

Potential Role of Glycogen Synthase Kinase-3 in Skeletal Muscle Insulin Resistance of Type 2 Diabetes

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Glycogen synthase (GS) activity is reduced in skeletal muscle of type 2 diabetes, despite normal protein expression, consistent with altered GS regulation. Glycogen synthase kinase-3 (GSK-3) is involved in regulation (phosphorylation and deactivation) of GS. To access the potential role of GSK-3 in insulin resistance and reduced GS activity in type 2 diabetes, the expression and activity of GSK-3 were studied in biopsies of vastus lateralis from type 2 and nondiabetic subjects before and after 3-h hyperinsulinemic ($300 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$)-euglycemic clamps. The specific activity of GSK-3 α did not differ between nondiabetic and diabetic muscle and was decreased similarly after 3-h insulin infusion. However, protein levels of both α and β isoforms of GSK-3 were elevated ($\sim 30\%$) in diabetic muscle compared with lean ($P < 0.01$) and weight-matched obese nondiabetic subjects ($P < 0.05$) and were unchanged by insulin infusion. Thus, both basal and insulin-stimulated total GSK-3 activities were elevated by approximately twofold in diabetic muscle. GSK-3 expression was related to *in vivo* insulin action, as GSK-3 protein was negatively correlated with maximal insulin-stimulated glucose disposal rates. In summary, GSK-3 protein levels and total activities are 1) elevated in type 2 diabetic muscle independent of obesity and 2) inversely correlated with both GS activity and maximally insulin-stimulated glucose disposal. We conclude that increased GSK-3 expression in diabetic muscle may contribute to the impaired GS activity and skeletal muscle insulin resistance present in type 2 diabetes. *Diabetes* 49:263–271, 2000

Glycogen synthesis represents a major pathway of glucose disposal in skeletal muscle after insulin stimulation (1). The rate of glycogen synthesis is impaired in type 2 diabetes (2). Several laboratories have previously shown that the activity of glycogen synthase (GS), the rate-limiting enzyme for glycogen syn-

thesis, is reduced in type 2 diabetes (3–5). This impairment in GS activity occurs in the presence of normal enzyme protein levels in diabetic muscle (6), although there is evidence that GS mRNA expression may be lower in muscle from diabetic subjects (7).

GS is activated by insulin, mainly through dephosphorylation of specific serine residues at sites designated 3a, b, and c, although dephosphorylation of additional sites may also play a role (8). Phosphorylation at sites 3a, b, and c, with subsequent deactivation of the enzyme, is catalyzed by glycogen synthase kinase-3 (GSK-3) (9–12), whereas dephosphorylation is catalyzed by PP1g, the glycogen-bound form of protein phosphatase 1 (13,14). Previous studies of possible defects in the regulation of GS have focused on the role of the PP1g in diabetic muscle. The limited evidence suggests that PP1g activity is not impaired in diabetic skeletal muscle, even when GS activity (fractional velocity) is decreased (15). Recently, it has become clear that GSK-3 is also subject to acute regulation. Insulin has been demonstrated to cause inactivation of GSK-3 *in vivo* (16) and in several cell types (17–20). This inactivation appears to be regulated by a protein kinase B, also known as Akt (17,21), which is a downstream target of phosphatidylinositol (PI) 3-kinase (22). Blockage of insulin stimulation of PI 3-kinase also eliminates the activation of GS in human myoblasts (23). Studies in cells over-expressing GSK-3 have shown that twofold activation of GSK-3 is sufficient to inhibit GS (12) and provided additional evidence supporting a physiological role for GSK-3 in the regulation of GS.

Two isoforms of human GSK-3, with molecular weights of 51 kDa (α) and 46 kDa (β), have been identified (24). The α - and β -isoforms share 85% homology at the amino acid level and are expressed ubiquitously, but functional differences between them are still unclear (25). Insulin administration results in phosphorylation of Ser-9 and Ser-21 in the β - and α -isoforms of GSK-3, respectively, which inhibits their activity (26). In intact cells, GSK-3 is also phosphorylated on tyrosine-216 (27); this modification activates GSK-3 (19,26,28). However, it is not clear if tyrosine phosphorylation is responsive to insulin or if it modulates GSK-3 activity *in vivo* (17,19).

The aim of the current study was to evaluate whether impairment of whole body glucose uptake or skeletal muscle GS activity in type 2 diabetes is related to alterations in the expression or activity of GSK-3. To determine this, we measured levels of protein expression of the two isoforms (α and β) of GSK-3 and investigated the effects of insulin infusion on GSK-3 phosphorylation and activity in skeletal muscle of lean and obese nondiabetic and obese type 2 diabetic patients.

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DTT, dithiothreitol; FV, fractional velocity; GDR, glucose disposal rate; GS, glycogen synthase; GSK-3, glycogen synthase kinase-3; IRS-1, insulin receptor substrate-1; OD, optical density; PI, phosphatidylinositol; PMSF, phenylmethylsulfonyl fluoride; PP1g, glycogen-bound form of protein phosphatase 1; R_{gl} , muscle-specific glycogen-associated phosphatase.

RESEARCH DESIGN AND METHODS

Materials. UDP-[¹⁴C]glucose was obtained from DuPont NEN (Boston, MA). [³²P]ATP was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Horseradish conjugated anti-rabbit IgG was obtained from Amersham (Arlington Heights, IL). SuperSignal Chemiluminescent Substrate was from Pierce (Rockford, IL). Bovine serum albumin (Fraction V) and glucose-6-phosphate were purchased from Boehringer Mannheim (Indianapolis, IN). Protein assay reagents and electrophoresis chemicals were purchased from BioRad Laboratories (Hercules, CA). Glycogen, pepstatin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and other reagents and chemicals were purchased from Sigma Chemical (St. Louis, MO). An affinity purified rabbit polyclonal antibody against phospho-Ser-9 GSK-3 β was obtained from Quality Control Biochemicals (Hopkinton, MA). Immunoaffinity purified sheep polyclonal IgG against phospho Ser21 GSK-3 α (51 kDa), affinity purified sheep polyclonal anti-GSK-3 α antibodies, anti-phospho-GSK-3 Shaggy protein kinase family (51/46 kDa), anti-GSK-3/Shaggy protein kinase family (51/46 kDa) mouse monoclonal IgG, and purified GSK-3 α and β from rabbit skeletal muscle were purchased from Upstate Biotechnology (Lake Placid, NY). An affinity purified chicken polyclonal antibody against GS was a kind gift from Dr. J.C. Lawrence, Jr. (University of Virginia).

Human subjects. Muscle biopsy samples were obtained from 17 nondiabetic and 11 type 2 diabetic subjects. All subjects had a 2-h, 75-g oral glucose tolerance test. Normal glucose tolerance was defined as a fasting glucose <7.0 mmol/l and 2-h glucose level <7.8 mmol/l (29). Insulin action was determined by a 3-h hyperinsulinemic (300 mU · m⁻² · min⁻¹)-euglycemic (5.0–5.5 mmol/l) clamp; the glucose disposal rate (GDR) was determined during the last 30 min of the clamp (30). Needle biopsies of vastus lateralis were performed before insulin infusion and at the end of the clamp (180 min). Serial glucose and insulin measurements (every 10 min for 30 min) were performed before each biopsy. Biopsy samples were immediately frozen and stored in liquid nitrogen. All biopsies were handled similarly. The experimental protocol was approved by the Committee on Human Investigation of the University of California, San Diego. Informed written consent was obtained from all subjects after explanation of the protocol. Subject characteristics are summarized in Table 1.

Immunoblotting of GSK-3. A total of 20 mg of muscle was homogenized in a buffer containing 20 mmol/l Tris-HCl, pH 7.5, 1.5 mmol/l EGTA, 1 mmol/l EDTA, 10 mmol/l NaF, 1 mmol/l dithiothreitol (DTT), 0.5 mmol/l sodium orthovanadate, 10 μ g/ml aprotinin, 20 μ g/ml leupeptin, 0.2 mmol/l PMSF, and 0.5% Triton X-100. Homogenates were centrifuged for 20 min at 10,000g. The levels of different isoforms of GSK-3 (α - and β -Shaggy protein kinase family) were determined by Western blotting using monoclonal antibodies raised against a peptide with a common sequence for GSK-3 α and β isoforms. Partially purified rabbit GSK-3 α and/or GSK-3 β were included on each gel as internal standards. Western blotting was performed on tissue homogenates by the method of Burnette (31). After SDS-PAGE (10%), proteins were transferred to nitrocellulose membranes. Nonspecific binding was reduced by incubation in a blocking solution containing Tris-buffered saline, 3% nonfat dry milk, and 0.05% Tween-20. Anti-mouse IgG conjugated with horseradish peroxidase was used as a secondary antibody. Proteins were visualized using the SuperSignal Chemiluminescent Substrate kit (Pierce) and exposed to specific supersensitive autoradiograph film (Hyperfilm, Amersham). Multiple exposures were performed to verify linearity of the images. The intensity of the bands was quantified by scanning densitometry using NIH Image software. Results were expressed in integrated optical density (OD) units per 20 μ g of total protein (OD units).

Assessment of GSK-3 phosphorylation state. Changes in the phosphorylation state of GSK-3 in response to insulin infusion were detected using phospho-specific antibodies that selectively interact with different phosphorylated forms of GSK-3. To determine the proportion of GSK-3 α phosphorylated on serine residues, an affinity purified sheep polyclonal antibody (UB1) raised against a phosphorylated peptide corresponding to residues 16–26 (RARTSpSFAEPG) of the human GSK-3 α was used. This antibody recognizes GSK-3 α phosphorylated on serine-21 and does not react with active dephosphorylated GSK-3. Serine phosphorylation of GSK-3 β was investigated with an affinity purified rabbit polyclonal antibody (QCB), which specifically recognizes the serine-9 phosphorylated form of GSK-3 β . The secondary antibodies were anti-sheep IgG for GSK-3 α and anti-rabbit IgG for GSK-3 β , both conjugated to horseradish peroxidase.

An anti-phospho-GSK-3 Shaggy protein kinase family monoclonal IgG was used to determine the portion of tyrosine-phosphorylated GSK-3 proteins. This antibody was raised to a tyrosine-phosphorylated peptide of a common sequence 203–218 of GSK-3/Shaggy enzyme; this region is highly conserved among GSK-3 proteins and should cross-react with different species homologues. This antibody does not recognize non-tyrosine-phosphorylated forms of GSK-3. Proteins were visualized and quantified as described above. The proportion of proteins that were phosphorylated were calculated as the ratio of intensity of specific bands after blotting with phospho-specific antibodies to the intensity of the bands with the same molecular weight after blotting with an antibody that recognizes both phospho-

rylated and non-phosphorylated GSK-3. The same membrane was blotted with each of the different antibodies.

Assay of GSK-3 activity. Activities of specific GSK-3 isoforms were measured in immunoprecipitates with either inhibitor 2 or phospho-GS peptide 2 [YRRAAVPPSPSLSRHSSPHOS(P)EDEEE] as a substrate as previously described (12,26). Homogenates from muscle biopsies were prepared as for immunoblotting of GSK-3. Homogenates were centrifuged for 20 min at 10,000g (4°C), and the resulting supernatants were passed through DE52 mini-columns. The flow-through was collected, and equal amounts of protein were incubated with sheep polyclonal antibody specific to GSK-3 α prebound to protein G-agarose at 4°C for 2 h. After immunoprecipitation of GSK-3 α , supernatants were incubated for 2 h with monoclonal antibodies against GSK-3 β , followed by incubation with protein A-agarose. Immunoprecipitates of GSK-3 α or β were washed three times by homogenization buffer and once with assay buffer (1 mmol/l DTT, 20 mmol/l Tris, pH 7.4). Kinase assays were performed on the immunoprecipitates in reaction mixtures that included 5 μ mol/l inhibitor 2 (substrate), 100 μ mol/l [γ -³²P]ATP, 10 mmol/l magnesium chloride, 0.1 mg/ml bovine serum albumin, 1 mmol/l DTT, and 20 mmol/l Tris, pH 7.4. Reactions were stopped after 30 min by adding Laemmli's buffer and boiling for 5 min. After separation of proteins by 10% SDS-PAGE, phosphorylation of inhibitor 2 was determined by autoradiography. Specific activities of GSK-3 α or GSK-3 β are expressed as arbitrary units (AU) from densitometry scanning of autoradiogram of inhibitor 2 phosphorylated/unit of immunoprecipitable GSK-3 measured by Western blot using the same gel. Thus, enzyme protein and activity were measured in the same gel. When phospho-GS peptide 2 substrate was used, reactions were stopped by adding 40% trichloroacetic acid and centrifuging; then, 30- μ l aliquots were spotted on Whatmann P81 paper squares. Squares were washed three times with 0.75% phosphoric acid and once with acetone, and they were counted in a liquid scintillation counter. ³²Pi incorporation into the negative control peptide (GS peptide-2 [Ala21]) was subtracted from values obtained using phospho-GS peptide 2. No activity was detectable with nonimmune serum. GSK-3-specific activities are expressed as nanomole of ³²Pi incorporated into phospho-GS peptide 2 per minute by the immunoprecipitated GSK-3 protein in each sample, expressed as OD units measured by densitometry scanning of Western blots. Total activities of GSK-3 isoforms are calculated as specific activities of enzymes expressed in 1 mg of total biopsy homogenates measured by Western blotting.

GS activity. The activity of GS was measured as described in detail previously (3,32). A total of 10 mg of muscle biopsy sample was homogenized at 4°C in 1 ml of extraction buffer (50 mmol/l HEPES, 10 mmol/l EDTA, 100 mmol/l NaF, 5 mmol/l DTT, 1 μ mol/l leupeptin, 1 μ mol/l pepstatin, and 200 μ mol/l PMSF, pH 7.5) with a POLYTRON (Brinkman Instruments, Canada) at setting 6 for 15 s. After centrifugation (3,000g for 20 min), supernatants were diluted with the extraction buffer to a final total protein concentration of 0.5 mg/ml. Protein concentration was determined by the Bradford method (33) with bovine serum albumin as a standard. GS activity was determined in diluted supernatants at a physiological concentration of substrate (0.3 mmol/l UDP glucose) and expressed as nanomoles of UDP glucose incorporated into glycogen per minute per milligram of total protein and as fractional velocity (FV) (the ratio of activity assayed at 0.1 mmol/l glucose-6-phosphate divided by the activity at 10 mmol/l glucose-6-phosphate). This is believed to be an indicator of changes in the phosphorylation state of GS in response to insulin (8).

Calculations and statistical analysis. Statistical analysis was performed using StatView II (Abacus Concepts, Berkeley, CA). Statistical significance was evaluated with Student's *t* test and Pearson correlation analysis. Paired analysis was used to determine the effect of the hyperinsulinemic clamp in the same patient. All data and calculated results are expressed as average \pm SE. To investigate relationships between GSK-3 protein expression and indicators of insulin resistance, regression analysis was performed using the Statistica computer program (StatSoft, Tulsa, OK). Significance was accepted at the *P* < 0.05 level. Because of limitations in tissue availability, not all analyses could be performed in all subjects. The number of subjects is given in the figure legends.

RESULTS

Experimental subjects. A total of 17 nondiabetic and 11 obese type 2 diabetic subjects participated in the study. To control for the possible influence of obesity, the nondiabetic group was divided into lean and obese (BMI >27) subgroups. The groups were matched for age (Table 1). Diabetic subjects displayed elevated fasting glucose and HbA_{1c} levels. Insulin resistance in that group was confirmed by lower GDRs during a 3-h hyperinsulinemic (300 mU · m⁻² · min⁻¹)-euglycemic (5.0–5.5 mmol/l) clamp (Table 1). The obese nondiabetic

TABLE 1
Characteristics of experimental subjects

	Nondiabetic subjects		Type 2 diabetic subjects
	Lean	Obese	
Age (years)	42 ± 3	45 ± 2	47 ± 2
BMI (kg/m ²)	23.5 ± 0.5	32.9 ± 1.5*	32.4 ± 1.2*
HbA _{1c} (%)	5.2 ± 0.1	5.2 ± 0.2	9.6 ± 1.0*†
Fasting serum insulin (pmol/l)	37 ± 8	125 ± 23*	167 ± 33*
Fasting plasma glucose (mmol/l)	5.3 ± 0.5	5.1 ± 0.1	9.4 ± 0.9*†
GDR (mg · kg ⁻¹ · min ⁻¹)	14.8 ± 0.9	8.2 ± 0.6*	6.2 ± 0.5*
GS FV basal	0.270 ± 0.019	0.225 ± 0.024	0.175 ± 0.017*
GS FV clamp	0.480 ± 0.024	0.439 ± 0.030	0.363 ± 0.028*

Insulin action was determined by a 3-h hyperinsulinemic (300 mU · m⁻² · min⁻¹)-euglycemic (5.0–5.5 mmol/l) clamp. GDRs were determined during the last 30 min of the clamp. GS FV was measured in homogenates of muscle biopsy samples of nondiabetic and diabetic subjects before and after the 3-h clamp. *P < 0.05 vs. lean nondiabetic subjects; †P < 0.05 vs. obese nondiabetic subjects.

group was matched by BMI with the diabetic group and was intermediate in insulin responsiveness between the lean nondiabetic and type 2 diabetic groups. GDRs were significantly lower and fasting insulin levels were higher in the obese nondiabetic group compared with the lean nondiabetic group (Table 1). In agreement with previously reported data (6), GS protein expression was not significantly different in skeletal muscle biopsies of obese nondiabetic (326 ± 36 OD units) and type 2 diabetic (327 ± 43 OD units) subjects compared with lean nondiabetic subjects (394 ± 51 OD units). However, GS activity, expressed as FV, was significantly reduced in muscle of diabetic patients both before and after the maximum hyperinsulinemic-euglycemic clamp compared with lean nondiabetic subjects. There was also a tendency (P < 0.07) for insulin-stimulated GS FV in type 2 diabetic subjects to be lower than in obese nondiabetic subjects (Table 1).

Protein levels of GSK-3 isoforms in human muscle of nondiabetic and type 2 diabetic subjects. Because it has been shown that GS inactivation correlates with phosphorylation by GSK-3 (9,11), the current studies were performed to determine whether the reduction in GS activity in skeletal muscle of diabetic patients could be due to increased total activity of GSK-3. Because one possible mechanism for this effect could be increased GSK-3 protein abundance, we measured levels of the enzyme in homogenates of biopsy samples by Western blotting.

Antibodies specific to GSK-3 (monoclonal antibodies raised against a peptide with a common sequence for α and β GSK-3 isoforms) recognized three bands with molecular weights of 46, 51, and 53 kDa (Fig. 1A). The protein at molecular weight ~46 kDa is the β isoform, and the ~51-kDa band has been identified as GSK-3α. Both the 51- and 53-kDa bands could be immunoprecipitated by an antibody specific for the GSK-3α isoform (Fig. 1A) (and is unrelated to the antibody used for Western blotting). The fact that the 53-kDa band is recognized by two different antibodies suggests that it is possibly another form of GSK-3α, which could result from dif-

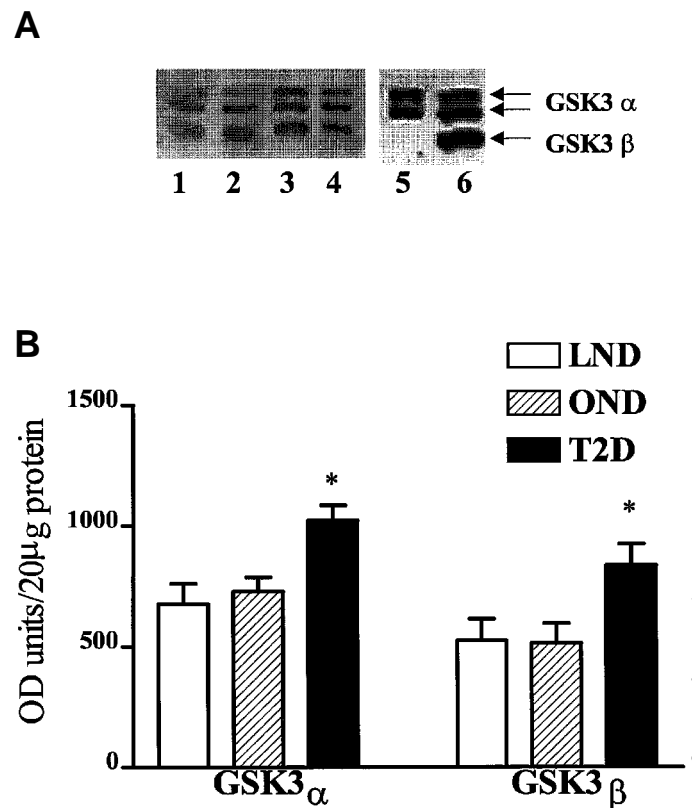


FIG. 1. Protein expression of GSK-3 isoforms in skeletal muscle biopsy samples of nondiabetic and type 2 diabetic subjects. A: Representative image of Western blot of total GSK-3 in obese nondiabetic (lanes 1 and 2) and diabetic (lanes 3 and 4) subjects. Blots were performed as described in RESEARCHDESIGN AND METHODS. Lane 5 is an immunoprecipitate of GSK-3 α ; lane 6 shows the total homogenate of skeletal muscle biopsy used for immunoprecipitation. B: Quantitation of Western blots performed with biopsy samples from 8 lean nondiabetic (LN) (\square), 8 obese nondiabetic (OND) (\square), and 11 type 2 diabetic (T2D) (\blacksquare) subjects. Results are expressed as integrated OD units per 20 μ g of total protein (average \pm SE). *P < 0.05 vs. lean and obese nondiabetic subjects.

ferences in posttranslational processing, such as phosphorylation. For subsequent analyses, the 51- and 53-kDa bands were considered collectively as GSK-3 α .

Quantitation of the intensity of GSK-3 bands revealed significant increases in protein levels of all isoforms of GSK-3 in skeletal muscle of type 2 diabetic patients (Fig. 1B). No difference in GSK-3 protein levels was seen between lean and obese nondiabetic subjects.

Enzyme activity of GSK-3 isoforms in nondiabetic and diabetic muscle. To investigate whether increased GSK-3 protein levels in diabetes result in increased total activity of either GSK-3 α or β isoforms, the specific activity of each isoform was measured in immunoprecipitates obtained using antibodies specific to each of the isoforms.

Activities of GSK-3 enzymes were measured against two substrates, inhibitor 2 and phospho-GS peptide 2, as described in RESEARCHDESIGN AND METHODS. The specific activities of GSK-3 α against phospho-peptide GS-2 did not differ significantly between normal and diabetic subjects in either the basal and insulin-stimulated states (Fig. 2A). Insulin infusion (180 min) caused a reduction of GSK-3 α specific activity (Fig. 2A). Diabetic (36 ± 11% reduction, P < 0.05 vs. basal), lean nondiabetic (35 ± 7%, P < 0.02 vs. basal), and obese non-

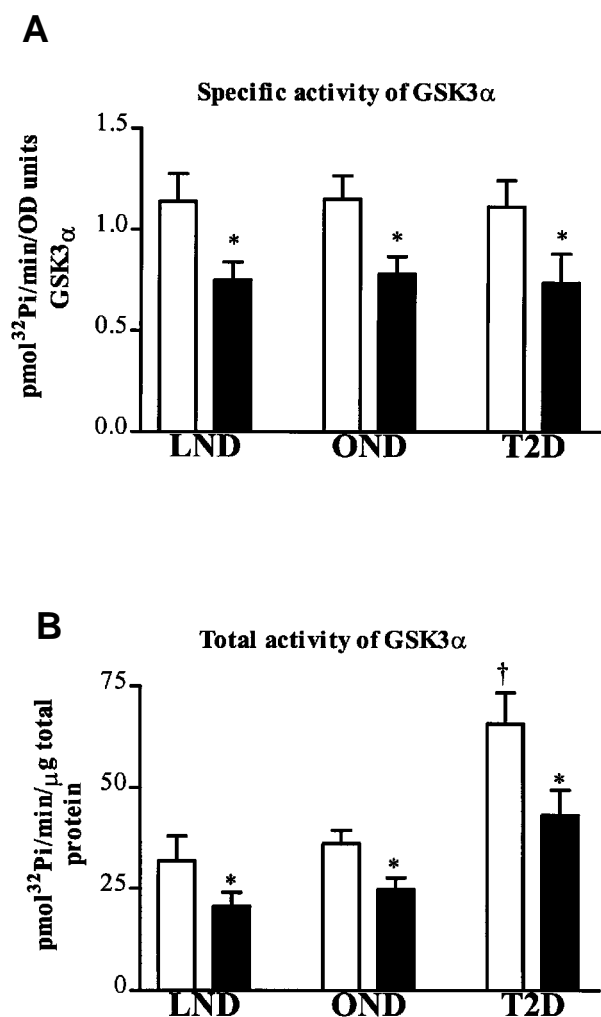


FIG. 2. Activities of GSK-3 α isoform in skeletal muscle of nondiabetic and type 2 diabetic subjects before (□) and after (■) hyperinsulinemic-euglycemic clamp. A: Specific activities of GSK-3 α were measured with phospho-GS peptide 2 as a substrate in eight lean nondiabetic (LND), eight obese nondiabetic (OND), and nine type 2 diabetic (T2D) subjects. B: Total activities of GSK-3 α . Units of activity are defined in RESEARCH DESIGN AND METHODS. Results are average \pm SE. * P < 0.05 vs. basal; $\dagger P$ < 0.05 vs. nondiabetic (lean or obese).

diabetic subjects ($34 \pm 9\%$, $P < 0.05$) all showed similar relative responses.

The total activity of the GSK-3 α isoform in the basal state measured with phospho-peptide GS-2 as a substrate was approximately twofold higher in type 2 diabetic subjects (Fig. 2B) than in lean nondiabetic and obese nondiabetic subjects. There was no consistent or significant change in GSK-3 α protein expression during the clamp (not shown). Thus, total activities were also reduced after clamp, and the total activity in diabetic muscle was still approximately twofold higher than that in lean ($P < 0.01$) and obese nondiabetic subjects ($P < 0.05$). Similar results were obtained with inhibitor 2 as a substrate.

The basal specific activities of GSK-3 β against inhibitor 2 in nondiabetic and diabetic subjects were not significantly different (Fig. 3A). Total basal activity in diabetic biopsies was elevated by two- to threefold compared with combined nondiabetic subjects ($P < 0.05$) (Fig. 3B). There was no consis-

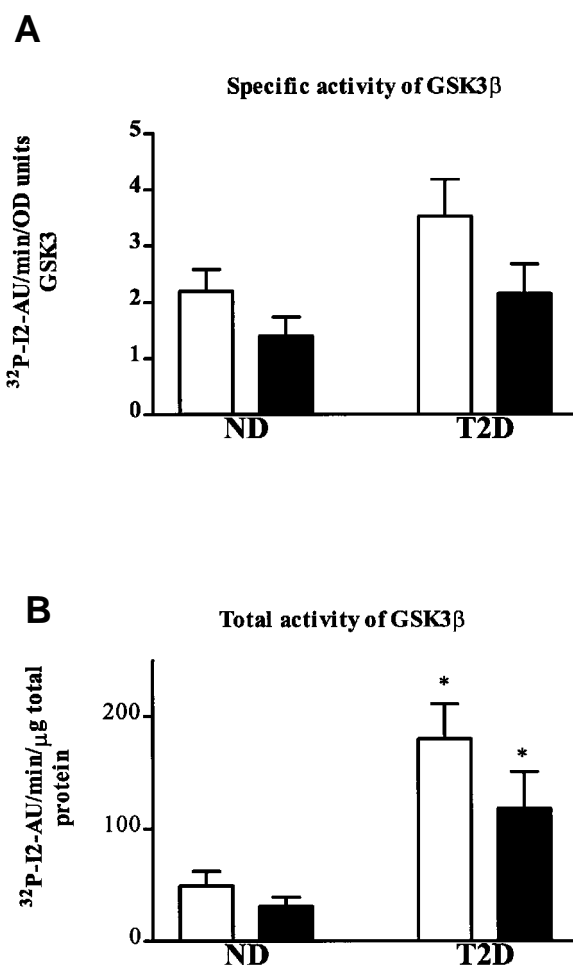


FIG. 3. Activity of GSK-3 β isoform in skeletal muscle of nondiabetic (ND) and type 2 diabetic (T2D) subjects before (□) and after (■) hyperinsulinemic-euglycemic clamp. A: Specific activities of GSK-3 β measured with inhibitor-2 as a substrate. Specific activity of GSK-3 β is expressed as arbitrary units of inhibitor-2 phosphorylated per unit of immunoprecipitable GSK-3 measured by Western blot using the same gel. Assay was performed in samples of 14 nondiabetic (7 lean and 7 obese) and 9 type 2 diabetic subjects. B: Total activities of GSK-3 β measured with inhibitor-2 as a substrate. * P < 0.05 vs. nondiabetic.

tent response of GSK-3 β activity to 180-min insulin infusion, and values were not significantly different (Fig. 3A). However, there was a tendency toward a decrease in diabetic subjects ($P = 0.1$).

Effect of insulin infusion on GSK-3 phosphorylation. An antibody specific for serine-phosphorylated GSK-3 α recognized bands of 51 and 53 kDa (Fig. 4). This is further evidence supporting the identification of the 53-kDa band as related to GSK-3 α . For subsequent analysis, results for the 51- and 53-kDa bands were combined. Under the same conditions that reduced the specific activity of GSK-3 α , insulin infusion increased serine phosphorylation of this isoform of the enzyme (Fig. 4). Total expression of GSK-3 α proteins was not significantly altered by insulin infusion (not shown), indicating that this effect is due to an increase in the stoichiometry of serine phosphorylation. Tyrosine phosphorylation of GSK-3 α was also detected by specific antibodies (Fig. 5). Insulin infusion resulted in no change in GSK-3 α tyrosine phosphorylation.

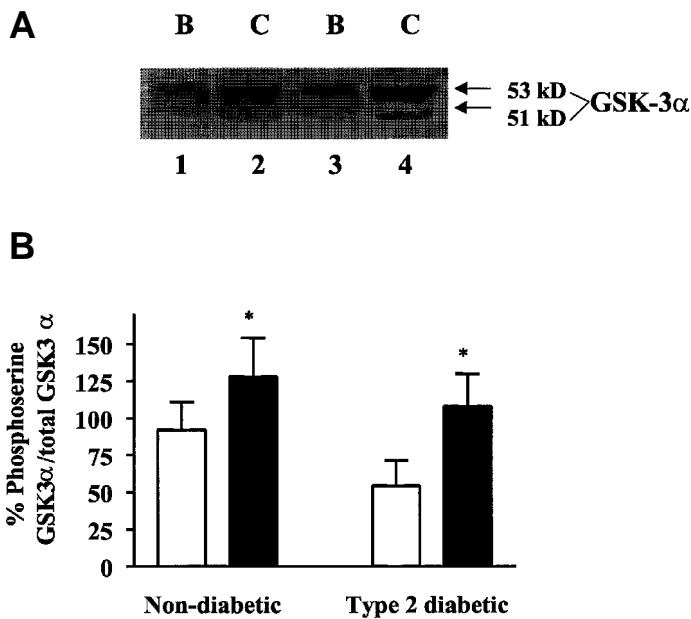


FIG. 4. Serine phosphorylation of GSK-3 α skeletal muscle of nondiabetic and type 2 diabetic subjects before and after hyperinsulinemic-euglycemic clamp. A: Representative Western blot, using anti-pSer-GSK-3 α antibody, of whole tissue homogenates from skeletal muscle biopsies of two nondiabetic subjects before (B) and after (C) clamp. B: Quantitation of serine phosphorylation of GSK-3 α (51- and 53-kDa isoforms combined) in nondiabetic ($n = 14$) and type 2 diabetic ($n = 8$) muscle before (\square) and after (\blacksquare) insulin infusion. Results are normalized to amount of enzyme protein, determined on the same membrane. Results are average \pm SE. * $P < 0.05$ vs. basal.

Serine-phosphorylated GSK-3 β was best detectable after immunoprecipitation (not shown). Serine phosphorylation of GSK-3 β was not significantly changed after 3 h of insulin, consistent with the lack of insulin effect on the specific activity of this isoform. Tyrosine phosphorylation of GSK-3 β was also unaltered following insulin infusion (not shown).

Relationship of GSK-3 to insulin action in vivo. To investigate the potential impact of differences in GSK-3 expression and activity on skeletal muscle glucose metabolism, especially in type 2 diabetes, we examined possible relationships between GSK-3 and quantitative measures of insulin action.

The major target of GSK-3 is GS, and a significant inverse correlation was observed between GSK-3 α protein expression and maximally insulin-stimulated GS FV for the entire population studied (Fig. 6A) ($r = -0.669$, $P < 0.002$). This relationship also held for the GSK-3 β isoform (Fig. 6B) ($r = -0.511$, $P < 0.01$). The same relationships were seen between total activity of each isoform and GS FV.

Although effects of GSK-3 on GS activity might be expected, there was also a highly significant inverse relationship between GSK-3 α or - β protein expression and the insulin-stimulated whole body maximal GDR (Fig. 7A and B) ($r = -0.606$, $P < 0.001$ for GSK-3 α ; $r = -0.553$, $P < 0.005$ for GSK-3 β) that resembled the relationships for GS. Thus, the higher the GSK-3 protein and activity levels in biopsy samples of skeletal muscle, the lower the insulin-stimulated whole body maximal GDR (Fig. 7).

We also found positive relationships between GSK-3 α and - β protein expression and fasting insulin levels ($r = 0.45$, $P <$

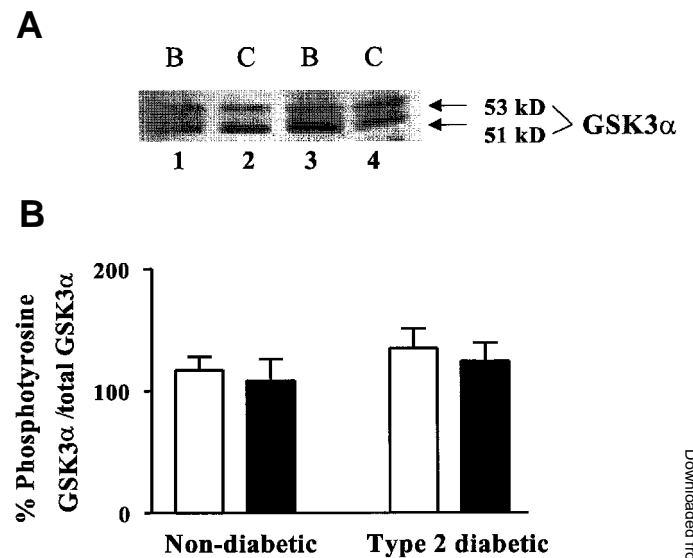


FIG. 5. Tyrosine phosphorylation of GSK-3 α skeletal muscle of nondiabetic and type 2 diabetic subjects before and after hyperinsulinemic-euglycemic clamp. A: Representative Western blot, using anti-pY-GSK-3 α antibody, of whole tissue homogenates from skeletal muscle biopsies of two nondiabetic subjects before (B) and after (C) insulin infusion. B: Quantitation of tyrosine phosphorylation of GSK-3 α (51- and 53-kDa isoforms combined) in nondiabetic ($n = 14$) and type 2 diabetic ($n = 8$) muscle before (\square) and after (\blacksquare) clamp. Results are normalized to amount of enzyme protein, determined on the same membrane. Results are average \pm SE.

0.05 for α ; $r = 0.42$, $P < 0.05$ for β), as well as with HbA_{1c} and fasting glucose levels ($r = 0.607$, $P = 0.001$ for α ; $r = 0.552$, $P = 0.002$ for β isoform), in the three groups combined.

DISCUSSION

Nonoxidative metabolism, primarily storage into glycogen, is the major fate of glucose in skeletal muscle in the insulin-stimulated state (1,5). This process is impaired in skeletal muscle of type 2 diabetic patients (2,4). The activity of GS, the key enzyme responsible for the final step of the process, has also been shown to be reduced in diabetic muscle (3). This difference is seen in the absence of any mutations in the GS sequence (34–37). Several groups have found normal levels of GS protein in type 2 diabetic muscle (6), an observation that we have confirmed. Another group reported that GS protein was elevated in diabetic subjects (38). The impaired nonoxidative glucose disposal and reduced GS activity characteristic of type 2 diabetes, in the presence of normal or elevated GS protein, suggests that activation/regulation of GS is defective in diabetes. GS is subject to regulation by both allosteric and covalent mechanisms (8). Because GS activity in diabetic muscle remains impaired independent of allosteric modulators (32), it is possible that changes in covalent modification are responsible for the behavior in the diabetic state. GS was one of the first enzymes shown to be regulated by phosphorylation/dephosphorylation (9). Enzymes believed to be responsible for this regulation are PP1 γ , which activates the enzyme, and GSK-3, which deactivates GS. The information about glycogen-associated PP1 is very limited (39), and it is still unclear whether it is a major enzyme responsible for regulation of GS. Total activity of PP1 (not necessarily associated with glycogen) in skeletal muscle has been shown to

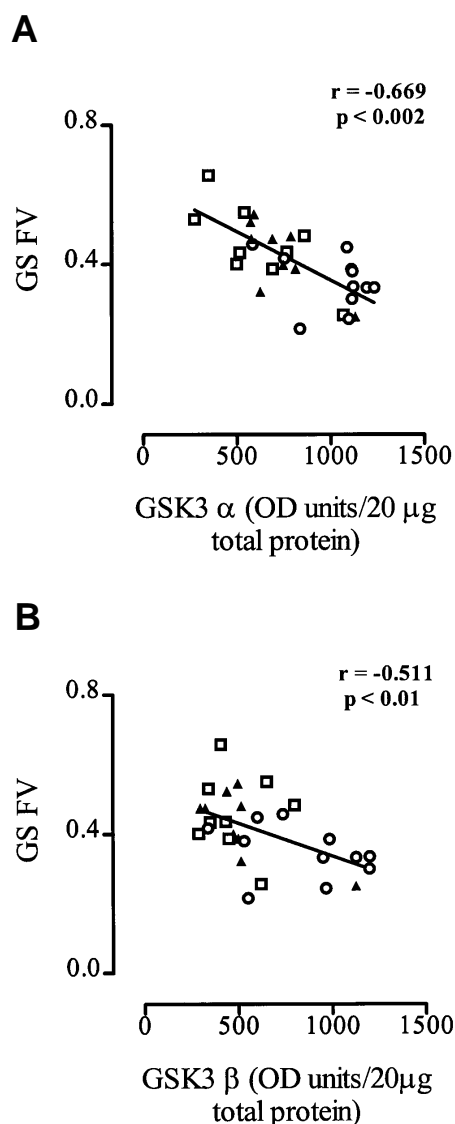


FIG. 6. Relationship between GSK-3 α and - β protein expression and insulin stimulation of GS in nondiabetic and type 2 diabetic subjects. A: GSK-3 α protein levels and GS FV determined in biopsy samples obtained at the end of the clamp. Samples were from lean nondiabetic (\square), obese nondiabetic (\blacktriangle), and type 2 diabetic (\circ) subjects. B: GSK-3 β protein levels and GS FV determined at the end of the clamp.

be rapidly activated in response to insulin infusion (40), but this activation was highly transient and returned to basal levels after 60 min of hyperinsulinemic-euglycemic clamp (15), even while GS remains activated. Activity of PP1 was shown to be reduced in skeletal muscle of insulin-resistant Pima Indians (39,40), but not in type 2 diabetic subjects (15). However, no differences in expression of PP1 catalytic subunit were found in insulin-resistant subjects (41). Thus, impaired activity of PP1 could be expected to result from derangement in control of its activity by regulatory subunits. However, studies in transgenic mice deficient in the muscle-specific glycogen-associated phosphatase (R_{gl}) showed normal activation of GS by insulin in R_{gl} null mutant mice, and therefore they argue that PP1 γ/R_{gl} is required for insulin stimulation of glycogen syntheses in skeletal muscle (39).

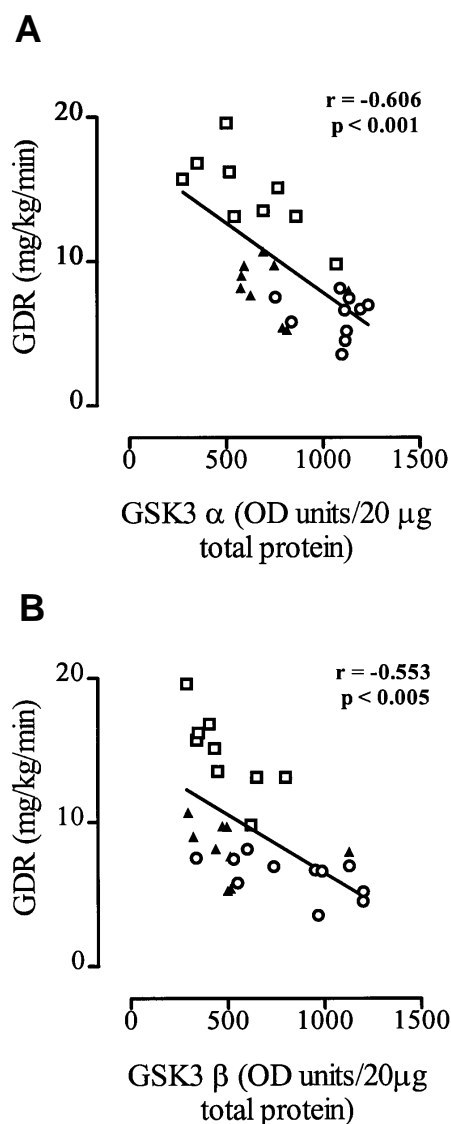


FIG. 7. Relationship between GSK-3 α and - β protein expression and in vivo insulin action in nondiabetic and type 2 diabetic subjects. A: GSK-3 α protein levels were determined in muscle biopsies, and GDR was determined over the last 30 min of the clamp. Samples were from lean nondiabetic (\square), obese nondiabetic (\blacktriangle), and type 2 diabetic (\circ) subjects. B: GSK-3 β protein levels in biopsy samples and GDR determined over the last 30 min of the clamp.

Because GSK-3 has been shown to be regulated by insulin (16,18), we investigated the possibility that differences in GSK-3 expression and/or activity might contribute to the defects in GS present in type 2 diabetes. To accomplish this, we measured GSK-3 protein and activity in skeletal muscle before and after a maximally stimulating hyperinsulinemic-euglycemic clamp.

The first notable observation of the current work is that human skeletal muscle expresses three different forms of GSK-3 (Fig. 1B). The 46-kDa form is the same size as that previously described for GSK-3 β (24) and is recognized by several antibodies specific for GSK-3 β . GSK-3 α has been reported to be 51 kDa (24), and this band is recognized by specific antisera (Figs. 2 and 4). The 53-kDa band was an unexpected observation, not previously reported or detected in

several cultured cell lines (42). However, this band was recognized in human skeletal muscle, through both immunoblotting and immunoprecipitation, by several different antibodies specific for GSK-3 α (Figs. 2 and 4), and it most likely represents the result of differential posttranslational processing of GSK-3 α , possibly phosphorylation.

The most important observation of our study was that the expression, at the protein level, of each of these forms of GSK-3 was elevated in the muscle of type 2 diabetic subjects (Fig. 1). This increase appeared to be a property of skeletal muscle of type 2 diabetic subjects, as it was not present in samples from insulin-resistant obese nondiabetic subjects. Because the substrates used in the current studies—which are primary physiological targets for GSK-3—can be acted on by other kinases (43), it was necessary to isolate the enzyme by immunoprecipitation to measure the activity due to GSK-3. Unlike the case for enzyme protein, the specific activities of both the α (Fig. 2A) and β (Fig. 3A) isoforms were similar in muscle from nondiabetic and type 2 diabetic subjects. Thus, total GSK-3 activity of each isoform, as well as the overall activity, was significantly elevated, by approximately twofold, in diabetic muscle. The consequence of such an increase would be to maintain GS in a more highly phosphorylated, less active state. Indeed, an elevation of GSK-3 activity similar to that in type 2 diabetes (twofold), attained by overexpression of GSK-3 in 293 cells (12), led to a reduction of GS activity in these cells (30–50%) that was similar to the reduction found in type 2 diabetic muscle.

Insulin infusion resulted in a reduction in the activity of the GSK-3 α isoform (Fig. 2), similar to what has been reported in other experimental systems (16,44). Along with no change in GSK-3 α protein, this would lead to reduction of inhibitory phosphorylation of GS with the activation of GS seen after the clamp (Table 1). The relative extent of the change in GSK-3 α specific activity did not differ significantly between nondiabetic and diabetic subjects. Thus, diabetic subjects retain responsiveness for the ability of insulin to reduce GSK-3 α activity. In support of this conclusion, the activity and insulin responsiveness of Akt, the kinase proposed to be involved in insulin-mediated phosphorylation and inactivation of GSK-3, was found to be normal in skeletal muscle biopsies of many of the same subjects as studied in the current report (45). A similar finding of normal activation of Akt by insulin in type 2 diabetes was obtained by Krook et al. (46), using muscle strips treated with physiological concentrations of insulin (2.4 nmol/l).

It is important to note that, although insulin causes a similar relative reduction in GSK-3 α specific activity in all groups, activity of this enzyme remains elevated in diabetic muscle by twofold after the clamp. It is interesting that GSK-3 β activity was not significantly altered by insulin infusion (Fig. 3), whereas in cultured L6 muscle cells and cultured human myoblasts, GSK-3 β activity was reduced after insulin treatment (23,47). One of the explanations may be that there are different time courses for insulin activation of the isoforms. Alternately, activation of GSK-3 β could be transient, returning to basal levels after 3 h of insulin infusion. In view of this possibility, it is important to consider that GSK-3 β activity in cultured cells was measured after 10 min of insulin treatment (23,47).

The activity of GSK-3 is differentially regulated by tyrosine and serine/threonine phosphorylation (26,27). Although it is accepted that serine phosphorylation inhibits GSK-3 activity

(17), there is some disagreement on the effect of tyrosine phosphorylation on activity (17,19,28). The observation that insulin infusion leads to a sustained (3 h) increase in serine phosphorylation of GSK-3 α (Fig. 4), together with deactivation of the enzyme (Fig. 2), shows that this mechanism is also operative in human skeletal muscle. There was no significant difference in serine phosphorylation normalized to GSK-3 protein between nondiabetic and type 2 diabetic subjects. Serine phosphorylation was similarly regulated by insulin in nondiabetic and diabetic muscle. These two findings suggest that serine phosphorylation of GSK-3 α and its regulation by insulin is intact in diabetic muscle. It seems likely that the major difference in diabetes with regard to GSK-3 is a quantitative one, due to increased protein expression and activity of the enzyme, with essentially normal regulation. Mutational analysis of the coding regions of GSK-3 α and β failed to find anything other than silent mutations in type 2 diabetic subjects (48), supporting the observation of normal structure and function.

The failure of insulin infusion to alter either tyrosine or serine phosphorylation of GSK-3 β agrees with the lack of effect on isoform activity (Fig. 3). This difference between isoforms in response to insulin could be indicative of different roles for GSK-3 α and β . Besides the potential differences in substrate specificity and time course of insulin action between GSK-3 α and β mentioned above, there is also evidence suggesting that subcellular localization may be important for specific functions of different GSK-3 isoforms (49,50).

What might be the role of GSK-3 in glucose disposal and insulin resistance in type 2 diabetes? Although correlations do not prove causal relationships, there are several interesting associations between GSK-3 protein and activity and aspects of glucose metabolism. The most expected would be the inverse relationship between GSK-3 and GS FV, strongly supporting a role of GSK-3 in determining GS activity. The fact that relationships exist between GSK-3 and insulin-stimulated GDR (Fig. 7) similar to those for GSK-3 insulin-stimulated GS FV (Fig. 6) indicates that the behavior of whole body glucose disposal reflects that of skeletal muscle. The positive correlation between GSK-3 expression or activity and three indicators of insulin resistance—fasting insulin, fasting glucose, and HbA_{1c}—does not indicate which is the causative event, but implies that elevated GSK-3 is a characteristic of impaired insulin action and glucose intolerance. Interestingly, the strongest relationship between GSK-3 expression and decrements in glucose disposal is seen in nondiabetic subjects (Fig. 7). One possible interpretation of this observation is that elevations in GSK-3 may predispose subjects to further decay in glucose utilization and the development of insulin resistance and type 2 diabetes. As we have shown previously, hyperinsulinemia and hyperglycemia can impair basal and insulin-stimulated GS FV, and this suggests the possibility of involvement of GSK-3 in these effects (51).

The results of the current investigation indicate that quantitative differences in the expression of GSK-3 are present in diabetic muscle and could contribute to impairments in glucose metabolism. Further support for a broader role of GSK-3 in the development of insulin resistance is provided in the recent report where GSK-3 was shown to phosphorylate insulin receptor substrate-1 (IRS-1), a key early molecule in insulin signaling (52). Considering that augmented serine phosphorylation of IRS-1 occurs in several insulin-resistant

conditions (52–54), it is possible that increased GSK-3 activity could also impair insulin action at steps proximal to GS, influencing other responses as well. Thus, GSK-3 may play a central role in the regulation of insulin action and glucose metabolism, and derangements in the diabetic state could have multiple negative impacts. These defects in GSK-3 regulation might represent a promising target for future therapeutic intervention for the control of insulin resistance in type 2 diabetes.

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