

EFFECT OF INSULIN DEPRIVATION ON MUSCLE MITOCHONDRIAL ATP
PRODUCTION AND GENE TRANSCRIPT LEVELS IN TYPE 1 DIABETIC SUBJECTS

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Running Title: Type 1 diabetes and Muscle Mitochondrial Function

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Abstract:

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 hours 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, non-esterified fatty acids, beta hydroxybutyrate and urinary nitrogen but no change in bicarbonate. Insulin deprivation decreased muscle mitochondrial ATP production rate 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 while those involved in vascular endothelial growth factor signaling, inflammation, cytoskeleton signaling and integrin signaling pathways were increased.

Conclusions: Insulin deficiency and associated metabolic changes reduce muscle mitochondrial ATP production rate 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 vascular endothelial growth factor, inflammation, cytoskeleton and integrin signaling pathways suggest that vascular factors and cell proliferation occurred that may interact with mitochondrial changes.

Reduced skeletal muscle mitochondrial ATP production (MAPR) has been reported to occur in association with insulin resistance in several prevalent conditions including, but not limited to, type 2 diabetes mellitus (T2D) (1-3) and offspring of people with T2D (4), obesity (5), and during aging (6-8). In addition, poor glycemic control in T2D 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 non-diabetic 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 post-absorptive insulin levels in both type 2 diabetic and non-diabetic people have similar muscle MAPR and mitochondrial DNA (mtDNA) abundance (2). Furthermore, whereas increasing insulin concentration resulted in an increase in MAPR in non-diabetic subjects, this response in MAPR did not occur in people with T2D. 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 T2D (13). Many other ATP-

dependent cellular processes that require 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 T1D 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 9 people with T1D in both the insulin-treated (I+) and insulin deprived (I-) conditions and obtained muscle biopsies to compare treatment effects on MAPR and expression of gene transcripts.

Research Design and Methods

People with type 1 diabetes mellitus were recruited with advertisements within the institution and in the Olmsted County, MN 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 patients with renal insufficiency, coronary artery disease or other vascular disease, neuropathy or poor wound healing and those taking beta blockers, tricyclic antidepressants and 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 I+ study day first followed by the I- 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 prior to 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 hours and stayed overnight until 1200 hours the next day. A retrograde catheter was inserted in 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. Following a standard dinner at 1800 hours 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 I+ day, an intravenous insulin infusion using regular human insulin was started and the plasma glucose was maintained between 4.44 to 5.56 mmol/L overnight until 1200 hours the next day. Plasma glucose was measured every 30-60 minutes and the insulin dose was adjusted every 30 min until midnight and every 15 minutes from midnight until the end of the study day. On the I- day, the insulin infusion was discontinued for 8.6 ± 0.6 hours. The muscle biopsy was not completed on one subject due to a plasma glucose level above 17 mmol/l and altered electrolytes and bicarbonate resulting in the study to be discontinued.

Blood samples for insulin, c-peptide, glucagon, amino acids, bicarbonate, beta-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 I- or a similar time point on I+. Vastus lateralis muscle samples were obtained at the end of the study (8.6 ± 0.6 hours following insulin withdrawal or similar time point on I+ under local anesthesia (lidocaine, 2%), with a percutaneous needle as described (17).

Analysis

Hormones and Substrates: Plasma insulin, glucagon, non esterified fatty acids (NEFA), betahydroxybutyrate, bicarbonate and glucose were measured as previously described (2,18,19). Plasma glucagon levels were measured by a direct double-antibody RIA (Linco Research Immunoassay, St. Charles, MO). Plasma levels of amino acids were measured by an HPLC 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 minutes at 10AM.

Mitochondrial ATP production rate (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 mM sucrose, 35 mM KH_2PO_4 , 10 mM Mg acetate, 5 mM 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 μM ADP. Substrates used were (in mM final concentration): 10 glutamate plus 1 malate (GM), 20 succinate plus 0.1 rotenone (SR), 1 pyruvate plus 0.05 palmitoyl-L-carnitine plus 10 α -ketoglutarate plus 1 malate (PPKM), 1 pyruvate plus 1 malate (PM), 0.05 palmitoyl-L-carnitine plus 1 malate (PCM), and 10 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 minutes 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 (ESTs) (HG-U133 plus 2.0 GeneChip arrays, Affymetrix, Santa Clara, CA).

GeneChip Data Processing: GeneChip data was subjected to invariant probe set normalization and perfect match (PM)-only model-based expression index for expression measurement by dChip (23). Genes that were called 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);

forward – CGCGATGCGCTCCAT,
reverse – CCAGTCGCCTGCTCTTCAT
and probe –
AGTGGGAACACCACCTCCAGATG,
Cox10; forward –
GATGAAGCTGCAAGTGTATGATTTG,
reverse –
GTGGTACTTACAACCAGAGCTGTGA
and probe – AATTTTGGCTCGACTATC,
ubiquinol cytochrome c reductase 6.4
kDa subunit (UQCR); forward –
CCTGGACTGGGTACCTTACATCA,
reverse –
AGGCACCAGAGCAGTCTGTGA and
probe – TGGCAAGTTTAAGAAGGAT,
ATP5F1; forward –
AGAAGTCACAACAGGCACTGGTT,
reverse –
CAATGTTATTCCTTTGCACATCAA
and probe – AGAAGCGCCATTACC,
UCP2; forward –
GATCTCATCACCTTTCCTCTGGATA,
reverse –TACTGGGCGCTGGCTGTAG
and probe-
TGACTTTCTCCTTGGATCTGTAACCGG
ACTTTA and UCP3; forward-
CTCAAGGAGAAGCTGCTGGACTA,
reverse-
GCTCCAAAGGCAGAGACAAAGT and
probe-
ACCTGCTCACTGACAACCTCCCCTGC.
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 represented at
mean±SEM. 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 (Table 2):

Average plasma levels of glucose,
glucagon, total and branched chain amino
acids were significantly higher during
insulin deprivation (I-) than during insulin
treatment (I+). Serum bicarbonate levels
were not significantly different between
the two groups ($p=0.1$), however beta-
hydroxybutyrate levels were significantly
higher in I- than in I+ ($p<0.001$). Urinary
nitrogen were measured in four subjects
that showed a greater loss during I-
(49.1 ± 7.7 mmol/h) than during I+
(31.5 ± 4.9) ($P<0.01$) and non esterified
fatty acid levels also tended to decrease.

Skeletal Muscle mitochondrial ATP
production (Figure 1): Vastus lateralis
muscle MAPR (N=7) using several
substrates such as glutamate plus malate
(GM) (7.7 ± 1.1 $\mu\text{mol}/\text{min}/\text{g}$ in I- vs.
 10.5 ± 1.3 in I+, $p<0.008$), pyruvate plus
malate (PM) (3.8 ± 0.6 $\mu\text{mol}/\text{min}/\text{g}$ in I- vs.
 5.0 ± 0.6 in I+, $p<0.01$), pyruvate plus
palmitoyl-L-carnitine plus α -ketoglutarate
plus malate (PPKM) (8.6 ± 1.1 $\mu\text{mol}/\text{min}/\text{g}$
in I- vs. 10.7 ± 1.6 in I+, $p<0.02$), α -
ketoglutarate plus glutamate (KG)
(5.7 ± 0.7 $\mu\text{mol}/\text{min}/\text{g}$ in I- vs. 7.6 ± 1.2 in I+,
 $p=0.01$) and palmitoyl-L-carnitine plus 1
malate (PCM) (3.6 ± 0.4 $\mu\text{mol}/\text{min}/\text{g}$ in I-
vs. 4.7 ± 0.4 in I+, $p=0.02$). Succinate plus
rotenone (SR) was the only substrate
used that did not reach statistical
significance difference between the two
groups (3.1 ± 0.7 $\mu\text{mol}/\text{min}/\text{g}$ in I- vs.
 3.9 ± 0.6 in I+, $p=0.06$). No statistically
significant correlation was found between
bicarbonate or beta-hydroxybutyrate and
MAPR using each of the six substrates.

Indirect Calorimetry (Figure 1): Oxygen
consumption (VO_2) and carbon dioxide
production (VCO_2) were significantly

higher during I- compared to I+ (N=8). The respiratory quotient during I- (0.76 ± 0.03) compared to I+ (0.77 ± 0.02) was not different ($p = 0.29$).

Gene Transcript levels - GeneChip Arrays: (Figure 2) 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). These 2,355 genes were used as “focus genes” for Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA), and the full list of 40,438 genes were used as reference genes for IPA. As shown in Figure 2, the top canonical pathways associated with up- or down-regulated genes in insulin-deprived compared to insulin-treated patients were displayed at the right and left panels of the volcano plot, respectively. The pathways that were up-regulated by insulin deprivation include integrin signaling, vascular endothelial growth factor (VEGF) 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 down-regulated by insulin deprivation (Figure 3, left panel). In contrast, insulin deprivation upregulated the expression of a smaller number of gene transcripts that are not directly involved in oxidative phosphorylation but are involved in other mitochondrial functions. Insulin deprivation down regulated 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 recorded in Supplementary Table 3.

mRNA Gene Chip Validation: (Figure 3) mRNA levels (corrected for 28S) of several mitochondrial enzymes and transporters were checked for validation of GeneChip array data. Levels of COX5B (0.81 ± 0.03 in I- vs 0.93 ± 0.06 in I+, $p < 0.05$), COX10 (0.59 ± 0.07 in I- vs 0.66 ± 0.07 in I+, $p = 0.05$). Ubiquinol cytochrome c reductase (UQCR) ($0.1.0\pm 0.06$ in I- vs 1.12 ± 0.09 in I+, $p < 0.05$) and ATP5F1 (1.04 ± 0.04 in I- vs 1.15 ± 0.07 in I+, $p < 0.05$) were lower in I- compared to I+.

In addition, mRNA (Real time PCR) of uncoupling protein (UCP) 2 levels, normalized to 28S, (1.86 ± 0.6 in I- vs 1.97 ± 0.9 , $p = 0.4$) and UCP3 levels (6.76 ± 2.3 in I- vs 5.3 ± 3.9 in I+, $p = 0.7$) were not significantly different between I+ and I- in T1 D subjects.

Discussion

Insulin-deprivation in T1D subjects, as expected, resulted in clear metabolic changes consistent with insulin deficiency including increased levels of glucose, amino acids, betahydroxybutyrate and urinary nitrogen. However, the current study demonstrated that insulin deficiency resulted in a significant reduction in muscle mitochondrial ATP production rate 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 following insulin deficiency, alterations in transcript levels involving several genes encoding mitochondrial proteins as well as several other

pathways including Vascular Endothelial Growth Factors (VEGF) and integrin signaling pathway occurred. Selected gene transcripts were analyzed by real time QPCR 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 T1D is consistent with previous findings (14,15). The current study demonstrated that increased O₂ consumption at the whole body level was associated with a decrease in MAPR. The dissociation between O₂ consumption and ATP production can occur if there is uncoupling of oxidative phosphorylation. There was no change in UCP 2 or UCP 3 mRNA abundance, suggesting no change in UCP2 or UCP3 protein expression. It is controversial whether UCP 2 and UCP3 are real uncoupling proteins in muscle as UCP 1 is in brown adipose tissue (25) and other uncoupling mechanisms or regulators of ATP production rate may be responsible for the changes. Although we can not exclude the possibility of uncoupling of oxidative phosphorylation in skeletal muscle during insulin deprivation no definitive data is currently available to support it. An alternative explanation that we favor is that increased VO₂, which represents whole body O₂ consumption, might have occurred in tissue (or tissues) other than skeletal muscle. Previous studies have shown that increased O₂ consumption during insulin deprivation is related to elevated glucagon levels (15). Moreover, inhibition of glucagon secretion by somatostatin during insulin deprivation in T1D reverses the increased O₂ consumption. Re-introduction of glucagon increased O₂ consumption in

those subjects demonstrating that high glucagon levels contributed to increased O₂ consumption during insulin deprivation (15). Glucagon has also been shown to increase O₂ consumption during insulin deficiency in non-diabetic healthy people (26). Since 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 O₂ consumption demonstrated at the whole body level occurs in the liver. Previous studies also have 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 O₂ consumption.

The current study supports a hypothesis that reduced insulin action results in reduced MAPR that was shown consistently using different substrates. The reduction in muscle MAPR results 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 following an eight hour infusion of insulin (1). In the current study, withdrawal of insulin from T1D subjects for a comparable period of time resulted in decline in MAPR.

We used a gene array approach to measure transcript levels of over 40,000 genes and the results support that insulin deficiency reduces the transcript level of many genes involved in mitochondrial function. 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, mitochondrial (TFAM), was also significantly lower in skeletal muscle of T1D subjects during insulin deprivation. TFAM is a key, nuclear encoded, transcription factor that regulates transcription mitochondrial genes (28). Since 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 up-regulation 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 up-regulated 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 T1D subjects during insulin deprivation (Figure 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 Tri Carboxylic 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 increases during insulin deficiency escape this block and provide 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 due to SDH downregulation, transmitting a signal from mitochondria to the cytosol (30-33). A recent report suggests that once in the cytosol, succinate inhibits HIF- α prolyl hydroxylase (PHD) leading to Hypoxia-inducible factor 1 α (HIF-1 α) 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 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 (Figure 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 non-hypoxic induction of HIF-1 α 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 due to lack of insulin action and that leads to stabilization of HIF- α and promote muscle mitochondria dysfunction. It can be related to the formation of more reactive oxygen species (ROS) in mitochondria during hyperglycemia and insulin deficiency (38-

40). The current evidence suggests that mitochondria themselves could be important players in 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 that insulin action, or lack of insulin, impact mitochondrial ATP production and gene transcript levels. However, the potential interaction with secondary metabolic and hormonal changes and mitochondrial functions can not 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 whole body level, there is no evidence of reduced glucose oxidation since RQ 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, presumably due to 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, beta hydroxybutyrate levels were elevated in T1D 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 mitochondrial ATP production rate which occurred in association with alterations in several genes involved in oxidative phosphorylation. This result demonstrates that reduced insulin action and associated metabolic changes can down regulate muscle mitochondrial oxidative phosphorylation. This reduction in muscle oxidative phosphorylation however, occurred in association with increased transcript levels of VEGF, HIF - 1 α , integrin, inflammation and cytoskeleton signaling pathways suggesting potential interaction between these important pathways and mitochondrial dysfunction and associated metabolic derangement.

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References

1. Stump CS, Short KR, Bigelow ML, Schimke JC, Nair KS: Effect of insulin on human skeletal muscle mitochondrial ATP production, protein synthesis, and mRNA transcripts. *Proc Natl Acad Sci USA* 100:7996-8001, 2003
2. Asmann YW, Stump CS, Short KR, Coenen-Schimke JM, Guo Z, Bigelow M, Nair KS: 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. *Diabetes* 55:3309-3319, 2006
3. Petersen KF, Dufour S, Shulman GI: Decreased Insulin-Stimulated ATP Synthesis and Phosphate Transport in Muscle of Insulin-Resistant Offspring of Type 2 Diabetic Patients. *PLoS Medicine / Public Library of Science* 2:E233, 2005
4. Felig P, Wahren J, Sherwin R, Hendler R: Insulin, glucagon, and somatostatin in normal physiology and diabetes mellitus. *Diabetes* 25:1091-1099, 1976
5. Ritov VB, Menshikova EV, He J, Ferrell RE, Goodpaster BH, Kelley DE: Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. *Diabetes* 54:8-14, 2005
6. Short KR, Bigelow ML, Nair KS: Age effect on muscle mitochondrial function and impaired glucose tolerance after a mixed meal (Abstract). *Diabetes* 52: S1, 2003
7. Short KR, Bigelow ML, Kahl JC, Singh R, Coenen-Schimke JM, Raghavakaimal S, Nair KS: Decline in skeletal muscle mitochondrial function with aging in humans. *Proc Natl Acad Sci USA* 102:5618-5623, 2005
8. Petersen KF, Befroy D, Sufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW, Shulman G: Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science* 300:1140-1142, 2003
9. Sreekumar R, Halvatsiotis P, Schimke JC, Nair KS: Gene expression profile in skeletal muscle of type 2 diabetes and the effect of insulin treatment. *Diabetes* 51:1913-1920, 2002
10. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ: Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *Proc Natl Acad Sci USA* 100:8466-8471, 2003
11. Mootha VK, Lindgren CM, Eriksson K-F, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC: PGC-1 α -responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34:267-273, 2003
12. Petersen KF, Shulman GI: Etiology of Insulin Resistance. *Am J Med* 119:S10-S16, 2006
13. Regensteiner JG, Sippel JM, McFarling ET, Wolfel EE, Hiatt WR: Effects of non-insulin-dependent diabetes on oxygen consumption during treadmill exercise. *Med Sci Sports Exerc* 27:875-881, 1995
14. Nair KS, Halliday D, Garrow JS: Increased energy expenditure in poorly controlled type I (insulin-dependent) diabetic patients. *Diabetologia* 27:13-16, 1984

15. Charlton MR, Nair KS: Role of hyperglucagonemia in catabolism associated with type 1 diabetes. Effects on leucine metabolism and the resting metabolic rate. *Diabetes* 47:1748-1756, 1998
16. Copeland KC, Kenney FA, Nair KS: Heated dorsal hand vein sampling for metabolic studies: a reappraisal. *Am J Physiol Endocrinol Metab* 263:E1010-E1014, 1992
17. Balagopal P, Schimke JC, Ades PA, Adey D, Nair KS: Age effect on transcript levels and synthesis rate of muscle MHC and response to resistance exercise. *Am J Physiol Endocrinol Metab* 280:E203-E208, 2001
18. Dhatariya KK, Bigelow ML, Nair KS: Effect of dehydroepiandrosterone replacement on insulin sensitivity and lipids in hypoadrenal women. *Diabetes* 54:765-769, 2005
19. Nair KS, Welle SL, Halliday D, Campbell RG: Effect of beta-hydroxybutyrate on whole-body leucine kinetics and fractional mixed skeletal muscle protein synthesis in humans. *J Clin Invest* 82:198-205, 1988
20. Jones BN, Gilligan JP: o-Phthaldialdehyde procolumn derivatization and reversed-phase high-performance liquid chromatography of polypeptide hydrolysates and physiological fluids. *J Chromatogr* 266:471-482, 1983
21. Short KR, Nygren J, Barazzoni R, Levine J, Nair KS: T₃ increases mitochondrial ATP production in oxidative muscle despite increased expression of UCP2 and -3. *Am J Physiol Endocrinol Metab* 280:E761-E769, 2001
22. Wibom R, Hultman E: ATP production rate in mitochondria isolated from microsamples of human muscle. *Am J Physiol Endocrinol Metab* 259:E204-E209, 1990
23. Li C, Wong WH: Model-based analysis of oligonucleotide arrays: Expression index computation and outlier detection. *Proc Natl Acad Sci USA* 98:31-36, 2001
24. Kropf S, Lauter J: Multiple tests for different sets of variables using a data-driven ordering of hypotheses, with an application to gene expression data. *Biometrical J* 44:789-800, 2002
25. Dalgaard LT, Pedersen O: Uncoupling proteins: functional characteristics and role in the pathogenesis of obesity and type II diabetes. *Diabetologia* 44:946-965, 2001
26. Nair KS: Hyperglucagonemia increases resting metabolic rate in man during insulin deficiency. *J Clin Endocrinol Metab* 64,5:896-900, 1987
27. Nair KS, Ford GC, Ekberg K, Fernqvist-Forbes E, Wahren J: Protein dynamics in whole body and in splanchnic and leg tissues in type I diabetic patients. *J Clin Invest* 95:2926-2937, 1995
28. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM: Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 98:115-124, 1999
29. Sato K, Kashiwaya Y, Keon CA, Tsuchiya N, King MT, Radda GK, Chance B, Clarke K, Veech RL: Insulin, ketone bodies, and mitochondrial energy transduction. *FASEB J* 9:651-8, 1995
30. Baysal BE: On the association of succinate dehydrogenase mutations with hereditary paraganglioma. *Trends Endocrinol Metab* 14:453-9, 2003
31. Eng C, Kiuru M, Fernandez MJ, Aaltonen LA: A role for mitochondrial enzymes in inherited neoplasia and beyond. *Nat Rev Cancer* 3:193-202, 2003

32. Rustin P, Rotig A: Inborn errors of complex II--unusual human mitochondrial diseases. *Biochim Biophys Acta* 1553:117-22, 2002
33. Pollard PJ, Wortham NC, Tomlinson IP: The TCA cycle and tumorigenesis: the examples of fumarate hydratase and succinate dehydrogenase. *Ann Med* 35:632-9, 2003
34. Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, Pan Y, Simon MC, Thompson CB, Gottlieb E: Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-a prolyl hydroxylase. *Cancer Cell* 7:77-85, 2005
35. Walmsley SR, Cadwallader KA, Chilvers ER: The role of HIF-1alpha in myeloid cell inflammation. *Trends Immunol* 26:434-9, 2005
36. Cowden Dahl KD, Robertson SE, Weaver VM, Simon MC: Hypoxia-inducible factor regulates alphavbeta3 integrin cell surface expression. *Mol Biol Cell* 16:1901-12, 2005
37. Heidbreder M, Qadri F, Jöhren O, Dendorfer A, Depping R, Fröhlich F, Wagner KF, Dominiak P: Non-hypoxic induction of HIF-3[alpha] by 2-deoxy-d-glucose and insulin. *Biochem Biophys Res Commun* 352:437-443, 2007
38. Hoppeler H, Vogt M, Weibel ER, Fluck M: Response of skeletal muscle mitochondria to hypoxia. *Exp Physiol* 88:109-19, 2003
39. Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT: Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc Natl Acad Sci USA* 95:11715-20, 1998
40. Doege K, Heine S, Jensen I, Jelkmann W, Metzen E: Inhibition of mitochondrial respiration elevates oxygen concentration but leaves regulation of hypoxia-inducible factor (HIF) intact. *Blood* 106:2311-7, 2005
41. Short KR, Nair KS, Stump CS: Impaired mitochondrial activity and insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med* 350:2419-2421, 2004
42. Petersen KF, Shulman GI: Etiology of insulin resistance. *Am J Med* 119:S10-6, 2006
43. Pehling GB, Tessari P, Gerich JE, Haymond MW, Service FJ, Rizza RA: Abnormal meal carbohydrate disposition in insulin-dependent diabetes. Relative contributions of endogenous glucose production and initial splanchnic uptake and effect of intensive insulin therapy. *J Clin Invest* 74:985-991, 1984
44. Shulman GI, Rothman DL, Jue T, Stein P, DeFronzo RA, Shulman RG: Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by ¹³C nuclear magnetic resonance spectroscopy. *N Engl J Med* 322:223-228, 1990

Table 1. Subject characteristics

Variable	mean \pm SD
Age (years)	31 \pm 3
Weight (kg)	79.5 \pm 4.0
BMI (kg/m ²)	25.7 \pm 1.0
Fat mass (%)	27.7 \pm 4.0
Duration of T1D (years)	17.3 \pm 3.2
Hemoglobin A1c (%)	7.4 \pm 0.40
C-peptide (nmol/l)	<0.03

Values are for 9 people with type 1 diabetes.

Table 2: Plasma hormone and metabolite concentrations (mean \pm SEM) .

	I+	I-	P-value	Number of subjects
Glucose (mmol/l)	5.3 \pm 0.1	16.8 \pm 0.7	p<0.0001	9
Glucagon (ng/l)	52.1 \pm 6.9	87.3 \pm 15.9	p=0.009	9
Total AA (mmol/l)	1828 \pm 132	2176 \pm 168	p=0.004	8
BCAA (mmol/l)	343 \pm 26	664 \pm 61	p<0.0001	8
NEFA (mEq/l)	0.51 \pm 0.15	1.10 \pm 0.16	p=0.056	7
Beta-hydroxybutyrate				
(mmol/l)	0.3 \pm 0.2	2.0 \pm 1.1	P<0.001	9
Bicarbonate (mmol/l)	22.8 \pm 1.7	21.0 \pm 3.5	P=0.1	9

I+, insulin treated state; I-, insulin deprived state; AA, total of all amino acids measured; BCAA, branched chain amino acid; NEFA, non-esterified fatty acids

Legends

Figure 1. Muscle mitochondrial ATP production rates (MAPR) and indirect calorimetry. *Upper panel:* MAPR (N=7) was significantly lower ($p < 0.05$) in I- (insulin deprivation, dark circles) compared to I+ (insulin treatment, empty circles) 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 1 malate (PCM). There was no significant difference with succinate plus rotenone (SR). Actual p values are given in the text.

Lower panel: whole body oxygen consumption (VO_2), carbon dioxide production (VCO_2) and respiratory quotient (RQ) during rest (N=8) were significantly higher ($p < 0.05$) (*) during I- compared to I+. There was no difference in respiratory quotient (RQ).

* $p < 0.02$, ** $p < 0.05$

Figure 2. Skeletal muscle gene transcript profiles measured using Affymetrix HG-U133 plus 2.0 GeneChips in insulin-treated and insulin-deprived T1D patients. Mitochondrial (Mito) genes are highlighted in red. A Volcano plot showing 2,355 differentially expressed genes, as well as the gene groups whose transcripts were significantly between the two studied groups, is shown (details in Supplement Tables 1-3). The gene groups whose transcripts are expressed at significantly lower levels during insulin-treatment (left panel) were enriched with genes involving in Oxidative Phosphorylation, Arachidonic Acid Metabolism, and Stilbene, Coumarine and Lignin Biosynthesis pathways. The more highly expressed transcripts during insulin-deprivation (right panel) were significantly enriched with genes involved in Integrin Signaling, VEGF Signaling, Actin Cytoskeleton Signaling, and Leukocyte Extravasation Signaling pathways.

Figure 3. mRNA levels by real-time QPCR (RTPCR) to validate Gene Chip 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 RTPCR) , COX10 (P= 0.041 for gene array, 0,050 for RTPCR), UQCR (P= 0.004 for gene array, 0,046 for RTPCR) and ATP5F1 (P=0.025 for gene array, 0,042 for RTPCR), corrected for 28S, were significantly lower during I- compared to I+ (N=9) (*= $p < 0.05$).

Figure 1.

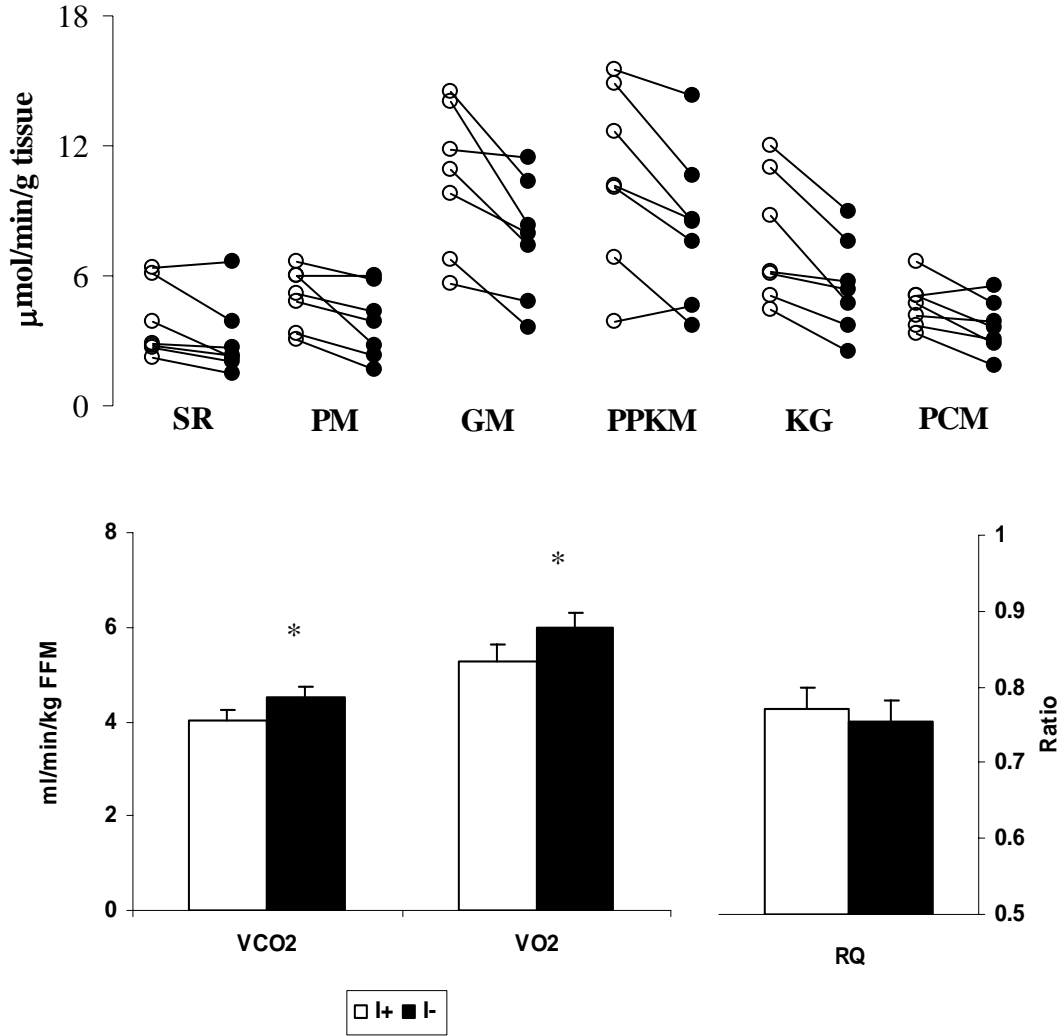


Figure 2.

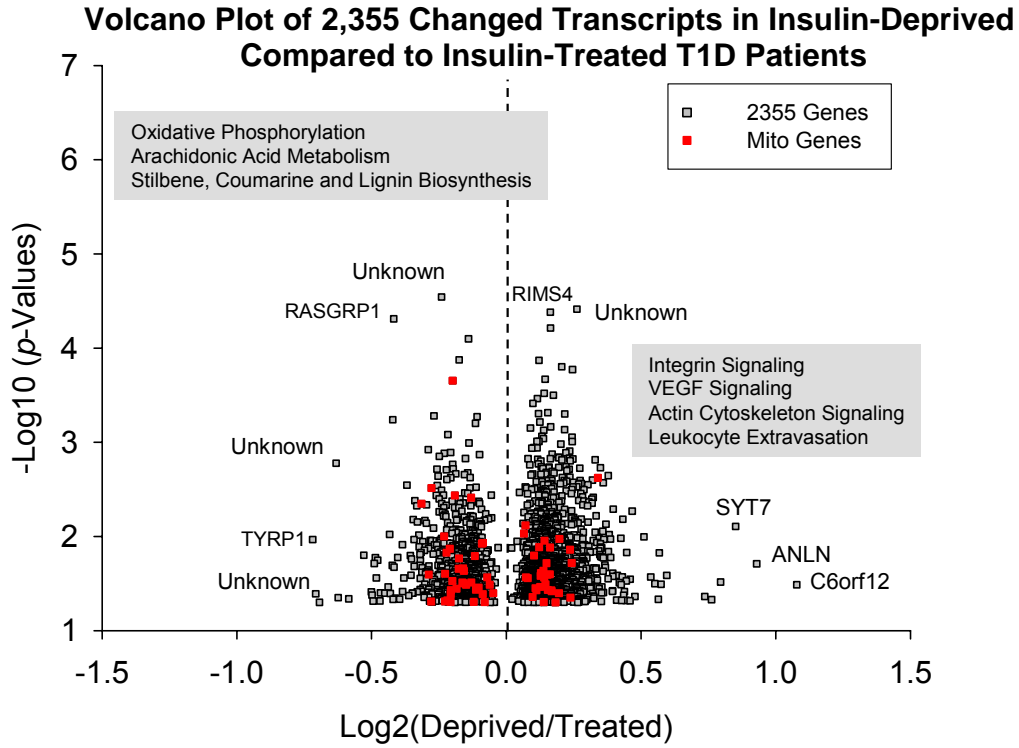


Figure 3.

