Impact of Genetic Versus Environmental Factors on the Control of Muscle Glycogen Synthase Activation in Twins

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Storage of glucose as glycogen accounts for the largest proportion of muscle glucose metabolism during insulin infusion in normal and insulin-resistant subjects. Studies in first-degree relatives have indicated a genetic origin of the defective insulin activation of muscle glycogen synthase (GS) in type 2 diabetes. The aim of this study was to evaluate the relative impact of genetic versus nongenetic factors on muscle GS activation and regulation in young and elderly twins examined with a 2-h euglycemic-hyperinsulinemic (40 mU · m^{-2} · min^{-1}) clamp combined with indirect calorimetry and excision of muscle biopsies. The etiological components were determined using structural equation modeling. Fractional GS activity; GS phosphorylation at sites 2, 2 + 2a, and 3a + 3b corrected for total GS protein; and GS kinase 3 (GSK3) activity were similar in both age groups, whereas total GS activity and protein were lower in elderly compared with younger twins. GS fractional activity increased significantly during insulin stimulation in both young and elderly twins. Conversely, there was a significant decrease in GS phosphorylation at site 3a + 3b and GSK3 activity during insulin stimulation in both age groups, whereas GS phosphorylation at site 2 and 2 + 2a only decreased on insulin stimulation in the younger twins. The increment in whole-body glucose disposal (R_d) and nonoxidative glucose metabolism (insulin − basal) correlated significantly with the increment in GS fractional activity. Fractional GS activity had a major environmental component in both age groups. GSK3 activity exhibited a genetic component in young (basal: $a^2 = 0.42$; insulin: $a^2 = 0.58$) and elderly (insulin: $a^2 = 0.56$) twins. Furthermore, GS phosphorylation at site 2 (insulin: $a^2 = 0.69$) in the elderly and at site 3a + 3b (insulin: $a^2 = 0.50$) in the young twins had a genetic component. In conclusion, GSK3 activity and GS phosphorylation, particularly at sites 2 and 3a + 3b, had major genetic components. Total and fractional GS activities per se were, on the other hand, predominantly controlled by environmental factors. Moreover, GS activity was intact with increasing age, despite a significant reduction in nonoxidative glucose metabolism. *Diabetes* 54:1289–1296, 2005

Insulin resistance in skeletal muscle tissue represents a major defect in type 2 diabetes and associated states of disease, including obesity, hypertension, and dyslipidemia (1). Storage of glucose as glycogen accounts for the quantitatively largest proportion of muscle glucose metabolism during insulin infusion in the physiological range in normal subjects, and similarly it represents a major defect of muscle glucose metabolism in insulin-resistant states (2–4). Previous studies have shown that muscle membrane glucose transport is a rate-limiting step in various normal and insulin-resistant conditions (5–7). Nevertheless, other studies have indicated that postmembrane enzymatic steps and in particular muscle glycogen synthase (GS) enzyme activity exert an independent rate-limiting step of insulin-stimulated muscle glucose metabolism in various nondiabetic and diabetic states (4,6,8,9).

Studies among first-degree relatives (4,10) and in human muscle cell cultures from type 2 diabetic patients (11) have indicated a genetic origin of the defective insulin activation of muscle GS in type 2 diabetes. In addition, a higher frequency of a polymorphism at the site of the muscle glycogen synthesis gene on chromosome 19 has been found in type 2 diabetic patients compared with control subjects (12,13). On the other hand, studies in elderly monozygotic twins discordant for type 2 diabetes showed that the defective muscle GS activation in type 2 diabetes also has a quantitatively major nongenetic component (14). Studies have demonstrated activation of muscle GS enzyme activity by reducing its phosphorylation state at site 2 (under supraphysiological insulin concentrations in animals) and at site 3a + 3b in healthy subjects and patients with type 2 diabetes (15,16). A key insulin-regulated enzyme responsible for the phosphorylation (and thereby inactivation) of muscle GS is GS kinase 3 (GSK3) (17).

Nongenetic factors of importance for the development of insulin resistance and type 2 diabetes are age, obesity, physical inactivity, and an adverse intrauterine environ-

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G6P, glucose-6-phosphate; GS, glycogen synthase; GSK3, GS kinase 3; HRP, horseradish peroxidase; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; TBST, Tris-buffered saline plus Tween.

ment associated with low birth weight (18). A more detailed understanding of the relative roles of the different etiological factors controlling muscle GS activity in humans may be crucial for our understanding of insulin resistance and type 2 diabetes. Twin studies represent a unique tool to determine the extent to which genetic versus nongenetic factors influence biological variables. We recently reported that insulin-stimulated nonoxidative glucose metabolism is controlled equally by genetic and nongenetic factors in a large and extensively characterized Danish twin population (19). The aim of this study was to evaluate the relative impact of genetic versus nongenetic factors on muscle GS activation and control in young and elderly twins studied using the euglycemic-hyperinsulinemic clamp technique with the excision of biopsies from skeletal muscles in the basal as well as the insulin-stimulated state.

RESEARCH DESIGN AND METHODS

Subjects were identified through the Danish Twin Register (19), as previously described. A random extract of monozygotic and same-sex dizygotic twin pairs born in Funen County from 1931 to 1940 (57–66 years of age) and from 1966 to 1975 (22–31 years of age) were contacted and interviewed. The exclusion criteria were: either twin from a pair not willing to participate; information of pre- or postmaturity (>3 weeks from expected time point) known diabetes; serious heart, liver, or kidney disease; medication with influence on glucose or lipid metabolism, including oral contraception that could not be withdrawn; and pregnancy/lactation.

A total of 184 twins (young monozygotic: 31 pairs, 1 single twin; young dizygotic: 19 pairs; elderly monozygotic: 20 pairs; and elderly dizygotic: 21 pairs, 1 single twin) were enrolled in the clinical examination. On an oral glucose tolerance test, 80.0% of the elderly monozygotic twins had normal glucose tolerance (NGT), 15.0% had impaired glucose tolerance (IGT), and 5.0% had previously unknown type 2 diabetes. Among the elderly dizygotic twins, 74.7% had NGT, 23.0% had IGT, and 2.3% had previously unknown type 2 diabetes. All younger dizygotic twins had NGT, and among younger monozygotic twins, 96.8% had NGT and 3.2% IGT. There was no significant difference in glucose tolerance status between monozygotic and dizygotic twins within each age group. Zygosity was determined by polymorphic genetic markers (20). The study was approved by the regional ethical committee, and the study was conducted according to the principles of the Helsinki Declaration.

Clinical examination. Subjects underwent a 2-day clinical examination separated by 1–2 weeks. The two twins in each pair were investigated simultaneously. The subjects were instructed to abstain from strenuous physical activity for 24 h and to perform a 10- to 12-h overnight fast before both examination days. Day 1 included a standard 75-g oral glucose tolerance test and anthropometric measures (i.e., the waist-to-hip ratio and BMI, calculated as weight in kilograms divided by height in meters squared). Body composition, i.e., lean body mass and fat mass, was determined by dual-energy X-ray absorptiometry scanning (21). Furthermore, indirect maximal aerobic capacity ($V\mathrm{O}_{2\mathrm{max}}$) was measured by bicycle testing (22).

On day 2, subjects underwent a 2-h hyperinsulinemic-euglycemic clamp as previously described (19). Briefly, a primed-continuous infusion of constant [3-^{3}H]-titrated glucose (bolus 22 μCi, 0.22 μCi/min) was initiated at 0 min and continued throughout the clinical investigation (basal period [120 min] and clamp period [120 min]). Steady state was defined as the last 30 min of the basal period. A primed-continuous insulin infusion (40 mU · m^{-2} · min^{-1}) was subsequently initiated and continued for 120 min. Steady state was defined as the last 30 min of the 2-h clamp period, when tracer equilibrium (i.e., constant specific activity) was anticipated. A variable infusion of glucose (180 g/l) enriched with titrated glucose (hot-GINF, 100 μCi/500 ml) maintained euglycemia during insulin infusion. Plasma glucose concentration was monitored every 5–10 min during the basal and clamp periods, using an automated glucose oxidation method (Glucose Analyser 2; Beckman Instruments, Fullerton, CA). Indirect calorimetry was performed during both steady-state periods using a computerized flow-through canopy gas analyzer system (Deltatrac; Datex, Helsinki, Finland). After an equilibrium period of 10 min, the average gas exchange rates recorded over the steady-state periods were used to calculate rates of glucose oxidation (23). Glucose disposal rate (R_d) was calculated at 10-min intervals during the steady-state periods, as previously described (19). Nonoxidative glucose metabolism was calculated as R_d − glucose oxidation, as determined by indirect calorimetry. Glucose turnover rates (mg · kg lean body mass^{-1} · min^{-1}) are presented as the mean values of the 30-min steady-state periods.

Biopsy handling. Muscle biopsies were taken during the basal and insulin-stimulated steady-state periods. The biopsies were obtained from the vastus lateralis muscle under local anesthesia (lidocaine) using a Bergström needle with suction applied. The biopsy specimens were quickly blotted on filter paper and frozen in liquid nitrogen. The biopsies were stored at −80°C until further processed.

Muscle lysate. We used ~50 mg of the biopsy specimens for muscle homogenate and lysate preparation. The muscle specimens were pulverized and homogenized, using a homogenizer (Polytron PT 3100; Kinematica) in a buffer (1:20 wt:vol) containing: 50 mmol/l HEPES (pH 7.5), 150 mmol/l NaCl, 20 mmol/l Na-pyrophosphate, 20 mmol/l β-glycerophosphate, 10 mmol/l NaF, 2 mmol/l Na-ortovanadate, 2 mmol/l EDTA, 1% Nonidet P-40, 10% glycerol, 2 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 3 mmol/l benzamindine. Homogenates were rotated end over end for 1 h at 4°C. The homogenate aliquots (30 μl) were frozen in liquid nitrogen and stored at −80°C for later analysis of GS activity. Muscle homogenate was then cleared by centrifugation at 17,500*g* at 4°C for 1 h. The supernatant (lysate) was harvested, divided into several aliquots, frozen in liquid nitrogen, and stored at −80°C for later analysis. Unless specified differently, all chemicals used were obtained from Sigma-Aldrich (St. Louis, MO).

Lysate protein content. Protein content in the lysate was measured in a microtiter-based assay using the bicinchoninic acid method (Pierce, Rockford, IL).

GS activity. To 30 μl of muscle homogenate we added 90 μl buffer (containing 50 mmol/l Tris, pH 7.8, 100 mmol/l NaF, and 10 mmol/l EDTA) and then measured GS activity by incorporation of [^{14}C]UDP-glucose (Perkin Elmer, Boston, MA) into glycogen (24,25). GS activity was determined in the presence of 0.17 and 8 mmol/l glucose-6-phosphate (G6P) and given as the percent of fractional velocity (100 × activity in the presence of 0.17 mmol/l G6P divided by the activity at 8 mmol/l G6P). Total GS activity is activity in the presence of 8 mmol/l G6P (mmol · min^{-1} · kg wet wt muscle). The interassay coefficient of variation (CV) for the GS activity measurements was 3%.

Muscle glycogen. Glycogen content was determined as glycosyl units after acid hydrolysis of ~5 mg wet wt muscle, as described previously by Lowry et al. (26), using an automatic spectrophotometer (Cobas FARA 2, Roche Diagnostic). The interassay CV for glycogen measurements was 3%.

Muscle protein expression. The muscle content of various proteins was determined by SDS-PAGE and Western blotting (criterion-gel system; BioRad, Herlev, Denmark). Muscle lysate proteins were separated using 26-well 7.5 or 10% Bis-Tris gels (BioRad) and transferred (semidry) to polyvinylidene difluoride membranes (Immobilion transfer membrane; Millipore, Glostrup, Denmark). After blocking, the membranes were incubated with primary antibodies followed by incubation in horseradish peroxidase (HRP)-conjugated secondary antibody (see specific procedure for the different antibody used below). After detection with ECL$^+$ (Amersham Pharmacia Biotech, Chalfont St. Giles, U.K.) and quantification using a charge coupled device image sensor and 1D software (Kodak Image Station E440CF; Kodak, Glostrup, DK), the protein content was finally expressed in arbitrary units relative to a skeletal muscle standard. Several (non–insulin-stimulated and insulin-stimulated) rat samples were run on each gel, and variation between gels was accounted for by expressing data relative to these samples. Analysis of assay linearity between signal and amount of protein loaded was verified for each antibody used. The interassay CV for Western blot analysis was in the range of 6–8%.

In muscle lysate from both basal and insulin-stimulated biopsies, the phosphorylation of GS protein was determined on the following sites: phosphorylation GS site 2 (Ser 7), phosphorylation GS site 2 + 2a (Ser 7 + 10), and phosphorylation GS site 3a + 3b (Ser 640 + 644) using antibodies raised in sheep (16). Protein content of GS was determined using an antibody raised in rabbit (27). All blots were performed using overnight blocking (Tris-buffered saline plus Tween [TBST] + 1% skim milk), followed by incubation in the appropriate primary for 2 h at room temperature (in TBST + 1% skim milk). A HRP-conjugated secondary antibody (rabbit anti-sheep HRP #P0448 or goat anti-rabbit HRP #P0448 [Dako, Glostrup, Denmark], both in TBST + 1% skim milk) was used to identify and quantify the appropriate GS band (~89 kDa).

Enzyme activity. We measured the activity of GSK3-α in muscle lysate from both rested and insulin-stimulated biopsies: activity of GSK3-α was measured after immunopurification using an anti–GSK3-α antibody (sheep anti–GSK3-α #06-391; Upstate Biotechnology, Waltham, MA) followed by coupling to protein G Sepharose beads (Pierce Chemical, Rockford, IL). A [^{32}P]ATP and p81 filter paper assay, with the (85 μmol/l) phospho-GS2 peptide (YRRAAVPP

TABLE 1
Clinical characteristics among young and elderly monozygotic and dizygotic twins

	Young	Old	*P*
n (M/F)	101 (56/45)	83 (37/46)	—
Age (years)	28.0 ± 1.8	61.9 ± 2.3	<0.001
BMI (kg/m^2)	24.1 ± 3.2	26.3 ± 4.4	<0.001
Waist-to-hip ratio	0.84 ± 0.09	0.89 ± 0.10	<0.001
Fat (%)	22.2 ± 7.0	28.0 ± 9.6	<0.001
Lean (kg)	54.8 ± 11.2	50.5 ± 11.9	0.01
Fat (kg)	16.4 ± 6.3	20.8 ± 8.7	<0.001
R_d basal	3.1 ± 0.5	3.1 ± 0.4	0.28
R_d clamp	11.8 ± 3.2	9.9 ± 3.4	<0.001
Δ R_d	8.7 ± 3.0	6.8 ± 3.3	<0.001
NOGM basal	0.9 ± 1.0	1.3 ± 0.9	0.005
NOGM clamp	7.1 ± 2.8	5.8 ± 3.2	0.003
Δ NOGM	6.2 ± 2.8	4.5 ± 3.0	<0.001
$V\text{O}_{2max}$ (ml · min^{-1} · kg^{-1})	39.5 ± 7.8	26.1 ± 7.5	<0.001

Data are the means ± SD. All metabolic rates are expressed as mg · kg lean body mass^{-1} · min^{-1}. *P* value expresses the significance level for comparisons between young and elderly twins. Δ, difference between insulin and basal values (i.e. insulin − basal); NOGM, nonoxidative glucose metabolism.

SPSLSRHSSPHQpSEDEEE; Schafer-N, Copenhagen) as substrate, was used to measure GSK3-α activity, as described previously (28,29). The interassay CV for the GSK3 activity measurements was 14%.

Statistical methods

Biometric modeling. The total phenotypic variance is the sum of the variance attributable to effects of both genotype and environmental factors. The etiological models that were tested included the following contributions to variance: genetic variance caused by additive genetic effects (V_A) and environmental variance due to an individual environment not shared with the co-twin (V_E) or a common environment shared among co-twins (V_C). In the current study, heritability (h^2) is equivalent to a^2, which is the proportion of the total variance (V_{total}) attributed to additive genetic variance (V_A). We used the MX software package, a program for linear structural equation modeling, to estimate the variance components and to compare the different models. Genetic modeling was conducted separately in the two age groups. Raw data for all phenotypes were adjusted for an effect of sex in a regression analysis, and all analyses were carried out on the residuals. The fit of each model was assessed by maximum-likelihood methods and resulted in a χ^2 goodness-of-fit index and probability value that tested the agreement between the observed and the predicted statistics. A small goodness-of-fit χ^2 value, a high *P* value, and a low AIC (Akaike's Information Criterion), which equals the χ^2 value minus 2 times the degree of freedom, indicate good correspondence and were used in comparisons of each model, leading to a best-fitting model.

For some of the variables (i.e., unadjusted GS phosphorylation at site 2 + 2a and total GS protein during insulin stimulation), the variances and/or means across twin pairs and/or zygosity were not equal and therefore did not fulfill the criteria for model fitting. To achieve similar means and variances, we performed double entry of the data and adjusted the degree of freedom accordingly, allowing application of the model-fitting analysis on data for insulin-stimulated total GS protein.

RESULTS

Clinical characteristics. The elderly twins had significantly higher BMI, waist-to-hip ratio, and fat percentage compared with the younger twins (Table 1). Furthermore, the elderly twins were more insulin resistant compared with the younger twins, with a significantly lower insulin-stimulated R_d (11.8 ± 3.2 vs. 9.9 ± 3.4 for young vs. old, respectively; $P < 0.001$) and nonoxidative glucose metabolism (7.1 ± 2.8 vs. 5.8 ± 3.2, $P < 0.001$). Similarly, Δ (insulin − basal) R_d and nonoxidative glucose metabolism were significantly higher in younger compared with elderly twins. Additionally, the younger twins had a higher $V\text{O}_{2max}$ compared with elderly twins (Table 1).

Impact of age on GS activity and regulation. Among the elderly twins, we found significantly lower GS total activity compared with younger twins in both the basal and insulin-stimulated steady-state periods. However, the fractional GS activity was similar among young and elderly twins. The total amount of GS phosphorylation at site 3a + 3b, 2, and 2 + 2a was significantly reduced in elderly compared with younger twins during the basal period and at site 3a + 3b and 2 during insulin stimulation (Table 2). However, when adjusting for the total amount of GS protein, the degree of phosphorylation at all sites was similar in young and elderly twins. Total GS protein content was lower in elderly twins during insulin stimulation compared with younger twins ($P = 0.03$), whereas, during the basal period total GS protein was similar in younger and elderly twins. Glycogen content, GS fractional activity as well as GSK3 activity were similar in the two age groups.

Impact of insulin on GS activity and regulation. GS fractional activity increased significantly during insulin stimulation in both young and elderly twins. Conversely, there was a significant decrease in adjusted and unadjusted GS phosphorylation at site 3a + 3b and in the activity of GSK3 during the clamp period (Table 2). GS phosphorylation at site 2 + 2a was significantly reduced during insulin stimulation in both age groups. However, when adjusting for total GS protein, the reduction only remained significant among the younger twins. GS phosphorylation at site 2 was similar in the basal and insulin-stimulated periods among both young and elderly twins, although there was a tendency toward an increase in adjusted GS phosphorylation at site 2 among the younger twins ($P = 0.06$). The increment in GS fractional activity and the decrease in GS phosphorylation and GSK3 activity were similar in both age groups. Glycogen and GS total activity were similar in the basal and insulin-stimulated periods in both young and elderly twins.

Phenotypic correlations. Insulin-stimulated R_d and nonoxidative glucose metabolism correlated significantly with fractional GS activity during the clamp period in both young (R_d: $r = 0.40$; nonoxidative glucose metabolism: $r = 0.47$) and elderly twins ($r = 0.32$, $r = 0.29$). Among the elderly twins, no significant correlations were seen between insulin-stimulated glucose metabolism and GS regulation, whereas insulin-stimulated R_d and nonoxidative glucose metabolism additionally correlated significantly with adjusted and adjusted GS phosphorylation at sites 2 + 2a and 3a + 3b during the clamp period in the younger twins (Table 3). Maximal aerobic capacity ($V\text{O}_{2max}$) correlated significantly with basal and insulin-stimulated total GS activity in both young and elderly twins. Furthermore, significant correlations were seen between $V\text{O}_{2max}$ and total GS protein in the elderly twins (Table 3). Finally, the increment in R_d and nonoxidative glucose metabolism (Δ values) correlated significantly with the increment in GS fractional activity in both young (R_d: $r = 0.46$, $P < 0.00001$; nonoxidative glucose metabolism: $r = 0.45$, $P < 0.0001$) and elderly ($r = 0.43$, $P < 0.0001$; and $r = 0.41$, $P < 0.001$) twins (Fig. 1).

Biometric modeling. In the younger twins, the data for insulin-stimulated glycogen, basal fractional GS activity, adjusted basal GS phosphorylation at sites 3a + 3b and 2, and adjusted GS phosphorylation at site 2 + 2a correlated

TABLE 2
GS activity and regulation among young and elderly monozygotic and dizygotic twins

			P		
			Young versus elderly	Basal versus insulin	
	Young	Elderly		Young	Elderly
Basal					
Glycogen	90.0 ± 24.7	87.7 ± 25.0	0.47	0.57	0.70
GS total protein	657.5 ± 327.4	609.7 ± 379.5	0.19	0.08	0.06
GS total activity	4.9 ± 1.0	4.1 ± 1.0	<0.001	0.37	0.75
GS fractional activity	16.8 ± 5.8	16.5 ± 6.1	0.79	<0.001	<0.001
pGS site 3a + 3b	152.8 ± 98.3	128.0 ± 97.9	0.02	<0.001	<0.001
pGS site 3a + 3b*	0.27 ± 0.23	0.25 ± 0.16	0.63	<0.001	<0.001
pGS site 2	641.1 ± 335.4	524.5 ± 294.7	0.01	0.88	0.85
pGS site 2*	1.05 ± 0.50	1.05 ± 0.59	0.81	0.06	0.14
pGS site 2 + 2a	2,150.8 ± 1,105.4	1,739.1 ± 972.3	0.003	<0.001	0.002
pGS site 2 + 2a*	3.96 ± 2.49	4.04 ± 3.33	0.41	0.003	0.25
GSK3 activity	5.0 ± 1.1	5.2 ± 1.2	0.22	<0.001	<0.001
Insulin					
Glycogen	91.6 ± 23.4	86.2 ± 4.2	0.18	—	—
GS total protein	579.8 ± 311.2	505.5 ± 346.7	0.03	—	—
GS total activity	5.0 ± 1.1	4.2 ± 1.0	<0.001	—	—
GS fractional activity	32.7 ± 9.2	32.2 ± 10.0	0.70	—	—
pGS site 3a + 3b	88.7 ± 56.7	62.9 ± 52.0	<0.001	—	—
pGS site 3a + 3b*	0.17 ± 0.11	0.15 ± 0.09	0.24	—	—
pGS site 2	626.5 ± 314.3	515.9 ± 289.1	0.006	—	—
pGS site 2*	1.21 ± 0.62	1.17 ± 0.57	0.74	—	—
pGS site 2 + 2a	1,481.5 ± 853.5	1,304.4 ± 833.2	0.10	—	—
pGS site 2 + 2a*	2.98 ± 1.87	3.33 ± 2.33	0.52	—	—
GSK3 activity	3.5 ± 0.8	3.6 ± 0.9	0.23	—	—

Data are means ± SD. *P* value expresses the significance level for comparisons between young and elderly twins and basal versus insulin stimulation in young and elderly twins, respectively. *Adjusted for total GS content. pGS, GS phosphorylation.

more tightly among dizygotic twins compared with monozygotic twins, which is why application of ACE modeling was not possible (data not shown). A major genetic component was seen for basal GSK3 ($a^2 = 0.58$), whereas, insulin-stimulated GSK3 had a major environmental component ($a^2 = 0.42$) among the younger twins (Table 4). The model for adjusted insulin-stimulated GS phosphorylation at site 3a + 3b had equal genetic and unique environmental components ($a^2 = 0.50$, $e^2 = 0.50$). Adjusted insulin-stimulated GS phosphorylation at site 2

TABLE 3
Phenotypic correlations between maximal aerobic capacity (Vo_{2max}), insulin-stimulated glucose disposal (R_d clamp), and nonoxidative glucose metabolism clamp and GS activity and regulation

	Glycogen	GS total protein	GS total activity	GS fractional activity	pGS site 3a + 3b	pGS site 3a + 3b*	pGS site 2	pGS site 2*	pGS site 2 + 2a	pGS site 2 + 2a*	GSK3 activity
Young											
Basal											
R_d clamp	−0.01	0.08	0.11	0.19	−0.03	−0.08	0.02	−0.06	−0.12	−0.17	−0.01
NOGM clamp	−0.11	0.07	0.19	**0.30**	−0.01	−0.05	0.13	0.07	−0.10	−0.15	−0.00
Vo_{2max}	0.17	0.26	**0.28**	−0.01	0.06	−0.05	0.13	**−0.20**	−0.03	−0.15	−0.13
Insulin											
R_d clamp	−0.04	−0.01	0.08	**0.40**	**−0.29**	**−0.20**	−0.09	0.08	**−0.30**	**−0.23**	−0.06
NOGM clamp	−0.14	−0.01	0.17	**0.47**	**−0.26**	**−0.21**	−0.02	0.14	**−0.30**	**−0.25**	−0.08
Vo_{2max}	0.10	0.16	**0.28**	0.08	0.07	−0.11	0.01	−0.19	−0.15	**−0.25**	**−0.20**
Elderly											
Basal											
R_d clamp	−0.06	−0.01	−0.12	−0.03	−0.02	0.12	−0.01	0.07	0.06	0.08	0.04
NOGM clamp	−0.07	−0.01	−0.10	−0.03	−0.07	0.06	−0.03	0.09	0.06	0.07	−0.02
Vo_{2max}	0.13	**0.37**	**0.38**	−0.19	0.18	−0.13	**0.24**	−0.18	0.21	−0.15	−0.03
Insulin											
R_d clamp	−0.02	−0.17	−0.16	**0.32**	−0.15	−0.02	−0.11	0.11	−0.20	−0.09	0.05
NOGM clamp	−0.06	−0.15	−0.12	**0.29**	−0.16	−0.07	−0.10	0.12	−0.15	−0.05	−0.01
Vo_{2max}	0.09	**0.39**	**0.45**	−0.05	0.21	−0.21	0.15	**−0.23**	0.22	−0.13	−0.13

Significant correlations ($P < 0.05$) are shown in bold. NOGM, nonoxidative glucose metabolism; pGS, GS phosphorylation.

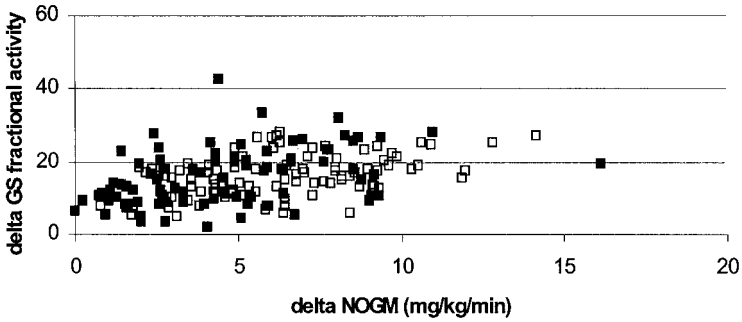

FIG. 1. Phenotypic correlation between Δ nonoxidative glucose metabolism and Δ GS fractional activity in young and elderly twins. The correlation coefficients were $r = 0.45$ for young and $r = 0.41$ for elderly twins. □, young, $P < 0.0001$; ■, elderly, $P < 0.0001$. NOGM, nonoxidative glucose metabolism.

had a major environmental component. The models for basal glycogen, basal and insulin-stimulated GS total protein and GS total activity, as well as insulin-stimulated fractional GS activity included only a unique environmental component (i.e., E model).

In the elderly twins, the data for insulin-stimulated muscle glycogen and fractional GS activity, adjusted basal GS phosphorylation at sites 3a + 3b and 2, and adjusted GS phosphorylation at site 2 + 2a during the basal state did not fulfill the criteria for ACE modeling because of a greater similarity within dizygotic compared with monozygotic twin pairs. A major genetic component was seen for GSK3 ($a^2 = 0.56$) and adjusted GS phosphorylation at site 2 ($a^2 = 0.69$) during insulin stimulation (Table 5). Basal glycogen had an equal genetic and environmental component ($a^2 = 0.49$, $e^2 = 0.51$). Data for total GS protein in the basal state fitted an E model, whereas insulin-stimulated total GS protein fitted a CE model, including a common environmental component. Similarly, data for both basal and insulin-stimulated total GS activity fitted CE models, whereas fractional GS activity fitted an E model. Adjusted GS phosphorylation at site 3a + 3b and 2 + 2a and basal GSK3 activity had exclusively environmental components.

DISCUSSION

The current results indicate a major environmental component in the control of total and fractional GS activity per se in both young and elderly nondiabetic twins. The design of the study enabled differentiation between GS activity per se and the regulation of GS (i.e., glycogen, GS phosphorylation, and GSK3). Muscle glycogen level had a major environmental etiological component in both age groups, except for a moderate genetic component during the basal state in younger twins. Interestingly, GSK3 activity exhibited a genetic component during both the basal and insulin-stimulated state in the younger twins ($a^2 = 0.42$ and $a^2 = 0.58$, respectively) and during insulin stimulation in the elderly twins ($a^2 = 0.56$). In contrast, GSK3 activity was not found to be a primary (i.e., genetic) defect in a recent study of human muscle cell culture from type 2 diabetic patients (30). To our knowledge, no other study has addressed the etiology of GS phosphorylation in humans. We demonstrated a genetic component in phosphorylation of GS at site 2 during the insulin-stimulated steady-state period that was nonsignificantly higher among the elderly compared with younger twins. Furthermore, insulin-stimulated GS phosphorylation at site 3a + 3b had a genetic component among the younger twins that was

TABLE 4
Best fitting models to GS and GS regulation fulfilling the criteria for application of ACE modeling in younger twins

		Components of variance			Goodness-of-fit tests		
Phenotype	Model	Additive genetic (a^2)	Common environment (c^2)	Unique environment (e^2)	χ^2	P	AIC
Glycogen							
Basal	E	—	—	1.00	2.23	0.14	0.23
GS total protein							
Basal	E	—	—	1.00	1.24	0.27	−0.76
Insulin	E	—	—	1.00	0.24	0.62	−1.76
GS total activity							
Basal	E	—	—	1.00	0.38	0.54	−1.62
Insulin	E	—	—	1.00	1.19	0.28	−0.81
GS fractional activity							
Insulin	E	—	—	1.00	3.60	0.06	1.60
pGS site 3a + 3b*							
Insulin	AE	0.50 (0.17–0.71)	—	0.50 (0.29–0.83)	0.03	0.85	−1.97
pGS site 2*							
Insulin	AE	0.35 (0.04–0.59)	—	0.65 (0.41–0.96)	0.00	1.00	−2.00
GSK3 activity							
Basal	AE	0.58 (0.34–0.75)	—	0.42 (0.25–0.66)	0.00	1.00	−2.00
Insulin	AE	0.42 (0.16–0.62)	—	0.58 (0.38–0.84)	0.00	1.00	−2.00

Data are proportion of total variance (95% CI) and are adjusted for sex. *Adjusted for total GS content. AIC, Akaike´s Information Criterion; pGS, GS phosphorylation.

TABLE 5
Best-fitting models to GS and GS regulation fulfilling the criteria for application of ACE modeling in elderly twins

Phenotype	Model	Components of variance			Goodness-of-fit tests		
		Additive genetic (a^2)	Common environment (c^2)	Unique environment (e^2)	χ^2	P	AIC
Glycogen							
Basal	AE	0.49 (0.15–0.72)	—	0.51 (0.28–0.85)	0.07	0.80	−1.94
GS total protein							
Basal	E	—	—	1.00	1.46	0.23	−1.54
Insulin	CE	—	0.47 (0.29–0.62)	0.53 (0.38–0.71)	0.28	0.60	−1.72
GS total activity							
Basal	CE	—	0.47 (0.20–0.67)	0.53 (0.33–0.80)	0.27	0.60	−1.73
Insulin	CE	—	0.51 (0.25–0.70)	0.49 (0.30–0.75)	1.46	0.23	−1.54
GS fractional activity							
Basal	E	—	—	1.00	2.11	0.15	0.11
pGS site 3a + 3b*							
Insulin	E	—	—	1.00	3.14	0.08	1.14
pGS site 2*							
Insulin	AE	0.69 (0.38–0.84)	—	0.31 (0.16–0.62)	0.49	0.48	−1.51
pGS site 2 + 2a*							
Insulin	E	—	—	1.00	2.84	0.09	0.84
GSK3 activity							
Basal	E	—	—	1.00	3.40	0.07	1.40
Insulin	AE	0.56 (0.18–0.77)	—	0.44 (0.23–0.82)	0.01	0.91	−1.99

Data are proportion of total variance (95% CI) and are adjusted for sex. *Adjusted for total GS content. AIC, Akaike´s Information Criterion; pGS, GS phosphorylation.

not seen in the elderly twins. No genetic component was demonstrated in site 2 + 2a phosphorylation in either young or elderly twins.

The lack of any significant heritability of total and fractional muscle GS enzyme activity per se found in this study may to some extent question the view from studies in first-degree relatives (4,10) and from human muscle cell culture studies in type 2 diabetic patients (11) of a genetic defect in this enzyme underlying the pathophysiology of type 2 diabetes. However, GSK3 activity and GS phosphorylation at site 2 had a major genetic component, in particular among elderly twins, indicating a role for genes in the regulation and activation of GS. Because levels of GSK3 activity and GS site 2 phosphorylation did not correlate significantly with GS fractional activity (data not shown) or measures of insulin sensitivity (i.e., R_d and nonoxidative glucose metabolism) in this study, and because the GS activity was under no detectable genetic influence, the metabolic relevance of this genetic influence on GS regulation may be questioned. Nevertheless, these results could be explained by environmentally induced modulation of a genetic component. Similarly, the apparent genetic effect on GSK3 activity in the current study could be secondary to currently unknown genetic and/or environmental regulators (i.e., gene-environment or gene-gene interactions), which may be abolished when performing experiments in standardized muscle cell cultures, explaining the somewhat diverging results. We speculate that a genetic regulator of GSK3, and therefore also to some extent of GS activity, may be located upstream of GSK3 in the insulin signaling cascade. Furthermore, we propose that one environmental modulator influencing GSK3 activity could be physical activity. Indeed, exercise has been shown to regulate GS activity/phosphorylation acutely (31,32), and more long-term training upregulates GS protein and total activity (33,34). In this study physical fitness, as judged by $V_{O_{2max}}$, was associated with total GS activity and protein content, but not with fractional GS activity. This suggests that physical fitness predominantly explains the nongenetic variation or component of total GS activity and protein content, and perhaps only to a smaller extent the predominant nongenetic component of fractional GS activity.

Subjects with known diabetes were not included in this study, and the current twin population may therefore be underrepresented with carriers of type 2 diabetes susceptibility genes. Only 13 (16%) elderly twins and 1 (1%) younger twin had first-degree relatives with type 2 diabetes. Furthermore, the elderly twins may, despite some family history of type 2 diabetes, represent a group selected for protective genes against type 2 diabetes because the majority of these twins have reached a mean age of 62 years without developing type 2 diabetes. This may also contribute to the apparent lack of heritability of fractional muscle GS activity in the current twin study. Nevertheless, even in studies of first-degree relatives of type 2 diabetic patients, the possibility that the defect of GS activation could be secondary to other molecular defects, e.g., insulin signaling (16,35) or impaired membrane glucose transport (5–7), cannot be excluded. Therefore, the current data suggest that muscle GS activity may not in itself be genetically determined, but it may in some metabolic states be reduced as a result of other genetic defects of muscle metabolism or insulin signal transduction.

Interestingly, we did actually find that nonoxidative glucose metabolism is controlled equally by genetic and nongenetic factors in the exact same twin population (19), a finding that indeed is consistent with the results of previous studies among first-degree relatives and genetically identical co-twins of patients with type 2 diabetes. In addition, the muscle GS fractional activities measured in this twin population correlated with nonoxidative glucose

metabolism to a quantitatively similar extent as in previous studies of first-degree relatives and co-twins of type 2 diabetic patients. With correlation coefficients of ~0.5, muscle GS fractional activity may only explain 25% of nonoxidative glucose metabolism, and it is moreover important to realize that a correlation does not explain the causality of an association between two variables. Indeed, if GS fractional activity is considered as a marker—and not the ultimate cause—of muscle insulin resistance, it may provide a further explanation of the divergent results in this and previous studies. Studies of the heritability of upstream regulators of muscle GS activity are needed to determine the exact step at which the well-documented genetic regulation of nonoxidative glucose metabolism and muscle glycogen synthesis is exerted.

Total GS activity and GS protein were reduced with age, whereas the fractional GS activity, which is supposed to reflect the degree of phosphorylation and covalent activation of the enzyme, was similar in young and elderly subjects during both the basal and insulin-stimulated states. There was a decline in GS phosphorylation at sites 2, 2 + 2a, and 3a + 3b with increasing age. However, when expressing the degree of phosphorylation in relation to the total amount of GS protein, no effect of age was seen on GS phosphorylation. This is consistent with the fractional GS activity measurements, suggesting that the "net" insulin signal transduction to activate muscle GS is intact with increasing age.

Despite the significant correlation between glucose disposal and GS fractional activity in both the young and elderly twins in this study, insulin activation of muscle fractional GS was similar in young and elderly twins and therefore cannot explain the highly significant reduction in nonoxidative glucose metabolism with age. This is an interesting observation to some extent resembling the above discussion concerning the discordant results of the relative impact of genes versus environment on nonoxidative glucose metabolism and fractional GS activity. Our results indicate that the age- or body fat–related decrease in insulin-stimulated glucose uptake and nonoxidative glucose metabolism may be caused in part by a decrease of total GS activity and GS protein and not of fractional GS activity. In support of these results, a recent study demonstrated reduced total GS activity and GS protein, but unchanged fractional GS activity, in obese glucose-tolerant subjects compared with lean control subjects (36). However, neither total GS activity nor GS protein content was associated with nonoxidative glucose metabolism in the current study. Therefore, the age-dependent reduction of nonoxidative glucose metabolism may, in addition to the decrease of total GS activity and protein content, be explained by other mechanisms, such as a reduction of peroxisome proliferator-activated receptor-γ cotranscriptional factor-1α (37) or of muscle membrane glucose transport per se (5–7,38).

The administration of insulin in physiological concentrations resulted in an increase in fractional GS activity and a concomitant decrease in GS phosphorylation at site 3a + 3b. In the current study, we found a significant decrease upon insulin administration in phosphorylation at site 2 + 2a in younger subjects, whereas, in the more insulin-resistant elderly nondiabetic subjects, no such effect of insulin on site 2 + 2a phosphorylation was seen. These findings indicate that the increased GS phosphorylation at site 2 + 2a (i.e., inactivation of GS) recently demonstrated in patients with type 2 diabetes compared with control subjects may be secondary or perhaps compensatory to diabetes per se (16).

The validity of twin studies to determine the impact of genetic versus nongenetic factors on a given phenotype has been challenged by the putative differential impact of the intrauterine environment in monozygotic and dizygotic twins compared with singletons. It was postulated that the measures of similarity (i.e., heritability) among monozygotic twins not only express genetic factors but may also express common fetal environmental factors because twins share the same uterine environment (39). However, in the current study, the birth weights were similar in all twin groups and did not correlate with the metabolic parameters, and therefore adjustments for this parameter were not performed or found appropriate. Furthermore, data not fulfilling the assumptions of equal means/variances or exhibiting negative correlations as well as higher correlations in dizygotic compared with monozygotic twins did not undergo biometric modeling, reducing the possibility of an erroneous influence of the intrauterine environment on the heritability estimates. Nevertheless, despite these precautions, the extent to which the current data from nondiabetic twins potentially influenced by intrauterine operating factors may be extrapolated to studies of singletons, such as first-degree relatives of type 2 diabetic patients, is currently unknown.

In conclusion, despite evidence of genetic factors influencing nonoxidative glucose metabolism, the current data indicated a predominant environmental component controlling fractional and total muscle GS activity in young and elderly twins. However, GSK3 activity and GS phosphorylation at sites 2 and 3a + 3b seemed to be regulated by genetic factors. Although nonoxidative glucose metabolism, total muscle GS activity, and protein content decreased with age, and total body fat increased with age, fractional muscle GS insulin activation was interestingly intact with aging. This, together with the heritability data, represents two examples of discordance between factors controlling the otherwise tightly correlated in vivo nonoxidative glucose metabolism and muscle fractional GS activity.

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