Reduced hepatic mitochondrial respiration following acute high-fat diet is prevented by PGC-1α overexpression

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Morris EM, Jackman MR, Meers GM, Johnson GC, Lopez JL, MacLean PS, Thyfault JP. Reduced hepatic mitochondrial respiration following acute high-fat diet is prevented by PGC-1α overexpression. Am J Physiol Gastrointest Liver Physiol 305: G868–G880, 2013. First published October 3, 2013; doi:10.1152/ajpgi.00179.2013.—Changes in substrate utilization and reduced mitochondrial respiratory capacity following exposure to energy-dense, high-fat diets (HFD) are putatively key components in the development of obesity-related metabolic disease. We examined the effect of a 3-day HFD on isolated liver mitochondrial respiration and whole body energy utilization in obesity-prone (OP) rats. We also examined if hepatic overexpression of peroxisomal proliferator-activated receptor-γ coactivator-1α (PGC-1α), a master regulator of mitochondrial respiratory capacity and biogenesis, would modify liver and whole body responses to the HFD. Acute, 3-day HFD (45% kcal) in OP rats resulted in increased daily energy intake, energy balance, weight gain, and adiposity, without an increase in liver triglyceride (triacylglycerol) accumulation. HFD-fed OP rats also displayed decreased whole body substrate switching from the dark to the light cycle, which was paired with reductions in hepatic mitochondrial respiration of multiple substrates in multiple respiratory states. Hepatic PGC-1α overexpression was observed to protect whole body substrate switching, as well as maintain mitochondrial respiration, following the acute HFD. Additionally, liver PGC-1α overexpression did not alter whole body dietary fatty acid oxidation but resulted in greater storage of dietary free fatty acids in liver lipid, primarily as triacylglycerol. Together, these data demonstrate that a short-term HFD can result in a decrease in metabolic flexibility and hepatic mitochondrial respiratory capacity in OP rats that is completely prevented by hepatic overexpression of PGC-1α.

SEVERAL MECHANISMS, including reduced metabolic flexibility, mitochondrial dysfunction, and ectopic lipid accumulation, have been postulated as causative for development of the metabolic syndrome, insulin resistance, and type 2 diabetes. Nonalcoholic fatty liver disease has been described as the liver manifestation of the metabolic syndrome and is independently associated with obesity, insulin resistance, cardiovascular risk factors, and type 2 diabetes. The onset and progression of nonalcoholic fatty liver disease have been postulated to occur as a result of decreased liver mitochondrial respiratory capacity (37) and are associated with metabolic inflexibility (3).

The term “metabolic flexibility” has been used by Kelley et al (23) and others (10) to describe the capacity of an organism to switch substrate utilization from lipid to carbohydrate (and vice versa) on the basis of substrate availability and hormonal signals. Metabolic inflexibility, then, represents the inability to switch substrate utilization and has been observed in skeletal muscle of obese and type 2 diabetic individuals (21, 22). The study of Kelley et al. (22) and subsequent work resulted in the recognition of a strong association between metabolic flexibility and mitochondrial dysfunction in skeletal muscle. Subsequent studies demonstrated that exercise training reversed mitochondrial dysfunction and metabolic inflexibility, with associated improvements in skeletal muscle insulin sensitivity (35). However, cumulatively, these studies did not address whether mitochondrial dysfunction was causal for metabolic inflexibility. Furthermore, these studies were focused solely on skeletal muscle and did not examine the potential impact of other tissues. Importantly, the liver accounts for up to 30% and 20% of total metabolic rate (MR) in humans and rodents (42), respectively; thus the liver plays a significant role in how the body responds to changing fuel needs.

Abhorrent or decreasing mitochondrial respiratory capacity is strongly associated with several physiological pathologies of the metabolic syndrome, in addition to cancer, aging, and neurodegenerative diseases. Obesity, a major cofactor within the metabolic syndrome, has a complex etiology involving genetic predisposition in an environment of excess energy intake (EI) and decreased energy expenditure. Excessive positive energy balance has been shown to result in increased mitochondrial oxidative stress and protein acylation in skeletal muscle and liver (20, 40), which are linked to reductions in various mitochondrial functions (20, 40). It is unclear whether an acute positive energy balance can reduce liver mitochondrial respiratory capacity and whether liver mitochondrial respiratory capacity is associated with whole body metabolic flexibility.

Peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) coregulates the transcriptional activity of multiple transcription factors and is responsible for increased skeletal muscle mitochondrial content and respiratory capacity following exercise (16). In the liver, considerable work has described an important role of PGC-1α in integrating nutritional and peripheral signals to control the change from the fed to the fasted state, especially regulation of hepatic gluconeo-
MATERIALS AND METHODS

PGC-1α expression in a rat model with access to running wheels (29) and hepatic mitochondrial adaptations in treadmill-trained mice (12). Conversely, a chronic high-fat diet (HFD) has been observed to reduce PGC-1α expression in the liver of sedentary wild-type mice (15). However, whether liver-specific PGC-1α overexpression can improve hepatic mitochondrial respiratory capacity in the face of HFD-induced mitochondrial pathology is unknown.

In the current study we investigate the impact of a 3-day HFD on whole body substrate utilization and hepatic mitochondrial respiratory capacity in rats predisposed to develop obesity. Furthermore, we studied whether liver-specific overexpression of PGC-1α would protect obese-prone (OP) rats from acute HFD-induced pathologies. We hypothesized that the short-term HFD would result in increased EI sufficient to produce changes in whole body substrate utilization and reduce hepatic mitochondrial respiratory capacity, which would be prevented by PGC-1α overexpression.

MATERIALS AND METHODS

Ethical approval. The animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver.

Animal selection. Male Wistar rats at 4 wk of age (125–150 g) were purchased from Charles River Laboratories (Wilmington, MA). Animals that were prone to develop obesity were selected from the population as previously described (32, 33). Briefly, all rats were acclimatized to the University of Colorado School of Medicine Center for Comparative Medicine for 1 wk, during which time they were individually housed (20–22°C, 12:12-h light-dark cycle) with free access to normal chow and water. The rats were fed a high-fat, high-sucrose diet (45% kcal fat, 17% kcal sucrose; D12451, Research Diets, New Brunswick, NJ) for 1 wk and then separated into tertiles based on average/weekly weight gain. Animals in the top tertile were allowed to acclimatize to the cages until day 6, animals in the lower two tertiles were removed from the study.

Adenoviral transduction and 3-day HFD. At 8 wk of age, the previously selected OP rats were fed an open-source, low-fat, low-sucrose control diet (10% kcal fat, 7% kcal sucrose; D12450L, Research Diets) to acclimatize for 2 ± 2 wk. Rats were split into J) low-fat diet (LFD), 2) HFD, 3) HFD + adenosine containing β-galactosidase cDNA (HFD-Bgal); and 4) HFD + adenosine containing PGC-1α cDNA (HFD-PGC) groups and subjected to the experimental paradigm outlined in Fig. 1. On day 0, all animals were injected with cyclosporine (15 mg/kg ip) to reduce the immune response. On day 1, the HFD-Bgal and HFD-PGC groups received an intravenous bolus through the tail vein of the appropriate adenosine virus at 3.33 × 10^6 virus particles/g body wt, which was diluted in normal saline to a final volume of 0.5 ml, as previously described (36). This adenoviral transduction method results in liver-specific amplification of the gene of interest (36). All rats were then placed in calorimetry cages and allowed to acclimatize to the cages until day 6. On day 6, appropriate animals were switched to the HFD. On day 8, the animals had access to the same food supplemented with 14C-labeled free fatty acids (FFA) (see below) for 24 h. On day 9, isoflurane was administered, and the rats were euthanized by bilateral thoracotomy at the end of the light cycle. The design employed a 3-day HFD, which has been shown previously to generate hepatic steatosis (27) and cause a new, lower steady-state respiratory quotient (RQ) (18). The HFD and HFD-Bgal groups were not found to have statistically different calorimetry or mitochondrial respiration data and were combined for purposes of data analysis. Daily animal weights and food intake were recorded after the animals were placed in the calorimeter. Blood was collected by heart puncture, and a portion of the liver was quickly put in ice-cold mitochondria isolation buffer. Additional liver tissue segments were collected for RNA isolation and fixation; the remaining liver was snap-frozen in liquid nitrogen. Fat pads were removed, weighed, and clamp-frozen for further analysis. Plasma glucose (Sigma, St. Louis, MO), triacylglycerol (TAG; Sigma), and nonesterified fatty acid (Wako Chemicals, Richmond, VA) were determined according to the manufacturers’ instructions.

Body composition analysis. Body composition was measured by MRI (model 900, EchoMRI, Houston, TX). Body fat percentage was calculated as fat mass divided by animal weight. Body composition was determined prior to initiation of the 3-day HFD and immediately before the animals were euthanized.

Metabolic monitoring. Energy utilization was determined by monitoring whole body O2 consumption (V̇O2), CO2 production (V̇CO2), and urinary nitrogen using the metabolic monitoring system developed by the Energy Balance Core Laboratory at the University of Colorado Denver Center for Human Nutrition Animal Satellite Facility, as described previously (32, 33, 49). MR was calculated with the Weir equation (MR = 3.941 × V̇O2 + 1.106 × V̇CO2 – 2.17 × N), and RQ was calculated as V̇CO2/V̇O2. Total energy expenditure (TEE) was calculated as the average of all MR measurements and extrapolated for presentation purposes to reflect the amount of energy expended over 24 h. Resting energy expenditure (REE) was extrapolated from the resting MR, which was calculated as an average MR over a 1-h period in the later part of the light cycle, when MR and RQ indicated minimal physical activity and food intake. Calorimetry data are represented as the average of the daily values across the 3-day HFD challenge. Energy balance was calculated from the difference between EI and TEE throughout the monitoring period. Each metabolic cage was equipped with an animal activity meter (Opto-Max, Columbus Instruments, Columbus, OH), which allows for determination of total, ambulatory, and nonambulatory activity by monitoring the number of beam breaks within a one-dimensional series of infrared beams. Activity was monitored continuously for 24 h.

24-h dietary FFA tracer. To assess dietary fatty acid oxidation, tissue retention and trafficking of dietary fat, and de novo lipogenesis, an in vivo, 24-h dual-tracer study was performed as previously described (19, 49). Rats were acclimatized to the calorimetry system, received adenosine, and were started on the 3-day HFD as described above. On day 8 (Fig. 1), animals received an intraperitoneal injection of [3H]H2O (200 μl, 1 mCi/ml) 1 h prior to the start of the dark cycle (i.e., at 1300), which allows for tracer equilibration with total body water, and measurement of incorporation of tritium in extracted lipid serves as an estimate of net retention of carbon via de novo lipogenesis. A

![Fig. 1. Experimental paradigm. Cyclo, cyclosporine; Adv, adenosine virus; FFA, free fatty acid.](http://ajpgi.physiology.org/)

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3:1 ratio of [1-14C]oleate to [1-14C]palmitate was blended into the LFD and HFD, resulting in specific activities of 0.34 and 0.90 μCi/g diet (3.43 and 1.98 μCi/g dietary fat), respectively. The labeled diet was given at the start of the final dark cycle (at 1400). Every 3 h, CO2 from each chamber was collected in 3.0-ml aliquots of a 2:1 mixture of methanol-methylbenzethonium hydroxide (catalog no. B2156, Sigma Chemical). The 14C content of these samples was then measured with a scintillation counter (model LS6500, Beckman).

At the end of the 24-h tracer study, rats were anesthetized with isoflurane and euthanized by exsanguination as the tissues were extracted.

**Tissue lipid analysis.** Liver TAG concentration was determined as previously described using a commercially available kit (catalog no. F6428, Sigma) (51). To determine total tissue lipid 14C and 3H incorporation, total lipids were extracted utilizing the Dole extraction method (6). Isopropanol-heptane-1 N H2SO4 (40:10:1) was used to homogenize 200 μg of liver and red gastrocnemius and 100 μg of fat pads. Phases were separated with normal saline, the upper phase was collected and dried under N2 gas, and 14C and 3H were determined by liquid scintillation counting. 14C per sample was converted to milligrams of dietary fat and normalized to sample weight in grams. 3H counts per sample were normalized to sample weight in grams. To determine 14C incorporation into specific liver lipid species, lipids were extracted utilizing a modification of the Folch chloroform-methanol method (9). The bottom layer (chloroform) was collected, and the individual lipid species were separated by thin-layer chromatography (70:30:1 hexane-diethylether-acetic acid). Lipid species were visualized by 2,7-dichlorofluorescin staining and collected for determination of 14C by liquid scintillation counting.

**Mitochondrial isolation.** Mitochondria were isolated from rat liver tissue as previously described (36). Briefly, tissue was homogenized in cold liver mitochondrial isolation buffer (220 mM mannitol, 70 mM sucrose, 10 mM Tris, and 1 mM EDTA, with pH adjusted to 7.4 with KOH) and centrifuged (1,500 g, 10 min, 4°C). The supernatant was centrifuged (8,000-6,000-4,000 g) for 10 min at 4°C, with the pellet resuspended in liver mitochondrial isolation buffer following each centrifugation. Protein concentration was determined by bicinchoninic acid assay.

**Mitochondrial respiration.** Mitochondrial VO2 was measured using a Clark-type electrode system (Strathkelvin Instruments, North Lanarkshire, Scotland). Incubations were carried out at 37°C in a 0.5-ml final volume containing 100 mM KCl, 50 mM MOPS, 10 mM K2PO4, 10 mM MgCl2, 0.5 mM EGTA, 20 mM glucose, and 0.2% bovine serum albumin, pH 7.4. Mitochondrial respiration of substrates was monitored at the following concentrations: 1 mM malate, 10 mM glutamate, 1 mM pyruvate, 4 μM L-palmitoylcarnitine, and 10 mM succinate. Mitochondria and substrates were added, and the coupled maximal respiratory rate (state 3) was initiated with the addition of ADP (100 μM). Respiration through electron transfer through complex I was measured with glutamate (+malate) and pyruvate (+malate), while convergent respiration through complexes I and II of glutamate and pyruvate was examined by the addition of succinate. Respiration of L-palmitoylcarnitine (+malate) was examined for complexes I and electron transfer flavoprotein electron contribution to Vo2, As with glutamate and pyruvate, succinate was added to experiments with L-palmitoylcarnitine (+malate) to examine convergent electron flow. Complex II respiration was monitored as V˙O2 of succinate in the presence of rotenone. Maximal uncoupled respiration was determined by titration of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (1 μM). Vo2 (in nmol/min) was normalized to mitochondrial protein in the respirometer cell.

**Citrate synthase activity.** Citrate synthase activity was determined in isolated mitochondria and whole tissue homogenate, as previously described (48).

**mRNA expression.** RNA was isolated from primary hepatocytes, tissue was stored in RNAlater using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA), and cDNA was produced using the ImProm-II RT system (Promega, Madison, WI). RNA and cDNA concentrations were determined using a spectrophotometer (model ND-1000, NanoDrop, Thermo Scientific, Wilmington, DE). Real-time quantitative PCR analysis was performed utilizing a Prism 7000 and TaqMan gene expression assay (Applied Biosystems, Foster City, CA). The relative mRNA expressions of pgc1a, diacylglycerol acyltransferase (dgat1 and dgat2), adipose triacylglyceride lipase [agpat6, glycerol-3-phosphate acyltransferase (gp4at)], and uncoupled protein 2 (ucp2) were determined utilizing a predesigned, 6-carboxyfluorescein-labeled primer/probe assay supplied by Applied Biosystems. SYBR Green assays for gpat1 [ATCCGGACCGT-GAAATGGGA (forward) and GGCAAACCATGCGCTTGGAC (reverse)] and cd36 [CTCTGACATTGCGAGTC (forward) and CAGGCTTCCTCCTIGGC (reverse)] were purchased from Sigma. All gene-specific values were normalized to relative 18S rRNA values.

**Western blotting.** Triton X-100 cell lysates were used to produce Western blot-ready Laemml samples. Samples were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with primary antibodies. PGC-1α antibody was purchased from Calbiochem (EMD Chemicals, Gibbstown, NJ); Mn-SOD (SOD2), sirtuin 3 (Sirt3), adipose triglyceride lipase (ATGL), and acetylated lysine antibodies from Cell Signaling Technologies (Danvers, MA); 4-hydroxynonenal (4-HNE) antibody from Alpha Diagnostics International (San Antonio, TX); and OXPHAT antibody from American Research Products (Waltham, MA). Individual protein loading was quantified using a densitometer (Bio-Rad), and protein loading was corrected by 0.1% amido-black (Sigma) staining to determine total protein, as previously described (36).

**Statistics.** As mentioned above, the HFD and HFD-Bgal groups were not observed to have significantly different calorimetry or mitochondrial respiration data and were combined as “HFD” for the purpose of data analysis and reporting. Therefore, there were 6 animals in the LFD and HFD-PGC groups and 11 animals in the HFD group. One-way ANOVA was used for comparison of three groups (P < 0.05), and Fisher’s least significant difference post hoc test was performed to determine differences between groups with SPSS (IBM, Somers, NY). Values are means ± SE.

**RESULTS**

**Hepatic PGC-1α overexpression.** There was no difference in PGC-1α mRNA or protein between the LFD and HFD groups; however, intravenous administration of recombinant adenovirus containing PGC-1α cDNA resulted in a 3.5-fold increase (P < 0.05) in hepatic PGC-1α mRNA compared with the other groups (Fig. 2A). The increase in PGC-1α mRNA resulted in a 20% increase (P = 0.06) in liver PGC-1α protein expression compared with the LFD and HFD groups (Fig. 2B). The increases in liver PGC-1α mRNA and protein following adenoviral transduction are comparable to effects we witnessed in Sprague-Dawley rats (36). The increases in PGC-1α expression were associated with modest, but significant, increases in the classic mitochondrial marker citrate synthase activity in liver homogenate compared with LFD and HFD rats (12% and 5%, respectively, P < 0.05; Fig. 2C). Additionally, a small increase in citrate synthase activity was observed in the HFD compared with the LFD group (P < 0.05). Citrate synthase activity can also be utilized as a marker of mitochondrial capacity when measured in isolated mitochondria (36). Interestingly, citrate synthase activity in isolated liver mitochondria tended to be lower in the HFD than LFD rats (Fig. 2D), while isolated mitochondrial citrate synthase activity was ~30% higher in the HFD-PGC than HFD group (P < 0.05). These data demonstrate that PGC-1α adenoviral transduction of OP
Anthropometry, calorimetry, and energy intake data

Table 1. Anthropometry, calorimetry, and energy intake data

<table>
<thead>
<tr>
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<th>LFD</th>
<th>HFD</th>
<th>HFD-PGC</th>
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<tr>
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<td>%Body fat</td>
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<td>3-Day change in %body fat</td>
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<td>1.72</td>
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<td><strong>Calorimetry</strong></td>
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<td>$V_O2$, ml/h</td>
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<td>0.0093</td>
<td>0.0094</td>
</tr>
<tr>
<td>$V_CO2$, ml/h</td>
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<td>0.0087</td>
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<td>5.71</td>
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<td>30.27</td>
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Values are means ± SE. LFD, low-fat diet; HFD, high-fat diet; HFD-PGC, HFD + adenovirus containing peroxisome proliferator-activated receptor-γ coactivator-1α; NEFA, nonesterified fatty acid; TAG, triacylglycerol; $V_O2$, $O2$ consumption; $V_CO2$, $CO2$ production; RQ, respiratory quotient ($V_CO2/V_O2$); TEE, total energy expenditure; REE, resting energy expenditure. *$P < 0.05$, LFD vs. HFD.

Fig. 2. Adenoviral peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) transduction produces increased PGC-1α expression and citrate synthase activity. Liver PGC-1α expression following adenoviral transduction was determined by RT-PCR and Western blot analysis, and citrate synthase activity was determined in liver homogenates and isolated liver mitochondria ($n = 6–11$). A and B: higher liver PGC-1α mRNA and protein expression in rats fed the high-fat diet (HFD) and treated with adenovirus containing PGC-1α cDNA (HFD-PGC) than in HFD rats. C and D: increased citrate synthase activity in liver homogenates and isolated liver mitochondria in PGC-1α-overexpressing (HFD-PGC) rats. *$P < 0.05$, LFD vs. HFD. †$P < 0.05$, HFD vs. HFD-PGC.
increase in plasma glucose levels in the HFD and HFD-PGC compared with LFD rats; however, this increase does not appear to be associated with liver PGC-1α overexpression, as no difference was observed between OP HFD and HFD-PGC rats (Table 1). Furthermore, serum nonesterified fatty acids were elevated in both HFD groups compared with the LFD group ($P < 0.05$), with no significant differences in serum TAG (Table 1). Also, the 3-day HFD was not sufficient to produce significantly elevated liver TAG in either HFD group compared with the LFD rats. This is in contrast to liver TAG data from other experiments utilizing OP rats generated in the same manner from our group, where a ~2-fold increase was observed after 5 days of a HFD (17) and a ~3.5-fold increase was observed following a 6 wk HFD compared with LFD animals (1.72 and 0.49 nmol/g, respectively, $P < 0.05$).

Energy utilization. Classically, administration of HFD increases whole body lipid oxidation, as indicated by a reduced RQ. Indirect calorimetry (both $\text{VO}_2$ and $\text{VCO}_2$) was used to monitor the effect of the 3-day HFD on substrate utilization, allowing for quantification of substrate utilization and energy expenditure. Neither the 3-day HFD nor PGC-1α overexpression resulted in significant changes in $\text{VO}_2$ or $\text{VCO}_2$ between the animal groups (Table 1). Furthermore, no differences were observed for TEE, REE, or activity level (data not shown) due to the animal groups (Table 1). Furthermore, no differences were observed following a 6-wk HFD compared with LFD animals (1.72 and 0.49 nmol/g, respectively, $P < 0.05$).

Fig. 3. Liver PGC-1α overexpression prevents development of metabolic inflexibility following the 3-day HFD. Respiratory quotient (RQ, $\text{CO}_2$ production $\text{VCO}_2/\text{O}_2$ consumption ($\text{VO}_2$)) during light and dark cycles was determined by indirect calorimetry ($n = 6-11$). A: 3-day HFD lowered RQ in HFD and HFD-PGC compared with LFD animals. B: liver PGC-1α overexpression prevented HFD-induced reduction in ΔRQ between light and dark cycles. $P < 0.05$, light cycle vs. dark cycle. $† P < 0.05$, HFD vs. HFD-PGC.
The 3-day high-fat diet (HFD) in OP rats had no impact on 
O2 consumption with succinate. Unlike the respiration of pyruvate and glutamate, the 
convergent electron flow with the addition of 
electrons through complex I and electron transfer flavoprotein, 
pression on isolated mitochondria uncoupled mitochondrial respiration from oxidative phosphorylation and allows for examination of maximal O2 consump-
tion. Experiments were performed in the presence of succinate between LFD and HFD-PGC animals for this substrate com-
nate and the complex I inhibitor rotenone (Fig. 4).

Results

Mitochondrial Respiration

Glutamate

Pyruvate

L-palmitoylcarnitine

Succinate

Mitochondrial Respiration

Glutamate

Pyruvate

L-palmitoylcarnitine

Succinate

Mitochondrial Respiration

Glutamate

Pyruvate

L-palmitoylcarnitine

Succinate

Dietary [14C]FFA tracer. On day 3 of the HFD challenge, the rate of dietary fatty acid oxidation and cumulative dietary fatty acid oxidation were determined by monitoring the expired 
14CO2 in effluent air (Fig. 5, A and B). The 3-day HFD resulted in an increased rate of dietary fatty acid oxidation (Fig. 5A) and cumulative dietary fatty acid oxidation (Fig. 5B) at all time points in the HFD and HFD-PGC animals compared with the LFD group (P < 0.05). PGC-1α overexpression did not result in a significant difference in dietary fatty acid oxidation between the HFD and HFD-PGC animals. Monitoring 14C content of isolated lipid from various tissues allows for the investigation of dietary lipid trafficking during the 24-h dietary tracer study. The 3-day HFD resulted in a 2.5-fold increase in total dietary fat stored in the liver lipid compared with the LFD (P < 0.05; Fig. 5C), with PGC-1α overexpression resulting in a further 40% increase in total dietary lipid compared with the HFD alone (P < 0.05). In the retroperitoneal fat pad, the accumulation of dietary lipid was ~3.25-fold higher in HFD
and HFD-PGC than LFD rats, with no difference between HFD and HFD-PGC animals \((P < 0.05, \text{LFD vs. HFD})\). \(\hat{P} < 0.05, \text{HFD vs. HFD-PGC}\).

To assess the distribution of dietary lipid in different lipid species in the liver, total extracted lipid was separated by thin-layer chromatography. The HFD increased dietary lipid accumulation in liver FFA, diacylglycerol, cholesterol ester, and phospholipid in the HFD and HFD-PGC compared LFD rats \((P < 0.05, \text{LFD vs. HFD})\). The 3-day HFD also resulted in a greater than twofold increase in dietary lipid accumulation in liver TAG compared with the LFD \((P < 0.05, \text{LFD vs. HFD})\). Interestingly, liver PGC-1\(\alpha\) overexpression produced an additional 50% increase in dietary lipid accumulation in liver TAG compared with HFD alone \((P < 0.05)\). These results demonstrate that while hepatic PGC-1\(\alpha\) overexpression did not alter whole body dietary fatty acid oxidation or dietary lipid accumulation in adipose tissue, the increase in PGC-1\(\alpha\) expression resulted in increased dietary lipid accumulation in the liver, primarily as TAG.

Mitochondrial antioxidant and acetylation. Increased mitochondrial oxidative damage and increased protein acetylation have been proposed as mechanisms by which decreased mitochondrial respiratory capacity can occur in dietary-induced metabolic disease \((24, 45)\). To assess how the 3-day HFD and PGC-1\(\alpha\) overexpression may impact hepatic mitochondrial oxidative stress, the mRNA expression of UCP2 and protein expression of SOD2 were examined. UCP2 is proposed to increase leak of H\(^+\) across the inner membrane from the intermembrane space back into the mitochondrial matrix, potentially lowering reactive oxygen species (ROS) production by decreasing the electrochemical gradient \((5)\). No difference in UCP2 mRNA expression was observed between LFD and HFD animals. However, PGC-1\(\alpha\) overexpression resulted in a threefold increase in UCP2 mRNA expression compared with the LFD \((P < 0.05, \text{LFD vs. HFD})\) and a twofold increase over HFD alone \((P < 0.05)\). SOD2, an enzyme that is localized to the mitochondrial matrix, functions as the initial step in the antioxidant cascade of the mitochondria by catalyzing the reduction of superoxide to hydrogen peroxide \((37)\). The protein
expression of SOD2 was ~40% lower in liver homogenate from HFD than LFD rats (P < 0.05; Fig. 6B). This loss of SOD2 protein expression was prevented in the HFD-PGC rats, which displayed ~60% greater SOD2 protein expression than HFD rats (P < 0.05). 4-HNE mitochondrial protein adduction was analyzed in isolated hepatic mitochondria to determine if changes in SOD2 and UCP2 resulted in differing levels of oxidative damage in mitochondria. Liver mitochondrial 4-HNE levels tended to be lower in the HFD-PGC animals but were not significantly different across any of the groups (data not shown). However, this relatively crude measure of oxidative damage may not be sensitive enough to detect small changes that may be occurring during this experimental paradigm.

As an additional potential modifier of mitochondrial respiratory capacity, hyperacetylation of mitochondrial proteins following chronic HFD has been shown to result in decreased activity of ETS complexes (24). SIRT3 is localized to the mitochondrial matrix and is the major enzyme responsible for controlling mitochondrial protein acetylation through catalyzing NAD⁺-dependent deacetylation of numerous substrates (24). In isolated liver mitochondria, PGC-1α overexpression resulted in a ~40% increase in Sirt3 protein expression compared with the LFD (P < 0.05; Fig. 6C), while there was no difference between the LFD and HFD groups. Western blot analysis of liver mitochondrial protein acetylation revealed that acetylation tended to be lower in the OP HFD-PGC rats but was not significant (data not shown). As with 4-HNE analysis, this crude analytical method may not be sensitive enough to detect small changes. These data suggest that PGC-1α-mediated increases in Sirt3 expression may function to protect mitochondrial respiratory capacity from HFD-induced reductions prior to the onset of detectable hyperacetylation.

**Hepatic TAG metabolism.** To investigate the increases in liver dietary lipid storage as TAG, mRNA and protein expression of genes involved in FFA uptake, glycerolipid synthesis, and lipid droplet (LD) physiology were assessed. The uptake of serum FFA by hepatocytes is facilitated by plasma membrane transporters, such as CD36/fatty acid translocase, which has increased hepatic expression in HFD-induced and obese models of fatty liver disease (14). However, no difference was observed in the protein expression of CD36 following the 3-day HFD or PGC-1α overexpression (Fig. 7A). The FFA esterification of glyceraldehyde 3-phosphate to form lysophosphatidic acid is the rate-limiting step in the glycerolipid synthesis pathway (55). Outer mitochondrial membrane-localized glyceraldehyde 3-phosphate acyltransferase (GPAT)-1 (GPAT1) and endoplasmic reticulum-localized GPAT4 represent 70–90% of GPAT activity in hepatocytes and oppose fatty acid oxidation as the alternate metabolic fate of acyl-CoAs (2, 39, 55). The 3-day HFD reduced GPAT1 mRNA expression ~60% and 70% in OP HFD and OP HFD-PGC rats, respectively, compared with OP LFD rats (P < 0.05; Fig. 7B), while the hepatic overexpression of PGC-1α resulted in a further ~35% reduction in GPAT1 mRNA expression compared with the HFD alone (P < 0.05). While GPAT4 mRNA expression tended to be lower in OP HFD than LFD rats (P = 0.069; Fig. 7C), only the OP HFD-PGC rats demonstrated a significant reduction in hepatic GPAT4 compared with the LFD rats (30%, P < 0.05).

The mammalian DGAT1 and DGAT2 catalyze the esterification of diacylglycerol as the terminal step in the biosynthesis of TAG (31). DGAT1 mRNA expression tended to be lower in OP HFD than OP LFD rats (P = 0.069; Fig. 7D), while PGC-1α overexpression resulted in a 30% increase compared with the HFD (P < 0.05). DGAT2 mRNA expression was reduced in OP HFD and OP HFD-PGC rats (20% and 35%, respectively, P < 0.05; Fig. 7E) compared with OP LFD rats, with no significant difference between the HFD groups. Once thought to be inert, unorganized collections of ectopic TAG, LDs are known to be organelles with associated proteins that control cellular TAG storage and hydrolysis (54). Expression of the LD protein plin2 (also termed adipocyte differentiation-related protein) has been observed to be elevated in steatotic livers (38). A significant difference in plin2 protein expression was only observed in
HFD-PGC rats compared with LFD and HFD rats (90% and 50%, respectively, \( P < 0.05 \); Fig. 7F). However, expression of plin5 (OXPAT) protein, a LD protein that has previously been observed to be under PGC-1\(\alpha\) transcriptional control in skeletal muscle (26), was not observed to be different between any of the groups (Fig. 7G). ATGL, another PGC-1\(\alpha\) transcriptional target in skeletal muscle (26), catalyzes the initial hydrolysis of TAG to diacylglycerol. Interestingly, PGC-1\(\alpha\) overexpression resulted in a \( \sim 2.7 \)-fold increase in liver ATGL protein expression compared with the LFD and HFD (\( P < 0.05 \); Fig. 7H). These data suggest that the liver overexpression of PGC-1\(\alpha\) allows for greater capacity to sequester lipid as TAG in LDs and to initiate hydrolysis of the stored TAG under appropriate conditions.
DISCUSSION

To our knowledge, our study is the first to examine the interaction of changes in whole body energy utilization and liver mitochondrial respiratory capacity in a pro-obesogenic animal following a short-term HFD challenge. It is also the first to determine whether increased liver-specific PGC-1α expression can influence substrate utilization through increased mitochondrial respiratory capacity in this acute HFD environment. The major findings of this study are that the 3-day HFD in OP rats resulted in a loss of whole body substrate switching during the light-to-dark cycle, which was associated with decreased hepatic mitochondrial respiration independent of the development of steatosis. Furthermore, the liver-specific overexpression of PGC-1α prevented the reduction of substrate switching between the dark and light cycles, as well as maintenance of enhanced hepatic mitochondrial respiration. Interestingly, these effects of PGC-1α were also paired with a greater accumulation of dietary lipid as liver TAG.

Metabolic inflexibility has been characterized as the inability to appropriately change fuel utilization in response to metabolic challenges, such as fasting, refeeding, and exercise (50). It is associated with pathophysiologies including obesity, accumulation of ectopic lipid, and insulin resistance (10). The development of metabolic inflexibility has previously been connected to reduced mitochondrial respiratory capacity in skeletal muscle (22, 35). Our group previously observed that the 3-day HFD was sufficient to produce a new steady-state RQ in OP rats (18) and that continuation of the HFD chronically results in reduced metabolic flexibility in sedentary OP rats (34). These studies match human results, where short-term HFD or overfeeding trials have been shown to impair metabolic flexibility in overweight or OP individuals (47, 53). A loss of the diurnal fluctuation in fuel utilization in this study further supports the concept that the HFD alters metabolic phenotypes. Interestingly, this onset of metabolic inflexibility occurs independent of hepatic lipid accumulation, which has been previously observed following the 3-day HFD in other outbred rats (46).

Research in metabolic flexibility has focused on skeletal muscle because of its large role in resting energy metabolism and its important role in whole body glucose disposal (21), as well as the observation that increased energy expenditure through exercise can improve metabolic responses in association with increased skeletal muscle mitochondrial content (35). However, the liver also has a large impact on energy metabolism in resting conditions. It is estimated that the liver and skeletal muscle account for an equal percentage of the resting MR (42). Therefore, liver metabolism should also have a large impact on whole body metabolic flexibility in a sedentary environment. Our group has demonstrated that voluntary wheel running results in greater mitochondrial content in the liver of obese rats (43, 44) and increased expression of the transcriptional coactivator PGC-1α in normal-weight rats (29). The role of PGC-1α in increasing skeletal muscle mitochondrial content and respiratory capacity following exercise is well documented (16), and we recently demonstrated that adenoviral PGC-1α overexpression in the liver results in increased hepatic mitochondrial content and oxidative capacity (36). In the current experiments we observed that previous liver PGC-1α overexpression was sufficient to protect whole body metabolic flexibility (RQ transition from the dark to the light cycle) following the 3-day HFD challenge in OP rats. It appears that this protection of metabolic flexibility is due to a further increase in lipid utilization during the light (resting) cycle, possibly due to increased hepatic fatty acid oxidation, as previously observed with liver PGC-1α overexpression in rats (36). Similar alterations in metabolic flexibility have been observed in human subjects following a 3-day HFD (52). The acute HFD resulted in similar 24-h RQ values, but a significantly lower RQ was observed during sleeping (resting) in subjects without a family history of type 2 diabetes than in those with a positive family history. In our study, the protection of metabolic flexibility in sedentary animals occurred through a liver-specific intervention. This raises the possibility that the previously observed improvements in systemic metabolic flexibility following exercise could be due to liver and skeletal muscle changes. The protection of metabolic flexibility could have also been related to the maintenance of liver mitochondrial respiratory capacity, greater uncoupling capacity, protection against oxidative stress, and increased dietary lipid sequestration as TAG. As previously mentioned, reduced skeletal muscle mitochondrial respiratory capacity has been observed to be strongly correlated with metabolic inflexibility (10, 22), and improvements in mitochondrial respiratory capacity due to exercise parallel improvements in metabolic flexibility (35). These links have not been studied in the liver. We have demonstrated that the 3-day HFD was sufficient to produce a significant reduction in hepatic mitochondrial respiration of multiple substrates in various respiratory states in OP rats. Importantly, these deficits were prevented by prior liver-specific PGC-1α overexpression. The increases in liver mitochondrial respiration of palmitoylcarnitine and pyruvate are in agreement with our previous findings of increased mitochondrial fatty acid and pyruvate oxidation following hepatic PGC-1α overexpression (36). Together, these findings further support the role of PGC-1α in coordinating transcriptional regulation of mitochondrial pathways involved in substrate oxidation, the TCA cycle, and oxidative phosphorylation to increase liver mitochondrial capacity.

The rate of mitochondrial respiration is under complex substrate, allosteric, and posttranslational control. The posttranslational modification of proteins involved in β-oxidation, the TCA cycle, and oxidative phosphorylation have been described in various models of obesity and type 2 diabetes. Oxidative damage due to increased overacetylation of proteins and ROS production are two mechanisms by which mitochondrial respiratory capacity is hypothesized to be reduced in obesity and type 2 diabetes. In the current study no direct evidence for increased overacetylation or ROS was observed, perhaps because of the relatively short duration of the HFD challenge (15). However, expression of genes relevant to the protection of tissues and organelles from these pathologies was altered in the livers of the OP rats. The class III NAD+-dependent deacetylase Sirt family isoform Sirt3, a PGC-1α transcriptional target that is localized to the mitochondria and is critical to the regulation of mitochondrial lipid, nitrogen, carbohydrate, ROS, and oxidative phosphorylation metabolism (15, 40), was increased in isolated liver mitochondria of PGC-1α-overexpressing rats. Additionally, UCP2 was increased in the liver of PGC-1α-overexpressing rats, potentially resulting in reduced ROS production through lowering of the mitochondrial membrane potential due to increased basal proton conductance (37), which is supported by the increase in basal respiration, a relative marker of elevated proton conductance (5). Also, mitochondrial ROS levels can be controlled through a highly regulated antioxidant system, which contains the PGC-1α target SOD2 (4). In this...
studied, liver SOD2 expression correlates tightly with the changes in mitochondrial respiration capacity, as well as whole body metabolic flexibility. However, all these observations are associative and require further investigation.

In the current study the protection of whole body metabolic flexibility and liver mitochondrial respiratory capacity by hepatic PGC-1α overexpression was associated with increased hepatic storage of dietary lipid as TAG. Although we did not measure an increase in whole body dietary fatty acid oxidation, our previous study would suggest that liver fatty acid oxidation was increased. This increased fatty acid oxidation paired with an increased capacity to store dietary fatty acids in TAGs could have protected hepatic mitochondrial function by preventing an intracellular accumulation of fatty acids that have previously been shown to reduce liver mitochondrial respiration (11).

The mechanisms by which PGC-1α controls hepatic TAG storage are incompletely known. The increased dietary TAG storage following PGC-1α overexpression is likely not due to increased fatty acid uptake (CD36) or changes in the rate-limiting step of glycerolipid synthesis (GPAT1 and GPAT4) (2, 39, 55). However, differences in DGAT, the final step in the TAG synthesis pathway, may play a role. Recent findings suggest that DGAT1 is responsible for incorporation of exogenous fatty acid incorporation into TAG, while DGAT2 functions to incorporate de novo synthesized fatty acids (41, 56). Additionally, PGC-1α overexpression in skeletal muscle has been observed to increase DGAT1 expression (8). Therefore, the increase in liver dietary TAG following the 3-day HFD in PGC-1α-overexpressing rats may be due in part to increased incorporation of exogenous dietary FFA into TAG through PGC-1α-mediated maintenance of liver DGAT1 expression. Once synthesized, cellular TAG is sequestered in phospholipid monolayer organelles, LDs, which have associated proteins that control TAG metabolism through modulation of storage and hydrolysis (54). Plin2 has been observed to be increased in various tissues under elevated lipid conditions (54), including liver (38), where it is believed to facilitate TAG storage by limiting interaction of cellular lipases with the LD (1). Our observation of increased plin2 expression in the liver of PGC-1α-overexpressing rats is consistent with plin2 expression being under the control of a PGC-1α transcriptional target PPARα (7). Finally, we observed an increase in hepatic ATGL expression in PGC-1α-overexpressing rats, which is supported by the ATGL increase in liver during the fed-to-fasted transition (28) and control of ATGL expression by PPARγ (25). While this increase in a lipolytic enzyme may seem counterintuitive to the observed increase in liver dietary FFA storage as TAG and maintenance of mitochondrial function, recent data suggest that ATGL expression and production of FFA are necessary for appropriate PGC-1α-mediated transcriptional coactivation of PPARα mitochondrial gene pathways (13). In conclusion, these data suggest that the capacity for PGC-1α overexpression to protect against lipid-induced changes in mitochondrial respiration may not be solely due to mitochondria-specific changes, but may also be linked to changes in TAG storage and LD proteins. The regulation of these pathways by PGC-1α deserves further study.

In conclusion, these data demonstrate that, in OP rats subjected to a 3-day HFD challenge, an associative large increase in positive energy balance is sufficient to result in whole body metabolic inflexibility, with concomitant decreased hepatic mitochondrial respiratory capacity, independent of excess lipid accumulation. Importantly, we show that systemic and hepatic metabolic pathologies are completely prevented by previous liver-specific overexpression of PGC-1α. These data provide new insight into the potential role of liver metabolism and, more precisely, liver mitochondrial respiratory capacity and lipid storage in the maintenance of systemic metabolic health and prevention of HFD-induced metabolic disorders.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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