

# A High-Fat Diet Coordinately Downregulates Genes Required for Mitochondrial Oxidative Phosphorylation in Skeletal Muscle

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**Obesity and type 2 diabetes have been associated with a high-fat diet (HFD) and reduced mitochondrial mass and function. We hypothesized a HFD may affect expression of genes involved in mitochondrial function and biogenesis. To test this hypothesis, we fed 10 insulin-sensitive males an isoenergetic HFD for 3 days with muscle biopsies before and after intervention. Oligonucleotide microarray analysis revealed 297 genes were differentially regulated by the HFD (Bonferroni adjusted  $P < 0.001$ ). Six genes involved in oxidative phosphorylation (OXPHOS) decreased. Four were members of mitochondrial complex I: NDUFB3, NDUFB5, NDUF51, and NDUFV1; one was SDHB in complex II and a mitochondrial carrier protein SLC25A12. Peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 (PGC1)  $\alpha$  and PGC1 $\beta$  mRNA were decreased by  $-20\%$ ,  $P < 0.01$ , and  $-25\%$ ,  $P < 0.01$ , respectively. In a separate experiment, we fed C57Bl/6J mice a HFD for 3 weeks and found that the same OXPHOS and PGC1 mRNAs were downregulated by  $\sim 90\%$ , cytochrome C and PGC1 $\alpha$  protein by  $\sim 40\%$ . Combined, these results suggest a mechanism whereby HFD downregulates genes necessary for OXPHOS and mitochondrial biogenesis. These changes mimic those observed in diabetes and insulin resistance and, if sustained, may result in mitochondrial dysfunction in the prediabetic/insulin-resistant state. *Diabetes* 54:1926–1933, 2005**

**A**t the molecular and structural level, mitochondrial biogenesis and mitochondrial function are altered in diabetes, as well as in insulin-resistant relatives of type 2 diabetic subjects (1,2). At the ultra-structural level, a reduction in the number, location, and morphology of mitochondria is strongly associated with insulin resistance (1). Two recent microar-

ray studies have shown that genes involved in oxidative phosphorylation (OXPHOS) exhibit reduced expression levels in the skeletal muscle of type 2 diabetic subjects and prediabetic subjects. These changes may be mediated by the peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 (PGC1) pathway. PGC1 $\alpha$ - and PGC1 $\beta$ -responsive OXPHOS genes show reduced expression in the muscle of patients with type 2 diabetes (3,4). In addition to the cellular energy sensor AMP kinase, the peroxisome proliferator-activated receptor cofactors PGC1 $\alpha$  (5–7) and possibly PGC1 $\beta$  (8) activate mitochondrial biogenesis and increase OXPHOS gene expression by increasing the transcription, translation, and activation of the transcription factors necessary for mitochondrial DNA (mtDNA) replication. Similarly, PGC1 $\alpha$  increases the transcription of enzymes necessary for substrate oxidation, electron transport, and ATP synthesis. Morphological and functional studies (1,9,10), combined with the recent microarray data, indicate that PGC1 is important in the development of type 2 diabetes.

Rates of ATP synthesis, measured in situ with magnetic resonance spectroscopy, are decreased in subjects with a family history of diabetes before the onset of impaired glucose tolerance (2,10). Based on these results, the prevailing view is that these defects have a genetic origin (2). One common feature of diverse insulin-resistant states is an elevation in nonesterified fatty acids (11). This gave rise to the concept of “lipotoxicity” and “ectopic fat” (12) and shifted attention toward the adipose tissue and increased free fatty acid concentrations as a potential foundation for insulin resistance (11).

Excess dietary fat has also been implicated in the development of obesity and diabetes (13). At energy balance, high-fat diets (HFDs) increase the flux of fatty acids through skeletal muscle for oxidation. The purpose of these experiments was to identify the transcriptional responses of skeletal muscle to an isoenergetic HFD in healthy young men using oligonucleotide microarrays. We found a HFD downregulated PGC1 $\alpha$  and PGC1 $\beta$  mRNA, as well as genes encoding proteins in complexes I, II, III, and IV of the electron transport chain. These changes were recapitulated and amplified in a murine model after a 3-week HFD, along with decreases in PGC1 $\alpha$  and cytochrome C protein. These studies implicate increased dietary fat in the defects in OXPHOS genes observed in diabetes and the prediabetic/insulin-resistant state.

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Received for publication 10 December 2004 and accepted in revised form 4 April 2005.

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

BHAD,  $\beta$ -hydroxyacyl-CoA dehydrogenase; HFD, high-fat diet; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; PGC1, peroxisome proliferator-activated receptor  $\gamma$  coactivator-1.

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## RESEARCH DESIGN AND METHODS

ADAPT is a short-term intervention study designed to examine inter-individual differences in the ability to increase fat oxidation after consuming an isoenergetic HFD (14,15). Ten healthy young men, aged  $23.0 \pm 3.1$  years and with BMIs of  $24.3 \pm 3.0$  kg/m<sup>2</sup> underwent physical examination, medical laboratory tests, and anthropometry. The subjects had high preference for dietary fat as indicated by food preference questionnaire ( $114.16 \pm 18.14$ ). This measure is highly correlated with self-selected fat intake (16). Participants presented to Pennington inpatient unit on day -4 and ate a weight-maintaining (35% fat, 16% protein, and 49% carbohydrate) diet prepared by the metabolic kitchen. On day -3, a euglycemic-hyperinsulinemic clamp was performed, and this diet was continued on days -2, -1, and 1. On day 1, participants ate the same weight-maintaining 35% fat diet, and total daily energy expenditure, fat oxidation, protein oxidation, and carbohydrate oxidation were measured at energy balance as previously described in a whole-room calorimeter (17,18). On days 2, 3, and 4 subjects ate a 50% fat, 16% protein, 34% carbohydrate diet with an isoenergetic energy clamp procedure. Each meal was served to the subjects and consumed within 20 min.

**Euglycemic-hyperinsulinemic clamp.** Insulin sensitivity was measured only at baseline (day -3) by euglycemic-hyperinsulinemic clamp (19) before HFD. After an overnight fast, glucose and insulin (80 mIU/m<sup>2</sup> BSA) were administered. The glucose disposal rate (M value) was adjusted for kilograms of lean body mass.

**Maximal aerobic capacity.** Maximal oxygen uptake was determined by a progressive treadmill test to exhaustion (20). The volume of O<sub>2</sub> and CO<sub>2</sub> was measured continuously using a metabolic cart (V-Max29 Series; Sensor-Medics, Yorba Linda, CA).

**Body composition.** Body fat mass and lean body mass were measured on a Hologic Dual Energy X-ray Absorptiometer (QDR 4500; Hologic, Waltham, MA).

**Indirect calorimetry.** Respiratory quotient and 24-h energy expenditure were determined in the whole-room calorimeter before and during 3 days of isocaloric HFD and confirmed an increase in fat oxidation (data not shown). Energy expenditure was set at 1.4 times the resting metabolic rate measured by metabolic cart and clamped across the 4-day chamber stay.

**Animal study.** Male C57BL/6J mice were housed at room temperature with a 12-h-light/12-h-dark cycle for 5 weeks. Six mice consumed control diet ad libitum (D12450B [Research Diets, New Brunswick, NJ]: 10% of energy from fat, 20% of energy from protein, and 70% of energy from carbohydrate), and seven mice consumed HFD (D12451 [Research Diets]: 45% of energy from fat, 20% of energy from protein, and 35% of energy from carbohydrate). All animals ate the control diet ad libitum for 2 weeks, and seven were switched to HFD for 3 additional weeks. Gastrocnemius muscles were dissected and snap-frozen in liquid nitrogen.

**RNA and DNA extraction.** Human and mouse total RNA from 50–100 mg of vastus lateralis and gastrocnemius muscle, respectively, was isolated with Trizol reagent (Invitrogen, Carlsbad, CA). Gastrocnemius was digested overnight in proteinase K (FisherBiotech, Houston, TX) at 55°C. DNA was extracted with phenol-chloroform. The quantity and integrity of the RNA and DNA were confirmed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

**Oligonucleotide microarrays.** RNA sample pairs (2 µg) from human subjects were labeled by reverse transcriptase with dCTP-Cy3 and dCTP-Cy5, respectively, and in inverse order (dye swap) using MICROMAX TSA Labeling and Detection kit (Perkin-Elmer, Wellesley, MA). Equal amounts of labeled cDNA probes were hybridized in duplicate to oligonucleotide slides containing 18,861 spots corresponding to 17,260 unique genes (Compugen, San Jose, CA) in hybridization chambers (GenomicSolutions, Ann Arbor, MI) for up to 72 h at 42°C. Detection and washing were performed at room temperature according to manufacturer's protocol (Perkin-Elmer). Oligonucleotide chips were spotted on to poly-L-lysine slides using a GeneMachine OmniGrid microarrayer (GenomicSolutions) equipped with a Stealth SPH32 printhead and Stealth SMP4 Micro Spotting Pins (Telechem International, Sunnyvale, CA). Oligonucleotides were stored in 384-well plates in 45% DMSO. Microarray slides were scanned using a GSI Lumonics ScanArray 5,000 scanner (Perkin-Elmer) at high intensities (~95% for Cy3, ~75% for Cy5) and low intensities (~55% for Cy3, ~35% for Cy5) applying ScanArray Express software and quantified using QuantArray (GenomicSolutions). All subsequent microarray analyses were performed using SAS version 8.2 (SAS, Cary, NC). A robust local regression procedure (LOWESS) was performed to remove the systematic variations in the measured gene expression levels so that differences in expression across the samples could be distinguished accurately and precisely (21). After normalization, gene shaving (22), bootstrapping (23), and cluster analysis were performed (24), and the slide effect, dye effect, variety effect, and duplicate design were taken into account in an ANOVA model (25). Resampling-based multiple pairwise comparison was used to identify the

TABLE 1

Characteristics of the study population before the high-fat diet

Age (years)	23.0 ± 3.1
Height (cm)	179.7 ± 6.3
Weight (kg)	78.9 ± 13.2
BMI (kg/m <sup>2</sup> )	24.3 ± 3.0
VO <sub>2max</sub> (ml · kg <sup>-1</sup> · min <sup>-1</sup> )	48.8 ± 3.3
Waist-to-hip ratio (AU)	0.88 ± 0.1
RQ—metabolic cart (AU)	0.87 ± 0.1
Fasting glucose (ml/dl)	78.4 ± 4.7
Fasting insulin (µl/ml)	4.6 ± 1.4
GDR (mg · kg FFM <sup>-1</sup> · min <sup>-1</sup> )	14.7 ± 4.1
Free fatty acids (mmol)	0.4 ± 0.1
Body fat (%)	16.2 ± 3.2
Fat cell size (µl)	0.93 ± 0.2

Data are means ± SD. GDR, glucose disposal rate; RQ, respiratory quotient.

differentially expressed genes before versus after the HFD. Differentially expressed genes were identified based on a Bonferroni adjusted  $P < 0.01$ .

**Real-time quantitative RT-PCR for RNA.** RNA sample pairs (1 µg) were reverse transcribed using iScript cDNA synthesis kit (BioRad, Hercules, CA). All primers and probes were designed using Primer Express version 2.1 (Applied Biosystems-Roche, Branchburg, NJ). Sequences of primers and probes are shown in Supplemental Table 1 (online appendix [available at <http://diabetes.diabetesjournals.org>]).

Real-time quantitative RT-PCR reactions (26) were performed as one-step reactions in ABI PRISM 7900 (Applied Biosystems) using the following parameters: one cycle of 48°C for 30 min, then 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. For all assays performed using SYBR Green I (Applied Biosystems-Roche), 18S was the internal control; and for all assays performed using Taqman primers and probe, RPLP0, which is the human equivalent of the murine 36B4 (27), was the internal control. Cyclophilin B was used for all murine assays. All expression data were normalized by dividing target gene by internal control.

**Real-time PCR for mtDNA and genomic DNA copy number.** Taqman primers and probes were designed using Primer Express version 2.1 (Applied Biosystems-Roche). Real-time PCR was carried out in ABI PRISM 7900 sequence detector (Applied Biosystems) using the following parameters: one cycle of 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Murine skeletal muscle genomic DNA copy number was measured at the UCP2 gene using a primer-probe set that amplifies genomic DNA. mtDNA copy number was measured from the cytochrome C oxidase II mtDNA gene. mtDNA copy number was calculated by taking the absolute value of  $\Delta\Delta Ct$  values between groups (control vs. HFD). Because amplification occurs exponentially (increasing twofold with each cycle of PCR), log base 2 of  $\Delta\Delta Ct$  as the copy number for each sample (28).

**Western immunoblotting.** Tissues were homogenized in buffer (50 mmol/l HEPES, pH 7.4, 2 mmol/l EDTA, 150 mmol/l NaCl, 30 mmol/l NaPO<sub>4</sub>, 10 mmol/l NaF, 1% Triton X-100, 10 µl/ml protease inhibitor, 10 µl/ml phosphatase I inhibitor, 10 µl/ml phosphatase II inhibitor, and 1.5 mg/ml benzamidine HCl). The whole homogenates were centrifuged for 25 min at 15,000g, and supernatants were stored at -80°C before Western immunoblotting. Homogenates were run on a 10% PAGE, transferred to polyvinylidene fluoride membranes, incubated with the primary antibodies PGC1 $\alpha$  (P3363; US Biological, Swampscott, MA) and cytochrome C (556433; BD Biosciences, San Jose, CA) and signal detected using the ECL detection system (Pierce, Rockford, IL). Glyceraldehyde-3-phosphate dehydrogenase (46999-555; Biogenesis, Kingston, NH) was used as an internal control, and brown adipose tissue was used as a positive control.

**Skeletal muscle enzyme activities.** Skeletal muscle samples were diluted 20-fold and homogenized in extraction buffer (0.1 mol/l KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>PHO<sub>4</sub> and 2 mmol/l EDTA, pH 7.2). Citrate synthase, cytochrome C oxidase, and  $\beta$ -hydroxyacyl-CoA dehydrogenase (BHAD) activities were determined spectrophotometrically as previously described (19). DNA was extracted from the same homogenate (total nucleic acid extraction; Epicenter, Madison, WI), and mtDNA was measured to adjust for differences in the content of mitochondria (29).

**Statistical analysis.** Statistical analysis was performed using two-tailed paired Student's  $t$  test for before versus after HFD (human) and unpaired Student's  $t$  test for control versus HFD (mouse) to establish effects of the intervention. All values are presented in figures and tables as sample (raw) means ± SE. Population characteristics are represented as means ± SD. Type

TABLE 2

Change in overnight fasting blood parameters after a 50% high-fat diet at energy balance and fixed activity level

Variable	BL1	BL2	HFD day 1	HFD day 2	HFD day 3
Glucose (mg/dl)	88.60 ± 7.38	89.77 ± 8.74	89.00 ± 9.76	89.40 ± 7.29	90.40 ± 9.03
Insulin (mIU/ml)	6.81 ± 3.02	6.98 ± 2.86	6.65 ± 3.00	6.07 ± 2.98*	6.89 ± 2.67
Free fatty acids (mmol)	0.26 ± 0.08	0.28 ± 0.10	0.22 ± 0.08	0.25 ± 0.08	0.27 ± 0.10

Data are means ± SE. The HFD contained 50% fat. Repeated-measures ANOVA was used to test for time effects;  $P = 0.847, 0.130,$  and  $0.047$  for glucose, insulin, and free fatty acids, respectively. \*Post hoc comparisons of each HFD day versus average of the two baseline (BL) values were significant ( $P < 0.05$ ; Dunnett's adjustment).

I error rate was set a priori at  $P < 0.05$ . Analysis was performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA).

## RESULTS

**HFD decreases expression of genes involved in OXPHOS.** The characteristics of the study population are presented in Table 1. Fasting glucose, insulin, and free fatty acids were unchanged as the diet was switched from "standard" fat content (35%) to HFD (50%) (Table 2). Intra-subject changes in skeletal muscle gene expression before versus after 50% HFD were identified using oligonucleotide microarrays and analyzed by sequential analysis that included LOWESS normalization, gene shaving, bootstrapping, and ANOVA. A cluster analysis grouped the regulated genes into five clusters based on three principal components (Supplemental Fig. 1 [available at <http://diabetes.diabetesjournals.org>]). The 3-day isoenergetic HFD significantly changed the expression of 297 genes ( $P < 0.01$ ; Supplemental Table 2). By the HFD, 163 genes were upregulated, and 135 were downregulated. Six were known to be involved in OXPHOS by visual inspection or through gene ontology analysis ( $P < 0.001$ ; Table 3). All of the OXPHOS genes were downregulated. Four genes are components of complex I, and one is a component of complex II. The remaining regulated gene is involved in mitochondrial solute transport.

**Confirmation of microarray expression of OXPHOS genes of complexes I and II in human skeletal muscle by quantitative real-time RT-PCR.** We sought to confirm expression of these six OXPHOS genes by real-time quantitative RT-PCR. All six microarray "hits" displayed the same downward trend with quantitative RT-PCR, and three genes were "confirmed" (Fig. 1A): NDUFB5 ( $3.19 \pm 0.26$  to  $2.12 \pm 0.20$  AU [arbitrary units],  $P < 0.01$ ), SDHB ( $0.26 \pm 0.02$  to  $0.19 \pm 0.02$  AU,  $P < 0.05$ ), NDUF51 ( $0.28 \pm 0.03$  to  $0.21 \pm 0.02$  AU,  $P = 0.05$ ), SLC25A12 ( $0.29 \pm 0.04$  to  $0.19 \pm 0.02$  AU,  $P = 0.0838$ ), NDUFB3 ( $0.39 \pm 0.05$  to  $0.26 \pm 0.034$  AU,  $P = 0.1355$ ), and NDUFV1 ( $0.36 \pm 0.05$  to  $0.30 \pm 0.04$  AU,  $P = 0.3191$ ). The magnitudes of these changes (~20–30%) are strikingly similar to the decreases demonstrated by microarray analysis of reduced skeletal muscle OXPHOS gene expression found by Patti et al. (4) and Mootha et al. (3) in diabetic subjects.

**Expression of OXPHOS genes of complexes III and IV in human skeletal muscle.** As a subsequent step in elucidating effects of the diet intervention on expression of genes involved in mitochondrial function, we examined mRNA for genes in complexes III and IV using quantitative RT-PCR (Fig. 1A). Cytochrome C (complex III) and Surfeit one (complex IV) expression levels were reduced ( $1.13 \pm 0.07$  to  $0.85 \pm 0.05$  AU,  $P < 0.01$ , and  $1.10 \pm 0.05$  to  $0.90 \pm 0.05$  AU,  $P < 0.01$ ).

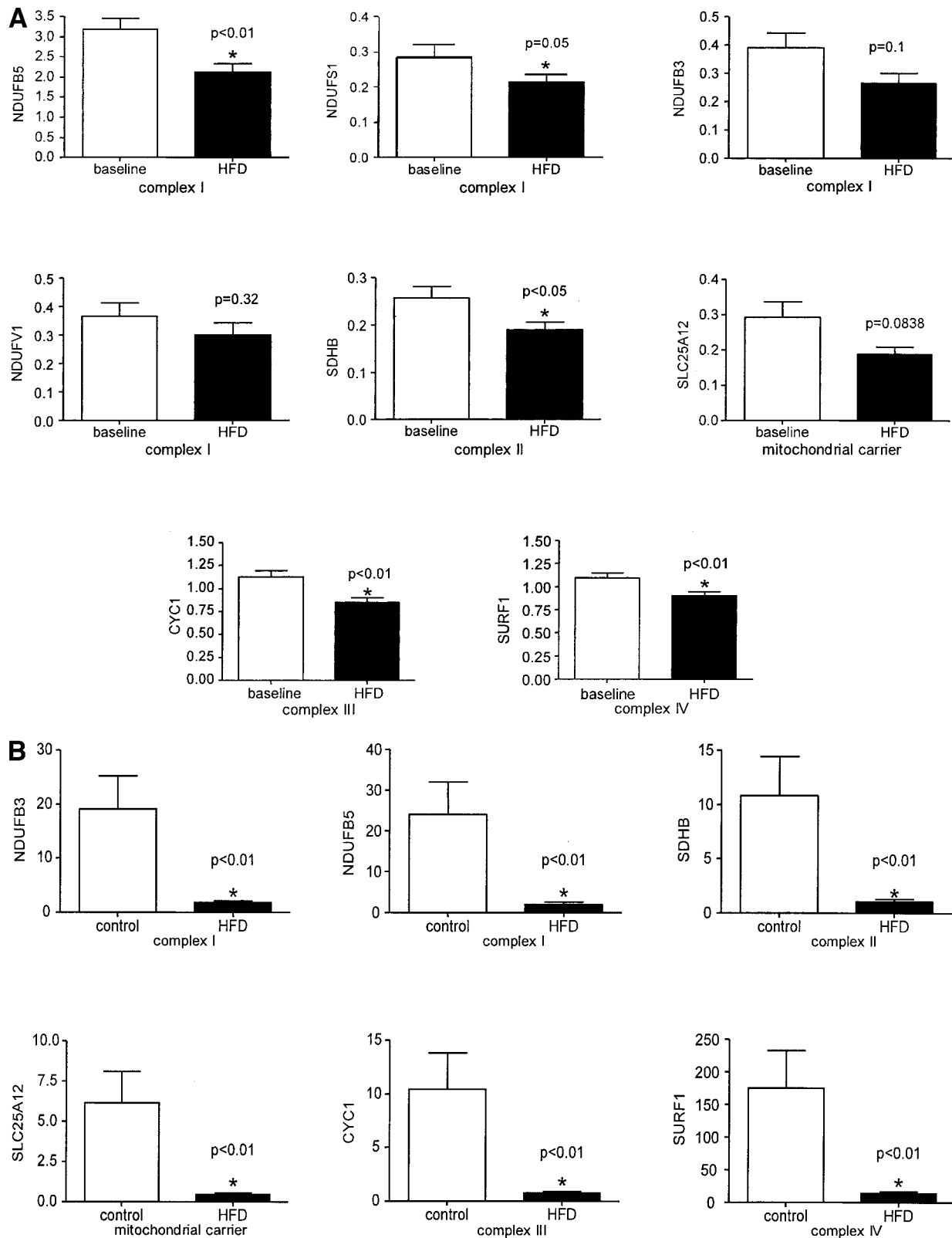
**Reduced expression of transcription factors and transcriptional cofactors in human skeletal muscle after a short-term HFD.** Because expression levels of genes involved in the function of mitochondria decreased, we examined expression of genes known to be involved in mitochondrial biogenesis. We observed a 20% and a 25% reduction in mRNA levels in PGC1 $\alpha$  and PGC1 $\beta$ , respectively (Fig. 2A); PGC1 $\alpha$  ( $1.44 \pm 0.08$  to  $1.13 \pm 0.06$  AU,  $P < 0.01$ ) and PGC1 $\beta$  ( $2.12 \pm 0.16$  to  $1.59 \pm 0.18$  AU,  $P < 0.05$ ). Mitochondrial transcription factor A, TFAM, a key activator of mitochondrial transcription and its genome replication, was not significantly changed ( $2.00 \pm 0.19$  to  $1.79 \pm 0.19$  AU,  $P = 0.3784$ ), nor was nuclear respiratory factor 1, NRF1 ( $1.89 \pm 0.13$  to  $1.56 \pm 0.16$  AU,  $P = 0.1398$ ) (Fig. 2A). **Expression of OXPHOS genes in murine skeletal muscle as a confirmation of the human experiments.** We next tested whether the changes in gene expression we found in the clinic were present in a murine model of HFD-induced obesity. We fed C57Bl/6J mice either a 10 or 45% fat diet for 3 weeks. We chose two murine genes from complex I; one gene each from complexes II, III, and IV; and one mitochondrial carrier protein from the human experiments. Decline in gene expression was of a greater magnitude than those seen in the human experiments. As measured by real-time quantitative RT-PCR, each gene was downregulated in high-fat-fed mice compared with controls (Fig. 1B): NDUFB5 ( $24.05 \pm 7.89$  to  $2.10 \pm 0.44$  AU,  $P < 0.01$ ), NDUFB3 ( $19.02 \pm 6.25$  to  $1.82 \pm 0.29$  AU,  $P < 0.01$ ), SDHB ( $10.84 \pm 3.58$  to  $1.05 \pm 0.20$  AU,  $P < 0.01$ ), SLC25A12 ( $6.14 \pm 1.99$  to  $0.45 \pm 0.11$  AU,  $P < 0.01$ ), CYC1 ( $10.41 \pm 3.40$  to  $0.79 \pm 0.13$  AU,  $P < 0.01$ ), and SURF1 ( $175.50 \pm 57.35$  to  $13.81 \pm 3.20$  AU,  $P < 0.01$ ).

TABLE 3

Microarray hits: oxidative phosphorylation

Gene	Fold change	Baseline value (%)	P
NDUFB3	↓ 1.4	71.4	<0.01
NDUFB5	↓ 1.9	52.6	<0.01
SDHB	↓ 2.4	41.6	<0.01
SLC25A12	↓ 1.8	55.5	<0.01
NDUFV1	↓ 1.9	52.6	<0.01
NDUFS1	↓ 2.4	41.6	<0.01

By microarray analysis, 297 genes were up- or downregulated after a HFD. Of those 297, 6 are known to be involved in oxidative phosphorylation or mitochondrial function. NDUFB3, NADH dehydrogenase (ubiquinone) 1 $\beta$  subcomplex, 3; NDUFB5, NADH dehydrogenase (ubiquinone) 1 $\beta$  subcomplex, 5; NDUFV1, NADH dehydrogenase (ubiquinone) flavoprotein 1; NDUFS1, NADH dehydrogenase (ubiquinone) Fe-S protein 1; SDHB, succinate dehydrogenase complex, subunit B; SLC25A12, solute carrier family 25 (mitochondrial carrier).



**FIG. 1.** HFD decreases mRNA for genes involved in OXPHOS in healthy young men and mice. **A:** Effect of a 3-day isoenergetic HFD (50% fat vs. 35% fat) in a cohort of healthy insulin-sensitive males ( $n = 10$ ) at baseline and after intervention on the expression of genes in complex I (NDUFB3, NDUFB5, NDUFV1, and NDUFS1), complex II (SDHB), complex III (CYC1), complex IV (SURF1), and a mitochondrial carrier protein (SLC25A12). mRNA was quantified by quantitative RT-PCR. Data are shown as means  $\pm$  SE and corrected for the expression of 18S and RPLP0. NDUFB3, NADH dehydrogenase (ubiquinone) one  $\beta$  subcomplex, 3; NDUFB5, NADH dehydrogenase (ubiquinone) one  $\beta$  subcomplex, 5; NDUFV1, NADH dehydrogenase (ubiquinone) flavoprotein 1; NDUFS1, NADH dehydrogenase (ubiquinone) Fe-S protein 1; SDHB, succinate dehydrogenase complex, subunit B; SLC25A12, solute carrier family 25 (mitochondrial carrier); CYC1, cytochrome c-1; SURF1, surfeit 1. **B:** Effect of a 21-day HFD (45% fat vs. 10% fat) in a cohort of C57Bl/6J mice (control,  $n = 6$ ; HFD,  $n = 7$ ) on genes in complex I (NDUFB3 AND NDUFB5), complex II (SDHB), complex III (CYC1), complex IV (SURF1), and a mitochondrial carrier protein (SLC25A12). Data are shown as means  $\pm$  SE and corrected for the expression of cyclophilin B.

**Reduced expression of genes involved in mitochondrial biogenesis in murine skeletal muscle after a 3-week HFD.** In parallel to the human experiment, we measured both PGC1 $\alpha$  and PGC1 $\beta$  mRNA in these same mice. A 90% reduction in mRNA levels was observed for both PGC1 $\alpha$  and PGC1 $\beta$  (Fig. 2B): PGC1 $\alpha$  ( $34.63 \pm 12.57$  to  $2.67 \pm 0.31$  AU,  $P < 0.01$ ) and PGC1 $\beta$  ( $25.75 \pm 9.03$  to  $1.85 \pm 0.30$  AU,  $P < 0.01$ ).

**Measurement of mtDNA copy number in murine skeletal muscle after a 3-week HFD.** It has been shown that the half-life of a mitochondrion in mammalian cells can range from 3 to 10 days, depending on the measurement technique (30). Given that the expression of both PGC1 $\alpha$  and PGC1 $\beta$  were decreased, we hypothesized that skeletal muscle mtDNA copy number might be decreased by the HFD; however, we found no differences between animals fed a HFD when compared with controls (Fig. 2B): mtDNA in control animals,  $1,166 \pm 112.50$ , and in high-fat animals,  $1,127 \pm 70.28$  AU,  $P = 0.76$ .

**Protein content of PGC1 $\alpha$  and cytochrome C and mitochondrial enzyme activity in murine skeletal muscle after a 3-week HFD.** PGC1 $\alpha$  and cytochrome C protein expression levels were reduced by ~40% in mice consuming a HFD (Fig. 2B): PGC1 $\alpha$  ( $1.31 \pm 0.19$  to  $0.84 \pm 0.07$  AU,  $P < 0.05$ ) and cytochrome C ( $1.35 \pm 0.17$  to  $0.76 \pm 0.09$  AU,  $P < 0.01$ ).

Whole-tissue homogenates were analyzed for citrate synthase, cytochrome C oxidase, and BHAD activity in whole-tissue homogenates and normalized for mitochondrial content using quantitative PCR of mtDNA as described by Ritov et al. (29). Citrate synthase activity and mtDNA from the same homogenate were correlated ( $R^2 = 0.51$ ,  $P < 0.01$ ). Animals fed a HFD had slightly, but not significantly, lower mitochondrial enzyme activity: citrate synthase,  $0.18 \pm 0.02$  to  $0.16 \pm 0.01$  AU,  $P = 0.26$ ; cytochrome C oxidase,  $0.41 \pm 0.11$  to  $0.38 \pm 0.10$  AU,  $P = 0.86$ ; and BHAD,  $0.07 \pm 0.01$  to  $0.06 \pm 0.004$  AU,  $P = 0.22$ .

## DISCUSSION

Numerous studies have implicated reduced mitochondrial biogenesis and OXPHOS in the pathogenesis of insulin resistance and type 2 diabetes (31). Our studies suggest that dietary fat is an important factor in the observed reduction in OXPHOS genes in insulin-resistant states. Microarray analysis and real-time quantitative RT-PCR results revealed a downregulation of OXPHOS genes in young men consuming a HFD, as well as transcription factors and cofactors. Additionally, we have shown that the reductions in genes involved in OXPHOS and mitochondrial biogenesis were recapitulated in an animal model of dietary-induced obesity and insulin resistance (32) and were of a much greater magnitude in mice compared with man.

Through the combined use of microarray technology, advanced bioinformatics, and confirmation of microarray results with quantitative RT-PCR, we were able to identify subtle (20–30%) changes in OXPHOS gene expression without a priori grouping of genes based on known function (3). The advantage of this approach is that genes that do not exhibit large changes in transcription but are clearly important in carbohydrate and energy metabolism (e.g., PGC1 $\alpha$ ) may be identified. HFD elicits a coordinated

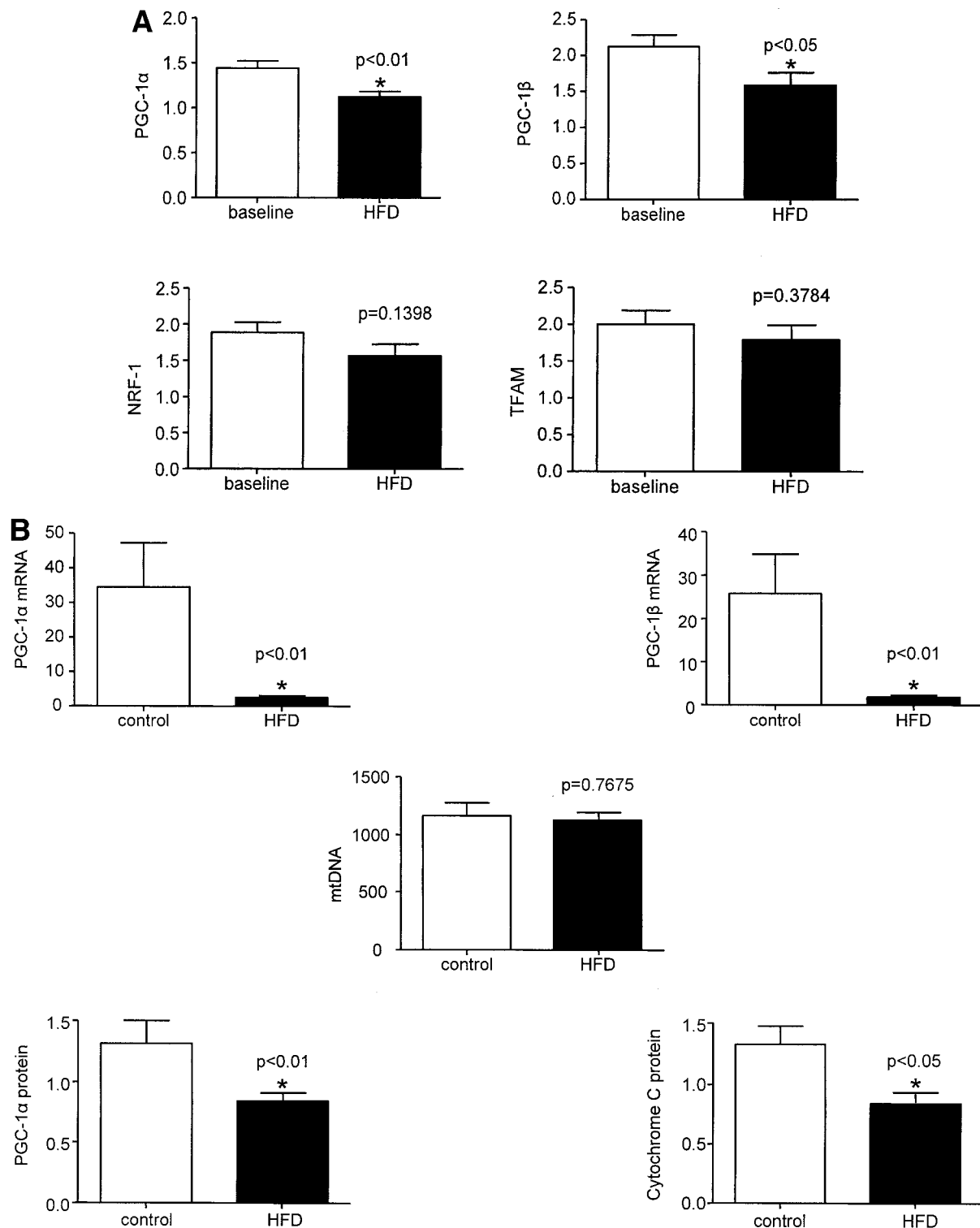
downregulation of genes involved in OXPHOS within the electron transport chain.

Our results support the hypothesis that HFDs and/or high-fat flux through the mitochondria reduce the expression of nuclear genes encoding mitochondrial proteins and transcription factors involved in mitochondrial biogenesis. Both PGC1 $\alpha$  and PGC1 $\beta$  were decreased by ~20% and accompanied by a 20% reduction in OXPHOS gene expression. Previous studies suggest a link between the downregulation of PGC1 and dysregulation of OXPHOS genes. Our results are consistent with this sequence of events, and three of our OXPHOS genes found by microarray analysis were also present in the analyses of Mootha et al. (3) and Patti et al. (4). Therefore, our findings expand the view beyond the relationship between PGC1 and OXPHOS genes. We move upstream to show that increased fatty acid flux through the mitochondria decreases PGC1 expression and associates with a downregulation of expression of OXPHOS genes. It remains unclear from this experimental data whether it is increased fatty acid mitochondrial oxidation per se or some other pathway triggered by fatty acids that is responsible for the effects on gene expression.

Puigserver and Spiegelman (5) demonstrated that PGC1 $\alpha$  is a master regulator of mitochondrial biogenesis and OXPHOS gene expression (33). PGC1 $\alpha$  co-activation of NRF1-mediated transcription leads to transcription and subsequent translocation of TFAM to the mitochondrion, thus increasing mtDNA transcription (34,35). In these studies, both coactivators PGC1 $\alpha$  and PGC1 $\beta$  were downregulated; however, we saw no significant change in two downstream targets, NRF1 and TFAM (Fig. 2A), suggesting alternate and compensatory regulation.

Although an increase in free fatty acid concentrations was not seen in this cohort, fatty acid flux through the muscle is by necessity increased in these subjects as demonstrated by a decrease in 24-h respiratory quotient (data not shown) to match fat intake in this experimental paradigm (14). Another explanation for the reduction in the expression of these genes is that HFD decreases insulin-stimulated gene expression. Fatty acids decrease insulin signaling both in vivo and in vitro. Recent microarray studies demonstrate an upregulation of OXPHOS genes after a short-term insulin infusion (36). A reduction in insulin signaling might reduce expression of these same genes. Our studies do not identify the exact mechanism of the reduction in PGC1 $\alpha$ , PGC1 $\beta$ , or their downstream targets. Rather, these studies point toward dietary fat, or increased lipolysis, as a potential source of the previously reported reduction in mitochondrial OXPHOS and subsequent mitochondrial dysfunction.

Importantly, mice fed a HFD for 3 weeks showed a similar pattern of changes in gene expression as in the shorter human experiments. The magnitude of the changes in gene expression was much larger in mice than in man. In light of the fact that 3 days of HFD is not long enough to cause changes in mtDNA copy number as mitochondrial turnover is relatively slow (30), we next tested the hypothesis that the changes in the transcriptional cofactors (e.g., PGC1 $\alpha$  and PGC1 $\beta$ ) would decrease mitochondrial number in mice fed a HFD for 3 weeks. We found large changes in PGC1 $\alpha$  and PGC1 $\beta$  mRNA, as well



**FIG. 2. HFD decreases expression of transcriptional cofactors involved in the regulation of OXPHOS expression and mitochondrial biogenesis in healthy young men and mice. A:** Effect of a 3-day isoenergetic HFD (50% fat vs. 35% fat) in a cohort of healthy insulin-sensitive males ( $n = 10$ ) at baseline and after intervention on the expression of genes involved in mitochondrial biogenesis. Data are shown as means  $\pm$  SE and corrected for the expression of ribosomal protein, large, P0. NRF1, nuclear respiratory factor-1. **B:** Effect of a 21-day HFD (45% fat vs. 10% fat) in a cohort of C57Bl/6J mice (control,  $n = 6$ ; HFD,  $n = 7$ ) on genes involved in mitochondrial biogenesis and function and mtDNA copy number per nuclear DNA copy number. Data are shown as means  $\pm$  SE and corrected for the expression of cyclophilin B for mRNA and glyceraldehyde-3-phosphate dehydrogenase for protein.

as decreases in both cytochrome C and PGC1 $\alpha$  protein levels, whereas mtDNA content remained unchanged after 3 weeks of HFD in mice.

In some ways, our inability to find changes in mtDNA

copy number are inconsistent with recent studies demonstrating a reduction in mitochondrial number in diabetes and insulin resistance (1,28) but similar to studies showing a decrease in OXPHOS gene expression (3,4). One possi-

bility is that “chronic” versus “acute” effects of high-fat flux through mitochondria are different. In the prediabetic and diabetic states, increased lipid flux has been maintained for a longer period of time. Therefore, additional studies of mitochondrial number and function via electron microscopy will be needed to fully rule out subtle changes in mitochondrial number or function with chronic HFD.

Our studies reveal a key question: “why would increased fatty acid flux decrease the expression of genes needed to oxidize these same fatty acids?”. Fasting is another “normal” physiological condition where fatty acid flux through skeletal muscle is increased. Surprisingly, fasting produces changes in gene expression that are strikingly similar to the pattern of fat-induced changes observed in our studies of HFDs. For example, Jagoe et al. (37) found that CASQ2 (calsequestrin 2), NDUFS1, glycogen synthase, and pyruvate dehydrogenase kinase isoenzyme 4, four genes found on our microarray “hit” list (Supplemental Table 2) and confirmed by quantitative RT-PCR (data not shown), were similarly regulated by fasting in rodents. This may explain the paradoxical decrease in systems needed to oxidize fatty acids (nuclear genes encoding mitochondrial proteins, PGC1 $\alpha$ ) when fat flux is increased during a HFD. In other words, the parallel results between fasting and HFDs suggest that fat flux through the skeletal muscle might be interpreted as a signal of fasting/starvation by the muscle cell itself. Signaling systems normally reserved for responding to energy deprivation (fasting) may be co-opted when dietary fat is increased. This hypothesis is also consistent with observed changes in the transcription of genes involved in nonoxidative metabolism (e.g., glycolysis) found on our microarray “hit” list (Supplemental Table 2).

In conclusion, HFDs in both insulin-sensitive humans and mice were associated with reduction in the expression of genes involved in oxidative capacity (e.g., genes of the electron transport chain), nuclear genes encoding mitochondrial proteins (e.g., mitochondrial carrier proteins), and those involved in mitochondrial biogenesis (e.g., PGC1 $\alpha$  and PGC1 $\beta$ ). These studies support the novel hypothesis that HFDs or high-fat flux explain the reduction in OXPHOS genes seen in aging, the prediabetic state, and in overt diabetes.

#### ACKNOWLEDGMENTS

This work was supported by U.S. Department of Agriculture Grant 2003-34323-14010. We thank Barbara Ukropcova, Iwona Bogacka, Anthony Civitarese, and Rachel Power for technical assistance. We also thank the study participants.

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