Original Article

Skeletal Muscle Mitochondrial Functions, Mitochondrial DNA Copy Numbers, and Gene Transcript Profiles in Type 2 Diabetic and Nondiabetic Subjects at Equal Levels of Low or High Insulin and Euglycemia

Yan W. Asmann,[1,2] Craig S. Stump,[1] Kevin R. Short,[1] Jill M. Coenen-Schimke,[1] ZengKui Guo,[1] Maureen L. Bigelow,[1] and K. Sreekumaran Nair[1]

We investigated whether previously reported muscle mitochondrial dysfunction and altered gene transcript levels in type 2 diabetes might be secondary to abnormal blood glucose and insulin levels rather than an intrinsic defect of type 2 diabetes. A total of 13 type 2 diabetic and 17 nondiabetic subjects were studied on two separate occasions while maintaining similar insulin and glucose levels in both groups by 7-h infusions of somatostatin, low- or high-dose insulin (0.25 and 1.5 mU/kg of fat-free mass per min, respectively), and glucose. Muscle mitochondrial DNA abundance was not different between type 2 diabetic and nondiabetic subjects at both insulin levels, but the majority of transcripts in muscle that are involved mitochondrial functions were expressed at lower levels in type 2 diabetes at low levels of insulin. However, several gene transcripts that are specifically involved in the electron transport chain were expressed at higher levels in type 2 diabetic patients. After the low-dose insulin infusion, which achieved postabsorptive insulin levels, the muscle mitochondrial ATP production rate (MAPR) was not different between type 2 diabetic and nondiabetic subjects. However, increasing insulin to postprandial levels increased the MAPR in nondiabetic subjects but not in type 2 diabetic patients. The lack of MAPR increment in response to high-dose insulin in type 2 diabetic patients occurred in association with reduced glucose disposal and expression of peroxisome proliferator–activated receptor-γ coactivator 1α, citrate synthase, and cytochrome c oxidase I. In conclusion, the current data supports that muscle mitochondrial dysfunction in type 2 diabetes is not an intrinsic defect, but instead a functional defect related to impaired response to insulin. *Diabetes* 55:3309–3319, 2006

Type 2 diabetes is characterized by both insulin resistance and reduced insulin secretion (1). Insulin is the pivotal hormone involved in the regulation of fuel metabolism, which is impaired in type 2 diabetic patients (2). Insulin regulates a variety of metabolic pathways, including glucose, amino acid, and lipid metabolism; protein turnover; and cell growth/maintenance and differentiation. Insulin regulates metabolic pathways in a variety of ways, including its effect on the activity of existing enzymes by modifying the phosphorylation states—thus altering the cellular location of multiple enzymes (3)—and its effect on the expression of many genes in part by acting at the level of mRNA translation, but also at the level of gene transcription (3–5).

The defects in insulin signaling genes may be important in the etiology of type 2 diabetes. In response to insulin infusion, the induction of gene transcripts encoding GLUT4 (6,7), insulin receptor substrate-1 and -2 (8), and p85α–phosphatidylinositol 3-kinase (9) is impaired in skeletal muscle of type 2 diabetic patients compared with nondiabetic subjects. Recent studies demonstrated that in nondiabetic subjects skeletal muscle mitochondrial ATP production is responsive to exogenous insulin infusion, suggesting that the insulin signaling pathway is involved in mitochondrial function (10). When increasing circulating insulin from low to high physiological concentrations, an increased muscle mitochondrial ATP production rate (MAPR) was observed in nondiabetic subjects but not in type 2 diabetic patients (10). It has also been reported that muscle mitochondrial protein synthesis is responsive to insulin in nondiabetic subjects (10) and in swine (11), but that insulin treatment failed to stimulate muscle mitochondrial protein synthesis in type 2 diabetic patients (12). Moreover, people with type 2 diabetes showed evidence of earlier muscle fatigue consistent with muscle mitochondrial dysfunction (12). There are also reports of association between insulin resistance and muscle mitochondrial dysfunction in aging (13,14), obesity (15), and insulin-resistant offspring of type 2 diabetic patients (16). Indications of impaired mitochondrial biogenesis have also been reported in type 2 diabetic patients (17).

Several recent investigations have examined human skeletal muscle gene transcript profiles in skeletal muscle

From the [1]Endocrinology Division, Mayo Clinic School of Medicine, Mayo Clinic and Foundation, Rochester, Minnesota; and the [2]Research Computing Facility, Mayo Clinic School of Medicine, Mayo Clinic and Foundation, Rochester, Minnesota.

Address correspondence and reprint requests to K. Sreekumaran Nair, Endocrinology Research Unit, Mayo Clinic, 200 First St. SW, Joseph 5-194, Rochester, MN 55905. E-mail: nair.sree@mayo.edu.

Received for publication 20 September 2005 and accepted in revised form 22 August 2006.

COX, cytochrome c oxidase; ETC, electron transport chain; FFM, fat-free mass; GCRC, General Clinical Research Center; MAPR, mitochondrial ATP production rate; mtDNA, mitochondrial DNA; mTOR, mammalian target of rapamycin; ND, NADH dehydrogenase; PGC-1α, peroxisome proliferator–activated receptor-γ coactivator-1α; PPIase, peptidyl-prolyl *cis*-trans isomerase; S6K, p70 S6 kinase.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

DOI: 10.2337/db05-1230

TABLE 1
Characteristics, including body composition, fasting glucose and insulin, nonesterified fatty acids, intramuscular triglyceride, and V_{O_2} peak in type 2 diabetic patients and nondiabetic subjects

Characteristic	Groups			
	Type 2 diabetic patients		Nondiabetic subjects	
	Means	*n*	Means	*n*
Age (years)	58.0 ± 3.5	13	54.6 ± 2.5	17
Weight (kg)	81.1 ± 2.7	13	81.6 ± 3.3	17
BMI (kg/m^2)	27.7 ± 1.0	13	26.6 ± 0.8	17
Fat (%)	33.1 ± 2.8	13	37.1 ± 2.5	17
FFM (kg)	51.1 ± 3.0	13	49.1 ± 3.0	17
Waist (cm)	95.8 ± 3.3	12	95.0 ± 2.8	17
Fasting glucose (mmol/l)	7.8 ± 0.5	13	4.9 ± 5.1	16*
Baseline insulin (pmol/l)	66.3 ± 16.3	13	28.2 ± 3.4	15*
Baseline plasma NEFA level (mmol/l)	0.45 ± 0.05	12	0.32 ± 0.03	16
Intramuscular triglyceride (mmol/g muscle)	19.3 ± 5.5	12	17.8 ± 6.5	12
V_{O_2} peak (ml/kg FFM per min)	34.2 ± 1.6	13	38.9 ± 1.6	17

Values are the means ± SEM. *Significant difference between type 2 diabetic patients and nondiabetic subjects. Only fasting glucose (P = 0.00014), and fasting insulin levels (P = 0.039) were different between two study subjects. NEFA, nonesterified fatty acid.

from patients with type 2 diabetes, since muscle is the predominant site of insulin-induced glucose disposal and the major site of insulin resistance in type 2 diabetes (18–22). Overall, patients with type 2 diabetes had impaired transcripts of genes involved in oxidative metabolism (20–22), glucose metabolism (22), and lipid metabolism (20). However, these studies were carried out either under postabsorptive states, when blood glucose and insulin levels were significantly higher in diabetic subjects compared with nondiabetic subjects (20,22), or after a short period (2 h) of euglycemic-hyperinsulinemic clamp, where the blood insulin levels may not be similar between the two study groups (21). Insulin treatment for 10 days normalized the impaired transcript levels of many genes involved in energy metabolism as well as other genes in type 2 diabetic patients (22). However, insulin treatment, though achieving normoglycemia, resulted in significantly higher blood insulin levels and may have contributed to alterations of transcript levels of additional genes in type 2 diabetic patients. Therefore, it is important to expose the skeletal muscle of both diabetic and nondiabetic subjects to similar levels of plasma insulin and glucose for a similar period in order to clearly determine the altered skeletal muscle gene transcripts in type 2 diabetes.

To determine whether there is a defect in the skeletal muscle mitochondrial oxidative phosphorylation pathway in type 2 diabetic patients, we compared the mitochondrial DNA (mtDNA) abundance, gene transcript levels, MAPR, and protein levels of key electron transport chain (ETC) genes in skeletal muscle of type 2 diabetic and nondiabetic subjects at postabsorptive (7-h low-dose insulin infusion) and postprandial (7-h high-dose insulin infusion) insulin levels and euglycemia. These studies were performed during somatostatin infusion to inhibit endogenous insulin secretion, thereby insuring that all subjects had comparable portal and peripheral insulin concentrations on the study days.

RESEARCH DESIGN AND METHODS

Patients with type 2 diabetes (n = 13, all Caucasians) and nondiabetic control subjects (n = 17, all Caucasians) without a family history of diabetes were matched for age, sex, physical activity history, BMI, and body composition (Table 1) for MAPR measurements. Type 2 diabetes was verified by clinical history, fasting glucose, and oral glucose tolerance testing. The duration of diabetes diagnosis (means ± SE) in the type 2 diabetic group was 5.7 ± 1.5 years. Diabetes was managed by lifestyle modification (exercise/diet) alone in five subjects, although none of these participants were actively engaged in regular exercise training. The other eight subjects with type 2 diabetes used hypoglycemic agents, including combinations of insulin (n = 2), metformin (n = 6), glimipride (n = 2), and glyburide (n = 1). Patients taking thiazolidinediones were excluded from the study. Fat mass and fat-free mass (FFM) were measured by using dual X-ray absorptiometry (DPX-IQ; Lunar, Madison, WI). Female subjects were studied on the luteal phase of their menstrual cycle. Physical activity history during the previous year was assessed using the Minnesota Leisure Time Activity questionnaire (23). Microarray studies and Western blot analysis were carried out only in eight each well-matched diabetic and nondiabetic subjects. Real-time PCR and mtDNA copy measurements were carried out in 12 type 2 diabetic/17 nondiabetic subjects, and 13 type 2 diabetic/15 nondiabetic subjects, respectively.

The study protocol was approved by the Mayo Clinic institutional review board, and all subjects provided their informed consent before entering the study. During an initial screening visit, subject eligibility was confirmed by a review of medical history, physical exam, blood testing, oral glucose tolerance test, and a standard treadmill stress test to exclude cardiovascular abnormality. At least 4 days before the inpatient study, peak oxygen uptake ($V_{O_{2peak}}$) was measured during leg cycling, as previously described (12). Two studies were performed for each subject at the Mayo Clinic General Clinical Research Center (GCRC). All subjects were on a standard weight-maintaining diet (carbohydrate/protein/fat 55:15:30% by calories) provided by the GCRC for 3 consecutive days before each inpatient study period. In type 2 diabetic patients, all oral hypoglycemics and long-acting insulins were discontinued 10 days before admission. All type 2 diabetic subjects were studied 10 days after discontinuation of diabetes therapy. Subjects were admitted to the GCRC at 1700 and ingested a standard meal at 1800 and a snack at 2200. Thereafter, they fasted until the end of the inpatient period the following day. Insulin was infused at either the low or the high dose (0.25 or 1.5 mU/kg of FFM per min, respectively) for 7 h (0600–1300) on the separate study days. All subjects were studied first with low-dose insulin and then with high-dose insulin infusion on the next day, with the exception of three subjects because of scheduling difficulties. The high-dose insulin infusions for these three subjects were performed 7, 49, and 219 days after the low-dose insulin infusions. Somatostatin (7 μg/kg of FFM per h) was infused on both study days to suppress endogenous secretions of insulin, growth hormone, and glucagons. Growth hormone (5 ng/kg of FFM per min) was replaced, but glucagons were not replaced because muscle has no glucagon receptors. Arterialized blood was collected using the heated hand vein method (24). Plasma glucose was measured every 10–15 min with a glucose analyzer (Beckman, Fullerton, CA). Euglycemia was maintained by dextrose (40% solution) infusion as previously described (25).

Muscle biopsies. Vastus lateralis muscle samples were obtained under local anesthesia (lidocaine, 2%) with a percutaneous needle, as previously described (26), from both nondiabetic and type 2 diabetic subjects after 3 and 7 h of low- or high-dose insulin infusions. Therefore, each subject had a total of four muscle biopsies. A portion of the muscle obtained at 7 h was kept on ice in saline-soaked gauze for immediate preparation of mitochondria for MAPR

studies. Otherwise, muscle samples were immediately frozen in liquid nitrogen and stored at −80°C.

Plasma analysis. Plasma insulin concentration was measured with a two-site immunoenzymatic assay (Access system; Beckman Instruments, Chaska, MN). Nonesterified free fatty acids were measured using an enzymatic colorimetric assay (NEFA C; Wako Chemicals, Richmond, VA).

MAPR. Muscle mitochondria were prepared by centrifugation, and MAPR was monitored with a bioluminescent technique (10,27). The reaction mixture included a luciferin-luciferase ATP monitoring reagent (BioThema, Haninge, Sweden), substrates for oxidation, and 35 μmol/l ADP. Substrates used were (in mmol/l final concentration): 10 glutamate plus 1 malate, 20 succinate plus 0.1 rotenone, 1 pyruvate plus 0.05 palmitoyl-L-carnitine plus 10 α-ketoglutarate plus 1 malate, 1 pyruvate plus 1 malate, and 0.05 palmitoyl-L-carnitine plus 1 malate, with blank tubes used for measuring background activity. All reactions for a given sample were monitored simultaneously at 25°C for 20–25 min and calibrated with the addition of an ATP standard using a BioOrbit 1251 luminometer (BioOrbit Oy, Turku, Finland). Mitochondrial integrity was monitored by measuring citrate synthase activity before and after membrane disruption by two freeze-thaw cycles and the addition of Triton X-100. All preparations used were 90–94% intact with no differences between groups.

Analysis of gene transcripts using GeneChips. Total RNAs were purified using an RNeasy Protect Mini Kit from Qiagen. The quality and quantity of total RNA was measured using the Agilent test on a Bioanalyzer (Agilent Technologies, Palo Alto, CA). Gene transcript profiles in both nondiabetic and type 2 diabetic subjects after low- or high-dose insulin infusions were studied by high-density oligonucleotide microarrays containing probes for 54,675 genes and expressed sequence tags (HG-U133 plus two GeneChip arrays; Affymetrix, Santa Clara, CA). Sample labeling, hybridization of test array, and hybridization of full-size arrays were performed by the Mayo Clinic Advanced Genomics Technology Center Microarray Lab using protocols described in the Affymetrix GeneChip expression analysis technical manual.

Real-time RT-PCR. The transcript levels of selected genes were examined by real-time quantitative PCR (Applied Biosystems PRISM 7700) as described previously (26). The primers for nuclear-encoded genes were designed to cover the boundaries of two adjacent exons, and the primers for mitochondrial-encoded genes were designed to expand the coding region and poly-A tail, thereby eliminating the possibility of amplifying DNA. The abundance of each target gene was normalized to the signal for 28S ribosomal RNA, which was coamplified within the same reaction well.

Western blot. Muscle tissues were homogenized in ice-cold cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with protease inhibitors (Mini Complete; Roche, Indianapolis, IN) and incubated on ice for 30 min. After high-speed centrifugation, supernatants from each sample were used for Western analysis. The same amount of protein was loaded (~40–60 μg) onto each lane. The densitometric analysis was performed using a Kodak Image Station 1000. Peroxisome proliferator–activated receptor-γ coactivator-1α (PGC-1α) antibody was purchased from Bethyl Laboratories (Montgomery, TX). Cytochrome c oxidase I (COX1) and citrate synthase antibodies were purchased from Molecular Probes and Chemicon International (Temecula, CA), respectively. Antibodies for phosphorylated and total Akt, p70 S6 kinase (S6K), and mammalian target of rapamycin (mTOR) were from Cell Signaling Technology. The specific phosphorylation sites measured were: Thr308-Akt, Thr389-S6K, and Ser2448-mTOR.

mtDNA quantification. DNA extractions were performed on frozen muscle samples from individual subjects using a QIAamp DNA mini kit (Qiagen). A real-time quantitative PCR system (PE Biosystems) was used to measure the mtDNA copy numbers, using mtDNA-encoded NADH dehydrogenase 1 (ND1) and ND4 genes (13). The abundance of each target gene was normalized to the signal for 28S ribosomal RNA, which was coamplified within the same reaction well.

Intramuscular triglyceride measurement. Intramuscular triglyceride concentrations were measured as previously described (28), except that the triglyceride was purified by thin-layer chromatography rather than high-performance liquid chromatography using triglyceride external standards.

Data analysis. We subjected the microarray data to invariant probe set normalization with a perfect-match–only model by dChip (29). We only considered genes for which there was at least a single measure >300. Differences between type 2 diabetic and nondiabetic subjects after low- or high-dose insulin infusions were evaluated using unpaired Student's *t* test with unequal variances. Differences within the same subject between low- and high-dose insulin infusions were measured using paired Student's *t* test. When adjusted for the multiple comparison errors (30), no single gene remained different between the two compared groups. Therefore, we opted to focus on significantly different functional groups rather than individual genes. The functions of the genes were assigned according to NetAffx gene ontology annotations (www.affymetrix.com/analysis). The identification of functional groups with significantly enriched gene numbers was performed using MAPPFinder 2.0 (http://www.genmapp.org) (31) and GOTree Machine (32). Functional groups with a P value ≤0.01 were considered statistically significant.

RESULTS

As expected, fasting glucose and insulin concentrations were significantly higher in type 2 diabetic than nondiabetic subjects (Table 1). No other parameters, including $V_{O_{2peak}}$ and intramuscular triglycerides, were significantly different between type 2 diabetic and nondiabetic subjects. The leisure time activity scores did not show significant differences between the two study groups (data not shown).

Plasma insulin glucose and glucose infusion rates. Similar insulin levels were noted in both type 2 diabetic and nondiabetic groups after 3 h of insulin infusion (Fig. 1*A*). After a 7-h high-dose insulin infusion, plasma insulin concentrations (292.8 ± 22.7 and 275.4 ± 8.0 pmol/l in nondiabetic and type 2 diabetic subjects, respectively) were significantly higher than those after the low-dose insulin infusion (43.4 ± 4.2 and 45.4 ± 2.2, respectively). During insulin infusions, plasma glucose was clamped to ~5.0 mmol/l for both type 2 diabetic and nondiabetic subjects by glucose infusion (Fig. 1*B–C*). The glucose infusion rate required to maintain similar plasma glucose was significantly higher in nondiabetic than type 2 diabetic subjects during both low- and high-dose insulin infusion ($P < 0.001$).

mtDNA abundance. There were no differences in mtDNA copy numbers between type 2 diabetic and nondiabetic subjects after 7 h of low- or high-dose insulin infusion using both ND1 and ND4 probes (Fig. 2).

Gene transcript levels. After a 7-h low-dose insulin infusion, 1,727 gene transcript levels were different between type 2 diabetic and nondiabetic subjects (details in online appendix Suppl-1, available at http://diabetes.diabetesjournals.org), of which 888 genes were expressed at higher levels in nondiabetic subjects, and 839 genes were higher in type 2 diabetic patients. The coordinately altered gene groups (Fig. 3*A* and online appendix Suppl-1a, Table 1a-1) included inflammatory responses, immune responses, and antigen processes (expressed at higher levels in type 2 diabetes), as well as mitochondrion, glucose metabolism, protein transport, and regulation of translation (expressed at lower levels in type 2 diabetes). These differentially expressed ontology groups in type 2 diabetes included a majority, but not all, of the genes from each group regulated toward the same direction (online appendix Suppl-1a, Table 1a-1). Pyruvate dehydrogenase kinase 4 (PDK4), fructose-2,6-biphosphatease 3 (PFKFB3), Cbp/p300-interacting transactivator 2 (CITED2), thioredoxin-interacting protein (TXNIP), and uncoupling protein 3 (UCP3) were among the most elevated genes in type 2 diabetic patients (Fig. 3*A*).

Among the very few mitochondrial genes that were expressed at a higher level in type 2 diabetes (online appendix Suppl-1a, Table 1a-1), COX subunit 5B (COX5B), and NDUFC2 (ND ubiquinone 1 subcomplex 2) are both members of the ETC complexes. We also measured levels of five additional ETC genes after low-dose insulin infusion, using real-time RT-PCR and Western blot. As shown in online appendix Suppl-1a (Table 1a-2) and Fig. 5*B*, most of these genes had significantly higher expression levels in type 2 diabetic subjects compared with nondiabetic subjects.

We found that 7 h of high-dose insulin normalized 903 (52.3%) of 1,727 transcripts (online appendix Suppl-1) that

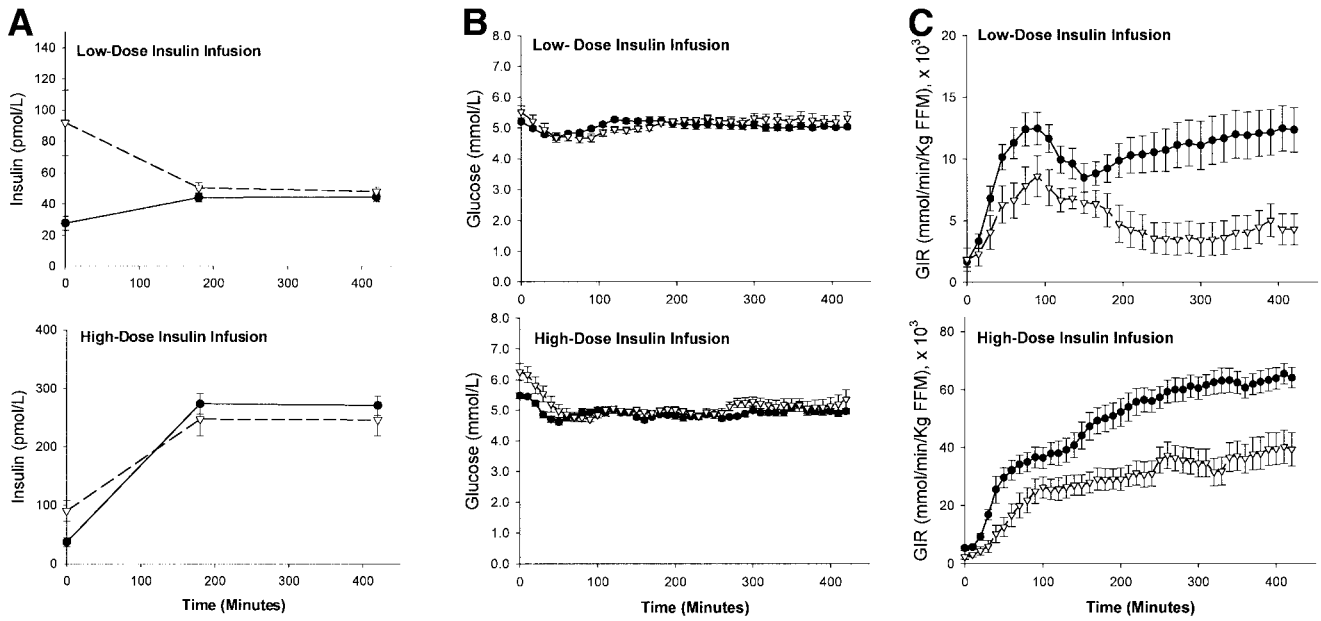

FIG. 1. Plasma insulin (*A*) and glucose (*B*) concentrations and glucose infusion rate (GIR) (*C*) during 7 h of low- or high-dose insulin infusion in 13 type 2 diabetic and 17 nondiabetic subjects. Top panels show results with the low-dose insulin infusion, and bottom panels show results with high-dose insulin infusion. ●, nondiabetic subjects; ▽, type 2 diabetic subjects.

were different between type 2 diabetic and nondiabetic patients during low-dose insulin infusion. Of the differentially expressed function groups during low-dose insulin, 56% of the transcripts were normalized by a high dose of insulin, as shown in online appendix table Suppl-1a. We noted that 187 of those 1,727 genes (Fig. 3*B* and online appendix Suppl-2) significantly altered by high-dose insulin are enriched with genes involved in protein folding/transport and transcription. The initial differences during low-dose insulin infusion, noted for transcripts of NSFL1 cofactor (NSFL1C), pentatricopeptide repeat domain 2 (PTCD2), and crystalline zeta-like 1 (CRYZL1), became greater during high-dose insulin infusion (Fig. 3*B*).

In addition to those 187 genes, 7 h of high-dose insulin infusion altered additional sets of genes in both type 2 diabetic (1,114 genes total, 2.04%) and nondiabetic (1,581, 2.89) subjects. There were 921 unique genes in type 2 diabetic subjects (Fig. 4*A*) that were altered by high insulin and enriched with functional gene groups upregulated by insulin (tubulin and microtubule, steroid hormone receptor, protein transport, and peptidyl-prolyl *cis*-trans isomerase [PPIase]) and gene groups downregulated by high insulin (transcription, translation, and calcium binding) (Fig. 4*C*). For nondiabetic subjects, there were 1,398 genes that were uniquely regulated by high-dose insulin infusion (Fig. 4*D*) and enriched with upregulated function groups (translation, transcription, ubiquitin conjugation, muscle development, and actin/cytoskeleton) as well as downregulated gene groups (transcription repressors, lyase, and cholesterol transporters).

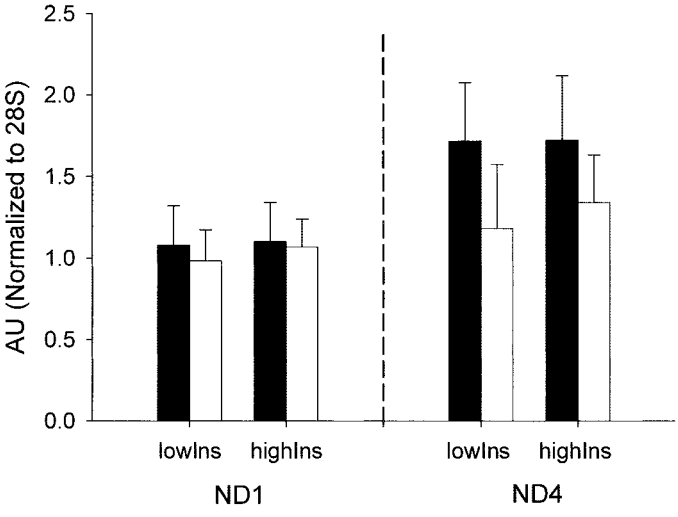

FIG. 2. mtDNA abundance (copy number) after 7 h of low-dose insulin infusion (lowIns) or high-dose insulin infusion (highIns) using primers and probes directed to mitochondrial-encoded genes ND1 (left panel) and ND4 (right panel). ■, type 2 diabetic subjects; □, nondiabetic subjects.

There were only 183 genes commonly regulated by high-dose insulin infusion (Fig. 4*B*) between the two study groups. Most of these 183 genes were regulated toward the same direction by insulin, except for DICER1, cell division cycle 2-like 5 (CDC2L5), and ubiquitin specific protease 5 (USP5) (Fig. 4*B*). Upregulated genes were significantly enriched with transcription factors, and downregulated genes enriched with transcripts related to ubiquitin cycle, transcription repression, and ion homeostasis. The details of the genes given in Fig. 4*A*–*C* can be found in online appendix Suppl-3, Suppl-4, and Suppl-5.

Effects of low- and high-dose insulin infusion on MAPR, transcripts, and protein concentrations of mitochondrial proteins. As shown in Fig. 5*A*, at a postabsorptive insulin level after low-dose insulin infusion, MAPR of type 2 diabetic subjects was not different from that of nondiabetic subjects, despite the lower expression of many mitochondrial genes, as indicated in Fig. 3*A* and online appendix Suppl-1a. There was no significant correlation between MAPR on either study day with intramuscular triglyceride content in either subject group.

Increasing insulin from the postabsorptive to postprandial level by 7-h high-dose insulin infusion showed a significant increase only in MAPR in nondiabetic subjects (Fig. 5*C*). mRNA levels of COX5B, succinyl dehydroge-

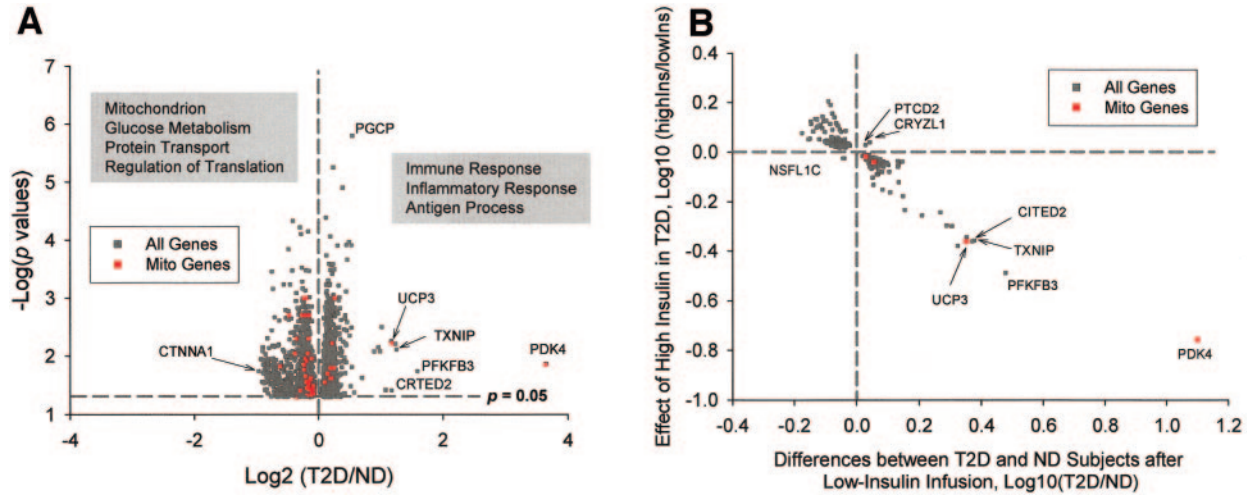

FIG. 3. Skeletal muscle gene transcript profiles measured using Affymetrix HG-U133 plus two GeneChips in eight type 2 diabetic (T2D) and eight nondiabetic (ND) subjects. Mitochondrial (Mito) genes are highlighted in red. *A*: Volcano plot of 1,727 differentially expressed genes between type 2 diabetic and nondiabetic subjects at postabsorptive insulin levels after a 7-h low-dose insulin infusion. The more highly expressed transcripts in nondiabetic subjects (left panel) were enriched with genes involved in mitochondrion, glucose metabolism, protein transport, and translation regulation. The more highly expressed transcripts in type 2 diabetic subjects (right panel) were significantly enriched with genes involved in immune response, inflammatory reaction, and antigen process. The most different transcripts between the two study groups included PDK4, CITED2, UCP3, TXNIP, and PFKFB3. Of the mitochondrial genes, 40 and 9 were expressed at lower or higher levels in type 2 diabetic patients, respectively (red dots). *B*: Effect of increasing insulin to postprandial levels by 7-h high-dose insulin infusion in type 2 diabetic patients. A total of 187 genes that were different (corrected) at postabsorptive insulin levels after a high-dose insulin infusion were significantly changed by increased insulin in type 2 diabetic subjects, using paired Student's *t* test. Genes more highly expressed in type 2 diabetes after low-dose insulin infusion were downregulated by high-dose insulin (upper-left quarter); genes expressed at lower levels in type 2 diabetes after low-dose insulin infusion were upregulated by high-dose insulin (bottom-right quarter). The differences of 184 of 187 genes, including 4 mitochondrial genes (red dots), were reduced by insulin in type 2 diabetic subjects, and only 3 genes (PTCD2, CRYZL1, and NSFL1C) were further differentiated by high-dose insulin administration between the two groups (bottom-left and upper-right quarters).

nase, COX3, COX4, and ND4 were significantly higher in type 2 diabetic than nondiabetic subjects. As shown in Fig. 5*D*, increasing insulin levels reduced the expression of COX1 and citrate synthase in type 2 diabetic subjects, but in nondiabetic subjects the levels of these two proteins increased.

Effect of high-dose insulin infusion on insulin signaling pathway. We measured the phosphorylation of Akt, S6K, and mTOR after 3 and 7 h of low- and high-dose insulin infusion in both nondiabetic and type 2 diabetic subjects. As shown in Fig. 6, 3 h of low-dose insulin infusion significantly increased the phosphorylation of Akt and S6K in nondiabetic but not in diabetic subjects, whereas 7 h of low-dose insulin infusion diminished the differences. When high-dose insulin was administrated for 3 or 7 h, Akt and S6K phosphorylation in both type 2 diabetic and nondiabetic patients was significantly decreased, whereas mTOR phosphorylation was significantly increased in both groups after 3 h of high-insulin administration, and selectively in the nondiabetic group after 7 h of high insulin.

Mitochondrial transcriptional factors. PGC-1α, a regulatory factor of mitochondrial biogenesis, was not different between the two study groups after 7 h of low-dose insulin infusion (Fig. 7*A*). A 7-h high-dose insulin infusion significantly decreased PGC-1α protein content in type 2 diabetic but not in nondiabetic subjects (Fig. 7*D*). PGC-1β mRNA levels were similar in type 2 diabetic and nondiabetic subjects at low levels of insulin (Fig. 7*B*), and high-dose insulin increased the levels, but no differences between type 2 diabetic and nondiabetic subjects were noted (Fig. 7*C*). After 7 h of both low- and high-dose insulin infusion, mitochondrial transcription factor A mRNA levels were lower in nondiabetic than in type 2 diabetic subjects (Fig. 7*C*).

DISCUSSION

The current study was conducted to determine whether the previously reported muscle mitochondrial defects in type 2 diabetic patients represent an intrinsic problem or occur secondary to altered glucose and insulin levels and/or are caused by insulin resistance. We observed that when plasma insulin and glucose concentrations were similar at postabsorptive levels, skeletal muscle ATP production and mtDNA copy numbers were similar in both type 2 diabetic and nondiabetic groups. The transcript levels of the majority (76.5%) of the genes encoding mitochondrial proteins were expressed at lower levels in skeletal muscle of type 2 diabetic patients at a postabsorptive insulin level (online appendix Suppl-1a). However, the mitochondrial gene transcripts (based on both gene array analysis, real-time PCR, and Western blot) encoding subunits of protein complexes involved specifically in the ETC were expressed at higher levels in type 2 diabetic than in nondiabetic subjects. Increasing insulin from the postabsorptive to a postprandial level caused an increase in MAPR in nondiabetic but not in type 2 diabetic subjects. This lack of enhancement of MAPR in type 2 diabetes was associated with a lower rate of glucose disposal and other evidence of insulin resistance. Increasing insulin levels also reduced PGC-1α, COX1, and citrate synthase expression in type 2 diabetic patients.

A major strength of the current study is that we maintained identical blood concentrations of glucose and insulin in type 2 diabetic and nondiabetic subjects using 7-h insulin infusion and euglycemia clamp during a somatostatin blockade. The above approach helped to eliminate the potential impact of variable insulin and glucose levels on muscle mitochondria in type 2 diabetic and nondiabetic subjects. The choice of a 7-h insulin infusion was largely

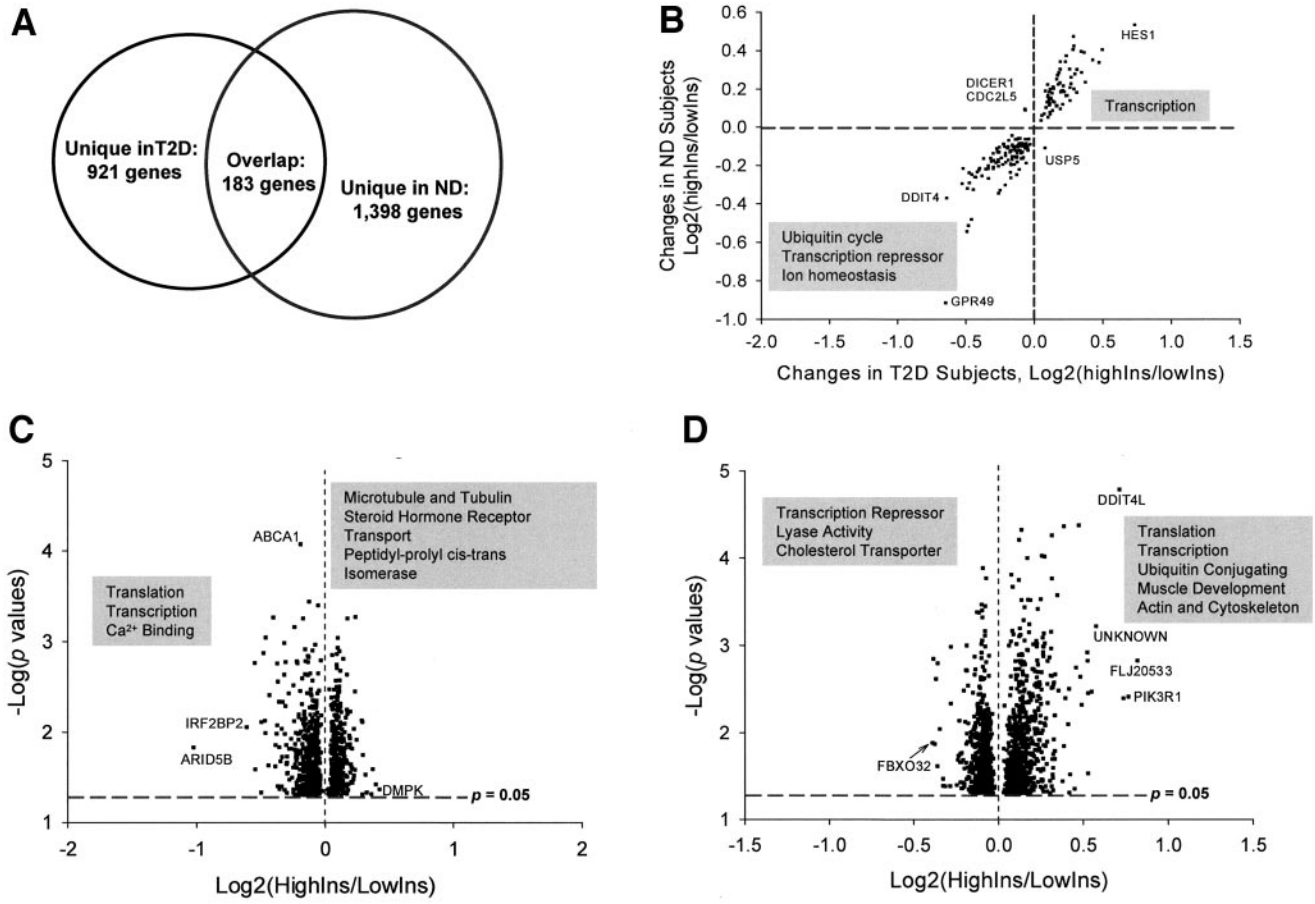

FIG. 4. The differential responses of transcript levels after increasing insulin levels from postabsorptive level to higher postprandial level in type 2 diabetic (T2D) and nondiabetic (ND) groups. *A*: A total of 921 unique genes in type 2 diabetes and 1,398 unique genes in nondiabetic subjects were changed by increased insulin levels. A common set of 183 genes from two study groups were regulated by increased insulin. *B*: A total of 183 genes changed in both type 2 diabetic and nondiabetic subjects from low- (lowIns) to high-dose (highIns) insulin. The upper-left quarter shows genes upregulated in nondiabetic subjects and downregulated in type 2 diabetic patients by high-dose insulin; the bottom-right quarter shows genes upregulated in type 2 diabetic patients and downregulated in nondiabetic subjects by high-dose insulin; the upper-right quarter shows genes upregulated in both nondiabetic and type 2 diabetic subjects by high-dose insulin; the bottom-left quarter shows genes downregulated in both nondiabetic and type 2 diabetic subjects by high-dose insulin. Only 3 of 183 genes (DICER1, CDC2L5, and USP5) were regulated toward different directions between the two study groups. The common upregulated genes between the two groups were enriched with transcription-related genes, whereas the common downregulated genes were enriched with ubiquitin cycle, transcription repressor, and ion homeostasis–related genes. *C* and *D*: Genes that were uniquely regulated by increased insulin in type 2 diabetic (*C*) and nondiabetic subjects (*D*). *C*: Volcano plot of 921 genes changed in type 2 diabetic subjects from low- to high-dose insulin. *D*: Volcano plot of 1,398 genes changed in type 2 diabetic subjects from low- to high-dose insulin. The right halves of the volcano plots are genes upregulated by insulin, and the left halves of the plots represent the genes downregulated by insulin. The significantly altered gene groups in both up- and downregulated groups of genes were indicated in the right and left halves of the plots, respectively.

based on a pilot muscle cell line study in our laboratory (B. Di Camillo, L. Greenlund, G. Toffalo, C. Cobelli, K.S. Nair, unpublished observations) showing that increases in many gene transcripts peaked and started to decline after 8 h of insulin exposure. Moreover, in a prior study, we found an increase of mitochondrial gene transcripts with 8 h of high-dose insulin infusion, whereas no significant changes occurred at 4 h (10). It is possible that insulin therapy for longer than 7 h may normalize the remaining differentially expressed genes between type 2 diabetic and nondiabetic subjects. This is supported by earlier work from our group showing that 10 days of insulin treatment in patients with type 2 diabetes normalized the transcript differences of most genes compared with nondiabetic control subjects (22), demonstrating that the dose and duration of insulin treatment play key roles in skeletal muscle gene regulation.

We also noted that during both low and high insulin levels, muscle mtDNA abundance was similar in both groups, which, together with the observation of similar MAPR at lower physiological concentrations of insulin, suggests there is not an intrinsic muscle mitochondrial defect in type 2 diabetic patients. To confirm the values for mtDNA abundance, we used two different probes and performed the measurements on muscle biopsy samples obtained from otherwise healthy subjects in both groups. A previous study reported lower muscle mtDNA content in type 2 diabetic patients, but their samples were obtained from legs undergoing amputation because of diabetes-related complications (33). It is possible that hypoxia caused by arterial occlusion or leg infection may affect mtDNA content and could explain the different results of these studies. Similar mtDNA abundance in type 2 diabetic and nondiabetic subjects observed in the current study is in contrast with an age-related decrease in mtDNA abundance reported in skeletal muscle of humans (13) and rodents (34). A reduced mtDNA in aging occurs in association with reduced MAPR, whereas baseline MAPR and mtDNA levels in type 2 diabetic and nondiabetic subjects are similar. The observed defect in type 2 diabetic patients

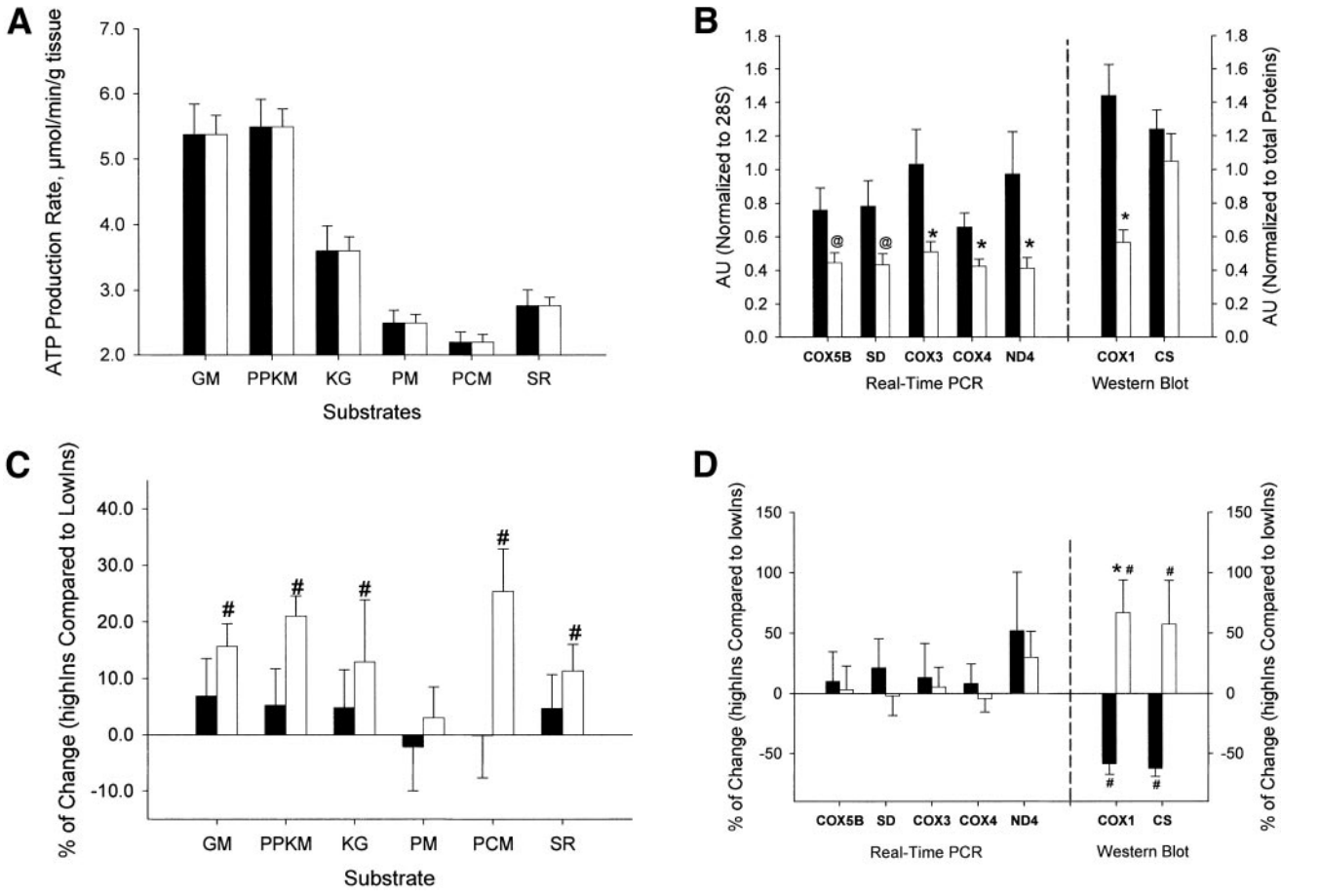

FIG. 5. Skeletal muscle ATP production rate (MAPR) and the effect of increasing insulin levels from a postabsorptive level to a higher postprandial level in type 2 diabetic and nondiabetic groups. Measurements were made in the presence of six different substrate combinations: pyruvate plus malate (PM), palmitoyl-L-carnitine plus malate (PCM), glutamate plus malate (GM), succinate plus rotenone (SR), pyruvate plus palmitoyl-L-carnitine plus α-ketoglutarate plus malate (PPKM), and α-ketoglutarate (KG). *A*: At postabsorptive insulin levels after a 7-h low-dose insulin infusion, there were no differences in MAPR between the two study groups. *B*: Expression levels of seven ETC genes were measured after low-dose insulin infusion, using real-time PCR and Western blot, showing significant differences between type 2 diabetic and nondiabetic subjects. *C*: Increasing insulin levels to postprandial levels by 7-h high-dose insulin (highIns) infusion significantly increased the MAPR in nondiabetic subjects using five of six substrates, but not in type 2 diabetic subjects. *D*: Increasing insulin to the postprandial levels significantly upregulated the protein expression levels of COX1 in nondiabetic but not in type 2 diabetic subjects. Increasing insulin to high levels reduced COX1 and citrate synthase levels in type 2 diabetic subjects but not in nondiabetic subjects. #$P < 0.05$, high vs. low insulin; *$P < 0.05$, type 2 diabetic vs. nondiabetic subjects; @$P = 0.07$ type 2 diabetic vs. nondiabetic subjects. ■, type 2 diabetic subjects; □, nondiabetic subjects. CS, citrate synthase; LowIns, low-dose insulin infusion.

is their inability to increase MAPR in response to increasing insulin levels. In type 2 diabetic patients, increasing insulin from a low to a higher level was associated with a lower glucose infusion rate, suggesting lower glucose disposal mostly in skeletal muscle (Fig. 1*C*). This reduced glucose disposal in muscle could be a potential explanation for the observed impairment in ATP production in response to increasing insulin levels. It is also known that oxygen consumption or thermic response to oral glucose (35), intravenous glucose, and insulin infusions (36) is also lower in type 2 diabetic patients, which is consistent with the current observation of a lack of increase in MAPR in type 2 diabetic patients.

We also compared the phosphorylation of insulin signaling proteins between type 2 diabetic and nondiabetic subjects after both 3 and 7 h of low-dose insulin infusion. After a 3-h infusion, both Akt and S6K phosphorylation were lower in the type 2 diabetic patients, and by 7 h the differences between the groups were diminished. Both Akt and S6K phosphorylation were reduced after 3 or 7 h of high-dose insulin infusion, which may be due to the desensitization by insulin. Interestingly, although no differences in mTOR phosphorylation were observed with low-dose insulin infusion, increasing insulin to a higher physiological concentration for 3 or 7 h increased phosphorylation of mTOR in both type 2 diabetic and nondiabetic subjects, but this increase was sustained at 7 h only in nondiabetic subjects. In general, the response of signaling proteins to insulin is consistent with reduced insulin sensitivity in type 2 diabetic patients in comparison with nondiabetic subjects. Moreover, the reduced glucose infusion rates needed to maintain similar glucose levels demonstrated lower insulin sensitivity in type 2 diabetic patients. Because insulin has been shown to enhance muscle mitochondrial biogenesis and MAPR (10), reduced insulin sensitivity is likely the basis of the lack of increase in MAPR in type 2 diabetic subjects on increasing insulin level. Moreover, our previous studies have shown that muscle mitochondrial protein synthesis was enhanced by insulin in nondiabetic subjects (10) and in swine (11), but not in type 2 diabetic patients (12). The current study demonstrated that the increment of phosphorylation of mTOR in skeletal muscle, which mediates insulin effects on protein synthesis, was higher in nondiabetic than in

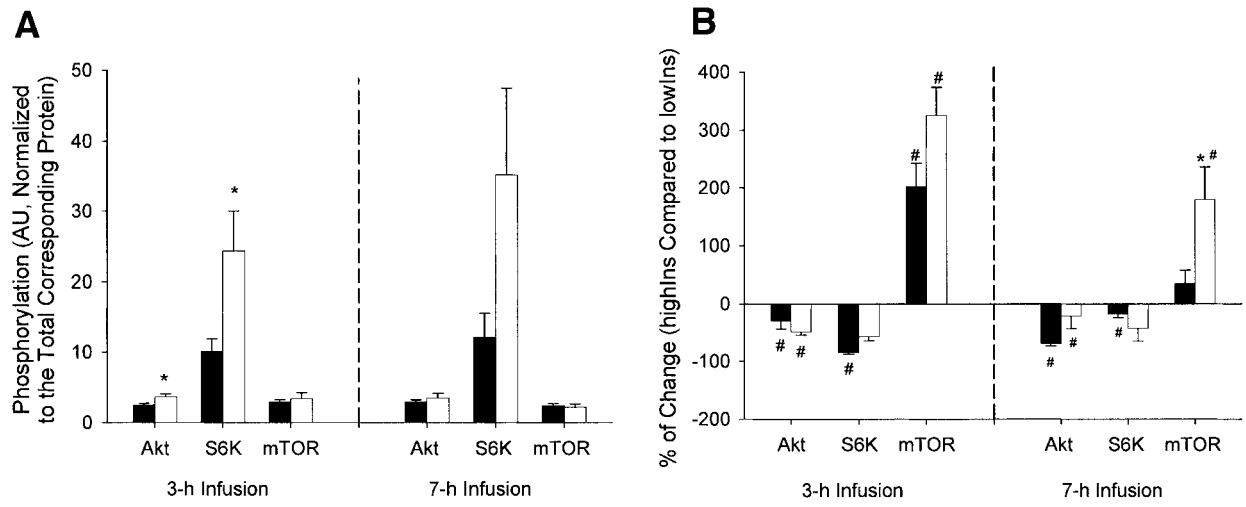

FIG. 6. Effect of insulin infusion on the phosphorylation of insulin pathway molecules. Measurements were made by Western blot. The phosphorylation signals were calculated as the ratios of phosphorylated and corresponding total proteins. *A*: Phosphorylation of insulin pathway molecules after 7 h of low-dose insulin infusion. A 3-h low-dose insulin infusion resulted in higher Akt and S6K phosphorylation in nondiabetic subjects compared with type 2 diabetic subjects, and 7 h of low-dose insulin normalized the differences. *B*: Changes in the phosphorylation of insulin pathway molecules from low- (lowIns) to high-dose (highIns) insulin. A 3- and 7-h high-dose insulin infusion decreased Akt/S6K phosphorylation in both type 2 diabetic and nondiabetic subjects, whereas it increased mTOR phosphorylation in both groups at 3 h and in only the nondiabetic group at 7 h. #$P < 0.05$, high- vs. low-dose insulin; *$P < 0.05$, type 2 diabetic vs. nondiabetic subjects. ■, type 2 diabetic subjects; □, nondiabetic subjects.

type 2 diabetic patients with increasing insulin levels. This may explain the lack of stimulation of muscle protein synthesis observed in type 2 diabetic patients (10–12).

The main objective of our study was to compare type 2 diabetic and nondiabetic subjects while on similar plasma insulin and glucose concentrations and to determine the effect of increasing insulin from low to high physiological concentrations. The responses, in general, to increasing insulin levels were significantly lower in type 2 diabetic than nondiabetic subjects. At the gene transcript level, increasing the insulin concentration affected the expression level of a substantially larger number of genes in nondiabetic (1,581 transcripts) than in type 2 diabetic patients (1,104 genes), which indicates the less robust response to insulin in type 2 diabetic patients. In the type 2 diabetic patients, the major impact of increasing insulin

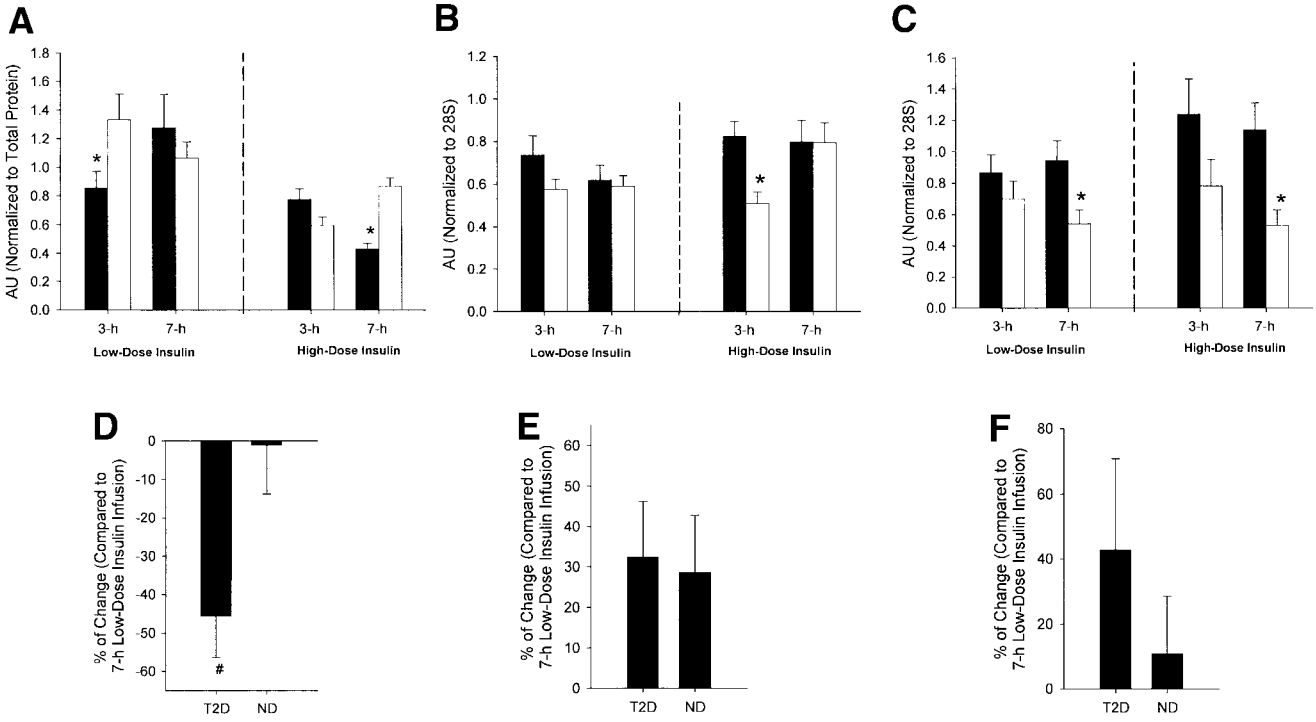

FIG. 7. Effect of insulin infusion on PGC-1α, PGC-1β, and mitochondrial transcription factor A (TFAM) levels measured by Western blot and real-time PCR. PGC-1α protein levels (*A*), PGC-1β mRNA levels (*B*), and TFAM mRNA levels (*C*) after 3 and 7 h of low- or high-dose insulin infusion. The effect of increasing insulin levels from low to high insulin for 7 h on PGC-1α protein level in type 2 diabetic (*E*), PGC-1β mRNA levels (*F*), and TFAM mRNA levels (*G*). #$P < 0.05$, high vs. low insulin; *$P < 0.05$, type 2 diabetic vs. nondiabetic subjects. ■, type 2 diabetic subjects; □, nondiabetic subjects. T2D, type 2 diabetic subjects; ND, nondiabetic subjects.

TABLE 2
Confirmation of the microarray results based on data in literature (human and animal studies)

Gene	Microarray results (nondiabetic/type 2 diabetic)	*P* values of microarray results	Literature findings that agreed with microarray findings (ref.)
PDK4	0.08	0.014	48,49
LPL	1.60	0.016	50
TXNIP	0.42	0.006	51,52
CEBPD	0.70	0.033	53
FBXO32	0.70	0.004	54
SOCS6	1.77	0.025	55

was to increase mRNAs for genes involved in substrate transport, microtubule formation, steroid hormone receptor activity, and PPIase activity, whereas gene groups for the more typical functions of insulin, such as transcription, translation regulation, and calcium binding, were reduced (Fig. 4*C*). This is an intriguing observation because genes involved in protein transport were lower in type 2 diabetic patients after low-dose insulin infusion (Fig. 3*A*), showing that higher insulin levels were needed to normalize this defect. Reduced tubulin and number of microtubules has been reported in diabetic rats (37). PPIases are a major class of protein-folding helper enzymes (38), and type 2 diabetes is associated with defective protein folding or protein misfolding (39). The compensation of these functions by increased insulin levels supports the beneficial effect of glycemic control by insulin treatment in diabetes. In nondiabetic subjects, high-dose insulin infusion upregulated transcripts evolved in translation and transcription regulation, ubiquitin conjugation, and muscle/actin/cytoskeleton development, and it downregulated transcripts related to lyase, transcription repressor, and cholesterol transporter. Transcription and translation regulations are well-known functions of insulin. Changes in transcript levels of actin as well as cytoskeleton and muscle development are likely the results of the anabolic effect of insulin.

In the current study, we focused on the effects of insulin infusion on functional gene groups rather than individual genes. By doing so, we were able to understand the different gene transcript profiles between nondiabetic and type 2 diabetic subjects in the context of gene function, and at the same time we increased our confidence in our microarray results because individual genes identified as differentially expressed were not adjusted for multiple comparison errors. Many of the genes that we observed as differentially expressed between nondiabetic and type 2 diabetic subjects during low-level insulin infusion have been previously reported by other studies (Tables 2 and 3). In addition, the microarray results of several genes were confirmed using real-time PCR (Table 3). Our finding of higher transcript levels of genes involved in immune and inflammatory responses after low-dose insulin infusion agreed with reports that circulating inflammatory cytokines and other inflammation-sensitive plasma proteins are elevated in insulin-resistant states, including type 2 diabetes and obesity (40–42). Our observation of higher expression of genes involved in inflammation is of substantial interest in view of previous reports of association of low-grade systemic inflammation to the development of type 2 diabetes (43). In addition, genomewide linkage scans for diabetes-related phenotypes demonstrated association of multiple genes with both type 2 diabetes and inflammation (44–46).

Our observation of lower levels of transcripts of the nuclear-encoded mitochondrial genes involved is also consistent with findings by Patti et al. (20) and Mootha et al. (21). However, it is of interest that some of the gene transcripts directly involved in ETC are expressed at higher levels in skeletal muscle of type 2 diabetic patients at postabsorptive insulin levels, which may explain why there are no differences in MAPR between type 2 diabetic and nondiabetic subjects at low insulin levels. Furthermore, the lack of enhancement of MAPR in response to increasing insulin levels in type 2 diabetes may be related to the decreased levels of proteins involved in ATP production, such as citrate synthase and COX1. After a 3-h low-dose insulin infusion, PGC-1α protein level was lower in type 2 diabetic patients, whereas after a 7-h infusion, when equal plasma insulin and glucose levels were achieved, the PGC-1α levels were similar between the two study groups (Fig. 7*A*). However, increasing insulin levels from low to high resulted in a decline of PGC-1α expression in type 2 diabetic but not in nondiabetic subjects (Fig. 7*E*). Two previous studies reported impaired PGC-1α expression in type 2 diabetic patients (20,21). Patti et al. (20) performed the measurements after overnight fasting without controlling the blood insulin/glucose levels between study groups, and Mootha et al. (21) performed analysis after a 2-h hyperinsulinemic clamp. Both studies reported lower PGC-1α levels in type 2 diabetic patients that were similar to our observations after the 3-h low-dose insulin infusion. However, another study by Southgate et al. (47) reported a reduction in PGC-1α mRNA expression in skeletal muscle of nondiabetic people after a

TABLE 3
Quantitative real-time RT-PCR validation of the microarray results

Gene	Microarray results (nondiabetic/type 2 diabetic)	*P* values of microarray results	PCR results (nondiabetic/type 2 diabetic)	*P* values of PCR results
UCP3	0.44	0.006	0.39	0.001
COX5B	0.84	0.001	0.61	0.075
SD	1.16	0.011	0.56	0.045

3-h insulin infusion but not in muscle from type 2 diabetic patients. The differences in study protocols may explain the differences in PGC-1α responses to insulin in type 2 diabetic patients between the two studies. In the current study, both postabsorptive and postprandial insulin levels were lower than those of the Southgate et al. (47) study. In addition, the latter study exposed skeletal muscle of type 2 diabetic patients to higher insulin levels than nondiabetic subjects at the baseline state. We did not compare baseline and 3-h levels, but levels at 3 and 7 h. However, it is not entirely clear why PGC-1α levels decreased in muscle of type 2 diabetic patients but not in nondiabetic subjects after 7 h of high-dose insulin infusion. This decline of PGC-1α protein levels may explain why MAPR did not increase in type 2 diabetic patients when insulin was increased from low to high levels. We also noted that increasing insulin levels resulted in increased mTOR phosphorylation in nondiabetic but not in type 2 diabetic subjects, which may have an impact on muscle mitochondrial protein synthesis and thus the differences in citrate synthase and COX1 between type 2 diabetic and nondiabetic subjects. Of note, we also measured PGC-1β mRNA, which was enhanced nonsignificantly by insulin, and no differences between the two groups were observed.

In summary, the current study demonstrated that MAPR was similar in type 2 diabetic and nondiabetic subjects at postabsorptive insulin levels, but patients with type 2 diabetes had a blunted MAPR response, in comparison with nondiabetic subjects, to increasing insulin to postprandial concentrations. Although the majority of mitochondrial gene transcripts were expressed at lower levels in skeletal muscle from type 2 diabetic patients at low insulin levels, ETC gene transcript levels were higher in type 2 diabetic patients, suggesting a compensatory response to maintain MAPR. No difference in mtDNA abundance between type 2 diabetic and nondiabetic subjects was noted, suggesting that reduced muscle mitochondrial content is not a standard finding in type 2 diabetic patients. The current data support the hypothesis that the differences in muscle mitochondrial function between type 2 diabetic patients and nondiabetic subjects are attributable to multiple factors related to insulin action rather than an intrinsic mitochondrial defect.

ACKNOWLEDGMENTS

Support was provided by National Institutes of Health T32 Training Grants DK7352-25 (to Y.W.A.) and MO1 RR00585 (Mayo Clinic GCRC) and Grant RO1 DK41972; the David Murdock Professorship (to K.S.N.); and a Mayo Research Computing Facility research fund allocated by Dr. John E. Carpenter.

We thank the Mayo Clinic GCRC staff and our laboratory associates for assistance in performing these studies. We thank Michelle V. Hoeltzle from Mayo Clinic Advanced Genomics Technology Center Microarray Lab for performing sample labeling, hybridization of test array, and hybridization of full-size Affymetrix human genome U-133 plus 2.0 arrays.

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