Gene Expression Profile in Skeletal Muscle of Type 2 Diabetes and the Effect of Insulin Treatment

Raghavakaimal Sreekumar, Panagiotis Halvatsiotis, Jill Coenen Schimke, and K. Sreekumaran Nair

Type 2 diabetes is characterized by muscle insulin resistance. Nondiabetic first-degree relatives of type 2 diabetic patients have also been reported to have insulin resistance. A polygenic basis for pathogenesis of type 2 diabetes has been proposed. A gene expression profile was evaluated in the skeletal muscle of patients with type 2 diabetes while not on treatment for 2 weeks and after 10 days of intensive insulin treatment. Comparison of gene expression pattern with age-, sex-, and BMI-matched people with no family history of diabetes was performed using a microarray technique (Hu6800 arrays; Affymetrix, Santa Clara, CA). Only those gene transcripts showing ≥1.9-fold changes and an average difference in fluorescence intensity of ≥1,000 in all subjects are reported. Insulin sensitivity (SI) was measured using an intravenous glucose tolerance test. Of 6,451 genes surveyed, transcriptional patterns of 85 genes showed alterations in the diabetic patients after withdrawal of treatment, when compared with patterns in the nondiabetic control subjects. Insulin treatment reduced the difference in patterns between diabetic and nondiabetic control subjects (improved) in all but 11 gene transcripts, which included genes involved in structural and contractile functions, growth and tissue development, stress response, and energy metabolism. These improved transcripts included genes involved in insulin signaling, transcription factors, and mitochondrial maintenance. However, insulin treatment altered the transcription of 29 additional genes involved in signal transduction; structural and contractile functions; growth and tissue development; and protein, fat, and energy metabolism. Type 2 diabetic patients had elevated circulating insulin during the insulin-treated phase, although their blood glucose levels (98.8 ± 6.4 vs. 90.0 ± 2.9 mg/dl for diabetic vs. control) were similar to those of the control subjects. In contrast, after withdrawal of treatment, the diabetic patients had reduced SI and elevated blood glucose (224.0 ± 26.2 mg/dl), although their insulin levels were similar to those of the nondiabetic control subjects. This study identified several candidate genes for muscle insulin resistance, complications associated with poor glycemic control, and effects of insulin treatment in people with type 2 diabetes. *Diabetes* 51:1913–1920, 2002

From the Endocrinology Division, Mayo Clinic, Rochester, Minnesota.

Address correspondence and reprint requests to K. Sreekumaran Nair, MD, Mayo Clinic & Foundation, 200 1st Street SW, Rm 5-194 Joseph, Rochester, MN 55905. E-mail: nair.sree@mayo.edu.

Received for publication 11 June 2001 and accepted in revised form 7 March 2002.

COX, cytochrome c oxidase; D_2-, type 2 diabetic patients 2 weeks after withdrawing their insulin treatment; D_2+, type 2 diabetic patients after 10 days of insulin treatment; IGFBP-5, insulin-like growth factor binding protein-5; IVGTT, intravenous glucose tolerance test; MHC-I, myosin heavy-chain isoform-I; MT, metallothionein; RAD, Ras associated with diabetes; ROS, reactive oxygen species; SI, insulin sensitivity; SOD2, manganese superoxide dismutase-2; TCA, tricaboxylic acid; UCP-3, uncoupling protein-3.

Type 2 diabetes is a major cause of blindness, kidney failure, ischemic heart disease, loss of limb, stroke, and overall mortality. A total of ~140 million people worldwide are affected by this disease (1). Its prevalence in different populations ranges from >50% among Pima Indians to 2% among Indian tribes in Chile (2). In addition, a substantial increase in the prevalence of diabetes among a migrant population to a different environment has also been reported (2), suggesting a potential interaction between genetic and environmental factors. Decreased insulin-induced glucose disposal in skeletal muscle (insulin resistance) is a common pathophysiological trait implicated in the development of type 2 diabetes (3,4). Several lines of evidence clearly point to genetic factors as important determinants of insulin sensitivity (SI) (5). It has also been reported that first-degree relatives of people with type 2 diabetes exhibit insulin resistance to glucose disposal without being diabetic, further supporting the hypothesis that insulin resistance has a genetic basis (6–9). A number of candidate genes for insulin resistance have been evaluated, including insulin receptor substrate-1 (10), glycogen synthetase (11), UCP-3 (12), GLUT4 (13), hexokinase II (14), phosphatidylinositol 3-kinase (15), mitogen-activated protein kinase (15), serine-threonine kinase (16), Rad genes (17), and calpain-10 (18,19). Although the expression of several of these genes are altered in type 2 diabetes, none has emerged as the leading candidate for causing type 2 diabetes. It is highly likely that type 2 diabetes results from interactions between many genetic factors and the environment. The identification of genes involved will hopefully allow modifications of lifestyle to prevent or delay the onset of diabetes.

Genomic analysis techniques offer powerful tools to decipher the pathophysiology of type 2 diabetes at the molecular level, thereby leading to a better fundamental understanding of muscle insulin resistance and providing new therapeutic targets for treatment of this common disease. It is now possible to examine the transcript profile of several thousand genes simultaneously using microarray technology (20). In the current study, we measured the gene transcript alterations in patients with type 2 diabetes 2 weeks after withdrawing their treatment (D_2-) and then again after 10 days of insulin treatment (D_2+). The diabetic subjects were compared with control subjects with no family history of diabetes.

RESEARCH DESIGN AND METHODS

Study design. The clinical characteristics of the subjects are shown in Table 1. The control subjects (n = 5) were matched for age, sex, and BMI with the

TABLE 1
Characteristics of subjects

Study population	Age (years)	BMI (kg/m^2)	Average glucose (mg/dl)	Insulin level (μU/ml)	SI (minimum model)
Control	52.4 ± 4.1	29.2 ± 1.5	90.0 ± 2.9	5.2 ± 0.1	5.96 ± 1.04*
Diabetic Patients (D_2-)	52.8 ± 3.5	29.1 ± 1.4	224.0 ± 26.2*	5.8 ± 0.6	1.82 ± 0.62
Diabetic Patients (D_2+)	52.8 ± 3.5	29.1 ± 1.4	98.8 ± 6.4	13.5 ± 2.9†	2.37 ± 0.8

Data are means ± SE. *$P < 0.001$; †$P < 0.05$ vs. other two groups. Five subjects were studied in each group (two men and three women).

diabetic subjects. The protocol was approved by the institutional review board of the Mayo Foundation, and informed consent was obtained before participation in the study. The five diabetic subjects who were studied on two occasions included two men and three women. Two of the diabetic patients were on glipizide (glucotrol; Pfizer, New York) and metformin (glucophage; Bristol-Myers Squibb, Princeton, NJ), one was on glynase (glyburide; Pharmacia & Upjohn, Kalamazoo, MI), and the other two were not taking any medication.

Two separate randomized studies were performed on each type 2 diabetic subject: one after withdrawing treatment for 2 weeks (D_2-), and one after intensive insulin treatment for 10 days (D_2+) (see Table 1 for glucose levels). There was an interval of 8 weeks between the two studies. All of the diabetic patients stopped taking their medications 2 weeks before the study. No treatment was given to patients for 2 weeks, during which time they were on poor glycemic control (D_2-). In the study in which insulin treatment (D_2+) was given, regular human insulin (Eli Lilly, Indianapolis, IN) was administered four times per day for 10 days. The insulin dose was adjusted to achieve a blood glucose level ranging between 80 and 110 mg/dl before meals. On day 10, an intravenous glucose tolerance test (IVGTT) was performed as previously described (21). After the IVGTT, the patients either continued on their insulin regimen (D_2+) or had no insulin (D_2-). The evening of the same day, all volunteers were admitted to the General Clinical Research Center (GCRC) of the Mayo Clinic in Rochester, Minnesota, and were studied after an overnight fast. During the insulin treatment phase, the last subcutaneous injection of insulin was given at 6:00 P.M. before their dinner. Then, at 9:00 P.M. an intravenous insulin infusion was started and continued overnight to maintain blood glucose levels of 80–100 mg/dl, according to the protocol described by White et al. (22). During the D_2- phase of the study, all subjects were given a constant intravenous normal saline (Baxter, Deerfield, IL) infusion at a rate of 1 ml · kg^{-1} · h^{-1} during the night before the study. Muscle needle biopsies were performed at 7:00 A.M. under local anesthesia, as previously described (23), and the samples were instantly frozen in liquid nitrogen and stored at −80°C for gene expression analysis. The control subjects were also admitted to the Clinical Research Center and were given a standard meal on the day before the study.

Analysis of glucose and insulin. Plasma glucose was measured by the glucose oxidase method using a glucose analyzer (Beckman, Fullerton, CA). Plasma insulin was assayed using a double-antibody chemiluminescence method with an Access immunoassay system (Beckman Coulter, Chaska, MN). SI was estimated using a minimum model, as proposed by Bergman and Cobelli (21).

Analysis of gene transcripts. Total RNA was isolated from frozen tissue using TRIZOL reagent (Life Technologies, Gaithersburg, MD), which was further purified using an affinity resin column (RNeasy; Qiagen, Chatsworth, CA). Total RNA (10 μg) isolated was converted to cDNA using the Superscript cDNA synthesis kit (Gibco-BRL, Gaithersburg, MD). Double-stranded cDNA was then purified by phase lock gel (Eppendorf, Westbury, NY) with phenol/chloroform extraction.

The purified cDNA was used as a template for in vitro transcription reaction for the synthesis of biotinylated cRNA using RNA transcript labeling reagent (Affymetrix, Santa Clara, CA). These labeled cRNAs were then fragmented and hybridized onto Hu6800 arrays (Affymetrix), which contain probes for ~6,800 human genes that are 25 bases in length (24). The labeled and fragmented cRNAs were hybridized onto the Hu6800 arrays as previously described (25). Briefly, appropriate amounts of fragmented cRNA and control oligonucleotide B2 were added along with control cRNA (BioB, BioC, and BioD), herring sperm DNA, and BSA to the hybridization buffer. The hybridization mixture was heated at 99°C for 5 min, followed by incubation at 45°C for 5 min, before injecting the sample into the microarray. Then, the hybridization was carried out at 45°C for 16 h with mixing on a rotisserie at 60 rpm. After hybridization, the solutions were removed, arrays were washed and stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR). After the washes, probe arrays were scanned using a microarray system confocal scanner (Hewlett-Packard).

The quality of the fragmented biotin-labeled cRNA in each experiment was evaluated before hybridizing onto the Hu6800 expression array, using both gel electrophoresis and hybridizing (fraction of the sample) onto a test-2 array as well as analyzing as a measure of quality control. The variability between two experiments when hybridizing about the same amount of fragmented, labeled cRNA from the same sample onto test-2 array was between 5 and 7%.

To validate the findings of gene transcript measurements by the microarray approach, we repeated the measurement of selected gene transcripts (myosin heavy-chain isoform-I [MHC-I], GLUT4, insulin-like growth factor binding protein-5 [IGFBP-5], manganese superoxide dismutase-2 [SOD2], and uncoupling protein-3 [UCP-3]) using a real-time PCR approach, as shown in Fig. 1 (26).

Data analysis. GeneChip 3.0 (Affymetrix, Santa Clara, CA) software was used to scan and quantitatively analyze the scanned image. Spotfire (Spotfire, Cambridge, MA) and Microsoft Excel software were also used for data analysis. For accessing the fold change (relative abundance) of mRNA expression in type 2 diabetic patients (D_2- or D_2+), each patient's gene expression profile was compared individually with that of age-, sex-, and BMI-matched control subjects from side-by-side experiments on the same lot of microarrays (each type 2 diabetic patient was compared with an age-, sex-, and BMI-matched control subject on both D2− and D2+ phases). The fold changes represent the average of all five possible pairwise comparisons among individual subjects on the basis of their age, sex, and BMI. The selection criteria for all of the genes reported were as follows: *1*) in absolute analysis, only those genes that gave an absolute call of "present;" *2*) only those genes that showed a difference call of either "increased" or "decreased;" *3*) an average difference of ≥1,000 in both control and diabetic patients (D2− and D2+); and *4*) only those genes that showed ≥1.9-fold changes in all five patients or five pairwise comparisons. To minimize the false-positive genes, only those transcript levels that altered and satisfied all of these four criteria were considered as significant and were reported in this article. One of the limitations of using this stringent approach is that we may not be able to pick up some of the low-abundance gene expression changes. Previous reports using similar microarray approaches used a 1.6-fold change as the criteria for determining a significant difference (27–29).

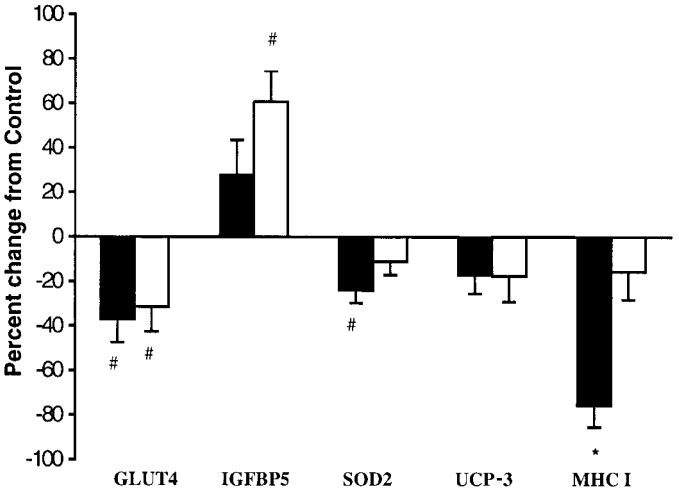

FIG. 1. Real-time PCR data (mRNA levels) of GLUT4, IGFBP5, SOD2, UCP-3, and MHC-I in D_2- patients (■) and D_2+ patients (□) (mean ± SE percent change from control subjects). The *P* values were significant for three genes (GLUT4, SOD2, and MHC-I) in the control versus D_2- comparison and also for GLUT4 and IGFBP5 in the control versus D_2+ comparison. *$P < 0.01$; #$P < 0.05$.

Statistics. The differences of plasma glucose, insulin levels, and SI were compared using a nonpaired *t* test when diabetic (D_2- or D_2+) and control groups were compared, and paired *t* test was used when two studies (D_2- or D_2+) in the same diabetic patients were compared. In the microarray and real-time PCR data analysis, the log average difference values and mRNA levels, respectively, were compared using a *t* test between individual diabetic patients (D_2- or D_2+), and corresponding age-, sex-, and BMI-matched control and the *P* values were shown along with the fold change (Tables 2–5 and Fig. 1).

RESULTS

Plasma glucose, insulin, and SI. Subjects with type 2 diabetes (D_2-) off insulin treatment had substantially higher plasma glucose compared with when they were insulin treated (D_2+) and compared with control subjects ($P < 0.001$) (Table 1). In contrast, plasma insulin levels were similar in the diabetic (D_2-) and control subjects. Plasma glucose levels were similar in the people with diabetes during insulin treatment (D_2+) and in control subjects, although insulin levels were higher in the diabetic subjects after insulin treatment (D_2+) than during the period when they were poorly controlled and than in control subjects ($P < 0.05$). Based on IVGTTs, estimation of SI by minimum model (21) demonstrated lower SI in the diabetic subjects than in nondiabetic control subjects ($P < 0.001$). Insulin treatment partially corrected this abnormality.

Gene transcript levels

D_2- patients versus control subjects. In type 2 diabetic patients (D_2-), 85 gene transcripts were altered in comparison with control subjects. Of these, 19 (22%) showed an increase and 66 (78%) displayed a decrease in transcript levels. These genes were classified into different groups on the basis of their functions and are shown in Tables 2 and 3.

D_2+ patients versus control subjects. A comparison between transcripts of the D_2+ patients and control subjects showed that 40 gene transcripts were altered in the D_2+ patients. Of these, 11 gene transcripts were the same as in D_2- patients (Table 4), with 3 remaining higher than the control group and 8 remaining lower. However, 29 gene transcripts were altered ≥1.9-fold on insulin treatment (Table 5), which was normal in D_2- subjects. Of these 29 transcripts, 16 were downregulated with insulin treatment, whereas 13 were upregulated.

Using a real-time PCR approach, we have validated five gene transcript expression levels that were altered ≥1.9-fold in microarray analysis (Fig. 1). For each of the five genes (MHC-I, GLUT4, IGFBP-5, SOD2, and UCP-3), mRNA levels showed trends similar to those of the microarray experiment. MHC-I, GLUT4, SOD2, and UCP-3 reduced their expression by about 80, 37, 24, and 17%, respectively, in type 2 diabetic (D_2-) patients compared with the control subjects. The 10 days of insulin treatment improved mRNA levels of these genes, as determined by both microarray and real-time PCR analysis. In contrast, IGFBP-5 showed a 28% increase in its expression in type 2 diabetic (D_2-) patients compared with the control subjects, and insulin treatment actually further enhanced the expression of IGFBP-5 (60%). This observation was parallel to that of microarray analysis.

DISCUSSION

The current study demonstrated that insulin not only upregulates but also downregulates transcripts of selected genes in human skeletal muscle. It is possible that some of these changes mediated by insulin are related to the changes in circulating substrates such as glucose, free fatty acids, and amino acids. The current results provide novel observations that may help to define the underlying molecular mechanisms of muscle insulin resistance, chronic complications of poor blood glucose control, and complications that may result from insulin treatment in people with type 2 diabetes. The transcripts of 85 genes were altered in poorly controlled type 2 diabetic subjects. Of these 85 gene transcripts, 74 genes (87%) had improved transcript levels after insulin treatment. These improved gene transcripts may represent the candidate genes responsible for some of the preventable chronic complications of type 2 diabetes. In contrast, insulin treatment did not alter 11 transcripts, which may represent the candidate genes for the pathogenesis of muscle insulin resistance in type 2 diabetes. However, insulin treatment resulted in alterations of 29 additional gene transcripts, which represent the effects of insulin treatment.

The results (Table 1) indicated that similar insulin levels in people with type 2 diabetes (D_2-) and the control subjects were associated with vastly different fasting glucose levels. An approximate 2.5-fold increase in circulating insulin was necessary in the diabetic subjects (D_2+) to achieve a plasma glucose level comparable to control subjects. The lower insulin responsiveness to glucose (SI) was confirmed to be the cause of this insulin resistance to glucose metabolism in the diabetic subjects (7). The primary goal in the treatment of diabetic subjects is to achieve normal glucose levels, although a relatively high circulating insulin level is necessary to achieve this goal. There are increasing indications that high circulating insulin may itself cause complications (30–33). For example, insulin is shown to increase vascular smooth muscle cell proliferation (31), thus promoting atherosclerosis. Insulin treatment itself either up- or downregulated 29 genes in muscle. The altered 29 gene transcripts after insulin treatment (Table 5) represent the effects of the high circulating insulin levels. The transcripts altered by insulin treatment include genes for growth factors as well as structural and contractile proteins. Several genes involved in protein metabolism, signal transduction, tissue development, and fatty acid metabolism are also affected by insulin treatment. It is not clear from the current study whether similar changes may occur in other tissues such as vessels, kidney, and retina after insulin treatment.

The gene transcripts that were altered during poor glycemic control (85 genes), but improved (74 genes) during insulin treatment, are candidate genes responsible for many chronic complications that occur in people with type 2 diabetes. Of these 74 genes that were improved during the insulin treatment, 15 (20%) showed an increase and 59 (80%) displayed a decrease in transcript levels. Of the 59 genes with decreased expression in diabetic patients, 15% were involved in structural/contractile function of skeletal muscle (myosin heavy chain isoform-I, troponin-T, myosin light-chain, α-actin, etc.), 20% were involved in energy metabolism (UCP-3, ATP synthase subunits, cytochrome c oxidase subunit Va [COX Va], COX VIIa, etc.), 8% were mediators of protein metabolism (elongation factors-1, ubiquitin, etc.), and 7% were involved in

TABLE 2
Differences in gene transcript levels of type 2 diabetic patients (D_2-) and insulin-treated type 2 diabetic patients (D_2+) in comparison with control subjects

Probe set	Fold Δ A	Fold Δ B	Gene name
Structural/contractile			
HG2743-HT2846	↑3.3*	(↑1.5)†	Caldesmon 1
HG1862-HT1897	↑2.0‡	(↑2.9)§	Calmodulin type I
HG2175-HT2245	↓4.9*	(↓1.4)†	Myosin heavy chain polypeptide
HG2260-HT2349	↓3.9‖	(↓1.4)†	Duchenne Muscular Dystrophy protein
M21984	↓3.2*	(↓1.7)†	Troponin-T
M21665	↓3.1*	(↓1.7)†	MHC-I
HG3514-HT3708	↓2.5*	(↓1.2)†	Tropomyosin (cytoskeletal)
X04201	↓2.4*	(↓1.9)*	Tropomyosin (1.3 kb)
X66276	↓2.4*	(↓2.0)§	Skeletal muscle C-protein
L21715	↓2.3‖	(↓2.1)§	Troponin-I fast-twitch
J04760	↓2.2*	(↓2.0)*	Troponin-I slow-twitch
M20642	↓2.1‡	(↓1.4)†	Myosin light chain
M20543	↓2.0‖	(↓1.5)†	Skeletal muscle α-actin
U35637	↓2.0‡	(↓1.5)†	Nebulin
X13839	↓1.9‡	(↓1.8)†	Smooth muscle α-actin
M33772	↓1.9†	(↓2.1)*	Troponin-C fast-twitch
Mitochondrial maintenance/chaperone			
X93511	↓2.4*	(↓1.3)†	Telomeric DNA binding protein
X83416	↓2.4§	(↓1.4)†	PrP
V00594	↓2.2‡	(↓1.6)†	MT
X82200	↓2.2‖	(↓1.8)†	Staf50
X65965	↓2.2‖	(↓1.2)†	SOD2
HG2855-HT2995	↑2.0*	(↑3.2)*	Heat shock protein, 70 kDa
Growth factors/tissue development/maintenance			
L27560	↑2.5*	(↑2.9)*	IGFBP-5
M55210	↓3.9*	(↓1.7)†	Laminin B2 chain
L08246	↓3.4‡	(↓2.2)§	MCL1
AB000897	↓2.8‖	(↓2.1)§	Cadherin FIB3
Insulin signaling/signal transduction/glucose metabolism			
HG2702-HT2798	↓5.6‖	(↓1.4)†	Serine/threonine kinase
D23673	↓2.8*	(↓1.3)†	IRS-1
L10717	↑2.9‡	(↓1.3)†	T-cell–specific tyrosine kinase
L33881	↓3.1†	(↓1.4)†	Protein kinase C-ι
K03515	↑2.3‡	(↓1.4)†	Neuroleukin
J04501	↓2.4‖	(↓1.6)†	Muscle glycogen synthase
U27460	↓2.3*	(↓1.2)†	UDP-glucose pyrophosphorylase
M91463	↓2.1§	(↓1.3)†	GLUT4
M32598	↓2.0*	(↓1.2)†	Muscle glycogen phosphorylase
Energy metabolism			
D16480	↑3.1*	(↑1.6)†	Mitochondrial enoyl-CoA hydratase
D10523	↑2.3‖	(↑1.2)†	2-Oxoglutarate dehydrogenase
AF001787	↓2.7§	(↓1.4)†	UCP-3
X13794	↓2.7‡	(↓1.4)†	Lactate dehydrogenase B
M83186	↓2.5‖	(↓1.7)†	COX VIIa
M19483	↓2.4‖	(↓1.4)†	ATP synthase β subunit
U65579	↓2.3‡	(↓2.0)‡	NADH dehydrogenase-ubiquinone
X69433	↓2.2‖	(↓1.2)†	Mitochondrial isocitrate dehydrogenase
U94586	↓2.2‖	(↑1.2)†	NADH-ubiquinone oxidoreductase
HG4747-HT5195	↓2.1*	(↓1.1)†	NADH-ubiquinone oxidoreductase MLRQ
M22760	↓2.1‡	(↓1.3)†	COX Va
U09813	↓2.0§	(↑1.2)†	ATP synthase subunit 9
X83218	↓2.0§	(↓1.1)†	ATP synthase
U17886	↓2.0§	(↓1.2)†	Succinate dehydrogenase (SDHB)
L32977	↓1.9§	(↑1.4)†	Ubiquinol cytochrome C reductase
Transcription factors/protein metabolism			
HG1428-HT1428	↓2.1‡	(↓1.4)†	Globin-β
HG3635-HT3845	↓3.8*	(↓1.6)†	Zinc finger protein, krupple
X69116	↓3.7*	(↓1.7)†	Zinc finger protein
X16064	↓2.2*	(↓1.5)†	Translationally controlled tumor protein
U37690	↓2.0‖	(↓1.3)†	RNA polymerase II subunit

Continued on following page

TABLE 2
Continued

Probe set	Fold Δ A	Fold Δ B	Gene name
U95040	↓2.1‡	(↓1.2)†	hKAP1/TIF1B
U65928	↓1.9*	(↓1.1)†	Jun activation domain binding protein
HG3214-HT3391	↑2.9*	(↓1.8)†	Metallopanstimulin 1
U73379	↑2.3‡	(↓1.3)†	Cyclin-selective ubiquitin carrier
X03689	↑2.3‡	(↓1.4)†	Elongation factor TU
M17886	↓2.1*	(↓1.7)†	Acidic ribosomal phosphoprotein P1
U49869	↓2.1*	(↓1.5)†	Ubiquitin
Z21507	↓2.0‡	(↓1.5)†	Elongation factor-1 Δ
U14968	↓1.9‡	(↑1.5)†	Ribosomal protein L27a
U14973	↓1.9‡	(↓1.4)†	Ribosomal protein S29

A, control versus D_2- patients; B, control versus D_2+ patients. *$P < 0.01$, †$P > 0.05$, ‡$P < 0.05$, §$P < 0.0001$, ‖$P < 0.001$, with P values calculated from t test on the average difference between control subjects and D_2- (A) or D_2+ (B) patients.

glucose metabolism. Some of the genes involved in glucose metabolism, such as GLUT4 (13), glycogen synthase, and glycogen phosphorylase, were previously shown to be altered in type 2 diabetes on an individual basis (11) and were improved during insulin treatment. A decline in expression of DNA maintenance/repair (8%), insulin signaling (3%), and transcription factor (12%) genes were also shown in people with type 2 diabetes and were improved during insulin treatment.

In diabetic subjects (D_2-), the expression level of SOD2 was decreased compared with healthy control subjects. The SOD2 gene encodes an intramitochondrial free-radical scavenging enzyme that is the first line of defense against superoxides produced by the mitochondria. Oxidative stress due to reactive oxygen species (ROS) have been implicated in a wide range of degenerative processes, including heart disease, Alzheimer's disease, and aging (34,35). It has been shown in mice that diminished expression of SOD2 resulted in hepatic lipid accumulation. SOD2 mutant mice exhibited inhibition of NADH-dehydrogenase, succinate dehydrogenase, inactivation of the tricaboxylic acid (TCA) cycle, and accumulation of oxidative DNA damage (36,37). We observed a parallel between our results and the data from these mouse studies in regard to reduced expression of NADH-dehydrogenase, succinate dehydrogenase, and some enzymes involved in the TCA cycle when SOD2 is downregulated. The decreased expression of telomeric DNA binding protein, primer recognition protein, metallothionein (MT), stimulated trans-acting factor-50, and SOD2 in D_2- patients were improved with 10 days of insulin treatment. MT has been postulated to detoxify metals and play a role in copper, zinc, and cadmium homeostasis as well as protect against ROS. We found that MT expression was decreased in poorly controlled diabetic subjects but that it was then improved with blood glucose control using insulin. A previous animal model demonstrated that MT disruption leads to increased sensitivity toward toxic metal and oxidative

TABLE 3
Additional genes altered in type 2 diabetes (D_2- and D_2+)

Probe set	Fold Δ A	Fold Δ B	Gene name	Class/function
HG2239-HT2324	↑11*	(↑1.3)†	Potassium channel protein	Ion channel
U90546	↑5.8*	(↑1.7)†	Butyrophilin 4	Immune system
X89267	↑3.6*	(↑1.5)†	Uroporphyrinogen decarboxylase	Heme biosynthesis
L24564	↑3.2‡	(↑1.8)†	Rad	Ras-oncogene associated with diabetes
D43951	↑3.1§	(↑1.3)†	KIAA0099	Unknown
D79986	↑2.3§	(↑1.3)†	KIAA0164	Unknown
J02611	↑2.1‡	(↑1.8)†	Apolipoprotein D	Lipid transport
L11238	↓3.2§	(↓1.6)†	Platelet membrane glycoprotein V	Homeostasis
X59405	↓3.0‖	(↓1.5)†	Membrane cofactor protein	Regulatory glycoprotein
X63575	↓2.8‡	(↓1.5)†	Calcium ATPase	Calcium homeostasis
M16714	↓2.6§	(↓1.6)†	MHC I lymphocyte antigen	Immune system
X00371	↓2.5*	(↓1.8)†	Myoglobin	Oxygen transport
X91103	↓2.5§	(↓1.2)†	Hr44	Immune system
X01060	↓2.4‡	(↓1.6)†	Transferrin receptor	Iron uptake
U90313	↓2.3§	(↓1.4)†	Glutathione-S-transferase	Glutathione metabolism
X75755	↓2.3‡	(↓1.3)†	PR264	Unknown
U33286	↓2.2§	(↓1.2)†	Chromosome segregation gene CAS	Cell proliferation/apoptosis
X15729	↓2.1§	(↓1.5)†	Nuclear p68 protein	Cell poliferation
X06700	↓2.1§	(↓1.7)†	Pro-α (III) collagen	Extracellular matrix protein
AB000220	↓1.9*	(↓1.4)†	Semaphorin E	Immune system

A, control versus D_2- patients; B, control versus D_2+ patients. *$P < 0.05$, †$P > 0.05$, ‡$P < 0.001$, §$P < 0.01$, ‖$P < 0.0001$, with P values calculated from t test on the average difference between control subjects and D_2- (A) or D_2+ (B) patients.

TABLE 4
Gene transcripts remain unaltered by 10 days of insulin treatment in type 2 diabetic patients

Fold Δ	Gene name
Structural/contractile genes	
↑2.9*	Calmodulin Type I
↓2.1*	Troponin I fast-twitch
↓2.1†	Troponin C fast-twitch
↓2.0*	Skeletal muscle C-protein
↓2.0†	Troponin I slow-twitch
↓1.9†	Tropomyosin
Stress response/energy metabolism	
↑3.2†	Heat shock protein, 70 kDa
↓2.0‡	NADH dehydrogenase-ubiquinone
Growth factor/tissue development	
↑2.9†	IGFBP-5
↓2.2*	MCL1
↓2.1*	Cadherin FIB3

*$P < 0.0001$, †$P < 0.01$, ‡$P < 0.05$, with P values calculated from t test on the average difference between control and D_2+ subjects.

stress (38). The reduced expressions of these antioxidant defenses in diabetic patients (D_2-) may partly account for many of the degenerative complications they experience.

The current study revealed an overall downregulation of gene transcripts involved in the energy metabolism pathway in diabetic patients (D_2-) (Table 2) and were improved with insulin treatment. This included alterations in genes associated with mitochondrial function, such as UCP-3, a mitochondrial transmembrane carrier that uncouples oxidative ATP phosphorylation in skeletal muscle, and COX Va and VIIa subunits, which are part of complex IV in the electron transport chain and various subunits of ATP synthase (subunits 9 and β). All of these genes are implicated in mitochondrial bioenergetics. Additionally, a decrease was observed in the expression levels of several other key enzymes involved in energy metabolism, such as NADH-ubiquinone oxidoreductase (NADHFS1), succinate dehydrogenase subunit B, ubiquinol cytochrome c reductase (UQCRFS1) (part of complex I, II, and III in the respiratory chain of mitochondria, respectively), and isocitrate dehydrogenase (TCA cycle). The expression of two gene transcripts (mitochondrial enoyl-CoA hydratase and 2-oxoglutarate dehydrogenase) were increased in poorly controlled diabetic patients. All of these abnormalities were improved by insulin treatment and normalization of blood glucose, except for NADH dehydrogenase-ubiquinone.

A total of 15 genes displayed an increase in expression related to type 2 diabetes (D_2-) (Tables 2 and 3) and were improved with insulin treatment. These included potassium channel protein (ion channel), metallopanstimulin 1, elongation factor TU (protein metabolism), and apolipoprotein D (lipid transport). There was an increase in the gene expression patterns of several other genes involved in glucose metabolism (neuroleukin), signal transduction (T-cell–specific tyrosine kinase), Ras associated with diabetes (RAD), protein metabolism (cyclin-selective ubiquitin carrier protein), immune system (butyrophilin-4), and heme biosynthesis (uroporphyrinogen decarboxylase), and their expression levels were improved with insulin treatment. RAD belongs to the Ras superfamily of GTP-binding proteins and has been implicated as a regulator of glucose uptake in a variety of cultured cell lines (39). It has been shown to be increased in the skeletal muscle of type 2 diabetic patients (17), which is consistent with our observation in this study.

After 10 days of intensive insulin treatment and normalization of blood glucose, 11 of the 85 altered gene transcripts noted in type 2 diabetes (D_2-) remained altered. These 11 genes, which may represent candidate genes for the pathogenesis of muscle insulin resistance in type 2 diabetes, included 6 gene transcripts involved in structural/contractile functions, 1 each in stress response, growth factor, and energy metabolism pathways and 2 gene tran-

TABLE 5
Gene transcripts altered (which was normal in D_2-) with 10 days of insulin treatment in type 2 diabetic patients

Fold Δ	Gene name
Structural/Contractile/ Growth factor	
↑2.2*	ACTN3
↑2.1*	CO-029
↓2.7†	SM22
↓2.6*	Sarcolipin
↓2.2*	β-Tropomyosin
↓1.9‡	Cytoskeletal gamma-actin
↑1.9*	IGF-II
Protein metabolism/Signal transduction	
↑2.0*	eIF-4C
↑1.9*	Ribosomal protein L21
↓2.6†	Ubiquitin carboxyl terminal hydrolase
↓2.3*	Ribosomal protein L37a
↓2.0*	Elongation factor 1 alpha-2
↑2.3*	AMP-activated protein kinase
↑2.1*	Protein tyrosine phosphatase, alpha
↓2.7†	Tyrosine kinase receptor
↓2.5†	Adenylyl cyclase
Immune system/energy metabolism/cell adhesion	
↑2.1*	Class II histocompatibility antigen DC-α-chain
↑2.3†	HK1
↓1.9*	NDUFV3
↓3.8*	Fibronectin
↓2.4†	HSPG2
Tissue development/fatty acid metabolism	
↑2.1†	Cadherin FIB2
↓2.4*	Fatty acid binding protein
Transposition/extracellular matrix/unknown	
↑2.0*	Transposon-like element
↓2.3*	Tenascin-X
↑3.0†	GOS2
↑2.6*	H4 Histone
↓2.1*	SCAMP1
↓2.0†	Phosphodieaterase 3B

*$P < 0.01$, †$P < 0.0001$, ‡$P < 0.05$; P values were calculated from t test on the average difference between control and D_2+ subjects. Metallopanstimulin 1 (↓1.8) was the only gene that showed <1.9-fold alteration in gene expression in D_2- or D_2+ patients compared with the control subjects, with a statistical significance in the above t test ($P = 0.04$).

scripts that were mediators of tissue development (Table 4). In contrast, the transcripts of 29 additional genes became altered after insulin treatment (Table 5). These changes presumably were related to the increased circulating insulin levels (Table 1) required to achieve normal glucose levels in people with type 2 diabetes with insulin resistance to glucose metabolism (40).

There were six genes (calmodulin type I, troponin-I [fast and slow twitch], troponin-C [fast twitch], tropomyosin, and skeletal muscle C-protein) involved in structural and contractile functions whose transcripts remained altered in the diabetic subjects, irrespective of their metabolic function, even after 10 days of insulin treatment. Muscle contraction results from the force generated between the thin-filament proteins (actin, tropomyosin, and troponin complex) and the thick-filament protein (myosin). Troponin is a complex of three subunits (troponin-I [TnI], troponin-C [TnC], and troponin-T [TnT]) and play a key role in excitation-contraction coupling in muscle and are coordinately activated during skeletal muscle development (41). The contraction of skeletal muscle begins with the phosphorylation of the light chain of myosin, a reaction catalyzed by myosin light-chain kinase. This kinase is activated by the binding of calmodulin, a ubiquitous Ca^{2+} binding protein (42). Calmodulin is known to act as an important mediator of intracellular Ca^{2+} signaling and is involved in the regulation of a vast number of fundamental cellular processes, including cell division, motility, and signal transduction (43). The gene transcript level of calmodulin, along with that of caldesmon 1, was increased in diabetic subjects. Abnormalities of these muscle contractile functions could potentially be responsible for defective insulin-mediated glucose disposal and may contribute to many chronic complications associated with diabetes (3,44,45). Of note, MHC-I transcript levels were low during poor glucose control but were improved by insulin treatment. It has been reported that in people with type 2 diabetes, the relative composition of type I fibers are reduced (46). Type I fibers have dominant expression of MHC-I. The current investigation suggests that this decreased MHC-I mRNA is related to poor diabetic control and may be reversed by insulin treatment.

The broad nature of insulin resistance to glucose metabolism in the skeletal muscle of type 2 diabetic patients suggests that there may be a primary defect in insulin signaling pathways. Several genes involved in insulin signaling and glucose metabolism pathways also showed altered expression. For example, IRS-1 and serine/threonine kinase were decreased 2.8- and 5.6-fold, respectively, in type 2 diabetic subjects (D_2-). GLUT4 and glycogen synthase expression were also decreased. The changes of many gene transcripts involved in the cascade of insulin intracellular signaling observed in the current study are consistent with several previous reports (5,16,47–50). Insulin treatment improved all of these abnormalities. In contrast, increased gene expression of IGFBP-5 persisted even after the insulin treatment. This increased IGFBP-5 may decrease free IGF-I availability at the tissue level. IGF-I has been shown to increase SI (51), and it is possible that reduced availability of free IGF-I at the tissue level may reduce SI.

We confirmed the results from microarray experiments by determining a similar trend for MHC-I, GLUT4, IGFBP-5, SOD2, and UCP-3 using a real-time PCR technique (Fig. 1), which is a more standard technique to measure mRNA abundance in small tissue samples. Even though all of the five transcript levels measured using the real-time PCR showed the same directional changes as the microarray data, the magnitude of these changes were not well-matched between the techniques. These variations could be attributed to the differences in the techniques and the variations in the sequence of oligonucleotide probes used in the two approaches.

In summary, the current study demonstrated that 85 gene transcripts involved in a variety of functions were altered in skeletal muscle of people with type 2 diabetes when their glycemic control was poor (D_2-). All but 11 of the gene transcripts improved after insulin treatment. These gene transcripts remaining altered are potential candidates for the pathogenesis of muscle insulin resistance in type 2 diabetes. Insulin treatment achieved desirable glucose levels, but altered an additional 29 gene transcripts. These alterations in gene transcripts may represent the response to increased circulating insulin levels necessary to maintain normal glucose levels in these patients. The current study demonstrated for the first time several candidate genes involved in the pathogenesis of muscle insulin resistance and complications associated with poor glycemic control and hyperinsulinemia related to treatment in people with type 2 diabetes. In doing so, it provides an opportunity for more focused investigations that may identify the genes responsible for the pathogenesis and complications related to type 2 diabetes and insulin treatment.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health Grant RO1 DK41973 and RR00585. Dr. P. Halvatsiosis was supported by a Nova-Nordisk scholarship.

We are indebted to the volunteers who participated in this study and to the skilled technical support from Maureen Bigelow. We gratefully acknowledge our colleagues Drs. Robert Rizza, Craig Stump, and Kevin Short for their the helpful comments and Dr. Stephen Iturria for statistical analysis.

REFERENCES

1. King H, Aubert RE, Herman WH: Global burden of diabetes, 1995–2025: prevalence, numerical estimates, and projections. *Diabetes Care* 21:1414–1431, 1998
2. Bennett PH: Type 2 diabetes among the Prima Indians of Arizona: an epidemic attributable to environmental change? *Nutr Rev* 57:S51–S54, 1999
3. Reaven GM: Pathophysiology of insulin resistance in human disease. *Physiol Rev* 75:473–486, 1995
4. Ferrannini E: Insulin resistance is central to the burden of diabetes. *Diabetes Metab Rev* 13:81–86, 1997
5. Moller DE, Bjorbaek C, Vidal-Puig A: Candidate genes for insulin resistance (Review). *Diabetes Care* 19:396–400, 1996
6. Nyholm B, Mengel A, Skaerbaek CH, Moller N, Alberti KGMM, Schmitz O: Insulin resistance in relatives of NIDDM patients: the role of physical fitness and muscle metabolism. *Diabetologia* 39:813–822, 1996
7. Warram JH, Martin BC, Krolewski AS, Soeldner JS, Kahn CR: Slow glucose removal rate and hyperinsulinemia precede the development of type II diabetes in the offspring of diabetic parents. *Ann Inter Med* 113:909–915, 1990
8. Shulman GI: Cellular mechanisms of insulin resistance. *J Clin Invest* 106:171–176, 2000

9. Kadowaki T: Insights into insulin resistance and type 2 diabetes from knockout mouse models. *J Clin Invest* 106:459–465, 2000
10. Carvalho E, Jansson PA, Axelsen M, Eriksson JW, Huang X, Groop L, Rondinone C, Sjostrom L, Smith U: Low cellular IRS 1 gene and protein expression predict insulin resistance and NIDDM. *FASEB J* 13:2173–2178, 1999
11. Thorburn AW, Gumbiner B, Bulacan F, Wallace P, Henry RR: 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, 1990
12. Krook A, Digby J, O'Rahilly S, Zierath JR, Wallberg-Henriksson H: Uncoupling protein 3 is reduced in skeletal muscle of NIDDM patients. *Diabetes* 47:1528–1531, 1998
13. Choi WH, O'Rahilly S, Buse JB, Rees A, Morgan R, Flier JS, Moller DE: Molecular scanning of the insulin-responsive glucose transporter (GLUT4) gene in NIDDM subjects. *Diabetes* 40:1712–1718, 1991
14. Pendergrass M, Koval J, Vogt C, Yki-Jarvinen H, Iozzo P, Pipek R, Ardehali H, Printz R, Granner D, DeFronzo RA, Mandarino LJ: Insulin-induced hexokinase II expression is reduced in obesity and NIDDM. *Diabetes* 47:387–394, 1998
15. Cusi K, Maezono K, Osman A, Pendergrass M, Patti ME, Pratipanawatr T, DeFronzo RA, Kahn CR, Mandarino LJ: Insulin resistance differentially affects the PI 3-kinase and MAP kinase mediated signaling in human muscle. *J Clin Invest* 105:311–320, 2000
16. Krook A, Roth RA, Jiang XJ, Zierath JR, Wallberg-Henriksson H: Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. *Diabetes* 47:1281–1285, 1998
17. Reynet C, Kahn CR: Rad: a member of the Ras family overexpressed in muscle of type II diabetic humans. *Science* 262:1441–1444, 1993
18. Horikawa Y, Oda N, Cox NJ, Li X, Orho-Melander M, Hara M, Hinokio Y, Lindner TH, Mashima H, Schwarz PE, del Bosque-Plata L, Horikawa Y, Oda Y, Yoshiuchi I, Colilla S, Polonsky KS, Wei S, Concannon P, Iwasaki N, Schulze J, Baier LJ, Bogardus C, Groop L, Boerwinkle E, Hanis CL, Bell GI: Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. *Nat Genet* 26:163–175, 2000
19. Permutt MA, Mizrachi EB, Inoue H: Calpain-10: the first positional cloning of a gene for type 2 diabetes? *J Clin Invest* 106:819–821, 2000
20. Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, Brown EL: Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol* 14:1675–1680, 1996
21. Bergman RN, Cobelli C: Minimal modeling, partition analysis, and the estimation of insulin sensitivity. *Fed Proc* 39:110–115, 1980
22. White NH, Skor D, Santiago JV: Practical closed-loop insulin delivery: a system for the maintenance of overnight euglycemia and the calculation of basal insulin requirements in insulin-dependent diabetics. *Ann Int Med* 97:210–213, 1982
23. Nair KS, Halliday D, Griggs RC: Leucine incorporation into mixed skeletal muscle protein in humans. *Am J Physiol (Endo Metab)* 254:E208–E213, 1988
24. Fodor SP, Read JL, Pirrung MC, Stryer L, Lu AT, Solas D: Light-directed, spatially addressable parallel chemical synthesis. *Science* 251:767–773, 1991
25. Mahadevappa M, Warrington JA: A high-density probe array sample preparation method using 10- to 100 fold fewer cells. *Nat Biotechnol* 17:1134–1136, 1999
26. Balagopal P, Schimke JC, Ades P, Adey D, Nair KS: Age effect on transcript levels and synthesis rate of muscle MHC and response to resistance exercise. *Am J Physiol (Endo Metab)* 280:E203–E208, 2001
27. Welle S, Brooks A, Thornton CA: Senescence-related changes in gene expression in muscle: similarities and differences between mice and men. *Physiol Genomics* 5:67–73, 2001
28. Campbell WG, Gordon SE, Carlson CJ, Pattison JS, Hamilton MT, Booth FW: Differential global gene expression in red and white skeletal muscle. *Am J Physiol Cell Physiol* 280:C763–C768, 2001
29. Lee CK, Klopp RG, Weindruch R, Polla TA: Gene expression profile of aging and its retardation by caloric restriction. *Science* 285:1390–1393, 1999
30. Nishimoto Y, Miyazaki Y, Toki Y, Murakami R, Shinoda M, Fukushima A, Kanayama H: Enhanced secretion of insulin plays a role in the development of atherosclerosis and restenosis of coronary arteries: elective percutaneous transluminal coronary angioplasty in patients with effort angina. *J Am Coll Cardiol* 32:1624–1629, 1998
31. Avena R, Mitchell ME, Neville RF, Sidawy AN: The additive effects of glucose and insulin on the proliferation of infragenicular vascular smooth muscle cells. *J Vascular Surgery* 28:1033–1038, 1998
32. Raal FJ, Panz VR, Pilcher GJ, Joffe BI: Atherosclerosis seems not to be associated with hyperinsulinaemia in patients with familial hypercholesterolaemia. *J Intern Med* 246:75–80, 1999
33. Glueck CJ, Lang JE, Tracy T, Smith SL, Wang P: Contribution of fasting hyperinsulinemia to prediction of atherosclerotic cardiovascular disease status in 293 hyperlipidemic patients. *Metabolism* 48:1437–1444, 1999
34. Doll M: Coronary heart disease-a free radical associated disease? What is the value of antioxidant substances? *Med Monatsschr Pharm* 20:66–70, 1997
35. McIntosh LJ, Trush MA, Troncoso JC: Increased susceptibility of Alzheimer's disease temporal cortex to oxygen free radical-mediated processes. *Free Radic Biol Med* 23:183–190, 1997
36. Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH, et al.: Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 11:376–381, 1995
37. Melov S, Coskun P, Patel M, Tuinstra R, Cottrell B, Jun AS, Zastawny TH, Dizdaroglu M, Goodman SI, Huang TT, Miziorko H, Epstein CJ, Wallace DC: Mitochondrial disease in superoxide dismutase 2 mutant mice. *Proc Natl Acad Sci U S A* 96:846–851, 1999
38. Beattie JH, Wood AM, Newman AM, Bremner I, Choo KH, Michalska AE, Duncan JS, Trayhurn P: Obesity and hyperleptinemia in metallothionein (-I and –II) null mice. *Proc Natl Acad Sci U S A* 95:358–363, 1998
39. Moyers JS, Bilan PJ, Reynet C, Kahn CR: Over expression of Rad inhibits glucose uptake in cultured muscle and fat cells. *J Biol Chem* 271:23111–23116, 1996
40. Luzi L, Petrides AS, De Fronzo RA: Different sensitivity of glucose and amino acid metabolism to insulin in NIDDM. *Diabetes* 42:1868–1877, 1993
41. Ohtsuki I, Maruyama K, Ebashi S: Regulatory and cytoskeletal proteins of vertebrate skeletal muscle. *Adv Protein Chem* 38:1–67, 1986
42. Means AR, Tash JS, Chafouleas JG: Physiological implications of the presence, distribution, and regulation of calmodulin in eukaryotic cells. *Physiol Rev* 62:1–39, 1982
43. Kamm KE, Stull JT: Activation of smooth muscle contraction: relation between myosin phosphorylation and stiffness. *Science* 232:80–82, 1986
44. Ginsberg HN: Insulin resistance and cardiovascular disease. *J Clin Invest* 106:453–458, 2000
45. Kahn BB, Flier JS: Obesity and insulin resistance. *J Clin Invest* 106:473–481, 2000
46. Marin P, Andersson B, Krotkiewski M, Bjorntorp P: Muscle fiber composition and capillary density in women and men with NIDDM. *Diabetes Care* 17:382–386, 1994
47. Almind K, Bjorbaek C, Vestergaard H, Hansen T, Echwald S, Pedersen O: Aminoacid polymorphisms of insulin receptor substrate-1 in non-insulin-dependent diabetes mellitus. *Lancet* 342:828–832, 1993
48. Heesom KJ, Harbeck M, Kahn CR, Denton RM: Insulin action on metabolism. *Diabetologia* 40:B3–B9, 1997
49. Imai Y, Fusco A, Suzuki Y, Lesniak MA, D'Alfonso R, Sesti G, Bertoli A, Lauro R, Accili D, Taylor SI: Variant sequence of insulin receptor substrate-1 in patients with noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* 79:1655–1658, 1994
50. Vestergaard H, Bjorbaek C, Andersen PH, Bak JF, Pedersen O: Impaired expression of glycogen synthase mRNA in skeletal muscle of NIDDM patients. *Diabetes* 40:1740–1745, 1991
51. Frystyk J, Skjaerbaek C, Bestbo E, Fisher S, Orskov H: Circulating levels of free insulin-like growth factors in obese subjects: the impact of type 2 diabetes. *Diabetes Metab Res Rev* 15:314–322, 1999