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Interactions between the consumption of a high-fat diet and fasting in the regulation of fatty acid oxidation enzyme gene expression: an evaluation of potential mechanisms

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First published November 17, 2010; doi:10.1152/ajpregu.00367.2010.—The consumption of high-fat diets (HFDs) and fasting are known to increase the expression of enzymes involved in fatty acid oxidation (FAO). However, it has been reported that the ability of physiological stressors to induce enzymes of FAO in skeletal muscle is blunted with obesity. In this regard, we sought to explore the effects and potential mechanisms of an HFD on the expression of FAO enzymes in the fed and fasted state. The consumption of an HFD increased the mRNA expression of medium-chain acyl-CoA dehydrogenase (MCAD), uncoupling protein-3 (UCP3), and pyruvate dehydrogenase kinase 4 (PDK4) in the fed state. Fasting increased the mRNA expression of PDK4, MCAD, and UCP-3, and the protein content of UCP-3 in chow but not HFD rats. HFDs did not increase carnitine palmitoyl transfer 1 (CPT-1) mRNA levels in the fed state and the expression of PDK4, MCAD, and UCP-3 in chow but not HFD rats. The consumption of a high-fat diet and fasting in the regulation of fatty acid oxidation enzyme gene expression: an evaluation of potential mechanisms.

Fasting increases the mRNA expression of carnitine palmitoyl transfer 1 (CPT-1) (10, 34) and pyruvate dehydrogenase kinase 4 (PDK4) (10, 30, 34), which are involved in mediating the entrance of fatty acids into the mitochondria and inhibiting glucose oxidation, respectively. Similarly, fasting also upregulates the expression of uncoupling protein-3 (UCP3) (5, 27, 42) and pyruvate dehydrogenase kinase 4 (PDK4) (10, 34) (31). For instance, fasting-induced increases in FAO are blunted in obese humans (20), while increases in CPT-1 and PDK4 mRNA levels following fasting, are attenuated in cardiac muscle from obese, insulin-resistant Zucker rats (47). Along a similar line, De Filippis et al. (9) recently demonstrated that exercise-induced increases in the mRNA expression of PGC-1α and cytochrome-c oxidase subunit IV were attenuated in skeletal muscle from obese individuals. Interestingly, these impairments were associated with reductions in the exercise-induced activation of AMPK, suggesting that alterations in the
activity of this enzyme could be associated with the metabolic inflexibility that has been reported in obese individuals.

While a blunted effect of physiological stressors on the induction of mitochondrial enzymes has been reported in skeletal muscle from obese, insulin-resistant rodents and humans, several laboratories have found that mitochondrial content and markers of FAO are increased in skeletal muscle from rodents fed a high-fat diet (HFD) (11, 17, 41). Thus, it is not clear whether a blunted effect of fasting on the induction of mitochondrial enzymes is due to already elevated levels of these proteins or whether underlying defects in the activation of the signaling pathways that control the expression of FAO enzymes are present. Similarly, the signaling pathways that may explain the induction of mitochondrial biogenesis following the consumption of HFDs have not been fully explored. Since perturbations in fatty acid metabolism are believed to be linked to the development of insulin resistance, a thorough examination of these questions is warranted. Within this context, the purpose of the present study was to explore the effects of an HFD, and the potential mechanisms therein, on the expression of FAO enzymes in the fed and fasted state. We hypothesized that this model of obesity and insulin resistance would result in blunted increases in genes involved in FAO, decreases in PGC-1 mRNA expression, and an attenuation of AMPK and p38 MAPK signaling in skeletal muscle.

MATERIALS AND METHODS

Materials. Reagents, molecular weight marker, and nitrocellulose membranes for SDS-PAGE were purchased from Bio-Rad (Mississauga, ON, Canada). ECL Plus was a product of Amersham Pharmacia Biotech (Arlington Heights, IL). Antibodies against phospho-p38 MAPK (cat. no. 9211), total MAPK (cat. no. 9212), p-AMPK (cat. no. 2531), total AMPK (cat. no. 2793), and p-PKA-substrate (cat. no. 9621) were purchased from Cell Signaling (Danvers, MA). Anti-α-actin antibodies (cat. no. A2172) were a product of Sigma (St. Louis, MO), and tubulin antibodies (cat. no. ab7291) were purchased from Abcam (Cambridge, MA). An antibody against β2-adrenergic receptor (cat. no. 9042) was a product of Santa Cruz Biotechnology (Santa Cruz, CA). Medium-chain acyl-CoA dehydrogenase (MCAD) (cat. no. M5726) and PDH E1α-subunit (cat. no. MSP03) antibodies were purchased from Mitosciences (Eugene, OR). Antibodies against PDK4 were obtained from Abcam (Cambridge MA). UCP3 primary antibodies were from Abgent (cat. no. PA1–055; Golden CO). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Glucose standards were obtained from NERL Diagnostics (East Providence, RI), and glucose reagents were from Diagnostic Chemicals (PEI, Canada). Rat insulin ELISA kits were purchased from Alpco Diagnostics (Salem, NH). Free fatty acids were determined by a colorimetric assay purchased from Wako Chemicals (Waco, TX). Epinephrine was measured in precentrifuged muscle homogenates (as described above). Phosphatidyl-dimethylethanolamine was added to each sample as an internal standard. Lipids were extracted from the homogenates by the method of Folch et al. (13), and lipid classes were separated and quantified by the HPLC method (38) with minor modifications. Real-time PCR. RNA was isolated from skeletal muscle using a Fibrous RNasey kit according to the manufacturer’s instructions. One microgram of RNA was used for the synthesis of complementary DNA (cDNA) using SuperScript II Reverse Transcriptase, oligo(dT) and dNTP. Real-time PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems). Taqman Gene Expres-
Assays were used to determine the expression of PGC-1α, PRC, PDK4, MCAD, UCP3, and CPT1. Primers and probes for PGC-1β were designed using Primer Express 3.0 software (40). Samples were run in duplicate in a 96-well plate format. Each assay (20 μl total volume) contained 1 μl of gene expression assay, 1 μl of cDNA template, 10 μl of Taqman Fast Universal PCR Master Mix, and 8 μl of RNase free water. For PGC-1β, each 20-μl reaction contained 12.5 μl of PCR Master mix, 0.225 μl each of forward and reverse primers, 0.05 μl of probe, and 3.0 μl of RNase free water. We used β-actin as our endogenous control since the expression of this gene did not change following 6 wk of HFD or fasting. Differences in gene expression between chow-fasted, HFD-fed, and HFD-fasted rats were expressed relative to the fed diet group. Fasting-induced changes in gene expression were also expressed relative to the fed condition within the same diet group. The 2−ΔΔCT method (26) was used for these analyses.

β-Hydroxyacyl-CoA dehydrogenase activity. β-HAD activity was measured by determining the production of NADH spectrophotometrically at 412 nm as previously described by our laboratory (14). Statistical analysis. Data are presented as means ± SE. Comparisons between the group means of diet (chow and HFD) and feeding state (fed and fasted) were made using a two-way ANOVA followed by a least significant difference post hoc test. Differences between fasting-mediated increases in gene expression between Chow and HFD rats were made using as Student’s t-test. Statistical significance was set at P < 0.05.

RESULTS

Physical characteristics and blood-borne factors. Final body weights of rats fed an HFD were significantly greater than Chow-fed animals (Table 1). Levels of plasma glucose and insulin were significantly greater in the HFD rats compared with Chow-fed animals, in both the Fed and Fasted condition. Fasting NEFA was elevated in the Chow-fed animals only, whereas NEFA levels were significantly elevated in rats fed an HFD in the Fed state and were not further increased by fasting. Contents of intramuscular triglycerides were significantly greater in the HFD animals compared with Chow-fed animals. β-HAD activity was increased in triceps muscles in HFD (31.5 ± 5.7 μmol-min⁻¹·g protein⁻¹) compared with Chow rats (18.3 ± 1.8 μmol-min⁻¹·g protein⁻¹) in the Fed state.

Changes in mRNA levels of FAO enzymes. Fasting increased the mRNA expression of CPT-1 in Chow-fed but not HFD rats. There were no differences in CPT-1 mRNA levels in the Fed state between the two groups (Fig. 1A). PDK4 mRNA expression increased with fasting in both diet groups compared with Chow-fed controls. PDK4 mRNA levels were elevated in the Fed state, however not significantly, and as a result the fold increase in PDK4 mRNA with fasting was less in the HFD compared with Chow-fed group (Fig. 1B). The HFD increased the mRNA content of MCAD in the Fed state compared with Chow-fed controls (Fig. 1C). However, compared with the Fed state in the same diet group, induction of MCAD expression by fasting was greater in Chow-fed animals. Fasting increased UCP3 mRNA expression in rats provided an HFD, and a similar trend was seen in Chow-fed controls (Fig. 1D).

Changes in protein content of FAO enzymes. PDK4 protein content was higher in triceps muscles from HFD rats in the Fed condition compared with Chow-fed rats (Fig. 2). Fasting increased UCP3 protein content in Chow but not HFD rats, where UCP3 levels were already elevated in the Fed state. There were no significant differences in MCAD protein content between any of the conditions.

Changes in the mRNA expression of PGC coactivators. PGC-1α expression was similar in all experimental groups (Fig. 3A). However, HFD significantly increased PGC-1β mRNA expression in the Fed state, whereas fasting led to greater increases in PGC-1β mRNA in Chow vs. HFD animals (Fig. 3B). PRC expression was not increased with the HFD, whereas fasting increased PRC mRNA in Chow-fed animals only (Fig. 3C).

Adrenergic signaling. There was a significant elevation of epinephrine in all fasted animals regardless of diet (Fig. 4A). However, no changes in the protein content of the β2-AR were seen in any group (Fig. 4B). Using a PKA substrate antibody, we detected immunoreactive bands that increased with fasting at ~140, ~120, and ~75 kDa (Fig. 4C). Similar to the effects on epinephrine levels, fasting in both diet groups induced a significant increase in proteins phosphorylated by PKA. There was no effect of diet on PKA substrate phosphorylation.

Effects of diet and fasting on p38 and AMPK phosphorylation. AMPK phosphorylation was increased by fasting in HFD animals, and a similar trend was seen in the Chow-fed group (P = 0.07) (Fig. 5A). On the other hand, p38 phosphorylation was significantly increased by fasting in Chow-fed animals only (Fig. 5B). When expressed relative to the Fed condition within the same diet group, the effect of fasting on p38 phosphorylation was approximately twofold greater in the Chow compared with the HFD group (Fig. 5C).

DISCUSSION

Fasting induces the expression of genes involved in FAO (10, 30, 34), which likely serve as an initial event leading to increased enzyme activity and enhanced FAO in skeletal muscle. In obesity there have been reports of impairments in the ability of physiological stressors, such as fasting (47) and exercise (9), to induce the expression of mitochondrial enzymes and to increase whole body FAO (20). Interestingly, the
expression of FAO enzymes in skeletal muscle from obese, insulin-resistant rodents is elevated. Thus it is not readily apparent whether the proposed metabolic inflexibility in obesity is simply a consequence of already elevated levels of FAO enzymes or whether there are impairments in the activation of the pathways controlling mitochondrial biogenesis. Similarly, the signaling pathways and transcriptional coactivators, which may mediate HFD-induced increases in skeletal muscle mitochondrial enzymes have not been fully explored.

In this regard, we preconditioned rat muscle in an environment of HFD and investigated changes in mRNA levels and protein content of enzymes involved in FAO in response to fasting. Consistent with previous findings (11, 17, 41), we found that the consumption of an HFD increased the mRNA levels (MCAD), protein contents (UCP3, PDK4), and activity (HAD) of enzymes involved in FAO in skeletal muscle in the fed state. While fasting increased PDK4, MCAD, and UCP3 mRNA and the protein content of UCP3 in chow-fed animals, there were no further increases in these parameters with fasting in HFD rats, and, subsequently, the absolute levels of these variables between diet groups were similar. Thus, it would appear that an already elevated reliance on fatty acids as a fuel source (17, 41) in HFD rats precludes a further induction of certain FAO enzymes with fasting, a condition normally
associated with increases in FAO. While these findings argue against the notion of true impairments in the fasting response in terms of the induction of FAO enzymes, we did observe a reduced effect of fasting on the induction of CPT-1 in the absence of increases in the fed state in skeletal muscle from HFD rats. While our results build upon previous findings from Young et al. (47), who showed that fasting failed to induce CPT-1 and MCAD expression in heart muscle from obese Zucker rats, it should be noted that measurement of gene expression is limited in terms of the extrapolation to functional changes in the capacity for FAO.

Having demonstrated both diet and fasting effects on FAO enzymes in skeletal muscle, we sought to gain insight into the cellular mediators of these processes. PGC-1α garners enormous attention as a master regulator of metabolism for its fundamental and imperative role in mitochondrial biogenesis and for regulating the expression of genes controlling glucose and lipid oxidation (12, 23, 43, 45). Nonetheless, we observed no increase in PGC-1α expression with either fasting or HFD. This is consistent with several previous findings that showed no change (15, 17) in PGC-1α mRNA expression following the consumption of an HFD. In addition to PGC-1α, the control of gene expression vital to mitochondrial proliferation and respiration are also regulated by the related transcriptional coactivators PGC-1β (19) and PRC (3). Thus changes in the mRNA levels of these molecules could be associated with fasting and HFD-induced increases in FAO enzymes. In this light, we observed that fasting increased the mRNA content of PRC in chow-fed animals, but the fasting effect was absent in rats consuming the HFD. PRC appears to regulate metabolic genes in a similar manner as PGC-1α (3). Since PGC-1α remained unchanged, while PRC was increased by fasting in chow-fed animals, perhaps the metabolic perturbations induced by fasting were below the stimulatory threshold for the induction of

Fig. 2. Effects of fasting and an HFD on the protein content of PDK4 (A), MCAD (B), and UCP3 (C) in rat triceps muscles. Data are presented as means ± SE for 4–10 samples per group. Representative Western blots for PDK4, MCAD, UCP3, and tubulin are shown to the right of the quantified data. *Significant effect of fasting within a diet group; #significant effect of HFD in the fed state. P < 0.05.

R216 LIPID GENE REGULATION IN SKELETAL MUSCLE

AJP-Regul Integr Comp Physiol • VOL 300 • FEBRUARY 2011 • www.ajpregu.org
PGC-1α mRNA expression but sufficient for that of PRC. As PRC was the only PGC-1 family member to be induced by fasting, the attenuation of this effect by HFD may be linked to the blunted induction of CPT-1 mRNA levels with fasting in HFD rats. On the other hand, the induction of PGC-1β in skeletal muscle from HFD rats, in the absence of increases in PGC-1α or PRC implicates this transcriptional coactivator as potentially being involved in the mechanisms through which HFDs upregulate the expression of mitochondrial enzymes.

Alterations in the expression of metabolic genes in skeletal muscle, at least during exercise are thought to be regulated to a large extent by biochemical changes within the contracting muscle itself, such as decreases in high-energy phosphagens and increases in cytosolic Ca^{2+} levels (44). However, there is a growing body of evidence suggesting that humoral factors, such as catecholamines can also modulate the expression of skeletal muscle metabolic enzymes. For instance the β2-adrenergic agonist formoterol rapidly increases the mRNA expression of PDK4 in mouse skeletal muscle (29). In this context, we sought to determine whether changes in β-adrenergic signaling could explain, at least in part, the effects of HFDs and/or fasting, on the induction of mitochondrial enzymes. We found no differences in plasma epinephrine levels, in either the fed or fasted condition, between chow and high-fat fed rats. Similarly, the protein content of the β2-adrenergic receptor and the activation of PKA signaling by fasting were not different between the two groups. Collectively these results demonstrate that mechanisms distinct from upstream β-adrenergic signaling events do not account for HFD-induced increases in FAO enzymes or for the apparent blunted effect of fasting on the induction of PRC and CPT-1 in triceps muscle from HFD rats.

Considerable evidence implicates AMPK as a principle player in the regulation of PGC-1 expression and the control of mitochondrial biogenesis. For instance AMPK agonists, such as AICAR, have been shown to induce PGC-1α mRNA expression (22), and AMPK has been reported to directly phosphorylate and activate PGC-1α (18) leading to increases in mitochondrial enzyme gene expression. Given the similarities between PGC-1α, PGC-1β, and PRC in the control of mitochondrial biogenesis (reviewed by Scarpulla in Ref. 37), it is tempting to speculate that the activation of AMPK may parallel increases in the mRNA expression of PGC-1β and PRC and subsequently increases in FAO enzymes. Similar to what has been reported previously by de Lange et al. (10), fasting led to increased AMPK phosphorylation in chow-fed rats. Despite a blunted effect of fasting on the induction of CPT-1 and PRC
and increases in FAO enzymes in the fed state, AMPK phosphorylation was not different between chow and HFD rats. These findings demonstrate that alterations in AMPK are not involved in the mechanism(s) through which HFD leads to decreases in the fasting-mediated induction of PRC and CPT-1 nor the pathway through which HFDs increase FAO enzymes in skeletal muscle. The latter point is consistent with a recent report from Fillmore et al. (11) who demonstrated an additive effect of HFDs and chronic AMPK activation on increases in skeletal muscle mitochondrial content.

In addition to AMPK, p38 MAPK has been recognized as a key regulator of skeletal muscle mitochondrial biogenesis. For instance, the overexpression of MKK6, an upstream p38 MAPK kinase in mouse skeletal muscle, increases PGC-1 and markers of mitochondrial biogenesis (2). Congruently, the deletion of p38 MAPK/H9253 attenuates training-induced increases in skeletal muscle mitochondrial content.

In the present study, we found that fatty acids were increased with fasting in chow-fed rats, whereas in HFD rats NEFA levels were already elevated in the fed state and were not

Fig. 4. Effects of HFD on fasting-induced changes in plasma epinephrine levels (A), the protein content of the β2-adrenergic receptor (B), and the phosphorylation of PKA substrates (C). Data are presented as means ± SE for 4–10 samples per group. Representative Western blots for β2-adrenergic receptor, tubulin, and phosphorylated PKA substrates are shown (right). *Significant effect of fasting within a diet group. P < 0.05.
further increased with fasting. This pattern is strikingly similar to the effects of diet and fasting on the majority of FAO enzymes that were measured and in the absence of increases in AMPK, p38 or β-adrenergic signaling suggests that plasma fatty acids play a central role in mediating the effects of HFDs on skeletal muscle mitochondrial enzymes. This interpretation is consistent with previous work from Garcia-Roves et al. (15) in which they reported that elevations in plasma fatty acid levels achieved by HFDs in conjunction with heparin injections increased a wide range of mitochondrial enzymes in rat skeletal muscle. On the other hand, if increases in fatty acids were the sole mediator of the effects of fasting on metabolic genes/enzymes, then it would be expected that all genes measured would be higher in skeletal muscle from rats consuming an HFD in the fed condition. Since this was not the case, these findings demonstrate that the absence of fasting-induced elevations in fatty acids does not fully explain the impairment of certain genes (i.e., CPT-1, PRC) in rats fed an HFD and suggest the involvement of alternative mechanisms, i.e., p38 MAPK.

**Perspectives and Significance**

In summary, we have found that the consumption of HFDs increased the expression of FAO enzymes such as UCP3,
PDK4, and MCAD concomitant with elevated plasma fatty acid levels and the induction of PGC-1β, but independent of changes in AMPK, p38, and PKA signaling. Elevations in these transcript levels in the fed state likely preclude further increases with fasting in HFD rats since rates of FAO are reported to already be increased in this condition (17, 41). While the present study sheds important insight into the interactions between diet and fasting in the regulation of gene expression in fast-twitch skeletal muscle, it is yet to be seen whether a similar relationship exists in more oxidative fiber types that have a higher capacity for FAO. Not only do our findings provide novel information regarding the regulation of metabolic regulatory networks in skeletal muscle in obesity and insulin resistance, but they also reveal a fundamental issue regarding the effects of sample acquisition relative to the feeding state on the interpretation of metabolic enzyme mRNA data. In this regard, the analysis of skeletal muscle samples following an overnight fast could yield a dramatically different result than samples procured from an animal in the postprandial condition. These points should be carefully considered in the design and subsequent interpretation of gene expression studies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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