Essential Role for Uncoupling Protein-3 in Mitochondrial Adaptation to Fasting but Not in Fatty Acid Oxidation or Fatty Acid Anion Export* [S]

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Uncoupling protein-3 (UCP3) is a mitochondrial inner membrane protein expressed most abundantly in skeletal muscle and to a lesser extent in heart and brown adipose tissue. Evidence supports a role for UCP3 in fatty acid oxidation (FAO); however, the underlying mechanism has not been explored. In 2001 we proposed a role for UCP3 in fatty acid export, leading to higher FAO rates (Himms-Hagen, J., and Harper, M. E. (2001) Exp. Biol. Med. (Maywood) 226, 78–84). Specifically, this widely held hypothesis states that during elevated FAO rates, UCP3 exports fatty acid anions, thereby maintaining mitochondrial co-enzyme A availability; reactivation of exported fatty acid anions would ultimately enable increased FAO. Here we tested mechanistic aspects of this hypothesis as well as its functional implications, namely increased FAO rates. Using complementary mechanistic approaches in mitochondria from wild-type and Ucp3−/− mice, we find that UCP3 is not required for FAO regardless of substrate type or supply rate covering a 20-fold range. Fatty acid anion export and reoxidation during elevated FAO, although present in skeletal muscle mitochondria, are independent of UCP3 abundance. Interestingly, UCP3 was found to be necessary for the fasting-induced enhancement of FAO rate and capacity, possibly via mitigated mitochondrial oxidative stress. Thus, although our observations indicate that UCP3 can impact FAO rates, the mechanistic basis is not via an integral function for UCP3 in the FAO machinery. Overall our data indicate a function for UCP3 in mitochondrial adaptation to perturbed cellular energy balance and integrate previous observations that have linked UCP3 to reduced oxidative stress and FAO.

Uncoupling protein-3 (UCP3)3 is a member of the family of mitochondrial inner membrane anion carrier proteins which includes uncoupling protein-1 (UCP1), expressed exclusively in brown adipose tissue where it mediates adaptive thermogenesis via an inducible proton leak (1). UCP3 shares 57% amino acid homology with UCP1, has the same predicted tertiary structure, and like UCP1, possesses a nucleotide binding domain (2, 3). UCP3 protein is most abundant in skeletal muscle and is present to a lesser extent in brown adipose tissue and heart (4). In contrast to UCP1, the physiological role and underlying mechanism of action of UCP3 are as yet unresolved. A proposed function for UCP3 is in lipid metabolism (5, 6) and supportive evidence has accrued. In Gullah African-Americans an exon 6 splice junction polymorphism resulting in a truncated form of UCP3 was associated with lower fatty acid oxidation (FAO) as assessed by indirect calorimetry (7); similar results were found in Ucp3−/− mice (8). Early overexpression studies in mice and human muscle cells reported increased FAO (9, 10); however, these results may be confounded by nonspecifically increased basal proton leak due to supraphysiological UCP3 overexpression (11, 12). Physiological overexpression is associated with greater FAO in L6 myotubes (13) and elevated maximal activity of carnitine palmitoyl transferase-1, β-hydroxyacyl dehydrogenase, and citrate synthase and lower lipid storage in mouse skeletal muscle (14, 15). Although these studies functionally link UCP3 and increased FAO, the underlying mechanism remains unexplored, including whether the association between UCP3 and FAO may be related to reduced oxidative stress (13, 16–19).

Two hypotheses of UCP3-mediated fatty acid handling propose that UCP3 transports fatty acid anions from the mitochondrial matrix (20, 21). Schrauwen et al. (21) suggest the physiological outcome to be reduced matrix lipotoxicity. We, on the other hand, proposed a UCP3 export function that leads to increased FAO (20). Specifically, when fatty acyl supply is high, UCP3 would act in concert with mitochondrial thioesterase-1 (MTE-1), which cleaves long chain acyl-CoA into fatty acid anions and CoA (22, 23). Fatty acid anions, which cannot be reactivated in the matrix, would be exported by UCP3 to the cytosol to be reactivated then oxidized or esterified (Fig. 1).

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2 The online version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

3 The abbreviations used are: UCP3, uncoupling protein-3; FAO, fatty acid oxidation; WT, wild type; BSA, bovine serum albumin; ASP, acid-soluble product; MnSOD, Mn-superoxide dismutase; MTE-1, mitochondrial thioesterase-1; PDK, pyruvate dehydrogenase kinase; 4-HNE, 4-hydroxy-2-nonenal; PCarn, palmitoylcarnitine; PPAR, peroxisome proliferator-activated receptor.
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This mechanism would provide an overflow pathway when fatty acid supply exceeds oxidation capacity, liberating CoA to replenish the matrix CoA pool; a predicted functional consequence is that FAO rate is impaired in the absence or limited presence of UCP3 or enhanced with increased expression. Although supportive evidence has emerged (7–10, 13, 14), this hypothesis has not been explicitly tested. Here we tested the metabolic and mechanistic predictions that 1) UCP3 is associated with greater maximal but not submaximal FAO and 2) UCP3 exports fatty acid anions when fatty acid flux is high. Using a unique experimental approach that links mechanism with integrated mitochondrial metabolism, we find that mitochondria from wild-type (WT) and Ucp3−/− mouse equally export palmitate even when UCP3 protein expression is increased in WT mitochondria; thus, UCP3 does not play a unique role in the export process. UCP3 is also not required for FAO in mitochondria from fed mice regardless of substrate type and oxidation rate covering a ~20-fold span; thus, UCP3 is not required for the minimal mitochondrial machinery that oxidizes fatty acids. However, important limitations in FAO are observed in Ucp3−/− mouse mitochondria after increased in vivo fatty acid supply, pointing to a role for UCP3 in mitochondrial adaptation of FAO to fasting, possibly via mitigated oxidative stress.

EXPERIMENTAL PROCEDURES

Treatment of Animals—Male and female WT and Ucp3−/− mice (n = 30/genotype) were housed individually from weanling at 23 °C, lights on 0700–1900, and studied at 10–12 weeks of age. Ucp3−/− mice, backcrossed 10 generations into the C57Bl6/J background, have been described (14, 15). Mice had free access to chow (4.5% fat/weight; Charles River 5075). Subgroups (n = 16/genotype) were fasted for 18 h overnight with free access to water. A narrow age range and equal numbers of males and females were used. Experiments were paired by genotype (WT versus Ucp3−/−) or feeding status (fed versus fast). Animals were sacrificed at 08:00–10:00 for mitochondrial isolations. Animals were cared for according to the principles and guidelines of the Canadian Council on Animal Care and the Institute of Laboratory Animal Resources (National Research Council). The study was approved by the Animal Care Committee of the University of Ottawa.

Isolation of Mitochondria from Skeletal Muscle—Isolation of skeletal muscle mitochondria was performed essentially according to Chappell and Perry (24). All media were ice-cold, and the procedure was done on ice or at 4 °C. Briefly, pectoral, forelimb, and hindlimb muscles were rapidly dissected and placed in basic medium (BM: 140 mM KCl, 20 mM HEPES, 5 mM MgCl2, 1 mM EGTA, pH 7.0). Muscle was cleaned of connective tissue and fat, minced, and placed in 15 volumes of homogenizing medium (HM: BM with 1 mM ATP and 1% BSA (w/v)) containing one unit of protease (subtilisin A, Sigma P5459) per g of muscle wet weight. Tissue was homogenized using a glass/Teflon Potter-Elvehjem tissue grinder and fractionated by centrifugation at 800 × g (10 min), and the supernatant was collected and spun at 12,000 × g (10 min). The pellet was resuspended in BM and incubated on ice for 3 min (myofibrillar repolymerization). Samples were spun at 800 × g (10 min), and the supernatant was collected, then spun at 12,000 × g (10 min). The final pellet was resuspended in 220 μl of BM. Respiratory control ratio (state 3/state 4) of mitochondria fed pyruvate/malate was 7–8. Protein concentration was determined by a modified Lowry method with BSA as standard.

Mitochondrial Fatty Acid Oxidation—FAO was assessed as described (25, 26) with minor modifications. Labeled CO2 and carbon fixation in acid-soluble products (ASP) generated by fatty acid (palmitate, palmitoylcarnitine) oxidation were measured after a 30-min incubation (37 °C) of viable mitochondria in a sealed system. A 1500-μl aliquot of incubation medium (IM: 120 mM KCl, 1 mM EGTA, 5 mM KH2PO4, 5 mM MgCl2, and 5 mM HEPES, pH 7.4) supplemented with 1 mM ATP, 0.05 mM malate, 0.025 mM coenzyme A, and 0.5 mM carnitine was added to a 20-ml glass vial. Free CoA and carnitine were omitted for palmitoylcarnitine oxidation unless indicated. Substrates were added to vials in a 6:1 fatty acid-BSA complex and spiked with [1-14C]palmitoylcarnitine (10 nCi; PerkinElmer Life Sciences) or [1-14C]palmitate (10 nCi; Amersham Biosciences). Final concentrations of fatty acid substrate ranged from 1.2 to 38 μM. The 20-ml vial contained a microcentrifuge tube with 300 μl of 1 M benzethonium hydroxide to capture 14CO2. The reaction was initiated by adding mitochondria (0.5 mg/ml), then terminated after 30 min by adding ice-cold 12 n perchloric acid by syringe through the rubber cap. A fraction of the reaction medium was analyzed for [14C]ASP, whereas the remaining mixture was acidified, and gaseous 14CO2 was trapped and counted.

FIGURE 1. Role for UCP3 in FAO and fatty acid anion export. Different arms of the metabolic pathway are depicted by different shading; activation of palmitate (white), uptake of substrate (light gray), oxidation (medium gray), and putative hydrolysis/export of fatty acids by UCP3 (dark gray). Oxidation experiments using palmitate as substrate were supplemented with CoA and carnitine, enabling palmitate to be activated on the outer face of the outer mitochondrial membrane followed by uptake and oxidation of activated substrate. Putative export, re-activation, uptake, and oxidation are also possible. Oxidation experiments using palmitoylcarnitine as substrate were run in the presence and absence of CoA and carnitine; co-factor addition allowed re-activation and subsequent metabolism of putatively exported palmitate. Export experiments used palmitoylcarnitine in the absence of CoA and carnitine, preventing re-activation of exported palmitate; thus, generated and exported palmitate could be quantified. ACS, acyl-CoA synthase; CPT1, carnitine palmitoyltransferase 1; CAT, carnitine acylcarnitine transferase; CPT2, carnitine palmitoyltransferase 2; TCA, tricarboxylic acid cycle.
Western Blotting—UCP3 (1:1000; AbCam), MTE-1 (1:1500; a gift from Dr. Stefan Alexson), PDK4 (1:500, a gift from Dr. Robert Harris), MnSOD (1:2000; Santa Cruz sc-30080), and UCP2 (1:500; Santa-Cruz sc-6526) protein was determined in isolated mitochondria (UCP3 and MTE-1, 35 μg; PDK4, 8 μg; MnSOD, 15 μg; UCP2, 30–60 μg). Band intensity was measured by densitometry (Scion Image software) and expressed relative to the intensity of a band of similar Mr on the control (Coomassie-stained gel or Ponceau-stained membrane).

Palmitate Generation and Export Measurements—Generation and export of [1-14C]palmitate were determined essentially according to Gerber (27). Freshly isolated mitochondria (1 mg) were incubated with 19 μM palmitoylcarnitine as above and spiked with 200 nCi of [1-14C]palmitoylcarnitine. The reaction was terminated by removing half the suspension and mixing it with an equal volume of IM and 3 volumes of chloroform:methanol (2:1, v:v). The remaining suspension was filtered across a 0.45-μm nitrocellulose membrane (Millipore) presoaked for 40 min in IM containing 0.66 mg/ml BSA and 20 μM unlabeled palmitate. The filter was washed with 3 volumes of IM supplemented with 2 mg/ml BSA; nitrocellulose membrane incubation length with BSA and the chosen BSA concentrations and volumes of washing IM buffer stated above yielded the highest recovery rate (83 ± 4.3 (S.D.) %, n = 5) of [1-14C]palmitate added to a 1.0-mg suspension of nonfunctional mitochondria. Therefore, a ~17% loss of [1-14C]palmitate is accounted for by non-metabolic processes such as fatty acid binding to mitochondrial membranes. Three volumes of chloroform:methanol were then added to the filtrate. Sample blanks were run and processed as above to account for any contaminating [1-14C]-palmitate added to the filtrate. Lipids were extracted according to Folch (28). The organic fraction was collected and dried under nitrogen, resuspended in ethanol, and applied onto a 250-mm silica gel plate (Analtech). After migration in a hexane:diethyl ether:formic acid (50:50:1, v:v) solvent system, palmitate bands were scraped off, and radioactivity was counted.

Enzyme Activities—Activities of citrate synthase (EC 4.1.3.7) (29) and β-hydroxyacyl dehydrogenase (EC 1.1.1.35) (30) were measured at 25 °C in previously frozen mitochondria after 3 freeze-thaw cycles and exposure to 0.04% Triton X-100. Malate dehydrogenase (EC 1.1.1.37) activity (31) was determined at 25 °C in freshly isolated mitochondria before and after oxidation with 19 μM palmitoylcarnitine (PCarn). Mitochondria were assayed before and after the addition of 0.04% Triton X-100.

Oxidative Stress—Oxidative stress was assessed in mitochondrial extracts from fed and fasted mice by immunoblot detection of 4-hydroxynonenal (4-HNE)-modified proteins (the stable fluorophore resulting from lysine-HNE cross-links (32–34)) by polyclonal rabbit primary antibody (1:1000; Calbiochem). Reactive oxygen species production during PCarn oxidation was determined in freshly isolated mitochondria by measuring H2O2 generation using the fluorophore resulting from lysine-HNE cross-links (32–34)) and export fatty acid anions from the matrix. MTE-1, one of three such thioesterases identified (23), is the mitochondrial isoform specific for C14–C20 fatty acyl-CoA and the mitochondrial isoenzyme specific for C14–C20 fatty acyl-CoA moieties (37, 38). MTE-1 protein levels were similar in WT and UCP3 null mitochondria (Fig. 2A).

RESULTS AND DISCUSSION

Absence of UCP3 in Skeletal Muscle Does Not Impair Palmitate Oxidation Even at Elevated Fatty Acid Supply Rates—Wild-type and Ucp3−/− mice were congenic, with Ucp3−/− mice backcrossed 10 generations onto the WT C57Bl6/J background (14, 15) (Fig. 2A). According to the Himms-Hagen and Harper proposal (20), MTE-1 would work in concert with UCP3 to cleave fatty acyl-CoA into fatty acid anion and CoASH and export fatty acid anions from the matrix. MTE-1, one of three such thioesterases identified (23), is the mitochondrial isoform specific for C14–C20 fatty acyl-CoA moieties (37, 38). MTE-1 protein levels were similar in WT and UCP3 null mitochondria (Fig. 2B).

To directly test the functional implications of the hypothesis that UCP3 is involved in mitochondrial fatty acid efflux in conditions of elevated fatty acid supply, we first compared FAO rates in WT and Ucp3−/− mitochondria from fed mice-oxidiz-
ing substrate at low, intermediate, and high rates; experiments in isolated mitochondria allow investigation of a mitochondria-
autonomous mechanism of UCP3-mediated FAO. With palmitate as substrate, the entire FAO pathway is operational, including
putative export, reactivation, re-uptake, and oxidation arms (Fig. 1). Complete and incomplete oxidation rates were assessed,
respectively, as CO₂ release and ASP accumulation. FAO rates in mitochondria, optimized for coenzyme A, carnitine,
ATP, and malate concentrations, were similar to those reported (39, 40). In WT mitochondria, CO₂ production rates
increased linearly with palmitate concentration up to 9 µM, beyond which rates leveled off, indicating saturation of com-
plete FAO (Fig. 2C); ASP progressively increased, again consistent with saturation of complete FAO (Fig. 2D). CO₂ and ASP
production rates at low, medium, and elevated palmitate concentra-
tions were similar for WT and Ucp3⁻/⁻ mitochondria; thus, the absence of UCP3 does not limit palmitate oxidation over a wide range of oxidation rates in mitochondria from fed mice.

Palmitoylcarnitine Oxidation and Its Modulation by Free Carnitine in Skeletal Muscle Mitochondria—To further exam-
ine the role of UCP3 in FAO, we assessed PCarn oxidation at low and high concentrations. Palmitoylcarnitine uptake
bypasses carnitine palmitoyl transferase-1, and in the absence of added CoA, reactivation and metabolism of any exported
fatty acids cannot occur. At low PCarn concentrations (2 mM), CO₂ and ASP production rates were similar in WT and
Ucp3⁻/⁻ mitochondria (Fig. 3, A and B). A PCarn dose-
response analysis was performed to identify the concentration that yielded maximal CO₂ production with minimal variability
(not shown). Saturating PCarn (19 µM) increased CO₂ and ASP production rates by ~10-fold (p < 0.001) as compared with 2
µM PCarn, an effect observed equally in WT and Ucp3⁻/⁻
mitochondria.

In mitochondrial preparations from skeletal muscle, heart
and liver, extra-mitochondrial free carnitine facilitates export
of acetylcarnitine (41–43). The concept of carnitine-mediated
export has been extended to acylcarnitine (44, 45), although to
our knowledge there are no published data that demonstrate
long-chain acylcarnitine efflux from the mitochondrial matrix
and especially from intact cells or organs. On the other hand, it
is worth noting that C16 acylcarnitine can be detected in rat
plasma and skeletal muscle, and levels in both compartments increase in response to high fat feeding (46). Moreover, carnitine supplementation in an obese diabetic mouse model (BAP-
Agouti mice) increased both muscle and plasma levels of long
chain acylcarnitines (47). In neither of these two studies was
compartmentalization of long chain acylcarnitine species
within the muscle determined. The functional outcome of car-
nitine-driven export of acetylcarnitine and short-chain acylcar-
nitines (and the putative export of long chain acylcarnitines) is
similar to that proposed for UCP3-mediated fatty acid export;
that is, preservation of a sufficient pool of CoA in the matrix
under conditions of elevated fatty acid supply, thereby preventing limitation in FAO. We, therefore, examined the effect of carnitine on PCarn oxidation in Ucp3⁻/⁻ mitochondria. The addition of 0.5 mM carnitine expanded the ASP pool in WT and
Ucp3⁻/⁻ mitochondria to a similar extent (p < 0.01) (Fig. 3D)
and tended to decrease CO₂ production rates (Fig. 3C). These findings are in line with a role for carnitine in mitochondrial
CoA buffering, as short and medium chain acyl moieties are
commonly found in the ASP fraction (41, 48). If both carnitine
and UCP3 were involved in this buffering process, carnitine-
driven expansion of the ASP pool would be greater in Ucp3⁻/⁻
mitochondria; however, this was not the case, indicating that
this process was not functionally compensating for UCP3 absence.

Palmitate Is Generated and Exported from Skeletal Muscle
Mitochondria of WT and Ucp3⁻/⁻ Mice—We next determined
whether a fatty acid export process occurs in skeletal muscle
mitochondria and, if so, whether UCP3 plays a unique role. To
this end we supplied WT and Ucp3⁻/⁻ mitochondria with satu-
rating concentrations of [1-¹⁴C]PCarn during a 30-min incubation
period and assessed [1-¹⁴C]palmitate generation by thin layer
cromatography. The product ([1-¹⁴C]palmitate) was easily
identified from the substrate because the former migrated with a retention factor of 0.82 ± 0.01, whereas the latter remained at the origin. When supplied with 19 µM PCarn,
WT and Ucp3⁻/⁻ mitochondria generated palmitate at similar
rates (Fig. 4A). Localization of palmitate to mitochondria or
buffer (i.e. exported palmitate) was assessed by filtering the
reaction mixture across a nitrocellulose membrane. The major-
ity of generated palmitate was exported from both WT and
Ucp3⁻/⁻ samples, and this exported fraction was similar in both
mtotypes (Fig. 4A). Movement of palmitate from mitochon-
dria to buffer was not related to mitochondrial membrane
rupture since activity of a matrix marker, malate dehydrogen-
ase, remained minimal in mitochondria incubated under the
same conditions in the absence of TX-100 (Fig. 4B).

Our results characterize for the first time the generation and
export of long chain fatty acid anions in skeletal muscle mito-
chondria, processes recently described in cardiac mitochondria.
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(27, 49). Exported fatty acid anions are thought to be the product of an intramitochondrial thioesterase, such as MTE-1, which cleaves fatty acyl-CoA into fatty acid anion and free CoA (50). Without an export mechanism, released fatty acid anions would accumulate in the matrix. The possibility that UCP3 uniquely participates in the export mechanism (20, 27) is not supported, as palmitate export was independent of UCP3 protein; it is also noteworthy that adenine nucleotide translocator protein expression is not increased in muscle mitochondria from Ucp3−/− mice, whether fed or calorie-restricted for 2 weeks (51). The mechanisms and physiological implications of matrix fatty acid generation and export require further study, especially in light of the association between lipid metabolism and insulin resistance (45, 52).

Potential Reactivation of Exported Palmitate Increases CO2 Production in WT and Ucp3−/− Mitochondria—Because palmitate is produced and exported from mitochondria during PCarn oxidation, we examined whether exported fatty acid anions are reactivated outside mitochondria and contribute to total FAO rates. To this end, PCarn oxidation rates were measured in the presence and absence of free CoA, which is essential for reactivation (Fig. 1). Free CoA significantly and similarly increased CO2 production rates in WT and Ucp3−/− mitochondria (Fig. 4C). Free CoA had no effect on ASP production (Fig. 4D), which may indicate that the ASP pool is relatively large and, thus, less mobile or that the reactivated species were not contained within the ASP pool. The novel observation that free CoA increases PCarn oxidation to CO2 may be partially attributed to reactivation and oxidation of exported palmitate; however, the CoA-driven increase in PCarn oxidation was ~40-fold greater than the detected palmitate export rate. Thus, even assuming similar export rates for C12, C14, and C18 as for palmitate, increased PCarn oxidation in the presence of CoA cannot be entirely accounted for by reactivation and oxidation of exported fatty acids. Thus, CoA must also be regulating FAO by other, potentially more direct, means.

Fasting Increases UCP3 and MTE-1 Protein Content in WT but Not Ucp3−/− Mice—To test for a potential role for UCP3 in FAO when mitochondria undergo fasting-induced remodeling, WT and Ucp3−/− mice were fasted overnight (18 h) to achieve, in addition to elevated fatty acid supply to mitochondria, greater UCP3 protein abundance in WT mitochondria. In mitochondria from WT mice, UCP3 protein was significantly elevated by fasting (Fig. 5A), as previously shown in rats (53, 54). MTE-1 protein levels were also significantly higher (Fig. 5A). In contrast, MTE-1 protein levels were unchanged with fasting in Ucp3−/− mitochondria (Fig. 5A). Ucp3 and MTE-1 genes contain peroxisome proliferator-activated receptor (PPAR) response elements permitting their regulation by nuclear transcription factor PPARδ (55–57). To determine whether lack of MTE-1 up-regulation in Ucp3−/− mice indicated a globally impaired PPARδ response, we measured PDK4 protein, another PPARδ target gene product; significant and similar up-regulation of PDK4 protein was detected in mitochondria from fasted WT and Ucp3−/− mice (supplemental Fig. 1).

Palmitate and Palmitoylcarnitine Oxidation Rates Are Impaired in Mitochondria from Fasted Ucp3−/− Mice—The impact of an overnight fast on FAO rates in skeletal muscle mitochondria was assessed. With palmitate as substrate, CO2 production was unchanged with fasting in WT and Ucp3−/− mitochondria (Fig. 5B). However, ASP production was reduced from fed levels (p < 0.001) in Ucp3−/− but was unchanged in WT mitochondria (Fig. 5C). When mitochondria were supplied with PCarn, CO2 and ASP production rates in WT mitochondria from fasted mice increased by 32 ± 3% (p < 0.05) and 64 ± 9% (p < 0.001), respectively (Fig. 5, D and E). Conversely, no such fasting-induced up-regulation of CO2 or ASP production was observed in Ucp3−/− mitochondria. As in mitochondria from fasted mice, PCarn oxidation (CO2 and ASP production) in mitochondria from fasted WT and Ucp3−/− mice responded similarly to the addition of free carnitine or CoA (data not shown).

An increase in PCarn but not palmitate oxidation rate suggests that 18 h was sufficient to cause up-regulation of some matrix enzymes and/or cofactors involved in β-oxidation but not of carnitine palmitoyl transferase-1. We thus measured maximal activities of β-hydroxyacyl dehydrogenase and citrate synthase in skeletal muscle mitochondria from fed and fasted mice. Mitochondria from fed Ucp3−/− mice had significantly higher citrate synthase and β-hydroxyacyl dehydrogenase

![Image](348x26 to 376x38)
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Fasting Increases Palmitate Generation in Mitochondria from Ucp3<sup>−/−</sup> but Not WT Mice—Because FAO rates and capacity were impaired in mitochondria from fasted Ucp3<sup>−/−</sup> mice, we next determined whether this impairment resulted in higher generation of long chain fatty acid anions. Supplying PCarn to mitochondria from fasted mice, palmitate generation rates were increased by 38 ± 9% (<i>p</i> = 0.04) in Ucp3<sup>−/−</sup> mice when compared with the fed state (Fig. 6A), a response not observed in fasted WT mice. Interestingly, therefore, palmitate production can be increased independently from MTE-1 protein up-regulation, suggesting the existence of post-translational enzyme regulation, or to the activity of other, as yet unidentified, thioesterases. Palmitate export tended to increase with fasting in both genotypes, but this response was not statistically significant (Fig. 6B) and was unrelated to inner membrane damage (Fig. 6C).

Fasting Is Associated with Increased Oxidative Stress in Mitochondria from Ucp3<sup>−/−</sup> Mice—To gain further insight into a possible cause for the aberrant response to fasting in UCP3 null mitochondria, we tested whether fasting was associated with increased oxidative stress in skeletal muscle mitochondria by evaluating levels of 4-HNE-protein adducts. Oxidative stress was slightly but significantly lower (4 ± 0.6%; <i>p</i> = 0.005) in mitochondria from fed Ucp3<sup>−/−</sup> compared with fed WT mice (Fig. 7A). The slightly lower accumulation of 4-HNE adducts in the fed Ucp3<sup>−/−</sup> mice was not due to increased expression of MnSOD or compensatory expression of UCP2 protein (supplemental Figs. 2 and 3). Fasting, however, was associated with an increase (32 ± 4%; <i>p</i> = 0.013) in 4-HNE protein adducts in vivo response to fasting, originating from mitochondria, in muscle from Ucp3<sup>−/−</sup> mice.

FIGURE 5. Change in protein content of putative fatty acid export machinery and FAO in response to fasting in skeletal muscle mitochondria from WT and Ucp3<sup>−/−</sup> mice. A, Western blot analysis for UCP3 and MTE-1. Porcine-stained membranes served as loading controls. The bar graph shows protein levels in mitochondria from fasted mice normalized to mean levels from fed mice. CO2 (B and D) and ASP (C and E) production rates with palmitate (B and C) or palmitoylcarnitine (D and E) as substrate in mitochondria from fasted mice are shown. Maximal activity of citrate synthase (CS) (F) and β-hydroxyacyl dehydrogenase (β-HAD) (G) in mitochondria from fasted mice is shown. Panels B–G, values expressed as a fraction of rates in mitochondria from fed mice. *<i>p</i> < 0.05,<i> †p</i> < 0.01,<i> **p</i> < 0.001 versus fed state. ##<i>p</i> < 0.01, *<i>p</i> < 0.05 between genotypes. Error bars are S.E. All groups, <i>n</i> = 6.

activity when compared with mitochondria from fed WT mice (citrate synthase, 138.7 ± 4.8 versus 105.0 ± 8.9 μmol/min/mg, <i>p</i> < 0.05; β-hydroxyacyl dehydrogenase, 18.6 ± 0.6 versus 14.5 ± 0.6 μmol/min/mg, <i>p</i> < 0.01). No change with fasting was observed in WT mitochondria. In contrast, in Ucp3<sup>−/−</sup> mitochondria fasting reduced β-hydroxyacyl dehydrogenase activity (<i>p</i> = 0.013) and tended to decrease citrate synthase activity (<i>p</i> = 0.056) (Fig. 5, F and G). These lower maximal activities in Ucp3<sup>−/−</sup> mitochondria would limit the capacity for increased FAO with fasting. The absence of increased MTE-1 protein together with reduced enzyme activity of key oxidation markers and reduced/unchanged oxidation rates suggest an impaired in vitro response to fasting, originating from mitochondria, in muscle from Ucp3<sup>−/−</sup> mice.
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A

B

C

D

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UCP3<sup>−/−</sup> but not WT mitochondria (Fig. 7A). Increased oxidative stress (H<sub>2</sub>O<sub>2</sub> production) was not detectable in vitro in mitochondria from either fed or fasted mice under the conditions of the PCarn oxidation measurements (Fig. 7C). As a positive control for the p-hydroxy-phenylacetate assay that we used to detect H<sub>2</sub>O<sub>2</sub>, the electron transport chain inhibitor antimycin was added. Antimycin blocks the quinone reduction site (Qi) of complex III, thereby causing unstable semiquinone to accumulate at the Qo site. Robust H<sub>2</sub>O<sub>2</sub> generation was, therefore, expected due to electron leakage at Qi. Indeed, substantial H<sub>2</sub>O<sub>2</sub> rates were present in WT and Ucp3<sup>−/−</sup> mitochondria from fed and fasted mice. Fasting significantly reduced the rate of H<sub>2</sub>O<sub>2</sub> production in mitochondria from WT (17 ± 3% reduction; p = 0.036; Fig. 7D) but not Ucp3<sup>−/−</sup> mice. Fasting did not alter MnSOD protein levels or induce UCP2 protein expression in either genotype (supplemental Figs. 2 and 3).

That mitochondria from fasted Ucp3<sup>−/−</sup> mice showed clearly elevated levels of 4-HNE-modified proteins indicates increased lipid peroxidation and is consistent with previous reports of elevated oxidative stress in Ucp3<sup>−/−</sup> mitochondria (16, 18, 58) and cells (17, 19). A model has been proposed in which UCP3 is activated by lipid peroxides to mitigate reactive oxygen species production via mild uncoupling (59–61). Some observations have failed to support this model (62), whereas others demonstrate a disconnect between membrane potential and lower reactive oxygen species production with UCP3 overexpression (13). Although the antimycin condition in the present study may be considered to be non-physiologic, it is noteworthy that antimycin depolarizes the mitochondrial inner membrane. Thus, a putative protective role for up-regulated UCP3 protein in mitigating H<sub>2</sub>O<sub>2</sub> production in antimycin-treated WT mitochondria occurred in the absence of a highly energized inner membrane. Although an association between UCP3 and mitigated reactive oxygen species production/reduced oxidative stress appears to be solid and the current study links this function of UCP3 to FAO, the mechanistic basis for UCP3-mediated bioenergetic changes requires further investigation.

Concluding Remarks—Our results indicate overall that UCP3 is not an obligatory component of the minimal mitochondrial machinery that metabolizes fatty acids nor of the very poorly understood mechanism(s) mediating fatty acid export. However, analyses of the mitochondrial adaptation to fasting revealed clear genotypic differences; mitochondria of Ucp3<sup>−/−</sup> mice exhibited impaired FAO with consequential elevation in palmitate production and its matrix accumulation, possibly related to increased oxidative stress. Thus, our results indicate a role for UCP3 in the adaptation of FAO capacity to fasting and possibly more broadly to perturbed energy balance.

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