Glycogen synthase activity is reduced in cultured skeletal muscle cells of non-insulin-dependent diabetes mellitus subjects. Biochemical and molecular mechanisms.

R R Henry, … , K S Park, S E Nikoulina

Research Article

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Glycogen Synthase Activity is Reduced in Cultured Skeletal Muscle Cells of Non–Insulin-dependent Diabetes Mellitus Subjects

Biochemical and Molecular Mechanisms

Robert R. Henry,*‡ Theodore P. Ciaraldi,‡ Leslie Abrams-Carter,*‡ Sunder Mudaliar,* Kyong Soo Park,*
and Svetlana E. Nikoulina*‡

Department of Medicine, University of California, San Diego, La Jolla, California 92093; and ‡Veterans Affairs Medical Center, San Diego, San Diego, California 92161

Abstract

To determine whether glycogen synthase (GS) activity remains impaired in skeletal muscle of non–insulin-dependent diabetes mellitus (NIDDM) patients or can be normalized after prolonged culture, needle biopsies of vastus lateralis were obtained from 8 healthy nondiabetic control (ND) and 11 NIDDM subjects. After 4–6 wk growth and 4 d fusion in media containing normal physiologic concentrations of insulin (22 pM) and glucose (5.5 mM), both basal (5.21 ± 0.79 vs $9.01 \pm 1.25\%$, $P < 0.05$) and acute insulin-stimulated (9.35 ± 1.81 vs 16.31 ± 2.39, $P < 0.05$) GS fractional velocity were reduced in NIDDM compared to ND cells. Determination of GS kinetic constants from muscle cells of NIDDM revealed an increased basal and insulin-stimulated $K_{m_{0.1}}$ for UDP-glucose, a decreased insulin-stimulated $V_{max_{0.1}}$ and an increased insulin-stimulated activation constant ($A_{0.5}$) for glucose-6-phosphate. GS protein expression, determined by Western blotting, was decreased in NIDDM compared to ND cells (1.57 ± 0.29 vs 3.30 ± 0.41 arbitrary U/mg protein, $P < 0.05$). GS mRNA abundance also tended to be lower, but not significantly so (0.168 ± 0.017 vs 0.243 ± 0.035 arbitrary U, $P = 0.08$), in myotubes of NIDDM subjects. These results indicate that skeletal muscle cells of NIDDM subjects grown and fused in normal culture conditions retain defects of basal and insulin-stimulated GS activity that involve altered kinetic behavior and possibly reduced GS protein expression. We conclude that impaired regulation of skeletal muscle GS in NIDDM patients is not completely reversible in normal culture conditions and involves mechanisms that may be genetic in origin. (*J. Clin. Invest.* 1996. 98:1231–1236.) Key words: diabetes mellitus, non–insulin-dependent • glycogen synthase • enzyme activation • insulin resistance • muscle, skeletal

Introduction

Skeletal muscle is the principal glucose-consuming tissue in the insulin-stimulated state and a major site of impaired insulin action in non–insulin-dependent diabetes mellitus (NIDDM)[1] (1, 2).

Address correspondence to Robert R. Henry, VA Medical Center, San Diego (V111G), 3350 La Jolla Village Drive, San Diego, CA 92161. Phone: 619-552-8585, ext. 3648; FAX: 619-534-6653; E-mail: rrhenry@vapop.ucsd.edu

After insulin stimulation, glycogen synthesis is a dominant pathway of glucose disposal in skeletal muscle and is regulated by the rate-limiting enzyme glycogen synthase (GS) (3, 4). In NIDDM, resistance to insulin action is associated with impairments of both glycogen synthesis and GS activity. Previous studies from our laboratory (4, 5) have shown that both basal and insulin-stimulated muscle GS activity are reduced in NIDDM independent of impaired glucose uptake. These studies demonstrated that defective GS activity per se has a major role in impaired glycogen formation in NIDDM. In high risk patients with a strong family history of NIDDM, similar abnormalities of glycogen formation and GS activity have been shown to be early defects that are present before the development of overt diabetes (6–8). Although NIDDM has strong genetic determinants (9), recent evidence indicates that abnormalities of insulin action may also be acquired in origin and develop secondary to the presence of metabolic abnormalities such as hyperinsulinemia and hyperglycemia (10, 11).

Defects in muscle GS activity may result from decreased protein expression, abnormal protein structure, or impaired regulation by the specific phosphatases or kinases that affect covalent or allosteric modification of the enzyme. We have recently described the methodology to culture human skeletal muscle tissue obtained from needle biopsy specimens of vastus lateralis muscle (12). These muscle cultures display the morphological, biochemical, and molecular characteristics of intact skeletal muscle, including insulin stimulation of GS activity and glycogen formation. Using human skeletal muscle cultures from nondiabetic subjects, we have shown that cells exposed to chronic hyperinsulinemia exhibit diminished insulin-stimulated GS activity consistent with an acquired defect of activation (13). A similar situation may also occur in cells of NIDDM patients. Alternately, impaired glycogen synthesis could be due to primary defects in GS and would not be expected to resolve after prolonged culture in normal media conditions. A combination of these mechanisms may also occur. Recent support for the presence of an irreversible and possibly genetic defect in glycogen synthesis has been provided by studies of cultured fibroblasts isolated from NIDDM patients (14). In that report, NIDDM patients with two or more close relatives having NIDDM were found to have specific abnormalities of both basal and maximally insulin-stimulated glycogen synthesis in their fibroblasts.

In the current study, we sought to expand on these findings and address whether skeletal muscle GS activity of NIDDM subjects remains impaired during culture of this primary insu-

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1. *Abbreviations used in this paper:* FV, fractional velocity; G-6-P, glucose-6-phosphate; GS, glycogen synthase; NIDDM, non–insulin-dependent diabetes mellitus.

lin target tissue and, if so, by what mechanisms. To do this, kinetic assessment of GS activity, as well as protein expression and mRNA abundance, was compared in skeletal muscle cultures of nondiabetic and NIDDM individuals grown in normal glucose and insulin concentrations.

Methods

Materials. Culture growth media (SkGM Bullet Kit) and bovine insulin were obtained from Clonetics Corp. (San Diego, CA). Alpha-MEM, trypsin/EDTA, fungibact, glutamine, and Ham's F10 were purchased from Irvine Scientific (Santa Ana, CA). FBS was purchased from Gemini (Calabasas, CA). UDP-[^{14}C]glucose was obtained from DuPont-NEN (Boston, MA). Horseradish-conjugated anti–rabbit IgG and the enhanced chemiluminescence Western blot kit were obtained from Amersham Corp. (Arlington Heights, IL). Bovine serum albumin and glucose-6-phosphate were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Protein assay kits were purchased from Bio Rad Laboratories (Hercules, CA). RNA Stat 60 was obtained from Tel Test "B" Inc. (Friendswood, TX). The Riboprobe Gemini system from Promega Corp. (Madison, WI) was used to synthesize RNA probes. Restriction enzymes and random primer DNA labeling kits were purchased from GIBCO BRL (Gaithersburg, MD). Glycogen, pepstatin, leupeptin, PMSF, and other reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Human subjects. 8 healthy nondiabetic subjects on no medications and 11 patients with NIDDM participated in these studies. Glucose tolerance was determined after a 75-g oral glucose tolerance test (15). Characteristics of the study groups are summarized in Table I. Six of the NIDDM patients were treated with oral sulfonylureas, two with insulin, and three with diet therapy only. NIDDM patients had their medication withheld on the morning of biopsy. The experimental protocol was approved by the Committee on Human Investigation of the University of California, San Diego (San Diego, CA). Informed written consent was obtained from all subjects after explanation of the protocol.

Primary human muscle cell cultures. The method for skeletal muscle cell isolation and subculture has been described in detail previously (12). Briefly, percutaneous muscle biopsies were obtained from the lateral portion of the quadriceps femoris (vastus lateralis) using a 5-mm diameter side-cutting needle. Primary cultures were grown from satellite cells isolated from dissociated muscle tissue using modifications of the methods of Blau and Webster (16) and Sarabia et al. (17). After trypsinization, cells were resuspended in SkGM without added insulin, supplemented with 2% FBS, plated, and grown at 37°C in 5% CO_2. At confluence, cells were passed to multiple dishes and grown to 80% confluence (1–2 wk). They were then fused for 4 d in alpha-MEM containing 2% FBS, 1% fungibact, penicillin (100 U/ml), and streptomycin (100 μg/ml). All studies were carried out on cultures after first passage (12).

Glycogen synthase activity. The activity of GS was measured as described in detail previously (4, 5, 12). Cell cultures of human mus-

cle cells (in the myotube stage) were incubated for 1 h in media containing 5.5 mM glucose but without serum or insulin, followed by a 1-h treatment with 33 nM insulin. The cultures were then washed with cold PBS and 700 μl buffer (50 mM Hepes, 10 mM EDTA, 100 mM NaF, 5 mM DTT, 1 μM leupeptin, 1 μM pepstatin, and 200 μM PMSF, pH 7.5) was added to each 100-mm dish of cells. The myotubes were scraped from the dishes and sonicated using a high intensity ultrasonic processor (Fisher Instruments, Tustin, CA) at setting 8 for 30 s at 4°C. The sonicates were used for determination of protein and enzyme activity. GS fractional velocity (FV) was determined at physiologic concentrations of substrate (0.3 mM UDP-glucose) in parallel incubations with 0.1 and 10 mM glucose-6-phosphate. For kinetic studies, total cell homogenates were filtered through a Sephadex G-25 column and GS activity was measured at 0.1, 0.2, 0.6, 1.2, and 5.0 mM UDP-glucose in the absence or presence of 0.1, 0.2, 0.3, 0.5, 2.0, and 10 mM glucose-6-phosphate (G-6-P). Total cellular protein was determined by the Bradford method (18) with bovine serum albumin as a standard. GS activity is expressed as nanomoles of UDP-glucose incorporated into glycogen per minute per milligram of total protein or as FV (a percent of the ratio of activity at 0.1 mM G-6-P/10 mM G-6-P). The $K_{m_{0.1}}$ and $K_{m_{10}}$ are the Michaelis constants measured at 0.1 and 10 mM G-6-P, respectively, and expressed as millimoles UDP-glucose. The $V_{max_{0.1}}$ and $V_{max_{10}}$ are the maximum reaction velocities calculated from individual response curves at 0.1 and 10 mM G-6-P, respectively, and expressed as nanomoles per minute per milligram of protein.

Immunoblotting. Western blot analysis was performed by the method of Burnette (19) as detailed previously (12). Cell sonicates were solubilized in Laemmli's buffer and proteins separated on 8% SDS-PAGE with a 5% stacking gel and electrophoretically transferred to nitrocellulose. GS was identified using affinity-purified polyclonal antibody raised in rabbits against an oligopeptide (12-mer) specific for the carboxy terminal sequence of the enzyme (a gift from Dr. L. Groop, University of Lund, Malmö, Sweden). The secondary antibody for GS was anti–rabbit IgG conjugated with horseradish peroxidase. Proteins were visualized with an enhanced chemiluminescence Western blot detection kit, and exposed to autoradiograph film (XAR-5; Eastman-Kodak Co., Rochester, NY). The autoradiograms were quantified by scanning laser densitometry (Stratoscan 7000; Stratagene Inc., La Jolla, CA). Densitometric results were normalized to an internal standard (rabbit skeletal muscle glycogen synthase) and expressed as arbitrary units per milligram protein.

Ribonuclease protection assay. Total cellular RNA was extracted and purified by a single-step method using RNA STAT 60 according to manufacturer's instructions. Details of the RNase protection assay have been described previously (12). Briefly, a full length single-stranded RNA probe of 139 bases was used to hybridize to glycogen synthase mRNA, and a DNA probe was generated to identify the human β actin gene by slot blot. Bands were scanned and quantitated by densitometry with results expressed in arbitrary units as the ratio of the glycogen synthase optical density units to β actin signal. β actin was used to confirm that mRNA changes were specific and that equal amounts of total RNA were loaded on the gel. Subsaturating

Table I. Clinical Characteristics

Group	Age	BMI	FSI	FPG	2-h OGTT	
					Glucose	Insulin
	yr	*kg/m²*	*pM*	*mM*	*mM*	*pM*
Nondiabetic (*n* = 8)	42±2	23.9±1.0	32±12	5.5±0.1	6.3±0.5	254±84
NIDDM (*n* = 11)	50±2*	30.6±0.8*	108±15*	10.7±1.4*	16.0±2.5‡	271±85

BMI, body mass index; FSI, fasting serum insulin; FPG, fasting plasma glucose; OGTT, 75-g oral glucose tolerance test. *$P < 0.01$ vs nondiabetic subjects; ‡$P < 0.001$ vs nondiabetic subjects.

amounts of β actin (5 μg/lane) and multiple autoradiogram exposures were performed to ensure that band intensity was in the linear range and any differences would be readily apparent.

Calculations and statistical methods. The V_{max} and K_m of GS for UDP-glucose were determined by the least-squares fit to a rectangular hyperbola using the computer program PRIZM (GraphPAD Software for Science, San Diego, CA). The $A_{0.5}$ is the G-6-P concentration required for half-maximal activity of glycogen synthase measured at 0.2 mM UDP-glucose. The $A_{0.5}$ and Hill coefficient (h) were determined by fitting the data to a linear form of the Hill equation (20): $\log (V_a - V_0)/(V_{max \propto G\text{-}6\text{-}P} - V_a) = h \log A - \log A_{0.5\ G\text{-}6\text{-}P}$ where V_a is the measured velocity of the enzyme at subsaturating G-6-P concentrations, V_0 is the reaction velocity in the absence of G-6-P, $V_{max \propto G\text{-}6\text{-}P}$ is the calculated velocity at infinite G-6-P concentration and A is the concentration of G-6-P. The Hill coefficient is defined by the slope of the line and $A_{0.5}$ from the X-intercept. Statistical analyses were performed using StatView II (Abacus Concepts, Inc., Berkeley, CA). All data are expressed as mean±SEM. Statistical significance was evaluated using Student's t test and two-tailed P values calculated. Paired analysis was performed for comparisons of insulin exposure in the same sets of cells. Significance was accepted at the $P <$ 0.05 level.

Results

Glycogen synthase activity. GS activity was compared after fusion in cells from 8 nondiabetic subjects and 11 NIDDM patients. In NIDDM cell cultures, basal GS activity at 0.1 mM G-6-P was ∼ 47% lower than in cells of nondiabetic subjects (Table II). Total activity at 10 mM G-6-P tended to be lower, but was also not significantly so, in NIDDM cells. Basal GS FV was ∼ 40% lower in NIDDM ($P <$ 0.05). In agreement with our previous report (12), acute stimulation of myotubes from nondiabetic subjects with 33 nM insulin (1 h) resulted in significant elevations of GS activity at 0.1 mM G-6-P as well as FV (Table II). Maximal insulin stimulation also increased GS activity at 0.1 mM G-6-P and FV in cultures of NIDDM, but only to levels of activity that were significantly less than insulin-stimulated values in nondiabetic controls. Indeed, after insulin stimulation, GS activity in NIDDM cells was comparable to that present in the basal state of nondiabetic cells.

Glycogen synthase kinetics. To elucidate the mechanisms of reduced activity and impaired insulin stimulation of muscle GS

Figure 1. Substrate dependence of GS. Mean basal (*closed symbols*) and insulin-stimulated (*open symbols*) activation curves of GS in nondiabetic subjects (○) and NIDDM patients (□) as a function of increasing concentrations of UDP-glucose at subsaturating G-6-P concentration (0.1 mM). Cells were grown and differentiated in the presence of normal physiologic concentrations of glucose (5.5 mM) and insulin (22 pM). After a 1-h incubation in serum-free media, cells were assayed in the absence or presence of insulin (33 nM, 1 h). GS activity was measured in Sephadex G-25 filtrates as described in Methods. Results are from muscle cultures of six nondiabetic subjects and six NIDDM patients.

in NIDDM, the kinetic characteristics of the enzyme were assessed over a range of substrate (UDP-glucose) and allosteric activator (G-6-P) concentrations. To exclude the influence of endogenous G-6-P and other low molecular weight metabolites on GS activation by G-6-P, we performed kinetic studies in cell extracts after filtration through a Sephadex G-25 column.

Fig. 1 depicts the mean basal and insulin-stimulated reaction rates of GS at 0.1 mM G-6-P as a function of increasing UDP-glucose concentrations in nondiabetic and NIDDM muscle cultures. GS from NIDDM muscle displayed modest reductions in basal activity that were more pronounced at low levels of UDP-glucose (< 2 mM). Insulin-stimulated GS activity, however, was markedly impaired at all UDP-glucose levels in muscle cells of NIDDM. Consistent with these activation curves, both the basal and insulin-stimulated $K_{m_{0.1}}$ of GS were significantly higher in NIDDM than in nondiabetic muscle (Table III). Acute insulin stimulation (33 nM, 1 h) did not af-

Table II. Basal and Insulin-stimulated Glycogen Synthase Activity in Nondiabetic and NIDDM Muscle Cultures

	Basal state		Stimulated state	
	Nondiabetic	NIDDM	Nondiabetic	NIDDM
0.1 mM G-6-P	0.32±0.10	0.17±0.04	0.61±0.15*	0.30±0.08‡
10 mM G-6-P	3.15±0.53	2.75±0.34	3.51±0.44	2.86±0.34
FV (%)	9.01±1.25	5.21±0.79*	16.31±2.39*	9.35±1.81‡

Values are mean±SEM. Basal state, no insulin added to serum-free media; and stimulated state, 33 nM insulin added to serum-free media for 1 h. Glycogen synthase results at 0.1 mM and 10 mM G-6-P are expressed as nanomoles per minute per milligram protein and FV in percent. *$P <$ 0.05 vs basal activity in cultures of nondiabetic subjects; ‡$P <$ 0.05 vs stimulated activity in cultures of nondiabetic subjects; $n =$ 8 nondiabetic and 11 NIDDM cultures.

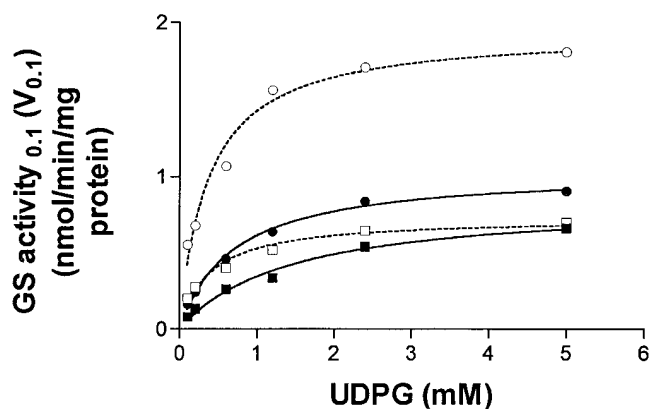*Table III. Effect of Acute Insulin Stimulation on Kinetic Constants of Glycogen Synthase in Nondiabetic and NIDDM Muscle Cultures*

Parameter	Basal state		Stimulated state	
	Nondiabetic	NIDDM	Nondiabetic	NIDDM
$K_{m_{0.1}}$	0.40±0.07	1.67±0.37*	0.38±0.05	0.69±0.06*‡
$V_{max_{0.1}}$	0.67±0.09	0.76±0.14	1.84±0.12§	1.04±0.28*
$K_{m_{10}}$	0.34±0.09	0.36±0.09	0.34±0.07	0.41±0.09
$V_{max_{10}}$	8.23±1.59	9.17±2.37	11.18±1.45	10.58±2.81
$A_{0.5}$	1.71±0.39	2.65±0.40	0.61±0.08§	2.07±0.40*
Hill coefficient	1.13±0.06	1.23±0.03	1.22±0.07	1.14±0.03

*$P <$ 0.05 vs corresponding value in cultures of nondiabetic subjects; ‡$P <$ 0.05 vs basal NIDDM values; and §$P <$ 0.05 vs basal nondiabetic values. $n =$ six nondiabetics and six NIDDM cultures.
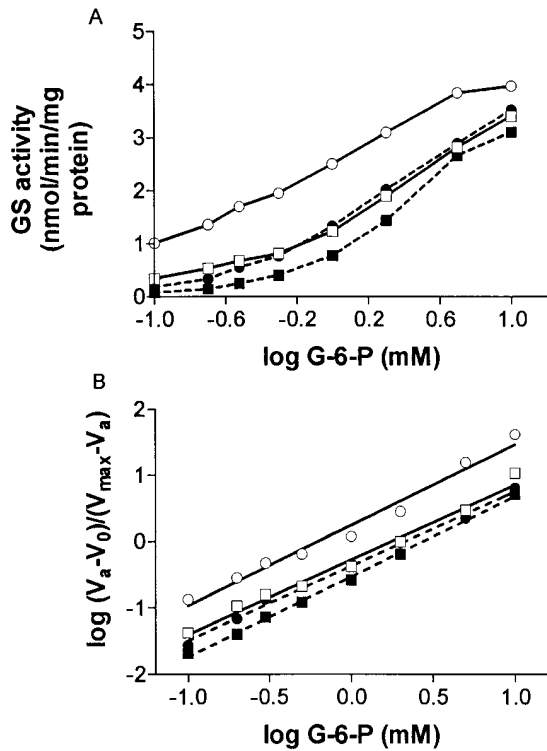
Figure 2. Allosteric activation of GS by G-6-P. (A) Mean basal (closed symbols) and insulin-stimulated (open symbols) activation curves of GS in nondiabetic subjects (○) and NIDDM patients (□) as a function of increasing concentrations of G-6-P and subsaturating concentrations of UDP-glucose (0.2 mM). (B) Hill plot of GS activation data. The Hill coefficient is defined by the slope of each line. Cells were grown and treated with insulin as described in Fig. 1. Results are from muscle cultures of six nondiabetic subjects and six NIDDM patients.

fect the $K_{m_{0.1}}$ in nondiabetic muscle, but lowered the abnormally elevated basal $K_{m_{0.1}}$ of NIDDM by 60%; this value was still significantly greater than the nondiabetic insulin-stimulated value, but similar to the nondiabetic basal value. The basal $V_{max_{0.1}}$ was comparable in the two groups, although the response to insulin-stimulation differed. In normal muscle cells, the basal $V_{max_{0.1}}$ increased ∼ 2.7-fold in response to insulin while it did not change in NIDDM cultures and remained significantly lower than in nondiabetic cultures (Table III).

Elevation of the G-6-P concentration from 0.1 to 10.0 mM lowered both the basal and insulin-stimulated $K_{m_{10}}$ of GS in NIDDM muscle to values similar to those in normal cells (Table III). Acute insulin did not influence the $K_{m_{10}}$ in either group. The basal $V_{max_{10}}$ (true V_{max}) was also comparable in both groups and equally unaffected by acute insulin administration.

Dose-response curves for allosteric activation of basal and insulin-stimulated GS in nondiabetic and NIDDM muscle cultures are shown in Fig. 2. The basal activation curve was slightly lower in the NIDDM compared with nondiabetics while the insulin-stimulated activation curve was markedly depressed in NIDDM and similar to the nondiabetic basal activation curve. When allosteric activation was compared using the $A_{0.5}$, the basal value tended to be higher in NIDDM, but not significantly so ($P = 0.08$). Acute insulin administration significantly decreased the $A_{0.5}$ of GS by 64% in nondiabetic muscle. The same treatment with insulin in NIDDM muscle resulted in a slight nonsignificant decrease in the $A_{0.5}$, which remained significantly greater than that of nondiabetics (Table III). The Hill coefficient was ∼ 1 in all study conditions of both groups, indicating that differences in allosteric cooperativity were not contributing to differences in GS activation.

Glycogen synthase mRNA and protein expression (Fig. 3). Myotubes from NIDDM patients expressed significantly lower immunoreactive GS protein than those from nondiabetic subjects (1.57±0.29 vs 3.30±0.41 arbitrary U/mg protein, $P <$ 0.05). Immunoreactive GS protein from NIDDM cells also displayed reduced electrophoretic mobility. In myotubes
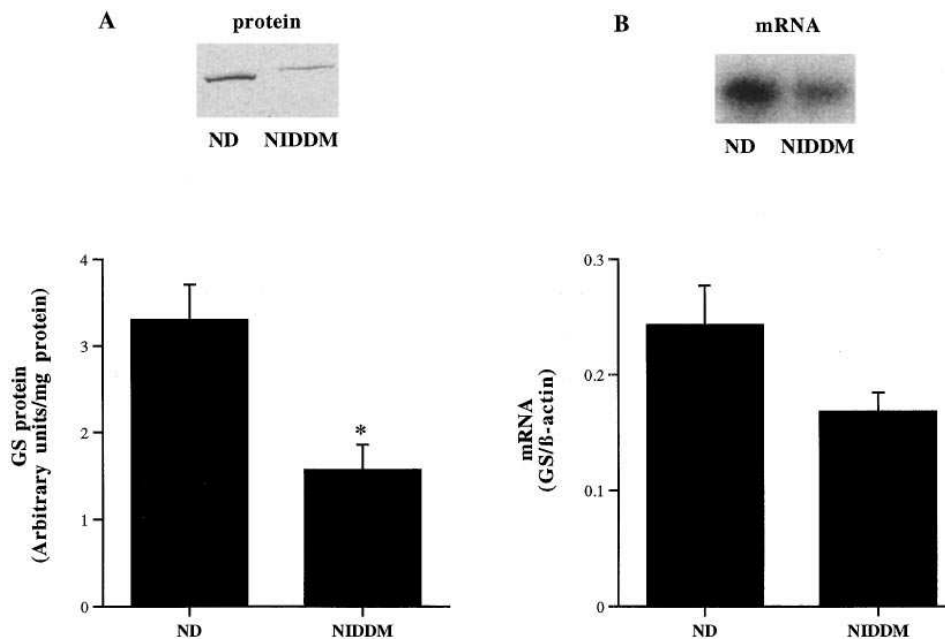

Figure 3. GS protein expression and mRNA abundance in nondiabetic and NIDDM muscle cultures. (A) Representative autoradiograph and mean±SEM of GS protein levels determined by Western blotting in 8 nondiabetic and 11 NIDDM muscle cultures. (B) Representative autoradiograph and mean±SEM of GS mRNA levels determined by RNase protection assay of four nondiabetic and four NIDDM muscle cultures. Cells were grown and fused in physiologic concentrations of glucose (5.5 mM) and insulin (22 pM). Western blotting was performed and results expressed in arbitrary units as described in Methods. 10 μg of total cellular protein was loaded in each lane. RNase protection assay was performed and results expressed in arbitrary units as described in Methods. 25 μg of total RNA was loaded in each lane. *$P < 0.05$ vs nondiabetic (ND) subjects.

of NIDDM subjects, mRNA levels tended to be lower (0.168 ± 0.017 vs 0.243 ± 0.034 arbitrary U), but the difference did not reach significance ($P = 0.08$) in comparison with myotubes from nondiabetic subjects.

Discussion

Decreased activity of GS, the key enzyme involved in nonoxidative glucose metabolism, has been a frequent observation in skeletal muscle of NIDDM patients (2, 4, 5, 21–23). Recent studies in cultured fibroblasts from NIDDM subjects have shown reduced rates of glycogen synthesis (14), consistent with the hypothesis that defects in this pathway may have genetic as well as acquired components. Attempts to identify significant mutations in the coding region of muscle-specific GS have not been successful (21, 22, 24–26). However, considerable evidence exists that supports an association between GS gene markers and NIDDM in various populations (27–30). These results suggest that GS may be involved in the development of NIDDM, but the inherited defect must be at some other loci that influences glycogen synthase activity per se.

One approach to study the potential genetic mechanisms of defective glucose use in skeletal muscle of NIDDM patients is to use a model system reflective of intact skeletal muscle that allows independent evaluation of the contribution of inherited factors from possible acquired complications of impaired metabolism such as hyperinsulinemia and hyperglycemia. In a series of recent publications, we have characterized human skeletal muscle cultures in our laboratory and demonstrated that these cells reflect the in vivo metabolic behavior of skeletal muscle (12, 13, 31). Using this technique, we undertook the present study to determine whether GS in skeletal muscle cell cultures of NIDDM subjects remained impaired after prolonged culture in normal glucose and insulin levels and, if so, by what mechanisms.

Even after removal of NIDDM muscle from the abnormal metabolic milieu of the diabetic state for 4–6 wk, the impaired GS activity observed in vivo was retained in cultured myotubes. Basal FV of GS was reduced by 42% in NIDDM compared to nondiabetic muscle. Insulin-stimulated GS activity at 0.1 mM G-6-P and FV were also reduced by 40–50% in NIDDM muscle (Table II). Insulin was able to stimulate GS in cells from both groups, but activity in NIDDM cells increased only to the basal level of nondiabetic cells. These results are in agreement with in vivo results from muscle biopsies reported by several laboratories (2, 4, 5, 21, 22), and provide additional evidence that muscle cultures reflect the metabolic behavior of intact skeletal muscle.

Reduced GS activity in NIDDM muscle could be the result of several abnormalities: an absolute reduction in the amount of the enzyme, changes in the intracellular levels of allosteric or covalent activators, impaired kinetic properties, or altered posttranslational modification of the enzyme. GS mRNA from muscle cultures of NIDDM subjects was reduced by $\sim 30\%$ compared with cells from nondiabetic subjects. This difference in GS mRNA just failed to reach statistical significance ($P = 0.08$), probably due to the small number of cultures studied (four of each group). GS mRNA has been reported to be reduced in muscle biopsy specimens from NIDDM patients by some (21, 22) but not all investigators (32). Thus, while transcriptional abnormalities may contribute to impaired skeletal muscle GS activity in NIDDM, additional studies in a larger number of NIDDM muscle cultures will be required to confirm whether this is a consistent defect.

GS protein expression, determined by Western blotting in NIDDM myotubes, was also reduced to $\sim 50\%$ of the value in nondiabetic control cells. Previously, the $V_{\mathrm{max}_{10}}$ (true V_{max}), measured at saturating levels of substrate and allosteric activator, has been used as an indictor of the total amount of glycogen synthase protein (5). However, the $V_{\mathrm{max}_{10}}$ activity of GS in these NIDDM muscle cultures was similar to that of the nondiabetic group (Table III) and did not correspond to changes in GS protein measured by immunoblotting. Majer et al. (26) have recently reported reduced immunoreactive GS protein in skeletal muscle biopsies from insulin resistant nondiabetic subjects despite a normal $V_{\mathrm{max}_{10}}$. Like us, these investigators used an antipeptide antibody directed against the COOH-terminal region of the GS protein (22) and concluded that the reduced immunoreactivity could reflect posttranslational modification(s) of the epitope that impaired antibody binding (26). The COOH-terminal region of GS protein contains several serine phosphorylation sites and it is possible that altered phosphorylation of these residues in NIDDM could influence the affinity of the GS-antibody reaction. Consistent with this possibility, differences in the electrophoretic mobility of glycogen synthase were also apparent in muscle from nondiabetic and NIDDM subjects (Fig. 3).

In keeping with our results, a reduction in the absolute amount of GS protein should result in reduced activity at 0.1 mM G-6-P (Table II). However, a reduction in protein alone should not alter FV, since if this were the only abnormality, the enzyme activity at both 0.1 and 10 mM G-6-P should be comparably reduced. In support of additional defects in NIDDM muscle, we found both basal and insulin-stimulated FV to be reduced, indicating that the enzymatic activity of the GS protein in NIDDM muscle was impaired. This abnormal behavior was evident from kinetic assessment of the enzyme. GS from NIDDM cells had a lower affinity (higher $K_{\mathrm{m}_{0.1}}$) for substrate (UDP-glucose) at physiologic G-6-P levels in the basal state. Acute insulin stimulation partially reversed this defect, but did not normalize the $K_{\mathrm{m}_{0.1}}$ (Table III). In nondiabetic muscle, insulin also improves allosteric activation by G-6-P with reduction of the activation constant ($A_{0.5}$). In NIDDM muscle, however, there was no significant change in the $A_{0.5}$ in response to insulin (Table III). As shown previously in muscle biopsy specimens (5), we have now confirmed that multiple kinetic defects of glycogen synthase persist in skeletal muscle cultures of NIDDM patients even in the absence of the metabolic derangements of diabetes.

These multiple kinetic defects could all be secondary to an increased glycogen synthase phosphorylation state (33). Skeletal muscle GS is regulated by both allosteric modification and phosphorylation/dephosphorylation (34). Insulin activates the enzyme by dephosphorylation, which is accompanied by a change in the affinity for G-6-P and UDP-glucose (35, 36). With increased phosphorylation of GS, there is decreased affinity of the enzyme for UDP-glucose and G-6-P (33). Furthermore, GS can be reactivated by binding G-6-P, which reverses the low affinity for UDP-glucose. The results of the current study are consistent with what is known about the regulation of this enzyme and indicate that the abnormal kinetic behavior in NIDDM muscle is possibly due to altered phosphorylation of the enzyme.

Because NIDDM muscle cultures are grown in conditions of normal glucose and insulin levels, and undergo multiple cell

doublings before fusion into multinucleated myotubes, it might be expected that any acquired component of defective GS activity would be reversed under such circumstances. Alternately, it is conceivable that an irreversible acquired defect could persist in culture. What is more probable is that abnormalities in muscle GS from NIDDM patients involve both an inherent and possibly genetic defect in combination with acquired adverse effects from metabolic derangements present in vivo, as we reported earlier (13).

In summary, GS activity is impaired in muscle cultures of NIDDM subjects and is not normalized after prolonged culture in normal physiologic conditions. In myotubes from NIDDM patients, defects in both basal and insulin-stimulated GS activity persist in culture and involve altered kinetic behavior and possibly reduced GS protein expression as well. We conclude that impaired GS activity in NIDDM involves multiple nonreversible component(s) that exist independent of extracellular metabolic disturbances. Defects occur at both pre- and posttranslational levels and may be genetic in origin.

Acknowledgments

References

1. Mandarino, L.J., K.S. Wright, L.S. Verity, J. Nichols, J.M. Bell, O.G. Kolterman, and H. Beck-Nielsen. 1987. Effect of insulin infusion on human skeletal muscle pyruvate dehydrogenase, phosphofructokinase, and glycogen synthase. Evidence for their role in oxidative and nonoxidative glucose metabolism. *J. Clin. Invest.* 80:655–663.

2. Bak, J.F., N. Moller, A. Saaek, and O. Pedersen. 1992. In vivo insulin action and muscle glycogen synthase activity in Type 2 (non–insulin-dependent) diabetes mellitus: effects of diet treatment. *Diabetologia.* 35:777–784.

3. Shulman, G.I., D.L. Rothman, T. Jue, P. Srein, and R.A. DeFronzo. 1990. Quantitation of muscle glycogen synthesis in normal subjects and subjects with non–insulin-dependent diabetes by ¹³C nuclear magnetic resonance spectroscopy. *N. Engl. J. Med.* 322:223–228.

4. Thorburn, A.W., B. Gumbiner, F. Bulacan, P. Wallace, and R.R. Henry. 1990. Intracellular glucose oxidation and glycogen synthase activity are reduced in non–insulin-dependent (type II) diabetes independent of impaired glucose uptake. *J. Clin. Invest.* 85:522–529.

5. Thorburn, A.W., B. Gumbiner, F. Bulacan, G. Brechtel, and R.R. Henry. 1991. Multiple defects in muscle glycogen synthase activity contribute to reduced glycogen synthesis in non–insulin dependent diabetes mellitus. *J. Clin. Invest.* 87:489–495.

6. Schalin-Jantti, C., M. Harkonen, and L.C. Groop. 1992. Impaired activation of glycogen synthase in people at increased risk for developing NIDDM. *Diabetes.* 41:598–604.

7. Vaag, A., J.E. Henriksen, and H. Beck-Nielsen. 1992. Decreased insulin activation of glycogen synthase in skeletal muscles in young nonobese Caucasian first-degree relatives of patients with non–insulin-dependent diabetes mellitus. *J. Clin. Invest.* 89:782–788.

8. Eriksson, J., A. Franssila-Kallunki, A. Ekstrand, C. Saloranta, E. Widen, C. Schalin, and L. Groop. 1989. Early metabolic defects in persons at increased risk for non–insulin dependent diabetes mellitus. *N. Engl. J. Med.* 321:337–341.

9. Ghosh, S., and N.J. Schork. 1996. Pespectives in diabetes. Genetic analysis of NIDDM. The study of quantitative traits. *Diabetes.* 45:1–14.

10. DeFronzo, R.A., and E. Ferrannini. 1991. Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care.* 14:173–194.

11. Beck-Nielsen, H., A. Vaag, P. Damsbo, A. Handberg, O.H. Nielsen, J.E. Henriksen, and P. Thye-Ronn. 1992. Insulin resistance in skeletal muscle of patients with NIDDM. *Diabetes Care.* 15:418–429.

12. Henry, R.R., L. Abrams, S. Nikoulina, and T.P. Ciaraldi. 1995. Insulin

13. Henry, R.R., T.P. Ciaraldi, S. Mudaliar, L. Abrams, and S.E. Nikoulina. 1996. Acquired defects of glycogen synthase activity in cultured human skeletal muscle cells: influence of high glucose and insulin levels. *Diabetes.* 45:400–407.

14. Wells, A.M., I.C. Sutcliffe, A.B. Johnson, and R. Taylor. 1993. Abnormal activation of glycogen synthesis in fibroblasts from NIDDM subjects. Evidence for an abnormality specific to glucose metabolism. *Diabetes.* 42:583–589.

15. National Diabetes Data Group. 1979. Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. *Diabetes.* 28:1039–1057.

16. Blau, H.M., and C. Webster. 1981. Isolation and characterization of human muscle cells. *Proc. Natl. Acad. Sci. USA.* 78:5623–5627.

17. Sarabia, V., L. Lam, E. Burdett, L.A. Leiter, and A. Klip. 1992. Glucose transport in human skeletal muscle cells in culture. Stimulation by insulin and metformin. *J. Clin. Invest.* 90:1386–1395.

18. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 71:248–254.

19. Burnette, W.N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195–203.

20. Henderson, P.J.F. 1992. Statistical analysis of enzyme kinetic data. *In* Enzyme Assays. A practical approach. R. Eisenthal and M.J. Danson, editors. Oxford University Press Inc., New York. 277–316.

21. Vestergaard, H., C. Bjorbaek, P.H. Andersen, J.F. Bak, and O. Pedersen. 1991. Impaired expression of glycogen synthase mRNA in skeletal muscle of NIDDM patients. *Diabetes.* 40:1740–1745.

22. Vestergaard, H., S. Lund, F.S. Larsen, O.J. Bjerrum, and O. Pedersen. 1993. Glycogen synthase and phosphofructokinase protein and mRNA levels in skeletal muscle from insulin-resistant patients with non–insulin-dependent diabetes mellitus. *J. Clin. Invest.* 91:2342–2350.

23. Bogardus, C., S. Lillioja, K. Stone, and D. Mott. 1984. Correlation between muscle glycogen synthase activity and in vivo insulin action in man. *J. Clin. Invest.* 73:1185–1190.

24. Chen, Y.H., L. Hansen, M.X. Chen, C. Bjorbaek, H. Vestergaard, T. Hansen, P.T.W. Cohen, and O. Pedersen. 1994. Sequence of the human glycogen-associated regulatory subunit of type 1 protein phosphatase and analysis of its coding region and mRNA level in muscle from patients with NIDDM. *Diabetes.* 43:1234–1241.

25. Orho, M., P. Nikula-Ijas, C. Schalin-Jantti, M.A. Permutt, and L.C. Groop. 1995. Isolation and characterization of the human muscle glycogen synthase gene. *Diabetes.* 44:1099–1105.

26. Majer, M., D.M. Mott, H. Mochizuki, J.C. Rowles, O. Pedersen, W.C. Knowler, C. Bogardus, and M. Prochazka. 1996. Association of the glycogen synthase locus on 19q13 with NIDDM in Pima Indians. *Diabetologia.* 39:314–321.

27. Groop, L.C., M. Kankuri, C. Schalin-Jantti, A. Ekstrand, P. Nikula-Ijas, E. Widen, E. Kuismanen, J. Eriksson, A. Franssila-Kallunki, C. Saloranta, and S. Koskimies. 1993. Association between polymorphism of the glycogen synthase gene and non–insulin-dependent diabetes mellitus. *N. Engl. J. Med.* 328:10–14.

28. Zouali, H., G. Velho, and P. Froguel. 1993. Polymorphism of the glycogen synthase gene and non–insulin-dependent diabetes mellitus. *N. Engl. J. Med.* 328:1568.

29. Kuroyama, H., T. Sanke, S. Ohangi, M. Furanta, H. Furata, and K. Nanjo. 1994. Single tandem repeat DNA polymorphism in the human glycogen synthase gene is associated with NIDDM in Japanese subjects. *Diabetologia.* 37:536–539.

30. Seldin, M.F., D. Mott, D. Bhat, A. Petro, C.M. Kuhn, S.F. Kingsmore, C. Bogardus, E. Opara, M.N. Feinglos, and R.S. Surwit. 1994. Glycogen synthase: a putative locus for diet-induced hyperglycemia. *J. Clin. Invest.* 94:269–276.

31. Ciaraldi, T.P., L. Abrams, S. Nikoulina, S. Mudaliar, and R.R. Henry. 1995. Glucose transport in cultured human skeletal muscle cells: regulation by insulin and glucose in nondiabetic and non–insulin-dependent diabetes mellitus subjects. *J. Clin. Invest.* 96:2820–2827.

32. Lofman, M., H. Yki-Jarvinen, M. Parkkonen, J. Lindstrom, L. Koranyi, C. Schalin-Jantti, and L. Groop. 1995. Increased concentrations of glycogen synthase protein in skeletal muscle of patients with NIDDM. *Am. J. Physiol.* 269:27–32.

33. Roach, P.J., and J. Larner. 1977. Covalent phosphorylation in the regulation of glycogen synthase activity. *Mol. Cell. Biochem.* 15:179–200.

34. Dent, P., A. Lavoinne, S. Nakielny, F.B. Caudwell, P. Watt, and P. Cohen. 1990. The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. *Nature (Lond.).* 348:302–308.

35. Skurat, A.V., Y. Wang, and P.J. Roach. 1994. Rabbit skeletal muscle glycogen synthase expressed in COS cells. Identification of regulatory phosphorylation sites. *J. Biol. Chem.* 269:25534–25542.

36. Zhang, W.A., A. DePaoli-Roach, and P. Roach. 1993. Mechanisms of multisite phosphorylation and inactivation of rabbit muscle glycogen synthase. *Arch. Biochem. Biophys.* 304:219–225.

action and glucose metabolism in non-diabetic control and NIDDM subjects: comparison using human skeletal muscle cell cultures. *Diabetes.* 44:936–946.