Effect of Insulin Deprivation on Muscle Mitochondrial ATP Production and Gene Transcript Levels in Type 1 Diabetic Subjects

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OBJECTIVE—Muscle mitochondrial dysfunction occurs in many insulin-resistant states, such as type 2 diabetes, prompting a hypothesis that mitochondrial dysfunction may cause insulin resistance. We determined the impact of insulin deficiency on muscle mitochondrial ATP production by temporarily depriving type 1 diabetic patients of insulin treatment.

RESEARCH DESIGN AND METHODS—We withdrew insulin for 8.6 ± 0.6 h in nine C-peptide–negative type 1 diabetic subjects and measured muscle mitochondrial ATP production and gene transcript levels (gene array and real-time quantitative PCR) and compared with insulin-treated state. We also measured oxygen consumption (indirect calorimetry); plasma levels of glucagon, bicarbonate, and other substrates; and urinary nitrogen.

RESULTS—Withdrawal of insulin resulted in increased plasma glucose, branched chain amino acids, nonesterified fatty acids, β-hydroxybutyrate, and urinary nitrogen but no change in bicarbonate. Insulin deprivation decreased muscle mitochondrial ATP production rate (MAPR) despite an increase in whole-body oxygen consumption and altered expression of many muscle mitochondrial gene transcripts. Transcript levels of genes involved in oxidative phosphorylation were decreased, whereas those involved in vascular endothelial growth factor (VEGF) signaling, inflammation, cytoskeleton signaling, and integrin signaling pathways were increased.

CONCLUSIONS—Insulin deficiency and associated metabolic changes reduce muscle MAPR and expression of oxidative phosphorylation genes in type 1 diabetes despite an increase in whole-body oxygen consumption. Increase in transcript levels of genes involved in VEGF, inflammation, cytoskeleton, and integrin signaling pathways suggest that vascular factors and cell proliferation that may interact with mitochondrial changes occurred. ***Diabetes*** **56:2683–2689, 2007**

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COX5B, cytochrome c oxidase subunit 5; CRU, Clinical Research Unit; HIF, hypoxia-inducible factor; IPA, ingenuity pathway analysis; MAPR, mitochondrial ATP production rate; NIH, National Institutes of Health; SDH, succinate dehydrogenase; TCA, tricarboxylic acid; TFAM, mitochondrial transcription factor A; UCP, uncoupling protein; UQCR, ubiquinol cytochrome c reductase; VEGF, vascular endothelial growth factor.

Reduced skeletal muscle mitochondrial ATP production rate (MAPR) has been reported to occur in association with insulin resistance in several prevalent conditions including, but not limited to, type 2 diabetes (1–3) and offspring of people with type 2 diabetes (4), obesity (5), and during aging (6–8). In addition, poor glycemic control in type 2 diabetic patients results in substantial changes in transcript levels of many genes involved in mitochondrial oxidative phosphorylation (9–11), but insulin treatment was shown to normalize many but not all of these alterations (2,9). It has been proposed that muscle mitochondrial dysfunction may cause insulin resistance (12), but an alternative hypothesis is that insulin resistance results in muscle mitochondrial dysfunction. Studies in nondiabetic people have shown that insulin infusion, while replacing glucose and amino acids, enhances muscle MAPR and transcript levels of both nuclear and mitochondrial genes involved in ATP production, indicating that insulin action per se can stimulate muscle mitochondrial function and gene activation (1). A recent study in type 2 diabetic patients demonstrated that at postabsorptive insulin levels, both type 2 diabetic and nondiabetic people have similar muscle MAPR and mitochondrial DNA abundance (2). Furthermore, whereas increasing insulin concentration resulted in an increase in MAPR in nondiabetic subjects, this response in MAPR did not occur in people with type 2 diabetes. This suggests that insulin resistance may contribute to muscle mitochondrial dysfunction. A reduced muscle mitochondrial capacity to produce ATP could contribute to the reduced peak oxygen uptake during aerobic exercise, as shown to occur with aging (7) and type 2 diabetes (13). Many other ATP-dependent cellular processes such as protein synthesis may also be adversely affected by reduced ATP production capacity.

It remains to be determined whether insulin deficiency causes a decrease in muscle MAPR. A reduced MAPR during insulin deprivation in type 1 diabetic patients would suggest that reduced insulin action reduces muscle mitochondrial function. However, it has been observed that insulin deprivation in type 1 diabetes results in increased oxygen consumption, suggesting an increased rate of oxidative phosphorylation (14,15). Therefore, in the current study, we determined whether acute withdrawal of insulin treatment in C-peptide–negative people with type 1 diabetes results in reduced MAPR and alterations in transcript levels of genes involved in mitochondrial function. We studied nine people with type 1 diabetes in both the insulin-treated and insulin-deprived conditions and

TABLE 1
Subject characteristics

Age (years)	31 ± 3
Weight (kg)	79.5 ± 4
BMI (kg/m^2)	25.7 ± 1
Fat mass (%)	27.7 ± 4
Duration of type 1 diabetes (years)	17.3 ± 3.2
A1C (%)	7.4 ± 0.4
C-peptide (nmol/l)	<0.03

Data are means ± SD. Values are for nine people with type 1 diabetes.

obtained muscle biopsies to compare treatment effects on MAPR and expression of gene transcripts.

RESEARCH DESIGN AND METHODS

People with type 1 diabetes were recruited using advertisements within the institution and in the Olmsted County, Minnesota, area. Informed written consent was obtained after a detailed review of the protocol, which had been approved by the Institutional Review Board of the Mayo Clinic and Foundation. All patients were screened with detailed history, physical examination, hematological, and biochemical profile. Exclusion criteria included renal insufficiency, coronary artery disease, other vascular disease, neuropathy, or poor wound healing and use of β-blockers, tricyclic antidepressants, or anticoagulants. Body composition was measured using dual-energy X-ray absorptiometry (DPX-L; Lunar, Madison, WI). Subject characteristics are given in Table 1.

Each subject performed two studies, the insulin-treated study day followed by the insulin-deprived study day, separated by 1–2 weeks. All subjects were placed on a weight-maintaining diet (energy content as carbohydrate:protein: fat = 55:15:30%) provided from the Mayo CTSA Clinical Research Unit (CRU) for 3 consecutive days before each inpatient study period. The insulin regimen of each participant was changed during this time. Those subjects on a multiple daily injection regimen (n = 7 of the 9 subjects) were instructed to use ultra rapid-acting insulin (aspart or lispro, recombinant insulins) before each meal and bedtime based on blood glucose, but long-acting insulin was discontinued for the 3 days before each overnight visit. Instructions were given to use short-acting insulin as needed to keep blood glucose concentration within the goal range of 4.4–6.6 mmol/l. Those subjects on an insulin pump (n = 2) using ultra rapid acting insulin continued their regimen until each admission. These subjects also had a target glucose range of 4.4–6.6 mmol/l.

On the evening before each study, subjects were admitted to the CRU at 1700 h and stayed overnight until 1200 h the next day. A retrograde catheter was inserted into a dorsal hand vein for sample collection, and the hand was kept in a heating pad. A second intravenous catheter was placed in the contralateral forearm for infusions. After a standard dinner at 1800 h, a fasting state was maintained, except for water, until the end of each inpatient visit.

In the morning, the hand with the retrograde catheter was kept in a "hot box" at 60°C to obtain arterialized venous blood (16). On the insulin-treated day, an intravenous insulin infusion using regular human insulin was started and the plasma glucose maintained between 4.44 and 5.56 mmol/l overnight until 1200 h the next day. Plasma glucose was measured every 30–60 min, and the insulin dose was adjusted every 30 min until midnight and every 15 min from midnight until the end of the study day. On the insulin-deprived day, the insulin infusion was discontinued for 8.6 ± 0.6 h. The muscle biopsy was not completed on one subject because of a plasma glucose level above 17 mmol/l and altered electrolytes and bicarbonate, resulting in discontinuation of the study.

Blood samples for insulin, C-peptide, glucagon, amino acids, bicarbonate, β-hydroxybutyrate, and free fatty acids were taken at three time points but reporting only the last sample representing the maximal period of insulin deprivation on the insulin-deprived day or a similar time point on the insulin-treated day. Vastus lateralis muscle samples were obtained at the end of the study (8.6 ± 0.6 h after insulin withdrawal or similar time point on the insulin-treated day under local anesthesia (lidocaine, 2%), with a percutaneous needle as described previously (17).

Hormones and substrates. Plasma insulin, glucagon, nonesterified fatty acids, β-hydroxybutyrate, bicarbonate, and glucose were measured as previously described (2,18,19). Plasma glucagon levels were measured by a direct double-antibody radioimmunoassay (Linco Research Immunoassay, St. Charles, MO). Plasma levels of amino acids were measured by a high-performance liquid chromatography system (HP 1090, 1046 fluorescence detector and cooling system) with precolumn *o*-phthalaldehyde derivatization (20).

Urinary measurements. Urinary nitrogen excretion rate was determined using timed collections. Urinary nitrogen content was analyzed with a Beckman GM7 Analox Microstat.

Indirect calorimetry. Respiratory gas exchange was measured using the DeltaTrac system (Sensormedics, Yorba Linda, CA) for 45 min at 1000 h.

MAPR. MAPR was measured as previously described (1,7). Briefly, freshly isolated mitochondrial preparation from muscle biopsy samples were used for the ATP production measurement. This mitochondrial pellet was suspended in buffer B (180 mmol/l sucrose, 35 mmol/l KH_2PO_4, 10 mmol/l Mg acetate, and 5 mmol/l EDTA) and used to measure MAPR with a bioluminescent technique as previously described (7,21,22). The reaction mixture included a luciferin–luciferase ATP monitoring reagent (BioThema, Haninge, Sweden), substrates for oxidation, and 35 μmol/l ADP. Substrates used were 10 mmol/l glutamate plus 1 mmol/l malate, 20 mmol/l succinate plus 0.1 mmol/l rotenone, 1 mmol/l pyruvate plus 0.05 mmol/l palmitoyl-L-carnitine plus 10 mmol/l α-ketoglutarate plus 1 mmol/l malate, 1 mmol/l pyruvate plus 1 mmol/l malate, 0.05 mmol/l palmitoyl-L-carnitine plus 1 mmol/l malate, and 10 mmol/l α-ketoglutarate 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 addition of an ATP standard using a BioOrbit 1251 luminometer (BioOrbit Oy, Turku, Finland). Muscle ATP production was measured in only seven people because muscle biopsy was performed in only eight and one of the ATP production measurements could not be completed because of equipment problem.

Analysis of gene transcripts using GeneChips. This measurement was performed as previously reported in muscle biopsy samples (2). Gene transcript profiles were measured by high-density oligonucleotide microarray containing probes for 54,675 transcripts and expressed sequence tags (HG-U133 plus 2.0 GeneChip arrays; Affymetrix, Santa Clara, CA).

GeneChip data processing. GeneChip data were subjected to invariant probe set normalization and perfect match–only model-based expression index for expression measurement by dChip (23). Genes that were identified as absent by dChip across all samples were excluded from further analysis. Differences between insulin-deprived and insulin-treated groups were evaluated by paired t test. When adjusted for the multiple comparison errors (24), no single gene remained different between the two compared groups. Therefore, we opted to focus on significantly altered pathways and functional gene sets rather than individual genes.

Real-time quantitative PCR. To validate the findings of the GeneChip results and to quantitate other genes of interest, transcript levels of selected genes were examined by real-time quantitative PCR (Applied Biosystems 7900) as previously described (7,17). RNA was extracted from frozen muscle samples using the RNeasy Fibrous Tissue kit (Qiagen) following the manufacturer's instruction. Total RNA was reverse transcribed using Taqman Reverse Transcription kit (Applied Biosystems). The primers were designed to cover the boundaries of two adjacent exons, thereby eliminating the possibility of amplifying DNA. The primers and probes used were as follows: cytochrome c oxidase subunit 5 (Cox5B), CGCGATGCGCTCCAT (forward), CCAGTCGCCTGCTCTTCAT (reverse), and AGTGGGAACACCACCTCCA GATG (probe); COX10, GATGAAGCTGCAAGTGTATGATTTG (forward), GT GGTACTTACAACCAGAGCTGTGA (reverse), and AATTTTGGCTCGACTATC (probe); ubiquinol cytochrome c reductase (UQCR) 6.4-kDa subunit, CCTG GACTGGGTACCTTACATCA (forward), AGGCACCAGAGCAGTCTGTGA (reverse), and TGGCAAGTTTAAGAAGGAT (probe); ATP5F1, AGAAGTCACAA CAGGCACTGGTT (forward), CAATGTTATTCCTTTGCACATCAAA (reverse), and AGAAGCGCCATTACC (probe); uncoupling protein (UCP)2, GATCTCAT CACCTTTCCTCTGGATA (forward), TACTGGGCGCTGGCTGTAG (reverse), and TGACTTTCTCCTTGGATCTGTAACCGGACTTTA (probe); and UCP3, CT CAAGGAGAAGCTGCTGGACTA (forward), GCTCCAAAGGCAGAGACAAAGT (reverse), and ACCTGCTCACTGACAACTTCCCCTGC (probe). The abundance of each target gene was normalized to 28S ribosomal RNA, which was coamplified in the same well. Primer and probe sequences to 28S were published elsewhere (17).

Statistics. Data are represented as means ± SE. Paired two-tailed t tests were used for outcome measures except when the hypothesis was one-sided (for previously shown directional changes), such as for indirect calorimetry, urinary nitrogen, and glucagon concentration. For such data, an a priori decision was made to use one-tailed t tests. Statistical significance was set at $P < 0.05$ for all comparisons.

RESULTS

Hormones and substrates. Average plasma levels of glucose, glucagon, and total and branched chain amino acids were significantly higher during insulin deprivation than during insulin treatment (Table 2). Serum bicarbon-

TABLE 2
Plasma hormone and metabolite concentrations

	I+	I−	*P* value	Subjects (*n*)
Glucose (mmol/l)	5.3 ± 0.1	16.8 ± 0.7	<0.0001	9
Glucagon (ng/l)	52.1 ± 6.9	87.3 ± 15.9	0.009	9
Total AA (mmol/l)	1828 ± 132	2176 ± 168	0.004	8
BCAA (mmol/l)	343 ± 26	664 ± 61	<0.0001	8
NEFA (mEq/l)	0.51 ± 0.15	1.10 ± 0.16	0.056	7
β-Hydroxybutyrate (mmol/l)	0.3 ± 0.2	2.0 ± 0.1.1	<0.001	9
Bicarbonate (mmol/l)	22.8 ± 1.7	21.0 ± 3.5	0.1	9

Data are means ± SE. I+, insulin-treated state; I−, insulin-deprived state; AA, total of all amino acids measured; BCAA, branched chain amino acid; NEFA, nonesterified fatty acid.

ate levels were not significantly different between the two groups ($P = 0.1$); however, β-hydroxybutyrate levels were significantly higher in the insulin-deprived group than in the insulin-treated group ($P < 0.001$). Urinary nitrogen was measured in four subjects that showed a greater loss during insulin deprivation (49.1 ± 7.7 mmol/h) than during insulin treatment (31.5 ± 4.9) ($P < 0.01$), and nonesterified fatty acid levels also tended to decrease.

Skeletal muscle mitochondrial ATP production. Vastus lateralis muscle MAPR ($n = 7$) using several substrates, such as glutamate plus malate (7.7 ± 1.1 μmol · min^{-1} · g^{-1} in insulin-deprived vs. 10.5 ± 1.3 in insulin-treated patients, $P < 0.008$), pyruvate plus malate (3.8 ± 0.6 μmol · min^{-1} · g^{-1} in insulin-deprived vs. 5.0 ± 0.6 in insulin-treated patients, $P < 0.01$), pyruvate plus palmitoyl-L-carnitine plus α-ketoglutarate plus malate (8.6 ± 1.1 μmol · min^{-1} · g^{-1} in insulin-deprived vs. 10.7 ± 1.6 in insulin-treated patients, $P < 0.02$), α-ketoglutarate plus glutamate (5.7 ± 0.7 μmol · min^{-1} · g^{-1} in insulin-deprived vs. 7.6 ± 1.2 in insulin-treated patients, $P = 0.01$), and palmitoyl-L-carnitine plus 1 malate (3.6 ± 0.4 μmol · min^{-1} · g^{-1} in insulin-deprived vs. 4.7 ± 0.4 in insulin-treated patients, $P = 0.02$) (Fig. 1). Succinate plus rotenone was the only substrate used that did not reach statistical significance difference between the two groups (3.1 ± 0.7 μmol · min^{-1} · g^{-1} in insulin-deprived vs. 3.9 ± 0.6 in insulin-treated patients, $P = 0.06$). No statistically significant correlation was found between bicarbonate or β-hydroxybutyrate and MAPR using each of the six substrates.

Indirect calorimetry. Oxygen consumption (Vo_2) and carbon dioxide production (Vco_2) were significantly higher during insulin deprivation than insulin treatment ($n = 8$) (Fig. 1). The respiratory quotient during insulin deprivation (0.76 ± 0.03) compared with insulin treatment (0.77 ± 0.02) was not different ($P = 0.29$).

Gene transcript levels: GeneChip arrays. A total of 40,438 transcripts were included in the analysis, of which 2,355 transcripts were differentially expressed between insulin-deprived and insulin-treated subjects ($P < 0.05$; Supplementary Table 1, available in an online appendix at http://dx.doi.org/10.2337/db07-0378) (Fig. 2). These 2,355 genes were used as "focus genes" for ingenuity pathway analysis (IPA; Ingenuity Systems, Redwood City, CA), and the full 40,438 genes were used as reference genes for IPA. In Fig. 2, the top canonical pathways associated with up- or downregulated genes in insulin-deprived compared with insulin-treated patients are displayed at the right and left panels of the volcano plot, respectively. The pathways that were upregulated by insulin deprivation include integrin signaling, vascular endothelial growth factor (VEGF)

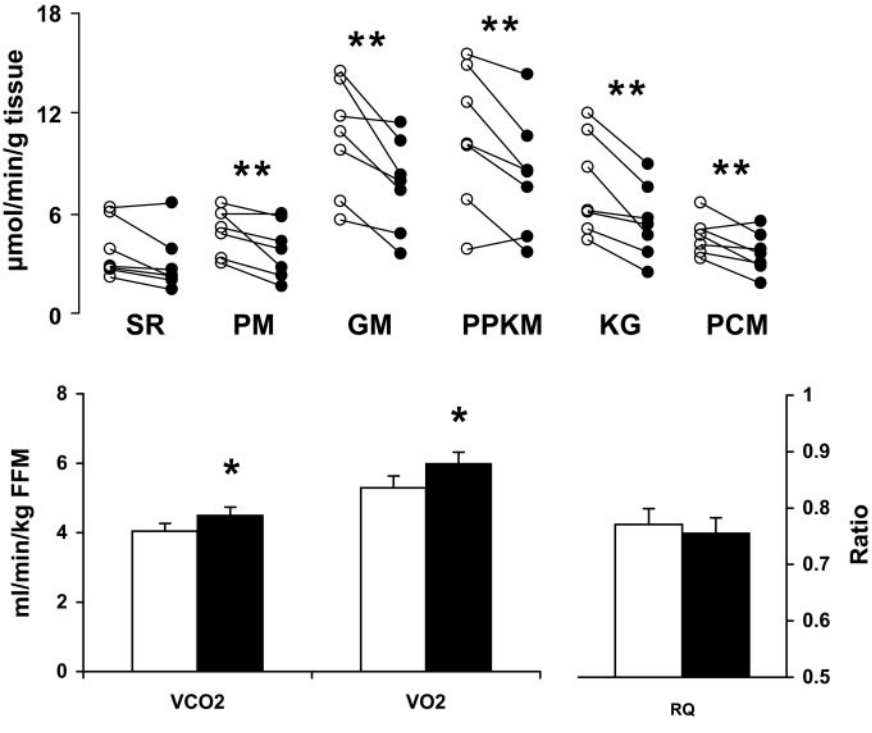

FIG. 1. Muscle MAPRs and indirect calorimetry. *Top*: MAPR ($n = 7$) was significantly lower (*$P < 0.05$) in insulin deprivation (I−, ●) compared with insulin treatment (I+, ○) using glutamate plus malate (GM), pyruvate plus malate (PM), pyruvate plus palmitoyl-L-carnitine plus α-ketoglutarate plus malate (PPKM), α-ketoglutarate plus glutamate (KG), and palmitoyl-L-carnitine plus one malate (PCM). There was no significant difference with succinate plus rotenone (SR). Actual *P* values are given in the text. *Bottom*: Whole-body Vo_2, Vco_2, and respiratory quotient (RQ) during rest ($n = 8$) were significantly higher (*$P < 0.05$) during insulin deprivation (I−, ■) compared with insulin treatment (I+, □). There was no difference in RQ. *$P < 0.05$, **$P < 0.02$.

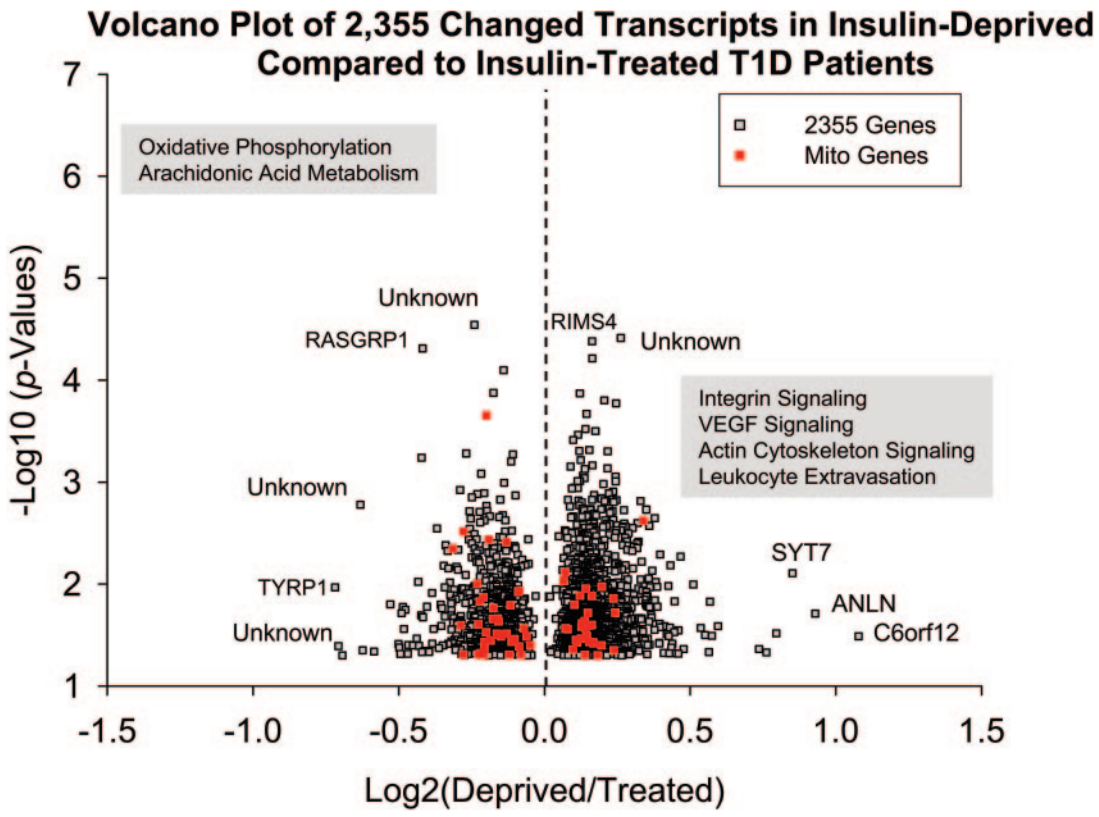

FIG. 2. Skeletal muscle gene transcript profiles measured using Affymetrix HG-U133 plus 2.0 GeneChips in insulin-treated and insulin-deprived type 1 diabetic patients. Mitochondrial (Mito) genes are highlighted in red. A volcano plot showing 2,355 differentially expressed genes and the gene groups whose transcripts were significantly between the two studied groups is shown (details in Supplementary Tables 1–3). The gene groups whose transcripts are expressed at significantly lower levels during insulin treatment (*left*) were enriched with genes involved in oxidative phosphorylation; arachidonic acid metabolism; and stilbene, coumarine, and lignin biosynthesis pathways. The more highly expressed transcripts during insulin deprivation (*right*) were significantly enriched with genes involved in integrin signaling, VEGF signaling, actin cytoskeleton signaling, and leukocyte extravasation signaling pathways.

signaling, actin cytoskeleton signaling, and leukocyte extravasation signaling. We observed significant alterations in 68 mitochondrial genes during insulin deprivation (Supplementary Table 2), of which the majority of genes directly involved in the oxidative phosphorylation pathway were downregulated by insulin deprivation (Fig. 3). In contrast, insulin deprivation upregulated the expression of fewer gene transcripts that are not directly involved in oxidative phosphorylation but are involved in other mitochondrial functions. Insulin deprivation downregulated the gene transcripts involved in arachidonic acid metabolism and stilbene, coumarine, and lignine biosynthesis pathways. The complete list of altered canonical pathways by insulin deprivation is available in Supplementary Table 3.

mRNA GeneChip validation. mRNA levels (corrected for 28S) of several mitochondrial enzymes and transporters were checked for validation of GeneChip array data (Fig. 3). Levels of COX5B (0.81 ± 0.03 in insulin-deprived vs. 0.93 ± 0.06 in insulin-treated patients, $P < 0.05$), COX10 (0.59 ± 0.07 in insulin-deprived vs. 0.66 ± 0.07 in insulin-treated patients, $P = 0.05$), UQCR (0.1.0 ± 0.06 in insulin-deprived vs. 1.12 ± 0.09 in insulin-treated patients, $P < 0.05$), and ATP5F1 (1.04 ± 0.04 in insulin-deprived vs. 1.15 ± 0.07 in insulin-treated patients, $P < 0.05$) were lower in insulin-deprived than in insulin-treated patients.

In addition, mRNA (real-time PCR) of UCP2 levels, normalized to 28S (1.86 ± 0.6 in insulin-deprived vs. 1.97 ± 0.9 in insulin-treated patients, $P = 0.4$) and UCP3 levels (6.76 ± 2.3 in insulin-deprived vs. 5.3 ± 3.9 in insulin-

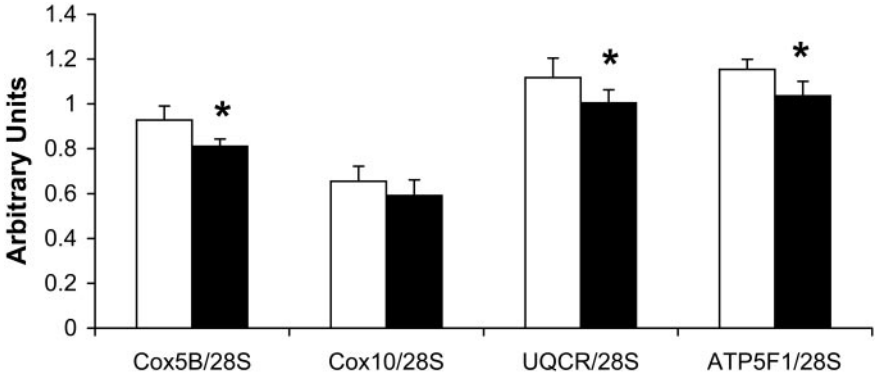

FIG. 3. mRNA levels by real-time quantitative PCR (RT-PCR) to validate GeneChip array data. All for gene transcripts changed in the same direction as in gene array data in insulin deprivation. mRNA levels of COX5B ($P = 0.004$ for gene array, 0.046 for RT-PCR), COX10 ($P = 0.041$ for gene array, 0.050 for RT-PCR), UQCR ($P = 0.004$ for gene array, 0.046 for RT-PCR), and ATP5F1 ($P = 0.025$ for gene array, 0.042 for RT-PCR), corrected for 28S, were significantly lower during insulin deprivation (I−, ■) compared with insulin treatment (I+, □) ($n = 9$) (*$P < 0.05$).

treated patients, $P = 0.7$) were not significantly different between insulin treatment and insulin deprivation in type 1 diabetic subjects.

DISCUSSION

Insulin deprivation in type 1 diabetic subjects, as expected, resulted in clear metabolic changes consistent with insulin deficiency, including increased levels of glucose, amino acids, β-hydroxybutyrate, and urinary nitrogen. However, the current study demonstrated that insulin deficiency resulted in a significant reduction in muscle MAPR demonstrated by measurements of maximal muscle ATP production capacity using six separate substrates. This muscle mitochondrial dysfunction was associated with alterations in transcript levels of several genes involving mitochondrial function and specifically a decrease in oxidative phosphorylation gene transcripts. Gene array analysis showed that after insulin deficiency, alterations in transcript levels involving several genes encoding mitochondrial proteins and several other pathways, including VEGFs and integrin signaling pathway, occurred. Selected gene transcripts were analyzed by real-time quantitative PCR that supported gene array results.

The reduced muscle MAPR during insulin deprivation occurred despite an increase in energy metabolism as noted by an increase in whole-body oxygen consumption. The finding of increased oxygen consumption during insulin deprivation in type 1 diabetes is consistent with previous findings (14,15). The current study demonstrated that increased O_2 consumption at the whole-body level was associated with a decrease in MAPR. The dissociation between O_2 consumption and ATP production can occur if there is uncoupling of oxidative phosphorylation. There was no change in UCP2 or UCP3 mRNA abundance, suggesting no change in UCP2 or UCP3 protein expression. It is controversial whether UCP2 and UCP3 are real UCPs in muscle because UCP1 is in brown adipose tissue (25), and other uncoupling mechanisms or regulators of ATP production rate may be responsible for the changes. Although we cannot exclude the possibility of uncoupling of oxidative phosphorylation in skeletal muscle during insulin deprivation, no definitive data are currently available to support it. An alternative explanation that we favor is that increased Vo_2, which represents whole-body O_2 consumption, might have occurred in tissue (or tissues) other than skeletal muscle. Previous studies have shown that increased O_2 consumption during insulin deprivation is related to elevated glucagon levels (15). Moreover, inhibition of glucagon secretion by somatostatin during insulin deprivation in type 1 diabetes reverses the increased O_2 consumption. Re-introduction of glucagon increased O_2 consumption in those subjects, demonstrating that high glucagon levels contributed to increased O_2 consumption during insulin deprivation (15). Glucagon has also been shown to increase O_2 consumption during insulin deficiency in nondiabetic healthy people (26). Because skeletal muscle does not have glucagon receptors and the liver is the main site of glucagon action, it is likely that much of the increased O_2 consumption demonstrated at the whole-body level occurs in the liver. Previous studies have also shown that insulin deprivation in type 1 diabetic patients is associated with increased synthesis rates of muscle protein (high energy consuming process) in the splanchnic bed (27), indicating increased O_2 consumption.

The current study supports a hypothesis that reduced insulin action results in reduced MAPR, which was shown consistently using different substrates. The reduction in muscle MAPR is consistent with a previous report demonstrating that insulin infusion enhances muscle MAPR (1) and therefore insulin deficiency causes inhibition of MAPR. These effects of insulin on MAPR occurred after an 8-h infusion of insulin (1). In the current study, withdrawal of insulin from type 1 diabetic subjects for a comparable period of time resulted in a decline in MAPR.

We used a gene array approach to measure transcript levels of over 40,000 genes, and the results support the reduction of the transcript level of many genes involved in mitochondrial function because of insulin deficiency. These results are confirmed by demonstrating similar directional changes by real-time PCR. As a group, the transcript levels of genes involved in oxidative phosphorylation were expressed at a significantly lower level during insulin deprivation. The mRNA transcript levels of many genes involved in MAPR, including ATP synthase, were expressed at lower levels during insulin deprivation. Interestingly, mitochondrial transcription factor A (TFAM) was also significantly lower in skeletal muscle of type 1 diabetic subjects during insulin deprivation. TFAM is a key, nuclear-encoded transcription factor that regulates transcription mitochondrial genes (28). Because TFAM expression is reduced, it is reasonable to assume that insulin deprivation also caused inhibition of transcription of mitochondrial genes. Most of the transcripts encoding mitochondrial proteins measured using the Affymetrix gene chip are nuclear encoded. It is intriguing that insulin deprivation caused upregulation of some genes encoding mitochondrial proteins, such as cytochrome c oxidase assembly protein, but it appears that none of the gene transcripts that are upregulated during insulin deprivation are directly involved in oxidative phosphorylation, per se. These upregulated gene transcripts may represent a compensatory response to the reduced mitochondrial oxidative phosphorylation capacity.

Of note, the gene transcripts involving the integrin signaling, VEGF, leukocyte extravasation, and cytoskeleton signaling pathways were higher in skeletal muscle of type 1 diabetic subjects during insulin deprivation (Fig. 3). The exact role of these pathways in regulating mitochondrial DNA abundance and mitochondrial dysfunction is not clear. We noted that many gene transcripts involved in tricarboxylic acid (TCA) cycle are altered by insulin deprivation. One plausible mechanism of upregulation of VEGF axis might be the deregulation of TCA cycle components. Insulin deficiency inhibits pyruvate dehydrogenase, but ketone bodies that increase during insulin deficiency escape this block and provide an alternative source of mitochondrial acetyl CoA (29). Also, succinate dehydrogenase (SDH) is a mitochondrial TCA cycle enzyme, and succinate, a TCA cycle metabolite, can accumulate because of SDH downregulation, transmitting a signal from mitochondria to the cytosol (30–33). A recent report suggests that once in the cytosol, succinate inhibits hypoxia-inducible factor (HIF)-α prolyl hydroxylase, leading to HIF-1α stabilization under normoxic conditions (34). Therefore, upregulation of succinate or other TCA cycle metabolites can increase expression of genes that facilitate angiogenesis and inflammatory responses during normoxia. Of note, upregulation of the leukocyte extravasation pathway observed in gene transcript analysis of the current study supports inflammatory response. On the

other hand, there are several reports suggesting that HIF can increase cell surface expression of integrin, promoting cytoskeleton signaling and proliferation (35,36). An upregulation of cytoskeletal signaling has been noted during insulin deprivation in the current study (Fig. 3). Overall, it is most likely (as noted from gene transcript data) that insulin deprivation leads the cascade of events that cause HIF activation in normoxic condition and promote mitochondrial dysfunction. Recent studies suggest that nonhypoxic induction of HIF-1α occurs during altered glucose metabolism (37). Another explanation might be that in the normoxic condition or normal oxygen concentration, cells can promote abnormal TCA cycle because of lack of insulin action, leading to stabilization of HIF-α and promoting muscle mitochondria dysfunction. It can be related to the formation of more reactive oxygen species in mitochondria during hyperglycemia and insulin deficiency (38–40). The current evidence suggests that mitochondria themselves could be important players in the oxygen-sensing pathway and can be dysregulated in the absence of insulin.

There are potential limitations in generalizing the results from this study to the decrease in insulin action in insulin-resistant states. Previous studies have shown an association between insulin resistance and muscle mitochondrial dysfunction, but it was unclear whether the insulin resistance resulted in or was secondary to mitochondrial dysfunction (41,42). The current results support insulin action, or lack of insulin, impacting mitochondrial ATP production and gene transcript levels. However, the potential interaction with secondary metabolic and hormonal changes and mitochondrial functions cannot be fully excluded based on the current study. It is possible that many metabolic changes, such as hyperglycemia and increased levels and metabolism of fatty acids, ketones, and amino acids, may have affected MAPR and gene transcript levels. At the whole-body level, there is no evidence of reduced glucose oxidation because respiratory quotient values were not different between insulin deprivation and insulin treatment. Previous studies have shown that glucose disposal during insulin deprivation is higher in type 1 diabetic patients, and presumably, because of the mass effect of increased glucose flux (43) and muscle being the main site of glucose disposal (44), it is likely that this increased glucose disposal occurred in muscle. Of interest, β-hydroxybutyrate levels were elevated in type 1 diabetic patients during insulin deprivation, but this short duration of insulin deprivation in these well-hydrated patients with normal kidney function did not cause any metabolic acidosis as indicated by normal bicarbonate level.

In summary, the current study demonstrated that insulin deprivation caused an inhibition of skeletal muscle MAPR that occurred in association with alterations in several genes involved in oxidative phosphorylation. This result demonstrates that reduced insulin action and associated metabolic changes can downregulate muscle mitochondrial oxidative phosphorylation. This reduction in muscle oxidative phosphorylation, however, occurred in association with increased transcript levels of VEGF, HIF-1α, integrin, inflammation, and cytoskeleton signaling pathways, suggesting potential interaction between these important pathways and mitochondrial dysfunction and associated metabolic derangement.

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