### Title

Elevated Free Fatty Acids Induce Uncoupling Protein 3 Expression in Muscle: A Potential Explanation for the Effect of Fasting




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# Elevated Free Fatty Acids Induce Uncoupling Protein 3 Expression in Muscle

## A Potential Explanation for the Effect of Fasting

**David S. Weigle, Leah E. Selfridge, Michael W. Schwartz, Randy J. Seeley, David E. Cummings, Peter J. Havel, Joseph L. Kuijper, and Hector BeltrandelRio**

**The newly described uncoupling protein 3 (UCP3) may make an important contribution to thermogenesis in humans because of its high level of expression in skeletal muscle. Contrary to expectations, fasting, a condition that reduces resting energy expenditure, has been reported to increase UCP3 expression in muscle. We have confirmed that a 10-fold increase in UCP3 mRNA levels occurs in rat quadriceps muscle between 12 and 24 h of food removal. A less consistent twofold increase in muscle UCP2 mRNA levels was observed in animals fasted for up to 72 h. Administration of recombinant leptin to prevent a fall in circulating leptin levels did not eliminate the fasting-induced increase in quadriceps UCP3 expression. Administration of a high dose of glucocorticoid to fed animals to mimic the increase in corticosterone induced by fasting did not reproduce the increase in UCP3 expression observed in fasted animals. In contrast, elevation of circulating free fatty acid levels in fed animals by Intralipid plus heparin infusion caused significant increases in the UCP3/actin mRNA ratio compared with saline-infused fed controls in both extensor digitorum longus (2.01 ± 0.34 vs. 0.68 ± 0.11, $P = 0.002$) and soleus muscles (0.31 ± 0.07 vs. 0.09 ± 0.02, $P = 0.014$). We conclude that free fatty acids are a potential mediator of the increase in muscle UCP3 expression that occurs during fasting. This seemingly paradoxical induction of UCP3 may be linked to the use of free fatty acid as a fuel rather than an increased need of the organism to dissipate energy.**
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It has long been known that uncoupling protein 1 (UCP1) is responsible for facultative thermogenesis in rodent brown adipose tissue under the control of the sympathetic nervous system (1). Newly described members of this protein family, UCP2 and UCP3, have also been shown to lower mitochondrial membrane potential when transfected into yeast (2–4), supporting the hypothesis that these homologs also uncouple oxidative phosphorylation and contribute to thermogenesis in vivo. UCP3 is of particular interest as a potential mediator of thermogenesis in humans because it is expressed at a high level in human skeletal muscle (5,6), a tissue that contributes significantly to resting energy expenditure (7). Consistent with this hypothesis, hypothyroidism in rats causes a reduction in muscle UCP3 expression, whereas hyperthyroidism increases muscle UCP3 expression relative to euthyroid animals (4). These changes parallel the well-known effects of hypothyroidism and hyperthyroidism on resting energy expenditure and therefore support a physiological role for UCP3. In view of these findings, the recent report that UCP3 (4), and possibly UCP2 (4,8), mRNA expression in rat skeletal muscle increase during fasting was unexpected, since fasting reduces energy expenditure in animals and humans (9,10). In the present study, we have confirmed that upregulation occurs and assessed the relative changes in muscle UCP2 and UCP3 expression with fasting. We have also investigated the mechanism underlying this paradoxical result by examining the impact on muscle UCP3 expression of leptin, glucocorticoids, and free fatty acids, circulating signals which help to mediate the transition from the fed to the fasted state.

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BBS, borate-buffered saline; EDL, extensor digitorum longus; UCP, uncoupling protein; PPAR-γ, peroxisome proliferator-activated receptor γ.

### RESEARCH DESIGN AND METHODS

**Animals.** Male, 8- to 10-week-old Sprague-Dawley rats were caged at thermoneutrality on a 12:12 h light-dark cycle and given unrestricted access to standard rodent diet blocks (caloric density 4.05 kcal/g, Teklad, Madison, WI) and water for at least 1 week prior to study according to one of the following protocols: *1*) to examine the time course of UCP2 and UCP3 mRNA induction during fasting, animals were caged in six groups of three animals each, standard diet was removed at 0800 on day 1 of the experiment, and animals were weighed daily. The three animals in each cage were decapitated at 0, 6, 12, 24, 48, or 72 h from the beginning of the fast, and quadriceps muscle was flash-frozen for subsequent RNA preparation. *2*) To examine the effect of leptin on UCP3 induction during fasting, six rats were given unrestricted access to standard diet and received daily 1 ml intraperitoneal injections of sterile 10 mmol/l borate-buffered saline (BBS) at 0800 and 1800. A second group of six rats was fasted beginning at 0800 on day 1 of the experiment and received identical twice-daily injections of BBS. A third group of six rats was fasted beginning at 0800 on day 1 and received twice-daily injections of recombinant human leptin (11) at a dose of 1 mg/kg in 1 ml of BBS. Beginning 2 h after their 0800 injections on day 3, rats were decapitated in random order for collection of trunk blood and quadriceps muscle. *3*) To examine the effect of glucocorticoids on UCP3 expression in fed animals, two control groups of six rats each were given twice-daily subcutaneous injections of saline at 0800 and 1800. One of these groups had unrestricted access to standard diet, and the other was fasted beginning at 0800 on day 1 of the experiment. A third group of six rats was fed ad libitum but received twice-daily subcutaneous injections of hydrocortisone sodium succinate (Upjohn, Kalamazoo, MI) at a dose of 50 mg/kg body wt. All rats were decapitated beginning at 1200 on day 2 for col-

lection of quadriceps muscle. 4) To test the hypothesis that free fatty acids induce UCP3 expression, jugular venous catheters were placed in three groups of six rats each, tunneled subcutaneously to the interscapular region, and led through a spring tether to a fluid swivel at the top of each cage. Catheters were kept patent by infusion of sterile saline, and animals were allowed to recover from surgery for 3 days, by which time their standard diet intakes had returned to baseline. Saline infusions were continued in an ad libitum–fed control group and a group that was fasted beginning at 0800 on day 1 of the experiment. The third group of animals was fed ad libitum but received an infusion of 20% Intralipid (Pharmacia, Clayton, NC) plus 50 U/ml heparin at a rate of 0.75 ml/h beginning at 0800 on day 1 of the experiment. All rats were decapitated beginning at 1200 on day 2 for collection of trunk blood and soleus and extensor digitorum longus (EDL) muscle. Plasma concentrations of free fatty acids and leptin were measured using kits supplied by Wako Diagnostics (Richmond, VA) and Linco Research (St. Charles, MO), respectively. All procedures were approved by the University of Washington Animal Care Committee.

**Northern analysis.** RNA was prepared by homogenizing tissues in a 10-fold volume excess of 4 mol/l guanidinium isothiocyanate, sedimenting the RNA through 5.7 mol/l cesium chloride, phenol chloroform extraction of RNA pellets, and ethanol precipitation. RNA was quantified spectrophotometrically at 260 nm, and the quality of preparations was verified by the detection of clear 28S and 18S ribosomal RNA bands at a 2:1 intensity ratio on an ethidium-stained 0.8% agarose gel. RNA was loaded at 15 μg per lane on a 0.8% agarose gel containing 37% formaldehyde, and electrophoresis was performed at 75 volts for 3 h. RNA was transferred to nylon membranes (Hybond-N, Amersham, Arlington Heights, IL) overnight, crosslinked to membranes by ultraviolet irradiation, and prehybridized for 1 h in ExpressHyb (Clontech) at 68°C. Blots were probed at 68°C with polymerase chain reaction–generated cDNA fragments of UCP or actin that were [$^{32}$P]-labeled by random priming (Megaprime kit, Amersham), and washed at 55–68°C in 0.25× standard sodium citrate with 0.25% sodium dodecyl sulfate. The UCP2 probe was produced from positions 443 to 1022 of the published mouse sequence (accession U69135), and the UCP3 probe was produced from positions 485 to 948 of the published human sequence (accession U84763). Hybridized blots were placed in a PhosphorImager cassette (Molecular Dynamics, Sunnyvale, CA) for 1–4 days, imaged, and quantified using Molecular Dynamics ImageQuant software. Differences among groups were assessed by analysis of variance with post hoc comparisons made using the Fisher PLSD test on StatView 4.5 software. Results were considered statistically significant at the $P < 0.05$ level.

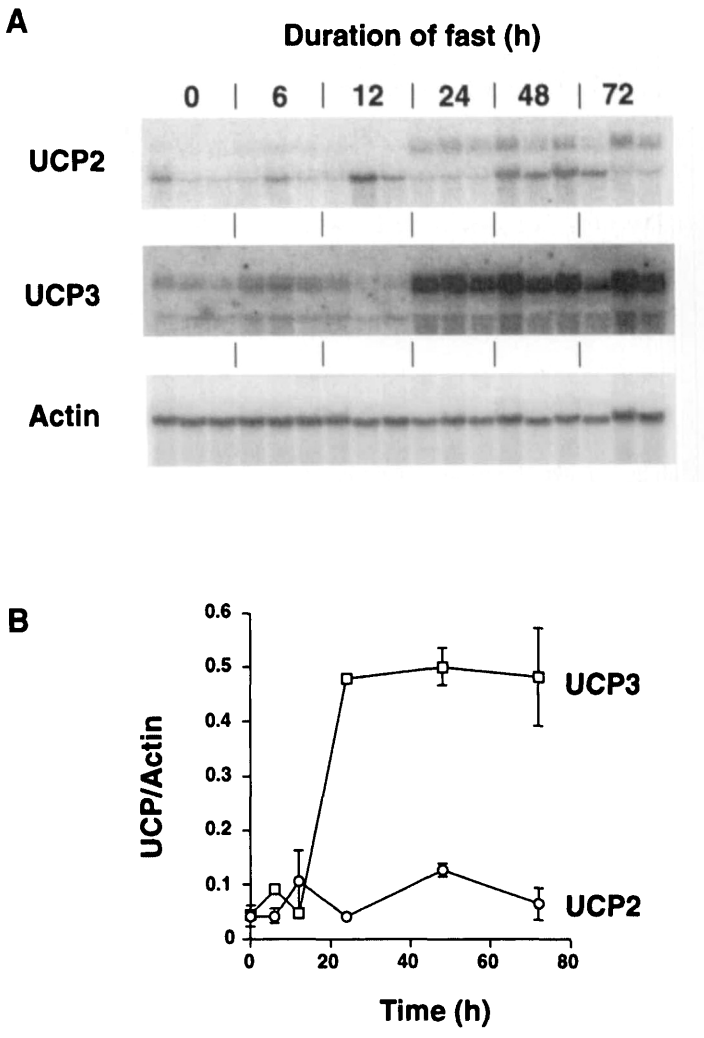


**FIG. 1. Induction of UCP2 and UCP3 expression during fasting. *A*: A Northern blot of quadriceps muscle RNA from 18 rats was probed with UCP2, UCP3, and actin cDNA fragments as described in the text. *B*: Ratios of UCP/actin mRNA band intensity determined with a phosphorimager were plotted against duration of fasting (mean ± SE of three rats per data point).**

## RESULTS

**Experiment 1.** The effect of a period of fasting of variable duration on UCP2 and UCP3 mRNA levels in quadriceps, a skeletal muscle composed of both fast-twitch and slow-twitch fibers, is shown in Fig. 1. A 10-fold induction of UCP3 expression was detected between 12 and 24 h of food removal. Although we also observed a significant increase in UCP2 mRNA levels after 48 h of fasting (Fig. 1 and additional data not shown), this effect tended to be inconsistent, with the maximal increase being twofold above baseline.

**Experiment 2.** To test the hypothesis that the induction of UCP3 expression in muscle by fasting resulted from a fall in leptin levels, we performed Northern analysis on quadriceps muscle from animals that were fed, fasted for 48 h, or fasted for 48 h with leptin replacement. In vehicle-treated animals, plasma leptin levels fell with fasting from 3.6 ± 0.6 to <0.5 ng/ml ($P = 0.0002$), whereas the mean level of human leptin immunoreactivity, which was below the detectability of the assay in the two vehicle-treated groups, was 48.1 ± 14.6 ng/ml 2 h after the last dose of human leptin in the fasted, leptin-treated animals ($P = 0.0013$). As shown in Fig. 2, administration of leptin, even at a dose that produced supraphysiological circulating levels, did not alter the highly significant increase in muscle UCP3 mRNA content induced by fasting.

**Experiment 3.** To determine whether the induction of UCP3 expression in muscle by fasting could be due to increased glucocorticoid levels, we performed Northern analysis on quadriceps muscle from animals that were fed, fed with cortisol administration, or fasted for 28 h. As shown in Fig. 3, administration of a pharmacological dose of hydrocortisone from the beginning of the 28-h interval produced no increase in UCP3 mRNA levels, despite the significant increase in UCP3 expression induced by a fast of the same duration.

**Experiment 4.** To determine whether muscle UCP3 induction varies according to fiber type, we compared the induction of UCP3 expression by 28 h of fasting in a muscle composed purely of fast-twitch glycolytic fibers (EDL) with the induction observed in a muscle composed exclusively of slow-twitch oxidative fibers (soleus). In EDL the UCP3/actin mRNA ratio in fed vs. fasted animals was 0.68 ± 0.11 vs. 2.44 ± 0.26 ($P = 0.0002$), and in soleus this ratio was 0.09 ± 0.02 vs. 0.28 ± 0.06 ($P = 0.036$). As shown in Fig. 4, elevation of circulating free fatty acid levels by Intralipid plus heparin infusion in fed rats resulted in a UCP3/actin ratio that was significantly greater in both EDL (2.01 ± 0.34, $P = 0.002$) and soleus muscles (0.31 ± 0.07, $P = 0.014$) than that observed in fed saline-infused control animals. This increase in UCP3 expression did not differ significantly from the increase induced by fasting in either mus-

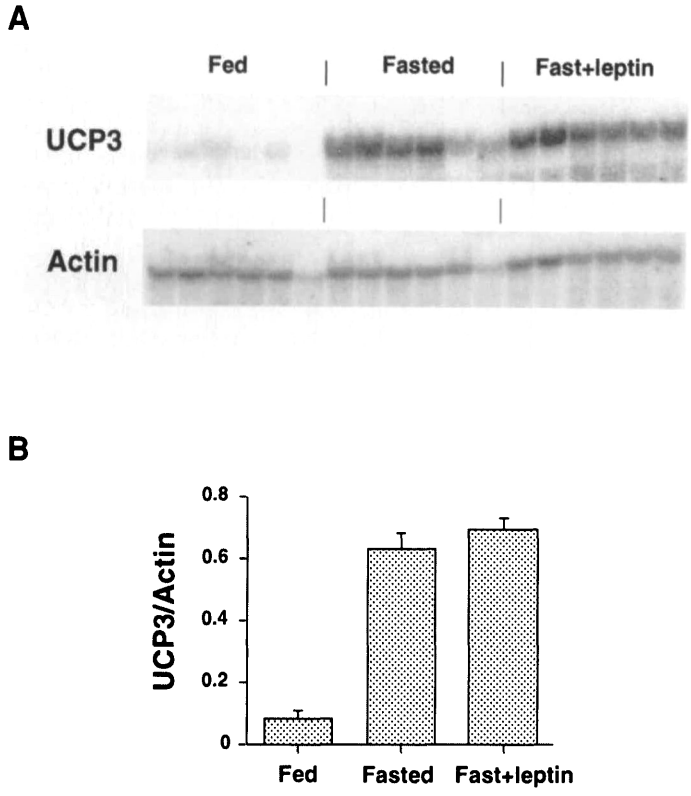


FIG. 2. Effect of leptin on fasting-induced UCP3 expression. *A*: A Northern blot of quadriceps muscle RNA from 18 rats was probed with UCP3 and actin cDNA fragments as described in the text. *B*: Ratios of UCP3/actin mRNA band intensity were determined with a phosphorimager. Bars represent the mean ± SE of six rats ($P < 0.0001$ for fasted and fast + leptin vs. fed, NS for fasted vs. fast + leptin).

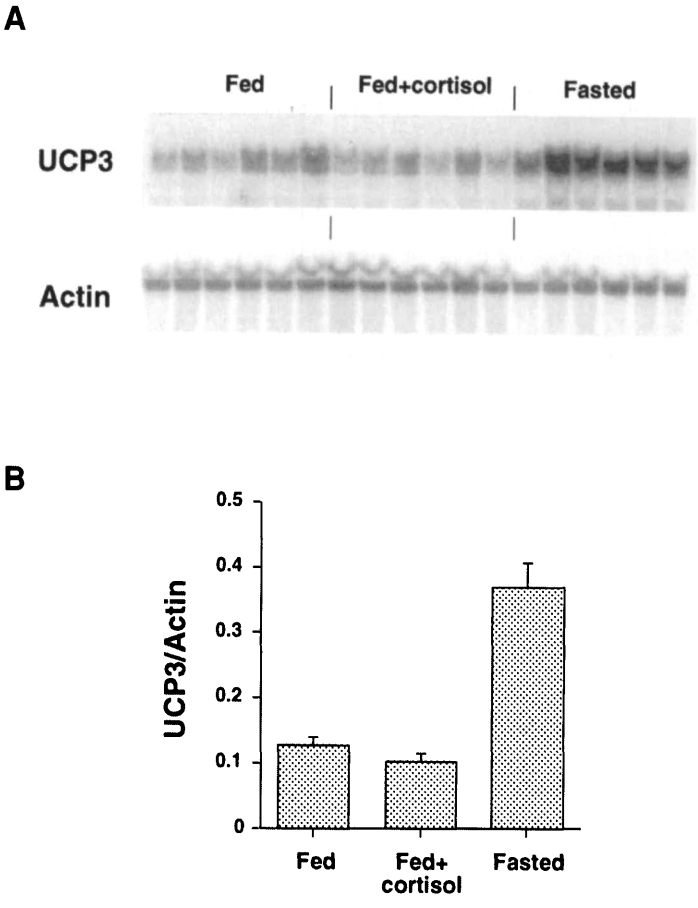


FIG. 3. Effect of hydrocortisone on UCP3 expression in fed animals. *A*: A Northern blot of quadriceps muscle RNA from 18 rats was probed with UCP3 and actin cDNA fragments as described in the text. *B*: Ratios of UCP3/actin mRNA band intensity were determined with a phosphorimager. Bars represent the mean ± SE of six rats ($P < 0.0001$ for fed and fed + cortisol vs. fasted, NS for fed vs. fed + cortisol).

cle type. Total energy intake (standard diet + Intralipid) in experimental animals during the 28-h infusion was 99.9 ± 7.7 kcal as compared with 90.7 ± 13.4 kcal in the fed saline-infused control animals. The mean free fatty acid level in fed animals was 0.26 ± 0.02 mmol/l as compared with 0.54 ± 0.05 mmol/l in fasted animals and 2.04 ± 0.23 mmol/l in animals that received infusions of Intralipid plus heparin.

## DISCUSSION

Our data confirm that 24 h of fasting causes a large and reproducible increase in UCP3 mRNA levels in skeletal muscle, whereas a 12 h fast has no such effect. The absence of a further increase in UCP3 expression between 24 and 72 h following food withdrawal suggests that the signal(s) mediating this increase in gene expression reach a maximal level early during the course of a fast. Although UCP2 mRNA levels also tended to increase by 48 h of fasting, this effect was much less striking and consistent than the induction of UCP3. The variability of the UCP2/actin mRNA ratio that we observed between 12 and 72 h of fasting is consistent with conflicting reports of the ability of fasting to induce UCP2 expression in muscle (4,8). Substantial differences therefore appear to exist in the metabolic factors regulating the expression of UCP2 and UCP3 in muscle.

The transition from the fed to the fasted state is marked by changes in a variety of circulating hormones and metabolic substrates. Based on recent evidence that the fall in leptin levels observed as early as 24 h after food withdrawal may coordinate a variety of neuroendocrine adaptations to fasting (12), we hypothesized that leptin deficiency might also explain the fasting-induced increase in UCP3 gene expression. Our data clearly demonstrate that administration of leptin at a dose that has been shown to affect both food intake and reproductive function (13,14) did not prevent the induction of UCP3 expression by fasting. This result is consistent with recent reports that leptin may actually increase UCP2 (15) and UCP3 (4) expression in adipose tissue and muscle of fed animals. Leptin seems unlikely to be a major regulator of UCP3 gene expression, however, since fasting leads to a significant increase in muscle UCP3 mRNA levels, despite a marked decrease in plasma leptin levels.

Circulating glucocorticoid levels increase in rats with as little as 3 h of fasting (16) and are known to be potent regulators of fuel metabolism and gene transcription. Although corticosterone is the major glucocorticoid found in the plasma of rats, hydrocortisone, triamcinolone, and dexamethasone are all biologically active in this species (17). We found that administration of hydrocortisone at a dose that has been reported to have a potent effect on gene transcription (17) did not result in an increase in muscle UCP3 mRNA levels in fed animals. Glucocorticoids therefore are unlikely candidates to mediate the fasting-induced increase in UCP3 gene expression. This result conflicts with a recent report that

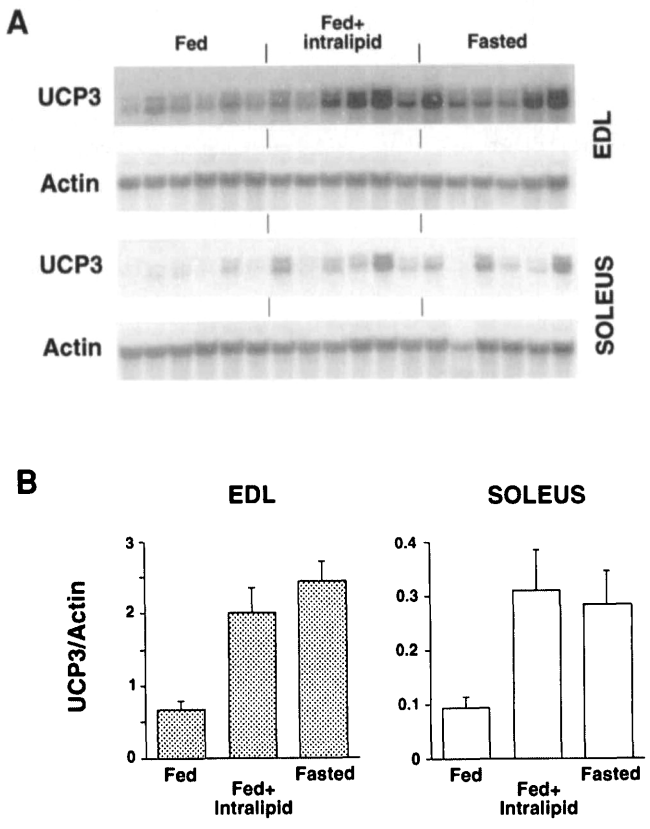


**FIG. 4. Effect of Intralipid + heparin infusion on UCP3 expression in fed animals. *A*: Northern blots of EDL and soleus muscle from 18 rats were each probed with UCP3 and actin cDNA fragments as described in the text. *B*: Ratios of UCP3/actin mRNA band intensity were determined with a phosphorimager. Bars represent the mean ± SE of six rats (EDL: $P = 0.0021$ for fed vs. fed + Intralipid, $P = 0.0002$ for fed vs. fasted, NS for fed + Intralipid vs. fasted. Soleus: $P = 0.0143$ for fed vs. fed + Intralipid, $P = 0.0355$ for fed vs. fasted, NS for fed + Intralipid vs. fasted).**

dexamethasone produced an increase in muscle UCP3 mRNA levels in mice 18 h after administration (4). However, the latter report was based on injections given to only three mice, and food intake data were not reported to confirm that the increase attributed to glucocorticoid administration was not actually due to stress-induced anorexia.

In the absence of ingested nutrients, the mobilization of stored fat provides a major source of fuel to meet ongoing energy requirements, especially in skeletal muscle. The onset of lipolysis leads to a significant elevation in circulating free fatty acid levels within 24 h of fasting, the interval over which maximal induction of UCP3 gene expression occurs. The similarity in the time course of these two events led us to hypothesize that elevated free fatty acids represented a signal, important for the induction of UCP3 expression in muscle. Our data provide direct support for this hypothesis, since we found that the increase in UCP3 mRNA levels produced by Intralipid infusion in fed animals was comparable to that observed during fasting. It is noteworthy that elevation of circulating free fatty acids produced similar increases in UCP3 gene expression in both EDL and soleus, muscles composed of divergent fiber types that ordinarily derive energy from different fuel molecules. This observation suggests that the effect of free fatty acids on UCP3 expression occurs in skeletal muscle throughout the body and could potentially have a large impact on whole-body fuel utilization. It has recently been reported that the 5′ region of the UCP1 gene contains a peroxisome proliferator-activated receptor γ (PPAR-γ) response element and that PPAR-γ is involved in the differentiation-dependent expression of UCP1 (18). Skeletal muscle is now known to express PPAR-γ (19). The possible existence of a PPAR-γ response element in the UCP3 gene could provide a mechanism by which free fatty acids, which are ligands for PPAR-γ, enhance UCP3 expression in muscle.

Plasma free fatty acid levels produced by Intralipid plus heparin infusion in this study were higher than those observed during fasting. The possibility must therefore be considered that an elevation of free fatty acids that precisely matched that which occurred during fasting would have resulted in a lesser induction of muscle UCP3. However, we anticipate that smaller elevations of free fatty acids would still be effective, since the widely used Intralipid plus heparin protocol appears to be without nonspecific effects (20). Furthermore, it is likely that the true circulating free fatty acid levels in our experimental animals were lower than the measured levels because of lipolysis that occurs in plasma samples from heparin-treated animals in vitro (21). Future studies using carefully defined infusions of individual free fatty acids will be required to establish the precise dose-response relationship for UCP3 induction.

Our data may help to resolve the paradox that fasting induces a potentially thermogenic protein in a tissue that comprises a large fraction of body mass at a time when overall energy expenditure by the organism is known to be decreased (9,10). We speculate that this induction of UCP3 may be linked to the utilization of free fatty acids as a fuel rather than an increased need of the organism to dissipate energy. This proposal is consistent with the ability of uncoupling proteins in the mitochondrial inner membrane to act as retrograde anion transporters that could assist in the removal of various charged species from the matrix of the mitochondria (22). This function might be critical to preserve mitochondrial ionic or osmotic equilibrium in the face of the great increase in fatty acid oxidation induced by fasting.

In summary, the striking induction of muscle UCP3 expression induced by fasting casts doubt on the role of this protein as a mediator of increased thermogenesis. In addition to measuring the impact of UCP3 on energy expenditure, future studies should address the possibility that UCP3 plays a direct role in the metabolism of free fatty acids by muscle during fasting.

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