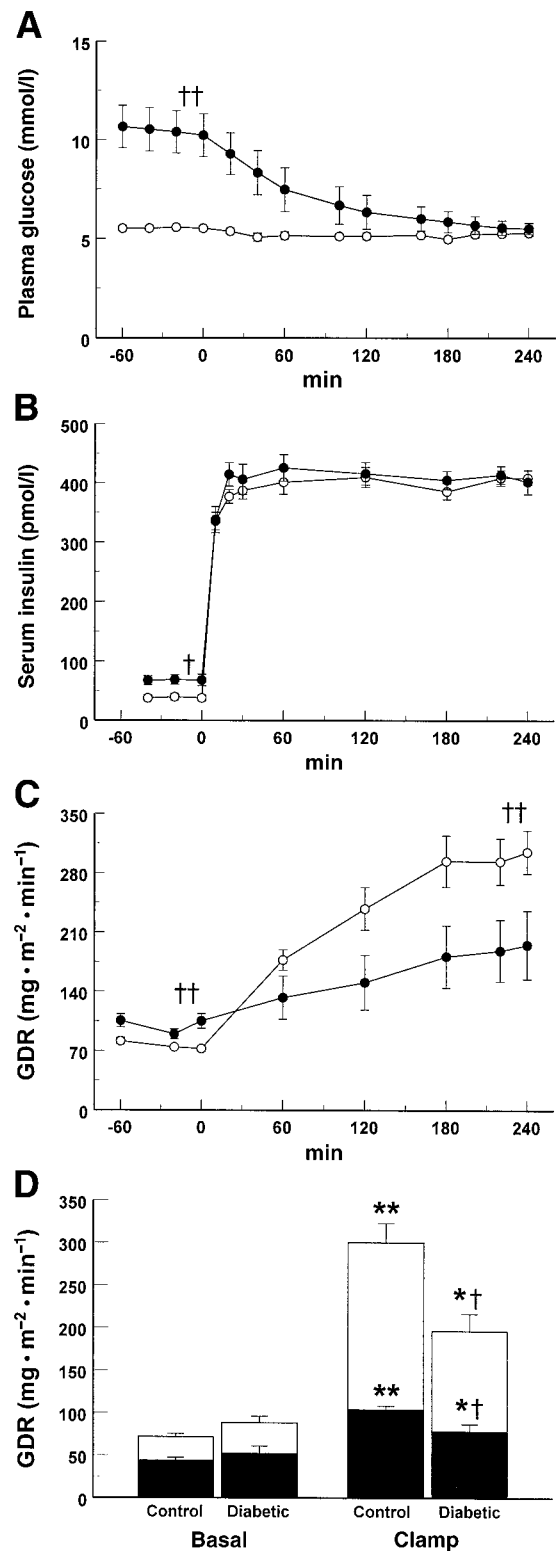


FIG. 1. Glucose metabolism during euglycemic-hyperinsulinemic clamp. Concentrations of plasma glucose (A) and serum insulin (B) and whole-body GDR (C) in 10 type 2 diabetic (●) and 10 control subjects (○) during euglycemic-hyperinsulinemic clamp. Mean values during the basal (–30 to 0 min) and the insulin-stimulated (clamp) (210–240 min) steady-state periods were compared. D: GDR in type 2 diabetic (*n* = 9) and control subjects (*n* = 8) is presented as oxidative (■) and nonoxidative (□) glucose metabolism during the basal and clamp steady state periods. Data are mean ± SE. **P* < 0.05 and ***P* < 0.01 for clamp vs. basal; †*P* < 0.05 and ††*P* < 0.01 vs. control subjects.

lower ($P < 0.01$) in the diabetic group versus the control group (Fig. 1C). Insulin increased GDR significantly in both groups, but the increase in GDR induced by insulin was significantly lower in diabetic subjects ($P < 0.01$). Indirect calorimetry data obtained from nine control and eight diabetic subjects showed no significant differences in glucose oxidation or nonoxidative glucose metabolism (glucose storage) in the basal state between the groups (Fig. 1D). During the insulin-stimulated state, glucose oxidation was 25% lower ($P = 0.02$) and glucose storage was 40% lower ($P = 0.03$) in diabetic compared with control subjects (Fig. 1D). Insulin increased glucose oxidation and glucose storage significantly in both groups, but the increase in glucose oxidation and glucose storage induced by insulin were both significantly impaired in type 2 diabetic subjects ($P < 0.05$). The lower glucose storage accounted for 75% of the reduction in GDR in the insulin-stimulated state in type 2 diabetic subjects. Thus, the reduced glucose uptake was primarily due to impaired insulin stimulation of glucose storage.

GS activity. To determine whether the failure of insulin to mediate normal muscle glucose storage was associated with a defect in the activation of GS, we examined muscle biopsies from m. vastus lateralis obtained in the basal and insulin-stimulated states. Physiological hyperinsulinemia increased GS activity significantly in control subjects, expressed both as the % I-form activity ($P < 0.01$) and as % FV ($P < 0.01$), whereas no significant change was observed in diabetic subjects either as the % I-form activity or as % FV (Fig. 2). The insulin-mediated increase of GS activity over basal were significantly reduced in diabetic compared with control subjects expressed as both the % I-form activity ($P = 0.03$) and as % FV ($P < 0.01$). In the basal state, GS activity (% I-form activity and % FV) was similar in the two groups, whereas in the insulin-stimulated state, GS activity expressed as both the % I-form activity ($P = 0.04$) and as % FV ($P < 0.01$) was significantly reduced in diabetic versus control subjects (Fig. 2). Both GS protein expression and total GS activity were similar in the two groups in both the basal and in the insulin-stimulated state (data not shown).

Insulin signaling through IRS-1, PI 3-kinase, Akt, and GSK-3. We next studied whether these defects of muscle glycogen synthesis could be attributed to defect(s) in the proximal or distal steps of the signal transduction cascade currently believed to mediate insulin activation of GS. We found that basal IRS-1-associated PI 3-kinase activity was significantly increased in patients with type 2 diabetes compared with control subjects ($P = 0.03$), whereas in the insulin-stimulated state no difference was observed between the groups (Fig. 3A). In control subjects, physiological hyperinsulinemia increased IRS-1-associated PI 3-kinase activity 1.4-fold, which was not significant ($P = 0.14$). In patients with type 2 diabetes, no change in IRS-1-associated PI 3-kinase activity in response to insulin was observed (Fig. 3A). The insulin-stimulated change in IRS-1-associated PI 3-kinase activity tended to be greater in control versus type 2 diabetic subjects ($P = 0.09$).

Activation of Akt requires phosphorylation at Ser473 and Thr308. Thus, in the present study we evaluated Akt activity by the phosphorylation of these two sites. Physiological hyperinsulinemia significantly increased phos-

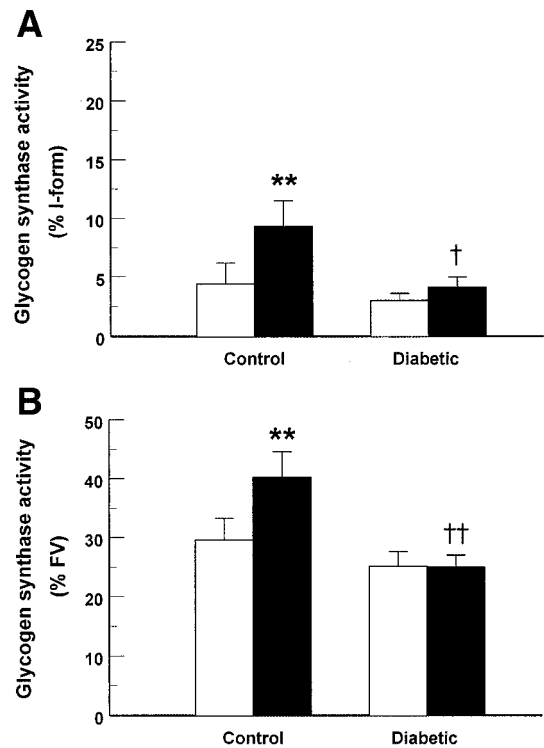


FIG. 2. Effect of insulin on glycogen synthase activity given as % I-form activity (A) and % FV (B) in skeletal muscle of 10 obese type 2 diabetic patients and 10 control subjects. Measurements were performed in muscle biopsies obtained from m. vastus lateralis during the basal (□) and insulin-stimulated (clamp) (■) steady-state periods of a 4-h hyperinsulinemic-euglycemic clamp at an insulin infusion rate of $40 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$. Data are mean \pm SE. ** $P < 0.01$ for clamp vs. basal; † $P < 0.05$ and †† $P < 0.01$ vs. control subjects.

phorylation of Ser473 and Thr308 in Akt in both the diabetic ($P < 0.01$) and control ($P < 0.01$) groups (Fig. 3B–C). There were no significant differences in basal or insulin-stimulated phosphorylation, or in the increase induced by insulin, between the two groups.

In rat skeletal muscle, supraphysiological concentrations of insulin inhibits both the GSK-3 α and the GSK-3 β isoforms in vivo (28). However, in human skeletal muscle, only GSK-3 α is inhibited by physiological concentrations of insulin (23,37). In the present study, we therefore compared the change in GSK-3 α activity only. We found that insulin resulted in a significant decrease in GSK-3 α activity in both the diabetic ($P < 0.001$) and control ($P < 0.01$) groups (Fig. 3D). There were no significant differences in activity under basal or hyperinsulinemic conditions, or in the decrease induced by insulin, between the two groups.

Phosphorylation of GS at sites 3a and 3b. For this study we developed a phospho-specific antibody that recognizes GS only when it is phosphorylated at both sites 3a and 3b. When this antibody, referred to here as anti-site 3a + 3b, was used against purified GS protein, a signal at the expected mass of $\sim 90 \text{ kDa}$ was only obtained when the GS protein was phosphorylated in vitro by GSK-3 β before electrophoreses. The signal was competitively inhibited by the doubly phosphorylated peptide RYPR-PASpVPPSPSLSR (residues 634–650 of human GS) but not by either singly phosphorylated peptides (Fig. 4A), showing that the anti-site 3a + 3b antibody requires phosphorylation of GS at both sites 3a and 3b.

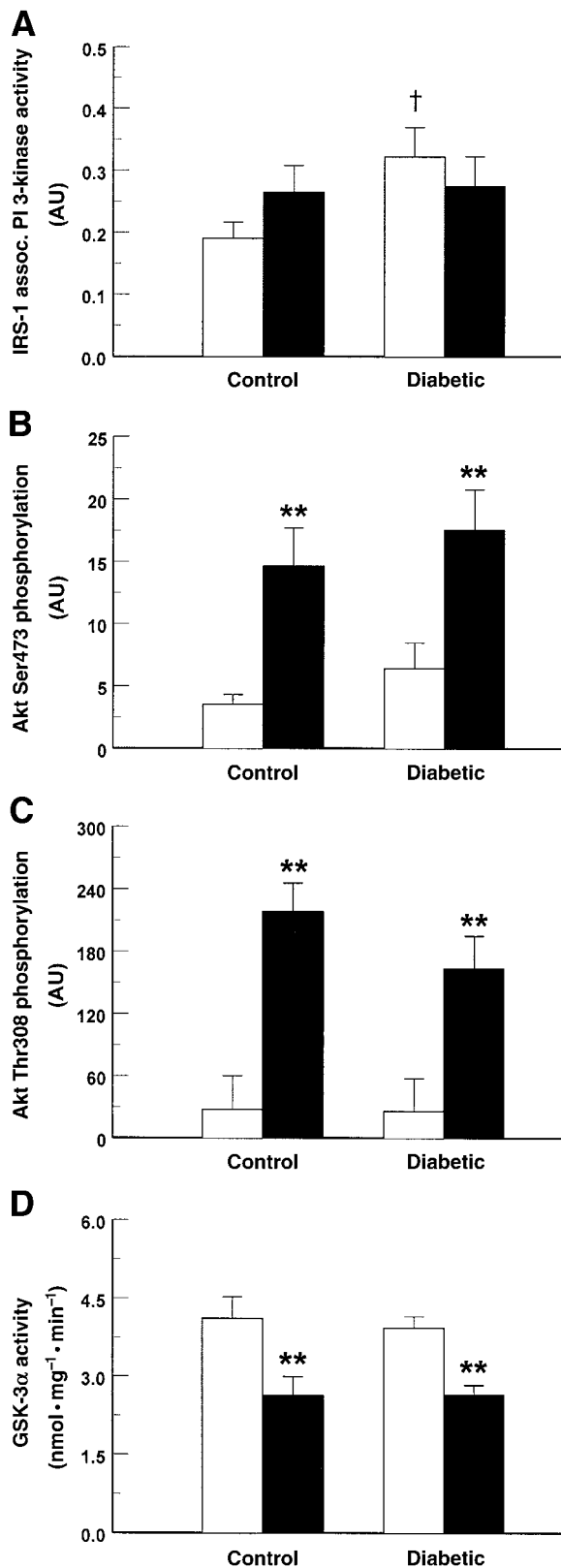


FIG. 3. Effect of insulin on IRS-1-associated PI 3-kinase activity (A), phosphorylation of Akt at Ser473 (B) and Thr308 (C), and GSK-3 α activity (D) in skeletal muscle of 10 type 2 diabetic patients and 10 control subjects. Measurements were performed in muscle biopsies obtained from m. vastus lateralis during the basal (□) and insulin-stimulated (clamp) (■) steady-state periods of a 4-h hyperinsulinemic-euglycemic clamp at an insulin infusion rate of 40 mU · m⁻² · min⁻¹. Data are mean \pm SE. ** P < 0.01 for clamp vs. basal values; † P < 0.05 vs. control subjects. AU, arbitrary units.

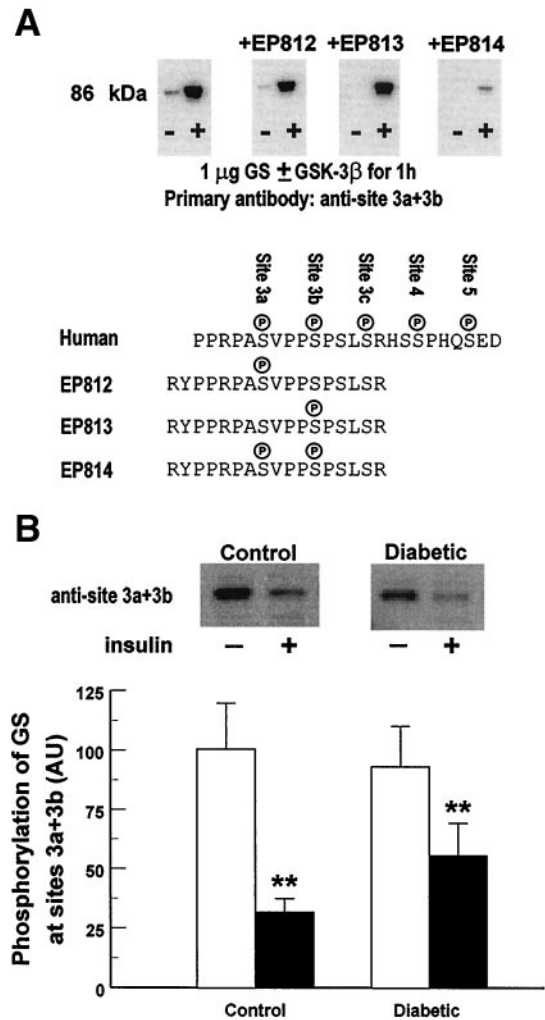


FIG. 4. Phosphorylation of GS at the COOH-terminal sites 3a and 3b. The specificity of the anti-site 3a + 3b antibody was tested by phosphorylating purified rabbit muscle GS with or without GSK-3 β before SDS-PAGE and Western Blotting (A). The signal obtained at the expected mass of ~90 kDa was competitively inhibited by simultaneously incubation with the doubly phosphorylated peptide RYPPRPASVPPSPSLSR (EP814) but not with either of the two singly phosphorylated peptides (EP812/EP813) (A). The effect of insulin on phosphorylation of GS at sites 3a and 3b (B) was determined in skeletal muscle of 10 type 2 diabetic and 10 control subjects. Measurements were performed in muscle biopsies obtained from m. vastus lateralis during the basal (□) and insulin-stimulated (clamp) (■) steady-state periods of a 4-h hyperinsulinemic-euglycemic clamp at an insulin infusion rate of 40 mU · m⁻² · min⁻¹. Data are means \pm SE. * P < 0.05 and ** P < 0.01 for clamp vs. basal values. AU, arbitrary units.

Administration of insulin in physiological concentrations caused a significant decrease in the amount of GS phosphorylated at sites 3a + 3b in both the diabetic ($P = 0.01$) and control ($P < 0.01$) groups (Fig. 4B). There were no significant differences in phosphorylation of sites 3a + 3b under basal or hyperinsulinemic conditions, or in the decrease induced by insulin, between the two groups (Fig. 4B).

Phosphorylation of GS at the NH₂-terminal sites (2 and 2a). We raised and affinity purified antibodies, using as antigens phosphopeptides corresponding to phosphorylation of site 2 and sites 2 + 2a. To test these phosphospecific antibodies, referred to here as anti-site 2 and anti-site 2 + 2a, purified GS was phosphorylated with AMPK, which phosphorylates site 2 (19), and/or CK1, which phosphorylates site 2 (19), and/or CK1, which phosphorylates site 2 (19), and/or CK1, which phosphorylates site 2 (19).

lates site 2a, but only when site 2 is phosphorylated (38). Initial experiments showed that the GS preparation was contaminated with an endogenous protein kinase that phosphorylated site 2 rapidly in the presence of ATP, so that further addition of AMPK had no effect. We suspected that AMPK might account, at least in part, for the endogenous site 2 kinase, particularly because GS has been reported to coprecipitate from skeletal muscle extracts using anti-AMPK antibodies (39). Western blotting of our GS preparation using anti-AMPK- α 1 and - α 2 antibodies (not shown) confirmed that both isoforms were present. Because AMPK is inactivated by protein phosphatase treatment, we treated the GS preparation with PP2A_C. As expected, this treatment greatly reduced the phosphorylation of site 2 by endogenous kinase(s). Figure 5A shows that after treatment with ATP alone, no signal migrating in the position of GS was obtained using the anti-site 2 antibody. A strong signal was obtained after phosphorylation by AMPK, but this was abolished by the inclusion of CK1, showing that the anti-site 2 antibody did not recognize GS that was phosphorylated at both sites. Using the anti-site 2 + 2a antibody, a weak signal was obtained after incubation with CK1 alone, but this was increased by the inclusion of exogenous AMPK. The weak signal obtained in the absence of AMPK may be due to the fact that the PP2A treatment was not 100% effective in removing the endogenous AMPK. No signal was obtained with dephosphorylated GS, or GS phosphorylated by AMPK alone (lanes 1 and 2). These results show that the anti-site 2 antibody is specific for GS phosphorylated at site 2 but not 2a, whereas the anti-site 2 + 2a antibody requires phosphorylation at both sites.

Using these phospho-specific antibodies we observed, the insulin infusion in the diabetic group caused a significant increase (61%) in the amount of GS phosphorylated at sites 2 + 2a ($P < 0.01$), whereas no change was found in the control group (Fig. 5B). The insulin-mediated increase in phosphorylation ($P < 0.01$) and the amount of GS phosphorylated at sites 2 + 2a in the insulin-stimulated state ($P = 0.01$) were also higher in diabetic compared with control subjects (Fig. 5B). However, insulin infusion caused no significant change in GS phosphorylation at site 2 alone, in either the diabetic or the control groups, and no difference between the two groups was observed in either the basal or the insulin-stimulated states (Fig. 5C).

DISCUSSION

The present study was carried out to investigate glucose metabolism, proximal and distal steps in insulin signaling, and phosphorylation of GS at regulatory sites in human skeletal muscle in vivo during the conditions of a hyperinsulinemic-euglycemic clamp. We also addressed the molecular mechanisms responsible for insulin resistance in type 2 diabetic patients compared with well-matched healthy control subjects. We report that administration of insulin in physiological concentrations in humans significantly decreases phosphorylation of GS at sites 3a + 3b in skeletal muscle in vivo. This response is associated with, and most likely mediated by, an activation of Akt and inhibition of GSK-3. It appears to be responsible for the activation of GS seen in normal muscle, because physiological hyperinsulinemia caused no changes in phosphor-

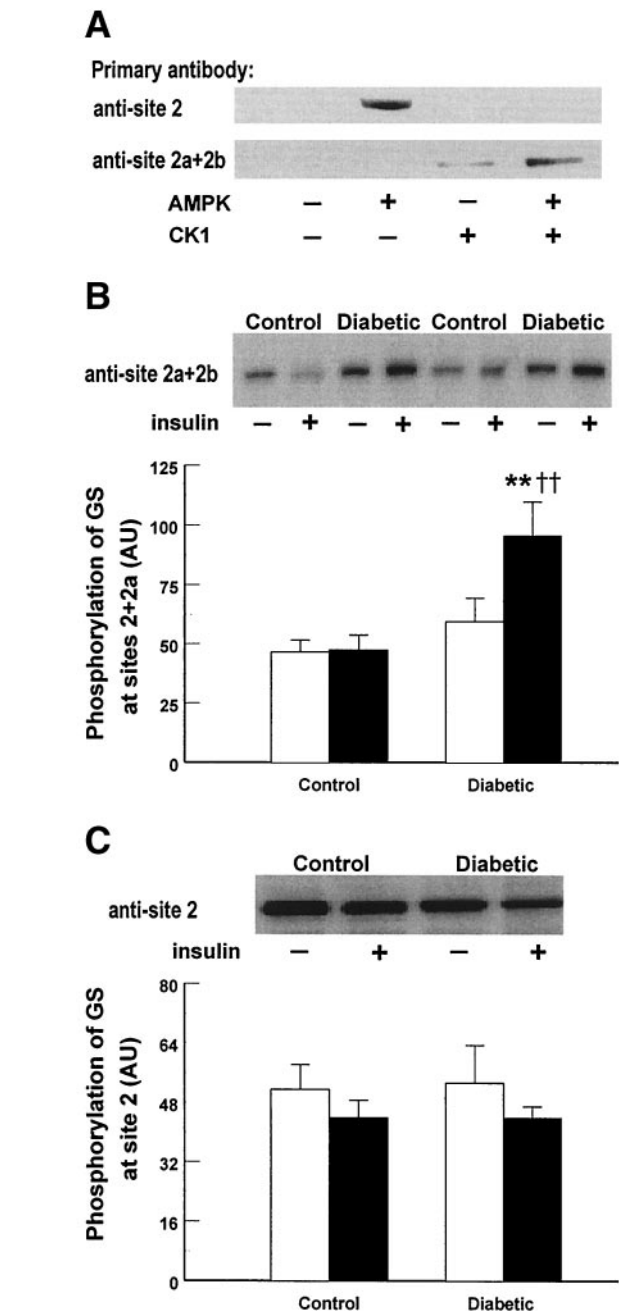


FIG. 5. Phosphorylation of GS at the NH₂-terminal sites 2 and 2a. The specificity of the anti-site 2 and anti-site 2 + 2a antibodies was tested by phosphorylating purified rabbit muscle GS (after dephosphorylation to inactivate the endogenous site 2 kinase) with or without rat AMPK and/or rat CK1 δ before SDS-PAGE and Western Blotting. The loading of GS protein was identical in all four lanes, but as the anti-site 2 antibody gave a slightly weaker signal than the anti-site 2 + 2a antibody, three times as much protein was loaded in the lower panel (A). The effect of insulin on phosphorylation of GS at sites 2 + 2a (B) and at site 2 alone (C) was determined in skeletal muscle of 10 type 2 diabetic and 10 control subjects. Measurements were performed in muscle biopsies obtained from m. vastus lateralis during the basal (\square) and insulin-stimulated (clamp) (\blacksquare) steady-state periods of a 4-h hyperinsulinemic-euglycemic clamp at an insulin infusion rate of 40 mU \cdot m⁻² \cdot min⁻¹. Data are mean \pm SE. ** $P < 0.01$ for clamp vs. basal values; †† $P < 0.01$ vs. control subjects. AU, arbitrary units.

ylation of GS at sites 2 and 2a in muscle from normal subjects. In patients with type 2 diabetes, the effects of insulin on Akt, GSK-3, and phosphorylation of GS at sites 3a + 3b remained intact. However, as reported previously

(23,40,41), the effect of insulin on glucose disposal and storage was significantly reduced and activation of GS was virtually absent in patients with type 2 diabetes. Interestingly, in type 2 diabetic subjects insulin infusion causes a marked increase in the phosphorylation of muscle GS at sites 2 + 2a. These data indicate that in type 2 diabetes increased phosphorylation of these NH₂-terminal sites counteracts the insulin activation of muscle GS mediated by dephosphorylation of sites 3a and 3b in the COOH-terminus. This abnormal phosphorylation of GS may explain (at least in part) skeletal muscle insulin resistance and thereby play a crucial role in the pathogenesis of type 2 diabetes.

Insulin signaling involves a cascade of events initiated by insulin binding to its cell-surface receptor. PI 3-kinase activation is necessary for insulin action on glucose transport, GS, protein synthesis, antilipolysis, and gene expression. Abnormalities in this proximal step of insulin signaling have been reported in obese nondiabetic subjects (40,41), type 2 diabetic subjects (40–42), and their first-degree relatives (43,44). The rather small (nonsignificant) increase in PI 3-kinase activity with insulin infusion observed in obese control subjects in this study supports the observation that obesity and elevated levels of FFA may inhibit the activation of this enzyme (40,45). However, the finding of increased basal PI 3-kinase activity not only in type 2 diabetic subjects (42), but also in obese first-degree relatives with impaired glucose tolerance (43), suggests that other factors may be more important to explain this abnormality in diabetes-related insulin resistance. Prior normalization of plasma glucose by overnight insulin infusion in obese type 2 diabetic subjects did not abolish this abnormality (42), which indicates that basal hyperglycemia is not the factor responsible for increased basal PI 3-kinase activity. Consistent with studies showing that activation of Akt is preserved under conditions in which PI 3-kinase is impaired in type 2 diabetic subjects both in vivo and in vitro (41,46,47), our data demonstrate that this abnormality has no impact on the insulin signaling to GS via Akt, GSK-3 α , and phosphorylation of sites 3a + 3b in GS. These results suggest that pathways that are independent of PI 3-kinase may be involved in activation of Akt and inhibition of GSK-3 by insulin or that only a small amount of PI 3-kinase activity is necessary to increase insulin signaling through these steps.

Previous in vitro studies have emphasized the role of GSK-3 in regulation of GS phosphorylation and activity (12). GSK-3 has therefore been suggested to play a key role in diabetes and obesity-related insulin resistance (10,12). In a single study, impaired insulin activation of GS in type 2 diabetic patients has been attributed to increased total activity of GSK-3 α caused by increased expression of GSK-3 α (23). However, in the same study the insulin-mediated inhibition of both total and specific GSK-3 α activity was significant and similar in obese nondiabetic and type 2 diabetic subjects, and to the same extent as observed in lean subjects (23). In the present study, we found no difference in either basal or insulin-mediated total activity of GSK-3 α between control and diabetic subjects, and the insulin-mediated inhibition of total GSK-3 α activity was significant and similar in both groups. Akt activation has been demonstrated to be responsible

for the inhibition of GSK-3 both in vivo and in vitro (10,12,22), and consistent with the observed inhibition of GSK-3 α , we found that insulin induced phosphorylation of Ser473 and Thr308 of Akt to the same extent in both groups. These results support recent reports finding no defect in the stimulation of Akt activity by physiological concentrations of insulin in muscle of type 2 diabetic subjects (41,47). Thus, our data strongly indicate that the insulin resistance associated with type 2 diabetes is not caused by a reduced signaling through either Akt or GSK-3 α .

Although insulin-mediated inhibition of GSK-3 α was normal in type 2 diabetic subjects, this did not a priori exclude impaired dephosphorylation of GS at sites 3a and 3b as the primary cause of impaired GS activity. Thus, although GSK-3 is the major kinase of sites 3a, 3b, and 3c in vitro (12), this has not been confirmed in vivo, and in fact it has been reported that sites 3a and 3b of GS can be phosphorylated by other unknown kinases (15) and that GSK-3 is not essential for GS activation by insulin in Rat-1 fibroblast expressing rabbit skeletal muscle GS (13). To further investigate this, we generated an antibody that specifically recognizes GS only when phosphorylated at both sites 3a and 3b. Our data show that administration of insulin actually decreased phosphorylation of GS at sites 3a + 3b equally and significantly in type 2 diabetic and obese nondiabetic subjects. The observed decrease in phosphorylation of GS at sites 3a + 3b (41–68%) is similar to previous findings of a ~50–60% decrease in a large cohort of elderly nonobese nondiabetic twins (J.F.P.W., unpublished data). The fact that GSK-3 sequentially phosphorylates sites 4, 3c, 3b, and 3a in GS in vitro (10,12) makes it likely that the observed inhibition of GSK-3 by insulin is followed by decreased phosphorylation at both sites 3a and 3b, although the present data do not directly prove this. With these data, we have provided evidence that, in healthy humans, insulin activation of skeletal muscle GS is associated with significant activation of Akt, inhibition of GSK-3, and dephosphorylation at sites 3a + 3b. These results support previous studies in animal muscle and in different cell models emphasizing the significance of this signaling pathway (10,12). However, our results also demonstrate that these effects of insulin are not sufficient for activation of GS in skeletal muscle of type 2 diabetic subjects. Although, the present study does not have the power to definitively exclude a little less dephosphorylation of GS at sites 3a and 3b in type 2 diabetic subjects, it is rather clear that this plays no major role for skeletal muscle insulin resistance in type 2 diabetes.

In our diabetic population there was no activation of GS by insulin infusion in physiological concentrations (40 mU \cdot m⁻² \cdot min⁻¹), which is consistent with observations in a recent study of poorly controlled obese diabetic subjects (48). Because this defect was not caused by defects in the signaling steps generally believed to be involved in insulin activation of GS, what mechanisms are involved? Studies on rat diaphragm muscle incubated with insulin in vitro and on rat cells expressing rabbit skeletal muscle GS have shown that insulin in supraphysiological concentrations (100–200 nM) promotes dephosphorylation of GS both at sites 2 and 2a in the NH₂-terminus and at sites 3a and 3b in the COOH-terminus (13,14,17,18). However, our data

indicate that insulin in physiological concentrations (0.4 nmol/l) in humans does not decrease phosphorylation of sites 2 or 2a in vivo. These data are consistent with the observations of Parker et al. (16) that insulin infusion in rabbits in vivo causes a significant decrease in phosphorylation only of the region containing sites 3a, 3b, and 3c in muscle GS. Thus, of the sites known to regulate GS activity (sites 2, 2a, 3a, and 3b), activation by physiological hyperinsulinemia in healthy human muscle seems to be mainly dependent on dephosphorylation of sites 3a and 3b. In patients with type 2 diabetes, this effect of insulin was intact but was counterbalanced by a simultaneous increase in the phosphorylation of the NH₂-terminal sites (sites 2 + 2a). The finding of increased phosphorylation of GS at sites 2 + 2a despite unaltered phosphorylation at site 2 alone indicates that phosphorylation at one site is rapidly followed by phosphorylation at the other site. This supports previous studies showing that prior phosphorylation of site 2 increases severalfold the phosphorylation of site 2a by CK1 in vitro (21,38). However, whether the observed defect is due to abnormalities in protein kinases or phosphatases, which regulates phosphorylation on both sites 2 and 2a, site 2 alone or even site 2a alone, cannot be determined from the present experiments.

Of the kinases known to phosphorylate site 2 in vitro (12,19,20), AMPK and PKC have recently been proposed to play a role in insulin resistance in type 2 diabetes (49–50), and a role for these kinases in the observed abnormal phosphorylation of GS in diabetic muscle seems attractive. Recent studies have indicated that muscle GS may also be a target for AMPK in vivo. Thus, in rat muscle GS coimmunoprecipitated with AMPK (39), and activation of AMPK by 5-aminoimidazole-4-carboxamide (AICA)-riboside, caused phosphorylation of GS (51). No effect of insulin on AMPK activity has been demonstrated yet, and normal basal activity of the catalytic subunits of AMPK was recently reported in muscle of patients with type 2 diabetes (52). Whereas it is unknown whether PKC phosphorylates muscle GS at site 2 in vivo, PKCs have in fact been reported to be involved in impaired insulin activation of GS in rat skeletal muscle. Thus, in muscle of insulin-resistant type 2 diabetic rats, it was shown that persistent PKC activation might contribute to impaired GS activation (53). In addition, it was recently shown that increased content of PKC- ϵ and - θ in the membrane fraction of denervated rat soleus muscle were accompanied by impaired insulin activation of GS despite a normal activation of Akt and inhibition of GSK-3 (54), exactly as observed in the present study. PKCs exist in several different isoforms in skeletal muscle (50), whereas AMPK exists as heterotrimers of three subunits (α , β , and γ), which each exists in two to three isoforms (49). The function of the different forms of PKC and AMPK in different compartments of skeletal muscle is less well understood, and the roles of specific PKC isoforms or AMPK heterotrimers to regulate muscle glycogen metabolism have not been reported. Further studies of the ability of PKC and AMPK to phosphorylate GS in skeletal muscle and in particular in insulin-resistant states are therefore warranted.

Insulin-mediated stimulation of the muscle-specific glycogen-associated type 1 phosphatase (PP₁ · G_M) was originally thought to be responsible for activation of GS by

dephosphorylation of site 2 and/or 2a in vitro and sites 3a, 3b, and 3c both in vivo and in vitro (55). However, recent reports using transgenic mice have questioned the ability of insulin to activate GS by PP₁ · G_M (56–57). Other type 1 protein phosphatases may be involved in the impaired insulin activation of muscle GS observed in type 2 diabetes. But as phosphorylation was unaltered at sites 3a + 3b and increased at sites 2 + 2a, this would require a phosphatase that selectively regulates site 2 and/or 2a phosphorylation.

In summary, the present study demonstrates that insulin signaling in vivo proceeds through Akt and GSK-3 and is associated with significant dephosphorylation of GS at sites 3a + 3b in skeletal muscle of both type 2 diabetic and healthy subjects. This likely mediates the activation of GS and the increase in glucose storage seen in healthy muscle during infusion of insulin in physiological concentrations. However, in muscle of type 2 diabetic subjects, these effects of insulin seem to be counteracted by a simultaneous marked increase in the phosphorylation of GS at sites 2 + 2a. These findings for the first time provide evidence for a specific phosphorylation-dependent defect of GS in skeletal muscle of type 2 diabetic patients, which could be involved in the pathogenesis of this human disease. This phosphorylation abnormality may be a target for the development of novel and more selective drugs for the treatment of type 2 diabetes.

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