Original Article

Reduced Expression of Nuclear-Encoded Genes Involved in Mitochondrial Oxidative Metabolism in Skeletal Muscle of Insulin-Resistant Women With Polycystic Ovary Syndrome

Vibe Skov,[1] Dorte Glintborg,[2] Steen Knudsen,[3] Thomas Jensen,[3] Torben A. Kruse,[1] Qihua Tan,[1,4] Klaus Brusgaard,[1] Henning Beck-Nielsen,[2] and Kurt Højlund[2]

Insulin resistance in skeletal muscle is a major risk factor for the development of type 2 diabetes in women with polycystic ovary syndrome (PCOS). In patients with type 2 diabetes, insulin resistance in skeletal muscle is associated with abnormalities in insulin signaling, fatty acid metabolism, and mitochondrial oxidative phosphorylation (OXPHOS). In PCOS patients, the molecular mechanisms of insulin resistance are, however, less well characterized. To identify biological pathways of importance for the pathogenesis of insulin resistance in PCOS, we compared gene expression in skeletal muscle of metabolically characterized PCOS patients ($n = 16$) and healthy control subjects ($n = 13$) using two different approaches for global pathway analysis: gene set enrichment analysis (GSEA 1.0) and gene map annotator and pathway profiler (GenMAPP 2.0). We demonstrate that impaired insulin-stimulated total, oxidative and nonoxidative glucose disposal in PCOS patients are associated with a consistent downregulation of OXPHOS gene expression using GSEA and GenMAPP analysis. Quantitative real-time PCR analysis validated these findings and showed that reduced levels of peroxisome proliferator–activated receptor γ coactivator α (PGC-1α) could play a role in the downregulation of OXPHOS genes in PCOS. In these women with PCOS, the decrease in OXPHOS gene expression in skeletal muscle cannot be ascribed to obesity and diabetes. This supports the hypothesis of an early association between insulin resistance and impaired mitochondrial oxidative metabolism, which is, in part, mediated by reduced PGC-1α levels. These abnormalities may contribute to the increased risk of type 2 diabetes observed in women with PCOS. *Diabetes* 56:2349–2355, 2007

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting 5–10% of reproductive-aged women (1). It is a heterogeneous condition with unknown etiology characterized by hyperandrogenism and anovulatory infertility and occurs in association with insulin resistance leading to compensatory hyperinsulinemia, which stimulates ovarian androgen production (1,2). Insulin resistance in PCOS patients confers a substantial risk for developing type 2 diabetes and cardiovascular disease (1–4), and the increase in incidence of PCOS parallels the increase in obesity making it an important threat to the Western world and developing countries in the future (1).

Skeletal muscle is the major site of insulin-stimulated glucose disposal (5), and muscular insulin resistance in this tissue therefore represents a major risk factor for type 2 diabetes in PCOS patients. This is reflected by impaired insulin-stimulated total, oxidative, and nonoxidative glucose disposal in PCOS (6) similar to the defects observed in type 2 diabetes (7,8). The mechanisms underlying skeletal muscle insulin resistance in PCOS in vivo are largely unknown but may include reduced insulin-mediated association of phosphatidylinositol 3-kinase (PI 3-kinase) with insulin receptor substrate-1 (IRS-1) and increased serine phosphorylation of the insulin receptor and IRS-1 (9–13). Similar abnormalities have been reported in skeletal muscle of patients with type 2 diabetes (7,8), but insulin resistance in patients with type 2 diabetes and their first-degree relatives is further characterized by impaired insulin activation of glycogen synthase (14), increased lipid content (15), and abnormalities in mitochondrial oxidative phosphorylation (OXPHOS) in skeletal muscle (16–22). Whether these abnormalities also exist in skeletal muscle from PCOS patients remains to be examined.

High-throughput technologies, such as DNA microarrays, are powerful tools that enable researchers to deter-

From the [1]Department of Biochemistry, Genetics, and Pharmacology, Odense University Hospital and Human Microarray Centre, University of Southern Denmark, Odense, Denmark; the [2]Department of Endocrinology, Odense University Hospital, Odense, Denmark; the [3]Medical Prognosis Institute Aps, Hørsholm, Denmark; and the [4]Institute of Public Health, University of Southern Denmark, Odense, Denmark.

Address correspondence and reprint requests to Vibe Skov, Department of Biochemistry, Genetics, and Pharmacology, Odense University Hospital, Sdr. Boulevard 29, 5000 Odense C, Denmark. E-mail: vibe.skov@ouh.regionsyddanmark.dk.

Received for publication 27 February 2007 and accepted in revised form 8 June 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 11 June 2007. DOI: 10.2337/db07-0275.

Additional information for this article can be found in an online appendix at http://dx.doi.org/10.2337/db07-0275.

CaMK, Ca^{2+}/calmodulin-dependent protein kinase; FDR, false discovery rate; FFA, free fatty acid; FSH, follicle-stimulating hormone; FWER, family-wise error rate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GenMAPP, gene map annotator and pathway profiler; GSEA, gene set enrichment analysis; IRS-1, insulin receptor substrate 1; LH, luteinizing hormone; NRF-1, nuclear respiratory factor 1; OXPHOS, oxidative phosphorylation; PCOS, polycystic ovary syndrome; PGC-1α and -β, peroxisome proliferator–activated receptor γ coactivator α and β; PI 3-kinase, phosphatidylinositol 3-kinase.

Data are available from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession no. GSE6798).

TABLE 1
Clinical and metabolic characteristics

	PCOS patients	Control subjects
n	16	13
Age (years)	30.8 ± 1.8	34.7 ± 2.0
BMI (kg/m^2)	34.1 ± 1.1	34.0 ± 1.8
Plasma glucose (mmol/l)	5.5 ± 0.1	5.5 ± 0.1
Serum insulin (pmol/l)	116 ± 16	43 ± 4*
Plasma triglycerides (mmol/l)	1.95 ± 0.23	0.86 ± 0.12*
Serum-free testosterone (mg/l)	0.17 ± 0.03	0.06 ± 0.01*
Serum LH-to-FSH ratio	1.55 ± 0.17	0.68 ± 0.06*
Plasma FFA (mmol/l)	0.47 ± 0.03	0.49 ± 0.04
Plasma FFA during clamp (mmol/l)	0.07 ± 0.01	0.02 ± 0.00*
Total glucose disposal during clamp (mg · m^{-2} · min^{-1})	121 ± 4	289 ± 23*
Glucose oxidation during clamp (mg · m^{-2} · min^{-1})	76 ± 5	124 ± 5*
Nonoxidative glucose metabolism during clamp (mg · m^{-2} · min^{-1})	45 ± 5	165 ± 22*
Lipid oxidation during clamp (mg · m^{-2} · min^{-1})	26 ± 2	7 ± 2*

Data are means ± SEM. *$P < 0.01$ PCOS vs. control subjects. Students *t* test for nonpaired data was used.

mine changes in transcript levels of thousands of genes simultaneously (23). Because many genes are dysregulated during the development and progression of a complex disease, biological pathway analyses using data from DNA microarray experiments may lead to a more comprehensive understanding of a disease at the molecular level. Recently, application of transcriptomics and proteomics in diabetes research has pointed to abnormalities in mitochondrial metabolism in skeletal muscle (19–22) and indicated that reduced expression of peroxisome proliferator–activated receptor γ coactivator-1α (PGC-1α) and -1β (PGC-1β) and nuclear respiratory factor 1 (NRF-1) could play a key role for these changes in insulin-resistant muscle (21,22). Whether insulin resistance in patients with PCOS is associated with the same or unique transcriptional changes in skeletal muscle remains to be determined.

To identify biological pathways in skeletal muscle associated with insulin resistance in PCOS, we applied high-density oligonucleotide arrays, two different approaches for global pathway analysis, and quantitative real-time PCR to compare gene expression profiles in skeletal muscle of insulin-resistant women with PCOS and well-matched healthy control women who were metabolically characterized by euglycemic-hyperinsulinemic clamp and indirect calorimetry.

RESEARCH DESIGN AND METHODS

Subjects, metabolic characterization, and muscle biopsy. Sixteen obese women of fertile age with PCOS and 13 healthy women, matched according to age and BMI, participated in the study (Table 1). These subjects were selected from a larger cohort who participated in a study reported recently (6). The subpopulation of PCOS patients represents the most insulin-resistant subjects from whom a basal muscle biopsy were obtained. Inclusion and exclusion criteria for PCOS and control subjects are as described previously (6). In brief, criteria for PCOS included oligoovulation (defined as irregular periods during <1 year in combination with a cycle length of >35 days), elevated free testosterone levels (>0.035 nmol/l), and/or hirsutism (total Ferriman-Gallwey score >7) and the absence of diabetes, hypertension, hyperprolactinemia, hypothyroidism, and adrenal enzyme defects. All PCOS subjects had A1C within the normal range (4.9–6.1%). Control subjects had regular menses, normal glucose tolerance, and no family history of diabetes. No subjects were taking medicines known to affect hormonal or metabolic parameters. Informed written consent was obtained from all subjects before participation. The study was approved by the local ethics committee and was performed in accordance with the Helsinki Declaration.

The euglycemic-hyperinsulinemic clamp studies were performed after an overnight fast as described in detail previously (6). In brief, a 2-h basal tracer equilibration period was followed by infusion of insulin at a rate of 40 mU · m^{-2} · min^{-1} for 3 h. The studies were combined with indirect calorimetry, and rates of total glucose disposal, glucose and lipid oxidation, and nonoxidative glucose metabolism were calculated as described previously (6). A muscle biopsy from each subject was obtained from the vastus lateralis muscle in the basal state after the 2-h basal tracer equilibration period using a modified Bergström needle with suction under local anesthesia. Muscle samples were immediately frozen in liquid nitrogen within 30 s. Serum levels of insulin, free testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and plasma glucose, triglyceride, and free fatty acids (FFAs) were assayed as described previously (6).

RNA extraction. Frozen muscle tissue was homogenized in Trizol with a Polytron Homogenizer, and total RNA was purified using TRIzol Reagent (Life Technologies, Gaithersburg, MD), including an extra step after phase separation with chloroform. Briefly, 1 vol phenol-chloroform-isoamylalcohol (25-24-1) (Sigma-Aldrich, St. Louis, MO) was added to the aqueous phase and spun at 12,000 rpm for 20 min at 4°C. Then, 1 vol chloroform was added to the aqueous phase and spun at 12,000 rpm for 20 min at 4°C. Quantity of RNA was determined with a spectrophotometer, and RNA integrity was assessed using Agilent 2100 Bioanalyser and degradometer software (24).

Amplified RNA preparation and microarray hybridization. One μg purified total RNA was converted to biotin-labeled aRNA using the MessageAmpTM II-Biotin single-round amplified RNA (aRNA) amplification kit according to the manufacturer's instructions (Ambion, Austin, TX). Labeled aRNA was fragmented as described in the Affymetrix manual (Affymetrix, Santa Clara, CA) and hybridized to Affymetrix HG-U133 Plus 2.0 chips. All glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 3′/5′ probe set hybridization ratios range from 0.92 to 1.42.

Data treatment and statistical analysis. The R statistical software (25) was applied for initial data processing and statistical analysis. Global background correction of probe intensities was performed using a method implemented in the robust multi-array average method (26), and nonlinear normalization of probe intensities was done using qspline (27). Gene expression index calculation was done using model-based index calculation (28). Only perfect-match probes were included in data analysis. Differences in gene expression between groups were calculated for each gene in the dataset by using Welch two sample *t* test. An uncorrected $P < 0.05$ was considered significant.

Global pathway analyses. Gene expression changes were integrated with gene sets and biological pathways using gene map annotator and pathway profiler (GenMAPP 2.0) (29) and gene set enrichment analysis (GSEA 1.0) (30). MAPPFinder 2.0 (31), a tool integrated in GenMAPP 2.0, was applied to examine significant pathways and gene ontology terms. In GSEA, a total of 169 gene sets were applied. All genes were ranked according to changes in gene expression between PCOS patients and control subjects using the *t* test in the GSEA software. Ten thousand gene permutations were used to obtain the nominal *P* value, and gene sets with a false discovery rate (FDR) <0.05 were suggested to be significantly regulated.

Quantitative real-time PCR. DNase I–treated RNA from 13 PCOS patients and 13 control subjects was reverse transcribed to single-stranded cDNA using TaqMan reverse transcription reagents and random hexamer primers (Applied Biosystems). Three women with PCOS were not included in the analysis because of a lack of RNA. TaqMan gene expression assays (Applied Biosystems) for nine selected genes (Supplemental Table 1, which is detailed in the online appendix [available at http://dx.doi.org/10.2337/db07-0275]) and TaqMan Universal Master Mix (Applied Biosystems) were used for quantification of gene expression using Applied Biosystems Prism 7700. Expression of target genes was normalized to the endogenous controls PPIA (cyclophilin A) and GAPDH. To perform the most appropriate validation of microarray data, bioinformatic approaches, such as NetAffx (http://www.Affymetrix.com), refseq (http://www.ncbi.nlm.nih.gov), and Ensembl (http://www.ensembl.org), were used to identify the Affymetrix probe set for each gene with the highest similarity to the TaqMan probe sequence (Supplemental Table 1).

RESULTS

Clinical and metabolic characteristics of the study subjects are presented in Table 1. Fasting levels of serum insulin, free testosterone, LH-to-FSH ratio, and plasma free triglycerides were elevated in women with PCOS compared with control subjects ($P < 0.01$). No difference

TABLE 2
Ranking of the 10 most downregulated pathways analyzed with MAPPFinder 2.0

MAPP name	Changed (*n*)*	Measured (*n*)†	On MAPP (*n*)‡	Changed (%)§	*z* score	Permute *P* value	FWER *P* value
Electron transport chain	35	91	105	38.46	6.39	<0.0005	<0.0005
Transforming growth factor-β signaling pathway	16	52	52	30.77	3.23	0.003	0.154
G-protein signaling	22	92	92	23.91	2.46	0.013	0.688
Calcium regulation in cardiac cells	32	149	149	21.48	2.23	0.024	0.8
Adipogenesis	28	130	131	21.54	2.20	0.037	0.858
Insulin signaling	33	159	159	20.75	2.12	0.038	0.871
Fas pathway and stress induction of heat shock protein regulation	10	38	38	26.32	1.98	0.06	0.963
Smooth muscle contraction	31	155	156	20.00	1.82	0.074	0.985
Hs G1 to S cell cycle reactome	15	67	67	22.39	1.73	0.091	0.998
Complement activation classical	5	17	17	29.41	1.68	0.151	1

A fold change ≤ -1.05 and a *P* value <0.05 were used as the criteria for gene expression changes between PCOS patients and control subjects. The statistical rating of the relative gene expression activity was provided by the *z* score. The *z* score was based on $N = 3,196$ genes linked to local MAPPs and $R = 477$ of these genes meeting the criteria for change in expression. *Number of genes changed. †Number of genes measured on the chip. ‡Number of genes on the MAPP. §Number changed divided by number measured.

in fasting plasma glucose or FFA levels was observed. In the basal state, rates of glucose and lipid metabolism were similar in the groups (data not shown). During the insulin-stimulated period, total glucose disposal was 58% lower in women with PCOS than in control subjects ($P < 0.01$), and this was primarily accounted for by a 72% reduction in nonoxidative glucose metabolism ($P < 0.01$), but also a 39% decrease in glucose oxidation ($P < 0.01$). Moreover, the ability of insulin to suppress lipid oxidation and plasma FFA levels was impaired in PCOS patients compared with control subjects ($P < 0.01$).

Gene expression analysis. By applying the R statistical software, we found that of the 54,675 transcripts represented on the array, 3,730 transcripts were downregulated and 2,407 were upregulated in skeletal muscle of PCOS patients compared with control subjects (uncorrected $P < 0.05$). Only 34 probe sets with a fold change ranging from -1.09 to -1.47 for downregulated genes and from 1.12 to 1.85 for upregulated genes (Supplemental Table 2) remained differentially expressed after controlling for multiple hypothesis testing using the Benjamini-Hochberg method (FDR <0.1) (32). Nearly one-half of these probe sets had unknown function, and the remaining probe sets did not appear to be of interest in the study of the pathogenesis of insulin resistance in PCOS.

Global pathway analysis revealed decreased expression of OXPHOS genes. We applied two pathway programs, GenMAPP 2.0 and GSEA 1.0, to perform a general search for pathways associated with insulin resistance in skeletal muscle of PCOS patients. Using MAPPFinder 2.0, the significantly downregulated pathways in muscle of PCOS patients were the electron transport chain, transforming growth factor-β signaling pathway, G-protein signaling, calcium regulation in cardiac cells, adipogenesis, and insulin signaling ($z > 2.0$) (Table 2). Nine pathways were significantly upregulated, and the 10th most upregulated pathway was insulin signaling ($z = 1.7$) (Table 3). Interestingly, two pathways, insulin signaling and calcium regulation in cardiac cells, were found among the top 10 of both the up- and downregulated pathways. We could not find evidence that a specific subgroup of genes involved in insulin signaling to glucose metabolism, protein synthesis, or mitogenesis was unambiguously regulated in one direction. Similarly, the genes involved in calcium regulation did not point to specific subgroups of down- or upregulated genes. When evaluating the family-wise error rate (FWER) values for up- and downregulated pathways, only the electron transport chain remained significant. In gene ontology, oxidoreductase activity/acting on NADH or NADPH and closely related pathways were the most

TABLE 3
The 10 most upregulated pathways analyzed with MAPPFinder 2.0

MAPP name	Changed (*n*)*	Measured (*n*)†	On MAPP (*n*)‡	Changed (%)§	*z* score	Permute *P* value	FWER *P* value
Integrin-mediated cell adhesion	21	98	99	21.43	3.39	0.003	0.161
Signaling of hepatocyte growth factor receptor	9	32	34	28.13	3.14	0.002	0.225
Regulation of actin cytoskeleton	26	142	146	18.31	2.89	0.002	0.342
Apoptosis	16	82	82	19.51	2.53	0.013	0.616
Tissues (muscle, fat, and connective)	16	82	84	19.51	2.53	0.015	0.616
Calcium regulation in cardiac cells	25	149	149	16.78	2.35	0.023	0.772
Glycogen metabolism	8	36	36	22.22	2.19	0.037	0.861
Smooth muscle contraction	25	155	156	16.13	2.13	0.039	0.888
Striated muscle contraction	8	38	38	21.05	2.01	0.066	0.944
Insulin signaling	24	159	159	15.09	1.73	0.092	0.989

A fold change ≥ 1.05 and a *P* value <0.05 were used as the criteria for gene expression changes between PCOS patients and control subjects. The statistical rating of the relative gene expression activity was provided by the *z* score. The *z* score was based on $N = 3,196$ genes linked to a MAPP and $R = 349$ of these genes meeting the criteria for change in expression. *Number of genes changed. †Number of genes measured on the chip. ‡Number of genes on the MAPP. §Number changed divided by number measured.

TABLE 4
The 10 most downregulated gene sets ranked according to the normalized enrichment score using GSEA 1.0

Name	SIZE	ES	NES	NOM *P* value	FDR *q* value	FWER *P* value
VOXPHOS	84	0.50	2.05	<0.0001	0.001	0.001
Electron transport chain	92	0.49	2.04	<0.0001	0.001	0.003
Fatty acid metabolism	25	0.51	1.65	0.011	0.130	0.531
MAP00280 valine, leucine, and isoleucine degradation	26	0.46	1.49	0.033	0.387	0.957
MAP00350 tyrosine metabolism	30	0.43	1.45	0.060	0.448	0.995
MAP00071 fatty acid metabolism	45	0.39	1.41	0.057	0.515	0.999
Mitochondria	437	0.28	1.37	0.004	0.579	1.000
hTERT DN	66	0.36	1.37	0.056	0.511	1.000
Human mitoDB 6 2002	412	0.28	1.35	0.007	0.506	1.000
CR repair	39	0.38	1.33	0.105	0.543	1.000

All genes on the chip were ranked by difference in expression between PCOS patients and control subjects using the *t* test. An enrichment score (ES) was calculated for each gene set. CR, caloric restriction; FDR q value, False Discovery Rate; FWER *P* value, Family Wise Error Rate; hTERT, human telomerase reverse transcriptase; hTERT DN, genes downregulated in hTERT-immortalized fibroblasts vs. non-immortalized controls; NES, enrichment score normalized for differences in gene set size; NOM, nominal.

significantly downregulated molecular function terms. Of interest, calcium ion transporter activity was the fourth most downregulated molecular function term, further indicating a role for calcium regulation in muscle of women with PCOS. Mitochondrion was the most downregulated cellular component, and mitochondrial electron transport/ubiquinol to cytochrome c was the most downregulated biological process (Supplemental Table 3). Upregulated gene ontology terms are depicted in Supplemental Table 4.

Applying GSEA on the same dataset, we found that only the gene sets VOXPHOS and electron transport chain were significantly downregulated (FDR <0.05). Moreover, the gene sets termed mitochondria and human mitoDB 6 2002 were among the 10 most downregulated pathways. Fatty acid metabolism was the third most downregulated pathway, and another fatty acid metabolism gene set was the sixth most downregulated pathway (Table 4). No gene sets were significantly upregulated (FDR <0.05) (Supplemental Table 5). Taking the FWER *P* value into account, only the VOXPHOS and electron transport chain gene sets remained significant. When evaluating the results from GenMAPP and GSEA, pathways representing OXPHOS genes were consistently downregulated, even when using the very stringent FWER *P* value.

Decreased expression of OXPHOS genes validated by quantitative real-time PCR. Earlier studies have implicated a role for alterations in OXPHOS in insulin resistance. Using two approaches for global pathway analysis of muscle transcripts, we consistently observed a downregulation of nuclear-encoded OXPHOS genes in muscle of PCOS patients. We therefore focused on this pathway in further analyses. To validate our microarray data, we used quantitative real-time PCR to examine gene expression levels of one gene from each of the five respiratory complexes (I–V) (NDUFA3, SDHD, UCRC, COX7C, and ATP5H) and one uncoupling protein (UCP2). In the microarray experiment, these genes were all downregulated at the single-gene level (uncorrected $P < 0.05$) in muscle from PCOS patients. Moreover, we studied gene expression of PGC-1α, PGC-1β, and NRF-1, which are known to be involved in the transcriptional control of mitochondrial biogenesis. In accordance with the results obtained from microarray analysis, the expression of four of five respiratory genes together with the expression of UCP2 was significantly downregulated in PCOS patients compared with control subjects ($P < 0.05$). Moreover, PGC1-α expression was significantly reduced in PCOS patients compared with control subjects ($P < 0.01$) (Fig. 1). No differences with respect to expression of PGC-1β and NRF-1 were found.

To explore the potential relationship between mRNA levels of PGC-1α and downregulation of OXPHOS genes, we performed simple correlation analysis. In the total population, PGC-1α mRNA levels correlated strongly with the expression of each of the five OXPHOS genes studied ($r = 0.59–0.89$; all $P < 0.001$) but not with UCP2 mRNA levels. In control subjects, the relationship between PGC-1α mRNA levels and expression of ATP5H, NDUFA3, and UCRC was preserved (all $P < 0.05$); whereas in PCOS patients, only NDUFA3 expression ($P < 0.01$) showed a significant association with PGC-1α levels.

DISCUSSION

Previous studies of insulin resistance in skeletal muscle of women with PCOS have focused on individual proteins and genes involved in insulin signaling (9–13). Using global approaches, such as transcriptomics and proteomics, it is possible to study the profile of a large number of distinct genes and proteins simultaneously. In the present

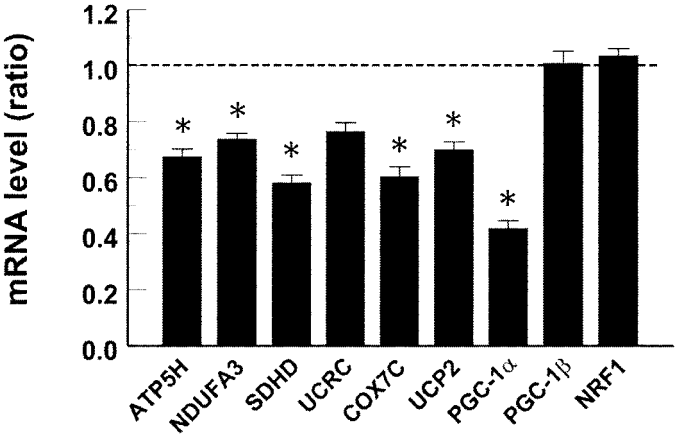

FIG. 1. Relative expression of nine selected genes in skeletal muscle of PCOS patients ($n = 13$) vs. control subjects ($n = 13$) determined by quantitative real-time PCR. Downregulated genes in PCOS patients have mRNA levels <1.0 (dotted line), and upregulated genes have mRNA levels >1.0. Data are means ± SE. *$P < 0.05$ PCOS vs. control subjects.

study, we used DNA microarrays to compare skeletal muscle transcripts between insulin-resistant PCOS patients and matched control subjects. We demonstrate a significant downregulation of nuclear-encoded genes involved in OXPHOS using two different approaches for global pathway analysis. Quantitative real-time PCR analysis validated our findings and showed that downregulation of PGC-1α is strongly associated with reduced expression of OXPHOS genes. These findings provide evidence for an association between insulin resistance and impaired mitochondrial oxidative metabolism in skeletal muscle of women with PCOS.

The most important finding of the study is that expression of nuclear-encoded genes involved in mitochondrial oxidative metabolism is decreased in muscle of women with PCOS. A similar decrease in OXPHOS gene expression has previously been reported in patients with type 2 diabetes and their first-degree relatives (20–22) but is, to our knowledge, a novel finding associated with insulin resistance in PCOS, where it is independent of obesity and type 2 diabetes. The findings of a more pronounced reduction in OXPHOS gene expression in patients with type 2 diabetes compared with their first-degree relatives (22) and that insulin treatment partly normalizes OXPHOS gene expression in poorly controlled patients with type 2 diabetes (20) raise the possibility that impaired oxidative metabolism is, at least in part, secondary to elevated circulating glucose levels. However, a lower mitochondrial ATP flux has been observed in insulin-resistant, glucose-tolerant, first-degree relatives of patients with type 2 diabetes (19). In our study, all PCOS subjects had normal A1C. Thus, although the etiological mechanisms of insulin resistance in PCOS and first-degree relatives of patients with type 2 diabetes may differ, this study provides further support for an association between reduced OXPHOS gene expression and insulin resistance in skeletal muscle at an early stage before hyperglycemia develops. In skeletal muscle of obese subjects and patients with type 2 diabetes, mitochondrial dysfunction seems to be caused by both a lower number of and decreased functional capacity of mitochondria (7,17,18). Further studies are needed to establish whether reduced OXPHOS gene expression is also associated with a lower content and function of muscle mitochondria in PCOS. Transcriptional profiling of ovarian tissues in PCOS did not show decreased expression of OXPHOS genes, suggesting that this may be specific for muscle and not necessarily an etiological factor in PCOS (33–35). Moreover, as in obesity and type 2 diabetes, it remains to be determined whether impaired OXPHOS in muscle of PCOS is a cause or consequence of insulin resistance (7).

In addition to mitochondrial dysfunction, insulin resistance in obese subjects, patients with type 2 diabetes, and their first-degree relatives is characterized by a lower proportion of oxidative, type 1 muscle fibers, reduced maximal oxygen consumption (Vo_{2max}), and increased intramyocellular lipid content (7,8). Moreover, lipid oxidation is impaired under basal conditions in obese and type 2 diabetic subjects (36,37). Recently, reduced Vo_{2max} was demonstrated in PCOS (4), and Mootha et al. (21) found that the expression of OXPHOS genes was strongly correlated with Vo_{2max}. Although we did not measure Vo_{2max}, these findings indirectly support our hypothesis of an impaired mitochondrial oxidative metabolism in muscle of PCOS. To our knowledge, no data are available concerning muscle fiber type composition, intramuscular lipid content, or leg lipid oxidation in PCOS. Using GSEA, we observed that two sets of genes representing fatty acid metabolism were the third and sixth most downregulated pathways in PCOS. Thus, it is likely that abnormalities in lipid metabolism also exist in muscle of PCOS patients. However, further studies are needed to address whether alterations in fiber type composition, increased lipid content, and impaired lipid oxidation in skeletal muscle are also components of insulin resistance in PCOS.

Expression of nuclear- and mitochondrial-encoded mitochondrial genes is thought to be coordinated by the transcriptional coactivators PGC-1α and -1β through activation of NRF-1 and -2 (38). In skeletal muscle, PGC-1α also stimulates expression of the insulin-sensitive glucose transporter GLUT4 and promotes an increased proportion of oxidative, type 1 muscle fibers (38). Patti et al. (22) provided evidence that downregulation of OXPHOS genes in muscle of patients with type 2 diabetes and their first-degree relatives is likely explained by reduced expression of PGC-1α and -1β, although these observations were made in separate study cohorts. They also found reduced levels of NRF-1 but only in patients with type 2 diabetes, indicating that this is not an early alteration associated with insulin resistance. In the present study, we show that decreased expression of PGC-1α is associated with reduced OXPHOS gene expression within the same cohort of insulin-resistant women with PCOS, whereas the expression of PGC-1β and NRF-1 was unaltered. Moreover, we provide evidence for a strong association between expression of PGC-1α and several subunits of the respiratory complexes. The correlations observed were stronger in control subjects than in PCOS subjects in whom a tight correlation between only PGC-1α and the complex I subunit NDUFA3 was seen. This could explain that, in particular, genes representing subunits of the respiratory complex I were downregulated in PCOS subjects. Our results strongly suggest that downregulation of OXPHOS genes are mediated mainly through reduced expression of PGC-1α and further point to an important role for PGC-1α in impaired oxidative metabolism in skeletal muscle insulin resistance in PCOS and the risk of type 2 diabetes in these patients.

Studies of cultured fibroblasts and myotubes from PCOS patients have provided evidence that constitutively enhanced serine phosphorylation of the insulin receptor and IRS-1 could play an important role in the pathogenesis of insulin resistance in PCOS (10–13). In cultured myotubes (11) but not in fibroblasts (39), increased serine phosphorylation of IRS-1 was associated with enhanced mitogenic signaling. In skeletal muscle, which is the major site of insulin-mediated glucose uptake, only a few significant abnormalities have been demonstrated in muscle of women with PCOS in vivo. This includes a transient decrease in insulin stimulation of IRS-1–associated PI 3-kinase activity, increased phosphorylation of extracellular signal-regulated kinase 1/2, and increased IRS-2 protein abundance (9,11). No differences in mRNA expression of IRS-1 or IRS-2 were found (9). Although similar abnormalities in proximal insulin signaling have been reported in obesity and type 2 diabetes (7,8), it has been hypothesized that the molecular mechanisms underlying insulin resistance in PCOS differ from those seen in these conditions (12). In the present study, we found that the insulin signaling pathway, as defined by GenMAPP, was among the 10 most up- and downregulated pathways in muscle of women with PCOS. A number of genes mediating meta-

bolic and mitogenic actions of insulin and modulators of insulin action were dysregulated; however, in none of these subpathways, the genes were uniformly up- or downregulated. In recent studies of muscle transcripts, dysregulation of the insulin signaling pathway was not detected in patients with type 2 diabetes (20–22). This indicates that changes in expression of genes or proteins involved in insulin signaling, whether compensatory or not, could play a greater role for insulin resistance in skeletal muscle in PCOS than in type 2 diabetes. It also emphasizes the need for further studies of expression and activity of proteins known to mediate and modulate insulin signaling in muscle of PCOS patients.

An intriguing finding of the study was that a calcium pathway termed calcium regulation in cardiac cells was significantly up- and downregulated (GenMAPP), and calcium ion transporter activity was a significantly downregulated gene ontology term in PCOS subjects. There is increasing evidence supporting a modulating role for Ca^{2+} influx, calmodulin, and Ca^{2+}/calmodulin-dependent protein kinase (CaMK) in insulin-stimulated glucose transport in skeletal muscle (40–43). In mitochondria, Ca^{2+} is important for activation of key enzymes to enhance ATP production, and an increase in cytosolic Ca^{2+} and activation of CaMK also induce mitochondrial biogenesis and GLUT4 expression via activation of different transcription factors, including NRF-1 and -2, and the coactivator PGC-1α (44–46). It is easy to infer that dysregulation of Ca^{2+} homeostasis could have a pronounced disturbing effect on insulin-stimulated glucose disposal and mitochondrial function. Abnormal Ca^{2+} homeostasis has been reported in skeletal muscle of patients with type 2 diabetes (47), and more recently, serum calcium was demonstrated to be independently associated with insulin resistance measured with euglycemic-hyperinsulinemic clamp (48). Although similar abnormalities in muscle transcripts of calcium pathway genes were not reported in studies of patients with type 2 diabetes (20–22), it is too early to conclude that the observed changes should play a unique role for insulin resistance in skeletal muscle of PCOS subjects. Further studies will be required to assess the precise implication of Ca^{2+} homeostasis in the pathogenesis of insulin resistance in PCOS.

In summary, using transcriptional profiling, we demonstrate that insulin resistance in skeletal muscle of women with PCOS is associated with reduced expression of genes involved in mitochondrial oxidative metabolism and that reduced expression of PGC-1α could play a role for these abnormalities. Moreover, our data indicate that transcriptional alterations in insulin signaling pathways, fatty acid metabolism, and calcium homeostasis may contribute to the potentially unique phenotype of insulin resistance in patients with PCOS. Future studies focusing on the interaction between these pathways in skeletal muscle may unravel the molecular mechanism of insulin resistance in PCOS and help develop strategies to prevent the increased risk of early onset of type 2 diabetes in these women.

ACKNOWLEDGMENTS

This study has received grants from the Novo Nordisk Foundation.

We acknowledge discussions of quantitative real-time PCR data with Lene Christiansen (University of Southern Denmark).

REFERENCES

1. Ehrmann DA: Polycystic ovary syndrome. *N Engl J Med* 352:1223–1236, 2005
2. Dunaif A: Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. *Endocr Rev* 18:774–800, 1997
3. Legro RS: Polycystic ovary syndrome and cardiovascular disease: a premature association? *Endocr Rev* 24:302–312, 2003
4. Orio F Jr, Giallauria F, Palomba S, Cascella T, Manguso F, Vuolo L, Russo T, Tolino A, Lombardi G, Colao A, Vigorito C: Cardiopulmonary impairment in young women with polycystic ovary syndrome. *J Clin Endocrinol Metab* 91:2967–2971, 2006
5. 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
6. Glintborg D, Hermann AP, Andersen M, Hagen C, Beck-Nielsen H, Veldhuis JD, Henriksen JE: Effect of pioglitazone on glucose metabolism and luteinizing hormone secretion in women with polycystic ovary syndrome. *Fertil Steril* 86:385–397, 2006
7. Hojlund K, Beck-Nielsen H: Impaired glycogen synthase activity and mitochondrial dysfunction in skeletal muscle: markers or mediators of insulin resistance in type 2 diabetes. *Curr Diab Rev* 2:375–395, 2006
8. Petersen KF, Shulman GI: Etiology of insulin resistance. *Am J Med* 119:S10–S16, 2006
9. Dunaif A, Wu X, Lee A, Amanti-Kandarakis E: Defects in insulin receptor signaling in vivo in the polycystic ovary syndrome (PCOS). *Am J Physiol Endocrinol Metab* 281:E392–E399, 2001
10. Li M, Youngren JF, Dunaif A, Goldfine ID, Maddux BA, Zhang BB, Evans JL: Decreased insulin receptor (IR) autophosphorylation in fibroblasts from patients with PCOS: effects of serine kinase inhibitors and IR activators. *J Clin Endocrinol Metab* 87:4088–4093, 2002
11. Corbould A, Zhao H, Mirzoeva S, Aird F, Dunaif A: Enhanced mitogenic signaling in skeletal muscle of women with polycystic ovary syndrome. *Diabetes* 55:751–759, 2006
12. Corbould A, Kim YB, Youngren JF, Pender C, Kahn BB, Lee A, Dunaif A: Insulin resistance in the skeletal muscle of women with PCOS involves intrinsic and acquired defects in insulin signaling. *Am J Physiol Endocrinol Metab* 288:E1047–E1054, 2005
13. Dunaif A, Xia J, Book CB, Schenker E, Tang Z: Excessive insulin receptor serine phosphorylation in cultured fibroblasts and in skeletal muscle: a potential mechanism for insulin resistance in the polycystic ovary syndrome. *J Clin Invest* 96:801–810, 1995
14. Hojlund K, Staehr P, Hansen BF, Green KA, Hardie DG, Richter EA, Beck-Nielsen H, Wojtaszewski JF: Increased phosphorylation of skeletal muscle glycogen synthase at NH2-terminal sites during physiological hyperinsulinemia in type 2 diabetes. *Diabetes* 52:1393–1402, 2003
15. Levin K, Daa SH, Alford FP, Beck-Nielsen H: Morphometric documentation of abnormal intramyocellular fat storage and reduced glycogen in obese patients with type II diabetes. *Diabetologia* 44:824–833, 2001
16. Hojlund K, Wrzesinski K, Larsen PM, Fey SJ, Roepstorff P, Handberg A, Dela F, Vinten J, McCormack JG, Reynet C, Beck-Nielsen H: Proteome analysis reveals phosphorylation of ATP synthase beta-subunit in human skeletal muscle and proteins with potential roles in type 2 diabetes. *J Biol Chem* 278:10436–10442, 2003
17. Kelley DE, He J, Menshikova EV, Ritov VB: Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* 51:2944–2950, 2002
18. 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
19. Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI: Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med* 350:664–671, 2004
20. 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
21. Mootha VK, Lindgren CM, Eriksson KF, 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-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34:267–273, 2003
22. 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 U S A* 100:8466–8471, 2003
23. Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, Brown EL: Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol* 14:1675–1680, 1996
24. Auer H, Lyianarachchi S, Newsom D, Klisovic MI, Marcucci G, Kornacker K: Chipping away at the chip bias: RNA degradation in microarray analysis. *Nat Genet* 35:292–293, 2003
25. Ihaka R, Gentleman R: R: A language for data analysis and graphics. *J Comp Graph Stat* 5:299–314, 1996
26. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP: Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4:249–264, 2003
27. Workman C, Jensen LJ, Jarmer H, Berka R, Gautier L, Nielser HB, Saxild HH, Nielsen C, Brunak S, Knudsen S: A new non-linear normalization method for reducing variability in DNA microarray experiments. *Genome Biol* 3:1–16, 2002
28. Li C, Wong WH: Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. *Genome Biol* 2:1–11, 2001
29. Dahlquist KD, Salomonis N, Vranizan K, Lawlor SC, Conklin BR: GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. *Nat Genet* 31:19–20, 2002
30. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP: Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 102:15545–15550, 2005
31. Doniger SW, Salomonis N, Dahlquist KD, Vranizan K, Lawlor SC, Conklin BR: MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. *Genome Biol* 4:R7, 2003
32. Benjamini Y, Hochberg Y: Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B* 57:289–300, 1995
33. Wood JR, Nelson VL, Ho C, Jansen E, Wang CY, Urbanek M, McAllister JM, Mosselman S, Strauss JF III: The molecular phenotype of polycystic ovary syndrome (PCOS) theca cells and new candidate PCOS genes defined by microarray analysis. *J Biol Chem* 278:26380–26390, 2003
34. Jansen E, Laven JS, Dommerholt HB, Polman J, van Rijt C, van den Hurk C, Westland J, Mosselman S, Fauser BC: Abnormal gene expression profiles in human ovaries from polycystic ovary syndrome patients. *Mol Endocrinol* 18:3050–3063, 2004
35. Diao FY, Xu M, Hu Y, Li J, Xu Z, Lin M, Wang L, Zhou Y, Zhou Z, Liu J, Sha J: The molecular characteristics of polycystic ovary syndrome (PCOS) ovary defined by human ovary cDNA microarray. *J Mol Endocrinol* 33:59–72, 2004
36. Kelley DE, Simoneau JA: Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus. *J Clin Invest* 94:2349–2356, 1994
37. Kim JY, Hickner RC, Cortright RL, Dohm GL, Houmard JA: Lipid oxidation is reduced in obese human skeletal muscle. *Am J Physiol Endocrinol Metab* 279:E1039–E1044, 2000
38. Finck BN, Kelly DP: PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest* 116:615–622, 2006
39. Book CB, Dunaif A: Selective insulin resistance in the polycystic ovary syndrome. *J Clin Endocrinol Metab* 84:3110–3116, 1999
40. Lanner JT, Katz A, Tavi P, Sandstrom ME, Zhang SJ, Wretman C, James S, Fauconnier J, Lannergren J, Bruton JD, Westerblad H: The role of Ca^{2+} influx for insulin-mediated glucose uptake in skeletal muscle. *Diabetes* 55:2077–2083, 2006
41. Brozinick JT Jr, Reynolds TH, Dean D, Cartee G, Cushman SW: 1-[N, O-bis-(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4- phenylpiperazine (KN-62), an inhibitor of calcium-dependent calmodulin protein kinase II, inhibits both insulin- and hypoxia-stimulated glucose transport in skeletal muscle. *Biochem J* 339:533–540, 1999
42. Shashkin P, Koshkin A, Langley D, Ren JM, Westerblad H, Katz A: Effects of CGS 9343B (a putative calmodulin antagonist) on isolated skeletal muscle: dissociation of signaling pathways for insulin-mediated activation of glycogen synthase and hexose transport. *J Biol Chem* 270:25613–25618, 1995
43. Bruton JD, Katz A, Westerblad H: The role of Ca2+ and calmodulin in insulin signalling in mammalian skeletal muscle. *Acta Physiol Scand* 171:259–265, 2001
44. Gunter TE, Yule DI, Gunter KK, Eliseev RA, Salter JD: Calcium and mitochondria. *FEBS Lett* 567:96–102, 2004
45. Ojuka EO: Role of calcium and AMP kinase in the regulation of mitochondrial biogenesis and GLUT4 levels in muscle. *Proc Nutr Soc* 63:275–278, 2004
46. Wu H, Kanatous SB, Thurmond FA, Gallardo T, Isotani E, Bassel-Duby R, Williams RS: Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. *Science* 296:349–352, 2002
47. Levy J: Abnormal cell calcium homeostasis in type 2 diabetes mellitus: a new look on old disease. *Endocrine* 10:1–6, 1999
48. Hagstrom E, Hellman P, Lundgren E, Lind L, Arnlov J: Serum calcium is independently associated with insulin sensitivity measured with euglycaemic-hyperinsulinaemic clamp in a community-based cohort. *Diabetologia* 50:317–324, 2007