In Vivo Effects of Uncoupling Protein-3 Gene Disruption on Mitochondrial Energy Metabolism*

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To clarify the role of uncoupling protein-3 (UCP3) in skeletal muscle, we used NMR and isotopic labeling experiments to evaluate the effect of UCP3 knockout (UCP3KO) in mice on the regulation of energy metabolism in vivo. Whole body energy expenditure was determined from the turnover of doubly labeled body water. Coupling of mitochondrial oxidative phosphorylation in skeletal muscle was evaluated from measurements of rates of ATP synthesis (using 31P NMR magnetization transfer experiments) and tricarboxylic acid (TCA) cycle flux (calculated from the time course of [13C]acetate). At the whole body level, we observed no change in energy expenditure. However, at the cellular level, skeletal muscle UCP3KO increased the rate of ATP synthesis from P, more than 4-fold under fasting conditions (wild type, 2.2 ± 0.6 versus knockout, 9.1 ± 1.4 µmol/g of muscle/min, p < 0.001) with no change in TCA cycle flux rate (wild type, 0.74 ± 0.04 versus knockout, 0.71 ± 0.03 µmol/g of muscle/min). The increased efficiency of ATP production may account for the significant (p < 0.05) increase in the ratio of ATP to ADP in the muscle of UCP3KO mice (5.3 ± 0.3) compared with controls (4.5 ± 0.4). The data presented here provide the first evidence of uncoupling activity by UCP3 in skeletal muscle in vivo.

Uncoupling protein-1 (UCP1) has been shown to regulate non-shivering thermogenesis in brown adipose tissue by allowing proton flux across the inner mitochondrial membrane to bypass ATP synthase (1, 2). The high sequence homology (57%) of UCP1 to UCP3, a protein expressed primarily in muscle, is suggestive of a common physiological function of the two proteins (3, 4). However, the results of in vitro and in vivo studies of UCP3 casts doubt on the hypothesis that the primary role of UCP3 is the regulation of energy efficiency in muscle (3–6). Hence, the functional role of UCP3 in the regulation of mitochondrial oxidative phosphorylation in skeletal muscle remains uncertain. For example, the up-regulation of UCP3 protein levels during fasting, when energy efficiency should be increased, is inconsistent with UCP3 acting as an uncoupling protein in vivo. Also, studies of energy metabolism in UCP3 knockout mice (UCP3KO) were unable to demonstrate any phenotypic changes in whole body energy metabolism (3, 4). These mice showed no differences in body composition, growth characteristics, exercise tolerance, fatty acid oxidation, or cold-induced thermogenesis. The clearest evidence to date that UCP3 might have the capacity to act as an uncoupling protein comes from measurements in isolated mitochondria. These studies established for the first time that oxidative ATP production in mitochondria isolated from the muscle of UCP3KO mice were more coupled compared with normal littermates (3, 4) and that proton leak over a range of mitochondrial membrane potentials was decreased (4). Conversely, overexpression of UCP3 in muscle uncoupled oxidative phosphorylation in isolated mitochondria (7). However, because these studies were performed in vitro under artificial concentrations of ADP and substrates, it is difficult to extrapolate these results to describe the physiologic function of UCP3.

To determine whether the lack of UCP3 in muscle causes changes in the coupling of skeletal muscle mitochondrial oxidative phosphorylation in vivo, we performed NMR saturation-transfer experiments to estimate rates of muscle ATP synthesis and isotopic labeling experiments to estimate rates of TCA cycle flux. In addition, we used the doubly labeled water method to determine total energy expenditure over days in the free moving mice (8). The ratio of ATP synthesis rates and TCA cycle flux rates provides an index of mitochondrial coupling in vivo (9, 10). Deletion of UCP3 in muscle may result in any one or a combination of several potential physiological perturbations. If UCP3 ablation increases the efficiency of mitochondrial ATP production, then ATP concentration could be maintained constant by a corresponding reduction in TCA cycle flux rate. However, if TCA cycle flux rates remain unchanged, then ATP production rates should increase, which must then be matched by an increase in ATP turnover. Finally, if UCP3 has no uncoupling function in vivo, then neither ATP synthesis nor TCA cycle flux rate would be affected. We show here that deletion of muscle UCP3 does result in significant perturbations of mitochondrial energy production in vivo.

EXPERIMENTAL PROCEDURES

Animals

Age- and weight-matched wild-type and UCP3KO mice were studied at 5–7 months of age. The generation of UCP3KO mice has been described previously (3). Animals were housed four per cage in a temperature-controlled room with a 12-h light/dark cycle with food and

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water available ad libitum unless noted otherwise. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and the protocol was approved by the Yale Animal Care and Use Committee.

**Doubly Labeled Water Method for Measurement of Whole Body Metabolic Rate**

**Dosage and Sampling**—Mice were injected intraperitoneally at 8 a.m. with a solution of D$_2$O (15 μl/g of mouse, 80% D$_2$O, and 20% H$_2$O, 150 mM sodium chloride). Tail tip bleeds were taken at 24-h intervals over the course of a week.

**Analysis for Deuterium Enrichment**—The deuterium atom percent enrichment of plasma water was determined by reacting 10 μl of the plasma with calcium carbide to form acetylene gas and analyzed by GC-MS (electron impact ionization) [11]. A small amount (10–15 mg) of freshly ground calcium carbide was added to a dry crimp-top vial and capped with a rubber septum. The plasma sample was injected into the vial through the septum and allowed to react for 5 to 15 min. A 5-μl sample of the gas phase within the vial was drawn into a Hamilton glass syringe and injected into the GC-MS. The GC temperature was isothermal at 180 °C, and ions m/z 26 and 27 were selectively monitored.

**Analysis for Deuterium and 18O Enrichment**—Deuterium and 18O isotopic enrichment of plasma water was determined by isotope ratio mass spectrometry (Metabolic Solutions, Inc., Nashua, NH) and are expressed as δV-SMOW (i.e. the difference from Vienna-standard mean ocean water). There was no difference in the kinetics of deuterium water turnover as determined by either method.

**Calculations**—Total energy expenditure was determined from the difference in the rates of loss of the deuterium and 18O isotopes following a bolus injection of doubly labeled water (i.e. D$_2$O/H$_2$O). Deuterium is lost as water, whereas 18O is lost either as water or as CO$_2$. The difference in the rates of isotopic loss (k$_D$ – rate of loss of D$_2$O; k$_H$ – rate of loss of deuterium) will be the rate of CO$_2$ output, which is proportional to whole-body metabolic rate (8).

The rate of CO$_2$ loss, rCO$_2$, was calculated as

$$rCO_2 = 0.4554 \times N(1.01 k_1 - 1.04 k_2) \text{ (Eq. 1)}$$

where N is total body water calculated as the mean of the corrected isotope dilution spaces of D$_2$O and H$_2$O at the time of the bolus injection.

Total energy expenditure (TEE) was calculated as

$$\text{TEE (kcal/day)} = 3.9 \times rCO_2/RQ + 1.11 rCO_2 \text{ (Eq. 2)}$$

Because it has previously been shown that there were no changes in the respiratory exchange quotient (RQ) as the result of UCP3 disruption in either the fed or fasted states [3, 4], we used a mean RQ of 0.79 to calculate total energy expenditure (3). The mean RQ of 0.79 may tend to overestimate the proportion of fat to carbohydrate metabolized over the 7-day period that total energy expenditure was estimated but will not mask any differences in total energy expenditure between the groups.

**ATP Flux Rates in Skeletal Muscle in Vivo**

All in vivo NMR experiments were performed on a Bruker (Billerica, MA) Biospec 7.0T system (horizontal/22-cm-diameter bore magnet). 31P NMR measurement of ATP flux was measured at 121.66 MHz using a 1-cm diameter inner coil (for 31P) and a 3-cm outer coil tuned to the proton frequency for scout imaging and shimming.

On the day of the NMR experiment, overnight-fasted mice were secured in a Plexiglas tube restrainer that allowed the right hind limb to be secured to the outside of the tube and centered over concentric surface coils. The mouse and probe assembly were then placed in the magnet isocenter.

Unidirectional rates of ATP synthesis from P$_i$, the product of the rate constant of ATP synthesis and the concentration of intracellular P$_i$. The unidirectional rate constant for ATP synthesis from P$_i$, $k_{ATP}$, was measured using the saturation-transfer experiment [9, 10]. The steady-state P$_i$ magnetization, $M_{P_i}$, was measured with selective irradiation of the 31P resonance, and compared with the equilibrium P$_i$ magnetization, $M_{P_i}^*$, with the selective irradiation placed symmetrically down field from the P$_i$ frequency, $T_1^*$, the spin lattice relaxation time for P$_i$ when ATP is saturated, was measured using a modified version of the inversion recovery experiment (180°-τ-90°-acquire-ID) in the presence of a steady-state saturation of γATP during the interpulse delay (ID) of 4 s and during the variable delay, τ.

P$_i$ concentration was calculated from the ratio of integrated peak intensities (T$_1$ corrected) of P$_i$ and β-ATP. The concentration of ATP was determined by HPLC as described below.

**TCA Cycle Flux Rates in Skeletal Muscle**

**Isotopic Measurement of TCA Cycle Flux Rates**—The mice were studied 4 days after a catheter was surgically implanted into their jugular veins. Following an overnight fast, the mice were placed in tube restrainers, and an infusion of [2-13C]acetate at a rate of 4 μg/g of mouse/min was begun. Blood samples for determination of acetate concentration and isotopic enrichment were obtained immediately prior to the end of the experiment by tail tip bleeds. TCA cycle flux was calculated from the time course of 13C enrichment in glutamate from perchoric acid extracts of freeze-clamped muscle sacrificed at various times after the start of the [2-13C]acetate infusion. At the end of the infusion period (variable from 2 to 90 min), the mice (controls, n = 12, UCP3KO, n = 12) were sacrificed and the muscles freeze-clamped in situ for the determination of glutamate concentration and 13C enrichment.

**Analysis of [2-13C]Acetate and [13C]Glutamate—Plasma acetate concentration and 13C isotopic enrichment were determined by GC-MS as described in detail elsewhere [12]. Briefly, 50 μl of plasma was spiked with an equal volume of an internal concentration standard containing 50 μM sodium acetate-d$_3$ acid. To this solution was then added 50 μl of 0.1 M HCl and ~50 mg of solid NaCl. The mixture was then coupled to 2,4-difluoroaniline (100 μl of reagent, 0.2 μl in hexane) using 1,3-dicyclohexy carbodiimide (100 μl of reagent, 0.2 μl in toluene). The solution was capped and mixed for 1 h. After 1 h, 1 ml of 1 M sodium bicarbonate was added, and the acetyl-2,4-difluoroaniline was extracted (3×) into ethyl acetate (1 ml). The organic layer was dried over sodium sulfate and concentrated under a stream of dry nitrogen gas for GC-MS analysis.

13C and d$_3$-acetate isotopic enrichments were corrected using standard curves at similar concentrations and processed as described above for the plasma samples. Analysis of the d$_3$-acetate internal standard yielded a background acetate concentration of 8.0 ± 0.04 nmol in the blank, which was less than 8% of the measured plasma acetate concentration. The plasma concentrations given are corrected for the acetate concentration in the blank.

Analyses were carried out using a Hewlett-Packard (H-P) 5937MSMS interfaced to an H-P 6890 gas chromatograph. The samples were then analyzed using electron ionization at 70 eV by monitoring ions m/z 171, 172, and 174. Glutamate concentration and 13C enrichment in the muscle were determined as follows. 100–200 mg of muscle was weighed, homogenized in cold perchloric acid (0.9 M), and neutralized with KOH. Glutamate concentration was measured in a 10-μl aliquot with a 2700 STAT Plus analyzer (Yellow Springs Instruments, Yellow Springs, OH) using a glutamate ion-specific electrode. The enrichment of glutamate at C-2, C-3, and C-4 was determined following purification from any glutamine by anion exchange chromatography as follows. The pH of the supernatant was adjusted to ~3, and the sample applied to a 1-ml mini-column of anion exchange resin (AG1-XS, Bio-Rad) in a Pasteur pipette. Glutamine was eluted with 5 ml of water, and glutamate was then eluted with 5 ml of acetic acid (0.5 M). The glutamate fraction was freeze-dried and reconstituted in 400 μl of D$_2$O for analysis of concentration and enrichment. The relative enrichment at each carbon position was determined by 13C-NMR spectroscopy and total enrichment by nuclear (CI ionization with 13C-labeled glutamine) as its trifluoroacetyl n-butyl ester derivative (m/z 356, 357, 358) [13].

**Calculation of TCA Cycle Flux Rates—**Rates of TCA cycle flux were calculated from the time course of 13C isotopic enrichment of plasma acetate and muscle glutamate C-2 and C-4 by iterative fitting of metabolic simulations to the data using the program Cwave [15]. Mass and isotope balance equations were derived using the model of TCA cycle as shown in Scheme 1. The isotopic enrichment and concentrations of plasma acetate, glucose, and free fatty acids are used as input drivers. Using these input parameters, flux rates are determined that provide the best fit to the observed time course of enrichment in [2-13C]- and [4-13C]glutamate.

The mass balance equations used were as follows.

$$dAcCoA/dt = V_a + V_{AcA} - V_{TCA} \text{ (Eq. 3)}$$

$$dOAA/dt = V_p - V_{an} \text{ (Eq. 4)}$$

$$dCit/dt = dKog/dt = dGlu/dt = 0 \text{ (Eq. 5)}$$
The isotope balance equations used were as follows,

\[
d^{[2-13]C}\text{AcCoA} \frac{dV}{dt} = V_{\text{dl}}(\text{FE}\ [2-13]C\text{Ac}) + V_{\text{TCA}}(\text{FE}\ [2-13]C\text{AcCoA}) \quad (\text{Eq. 6})
\]

\[
d^{[4-13]C}\text{Cit} \frac{dV}{dt} = V_{\text{TCA}}(\text{FE}\ [2-13]C\text{AcCoA}) - V_{\text{TCA}}(\text{FE}\ [4-13]C\text{Cit}) \quad (\text{Eq. 7})
\]

\[
d^{[4-13]C}\alpha\text{KG} \frac{dV}{dt} = V_{\text{glu}}(\text{FE}\ [4-13]C\text{Glu}) + V_{\text{TCA}}(\text{FE}\ [4-13]C\text{Cit}) - V_{\text{TCA}}(\text{FE}\ [4-13]C\alpha\text{KG}) - V_{\text{glu}}(\text{FE}\ [4-13]C\alpha\text{KG}) \quad (\text{Eq. 8})
\]

\[
d^{[4-13]C}\text{Glu} \frac{dV}{dt} = V_{\text{glu}}(\text{FE}\ [4-13]C\text{Glu}) - V_{\text{glu}}(\text{FE}\ [4-13]C\text{Glu}) \quad (\text{Eq. 9})
\]

\[
d^{[2-13]C}\text{OAA} \frac{dV}{dt} = 0.5V_{\text{TCA}}(\text{FE}\ [4-13]C\alpha\text{KG}) + 0.5V_{\text{TCA}}(\text{FE}\ [2-13]C\text{Cit}) + V_{\text{glu}}(\text{FE}\ [2-13]C\text{Pyr}) - V_{\text{TCA}}(\text{FE}\ [2-13]C\text{OAA}) \quad (\text{Eq. 10})
\]

\[
d^{[2-13]C}\text{Cit} \frac{dV}{dt} = V_{\text{TCA}}(\text{FE}\ [2-13]C\text{OAA}) - V_{\text{TCA}}(\text{FE}\ [4-13]C\text{Cit}) \quad (\text{Eq. 11})
\]

\[
d^{[2-13]C}\alpha\text{KG} \frac{dV}{dt} = V_{\text{glu}}(\text{FE}\ [2-13]C\text{Glu}) + V_{\text{TCA}}(\text{FE}\ [2-13]C\text{Cit}) - V_{\text{TCA}}(\text{FE}\ [2-13]C\alpha\text{KG}) \quad (\text{Eq. 12})
\]

\[
d^{[2-13]C}\text{Glu} \frac{dV}{dt} = V_{\text{glu}}(\text{FE}\ [2-13]C\alpha\text{KG}) - V_{\text{glu}}(\text{FE}\ [2-13]C\text{Glu}) \quad (\text{Eq. 13})
\]

where FE is the fractional enrichment calculated as the quotient of the mass of 13C-labeled molecules and the total mass of labeled and unlabeled molecules. The position of the 13C-label is denoted in brackets. Ac is plasma acetate, AcCoA is acetyl-CoA, Cit is citrate, Glu is glutamate, KG is alpha-ketoglutarate, Pyr is pyruvate, and TCA is the combined pools of plasma free fatty acid and glucose, and V\text{subscript} is the flux through the pathways as shown in the schematic, where V\text{dl} is the combined flux into acetyl-CoA from free fatty acids and glucose.

**Determination of ATP, ADP, and AMP Concentrations in Skeletal Muscle**—The concentrations of ATP, ADP, and AMP in the muscle of overnight-fasted UCP3KO mice and control mice were determined at the end of the acetate infusion or NMR experiments. Muscles were freeze-clamped in situ and then extracted with 0.9 N ice-cold perchloric acid. The concentrations of nucleotides in the supernatant were then determined by HPLC using a modification of the method described by Sabina et al. (14). ATP, ADP, and AMP in a 20-μl sample of the muscle extract were separated on a Supelcosil SAX1 (25 cm × 4.6 mm × 0.5 μm) column using a gradient of 5 mM ammonium phosphate, pH 2.8 (buffer A) and 750 mM ammonium phosphate, pH 3.9 (buffer B) at a flow rate of 1 ml/min. A linear gradient was developed over 14 min at 0% buffer B to 9% buffer B, then from 14 to 32 min from 9% buffer B to 100% buffer B. A Rainin Hypersil solvent delivery system (two pumps) with a Rainin Dynamax UV-1 absorbance detector (254 nm) controlled by Rainin Dynamax HPLC Method Manager was used for solvent programming and data collection. Peak identification was assigned by comparison of retention times to known external standards (AMP ~ 5.5 min, ADP ~ 25.5 min, ATP ~ 29.0 min). Nucleotide concentrations were calculated from the concentration standard curves of absorbance for the external standards.

**RESULTS AND DISCUSSION**

**Whole Body Metabolism**—During the 7 days that the mean rates of total energy expenditure were measured, the mice had free access to food and water and unrestricted movement. Deut
labeled water, the body water was calculated to be 64 ± 9% in the controls and 65 ± 8% in the UCP3KO (p < NS (not significant)). From the difference in the rates of decay of deuterium and 18O enrichments (Fig. 1), we calculated mean rates of CO2 production of 37 ± 2 ml/kg/min for the normal mice and 37 ± 1 ml/kg/min for the UCP3KO mice. From the mean rates of CO2 production, we calculated free range total energy expenditure of 325 ± 8 kcal/kg/day in the normal mice and 321 ± 15 kcal/kg/day in the UCP3KO mice. In agreement with previous results (3, 4), a lack of UCP3 in skeletal muscle did not cause any detectable phenotypic changes in energy metabolism at the whole body level.

ATP, ADP, and AMP Concentrations—Mean concentrations of ATP, ADP, or AMP in the UCP3KO mice were not significantly different from their littermates (Table I). However, the mean ratio of ATP to ADP determined for each individual mouse was significantly higher in the UCP3KO mice compared with the controls. As shown previously (3), the deletion of UCP3 in skeletal muscle increased the proportion of ATP to ADP in the tissue.

Coupling of ATP Synthesis and TCA Cycle Flux—In comparison with normal mice, mitochondria isolated from the muscle of UCP3KO mice have a lower rate of respiration under state 4 conditions (3) and decreased proton leak (4), indicating that UCP3 functions as an uncoupler of oxidative phosphorylation. However, the up-regulation of UCP3 protein in skeletal muscle under fasting conditions is counterintuitive to the need to increase the efficiency of energy production. To determine whether UCP3 functions as an uncoupler of mitochondrial ATP production in vivo, we used a combination of NMR and isotopic labeling methods to assess the role of UCP3 in the regulation of skeletal muscle mitochondrial energy production.

A representative 31P-NMR saturation-transfer experiment to determine mitochondrial ATP synthesis rates is shown in Fig. 2, and the mean results are presented in Table II. From these experiments, we determined that the rate constant of skeletal muscle ATP synthesis from Pi was 2-fold higher (p < 0.001) in the UCP3KO mice compared with the control mice. We observed no difference in the T1 relaxation time for Pi.

Results are expressed as the mean ± S.E. of ATP synthesis rates in the hind limb muscle of overnight-fasted wild-type (WT) and UCP3KO (KO) mice at rest

| Mouse type | Pi Measured | T1 Measured | k: Pi → ATP | Flux: Pi → ATP |
|------------|-------------|-------------|--------------|----------------|
| WT         | 0.82 ± 0.03 | 1.95 ± 0.25 | 0.09 ± 0.02  | 0.036 ± 0.010  |
| KO         | 0.63 ± 0.03 | 1.94 ± 0.16 | 0.19 ± 0.01* | 0.151 ± 0.024* |

* p < 0.001.

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FIG. 2. Typical 12-min spectra of the 31P-NMR saturation-transfer experiment for determination of unidirectional Pi to ATP flux in the hind limbs of awake mice.

FIG. 3. Rates of unidirectional ATP synthesis (upper plot), TCA cycle flux (TCA, middle plot), and the coupling index (lower plot) calculated as the ratio of the rates of ATP synthesis and TCA cycle flux in normal and UCP3KO mice after an overnight fast.

Table II

Saturation-transfer measurements of ATP synthesis rates in the hind limb muscle of overnight-fasted wild-type (WT) and UCP3KO (KO) mice at rest

The magnetization-transfer (WT, n = 8; KO, n = 12) and T1 (WT, n = 4; KO, n = 3) measurements were made as described under “Experimental Procedures.” Mz/Mo, ratio of steady-state to equilibrium magnetization; k, rate constant for unidirectional synthesis of ATP from Pi. Unpaired Student’s t test was used for the statistical analysis. Results are expressed as the mean ± S.E.
calculated that the rate of skeletal muscle of ATP synthesis under fasting conditions is ~4-fold higher in the UCP3KO mice compared with normal mice (Table II). A conservative estimate of flux rates, using the mean P concentration (0.64 ± 0.11 μmol/g of tissue), gave a 2-fold increase in the ATP synthesis rates in the UCP3KO mice (0.120 ± 0.10 μmol/mg of muscle/s) compared with normal mice (0.059 ± 0.10 μmol/g of muscle/s).

Steady-state plasma [2-13C]acetate enrichments (normal mice, 21.1 ± 4.6; UCP3KO, 18.7 ± 2.4, p = NS) and concentrations were reached within 5 min. The mean basal plasma glutamate 13C enrichments (controls, 0.006 mM, UCP3KO, 0.087 ± 0.006 mM) during the infusion of [2-13C]acetate, acetate concentrations rose to 0.109 ± 0.001 mM in the controls (p = 0.02 compared with basal) and to 0.124 ± 0.015 mM (p = NS compared with basal). Steady-state plasma acetate concentrations were not significantly different in the controls compared with the UCP3KO mice. Glutamate 13C enrichments reached a plateau of 8% within 30 min. From the time course of glutamate C3 and C4 enrichment, we calculate similar TCA cycle flux rates in both groups of mice (normal mice, 0.74 ± 0.04 μmol/g/min; UCP3KO, 0.71 ± 0.03 μmol/g/min).

The ratios of the rates of ATP synthesis to TCA cycle flux provide an in vivo index of mitochondrial coupling in the muscle (Fig. 3). Our results indicate that, under fasting conditions, the coupling of oxidative phosphorylation was 2–4-fold higher in the skeletal muscle of UCP3KO mice compared with wild-type mice. These results are consistent with earlier studies in rats in which an increase in UCP3 expression, induced by T3 treatment (9) or fasting (10), was associated with an increase in mitochondrial uncoupling. However, in contrast to these earlier studies where the observed increased mitochondrial uncoupling was 2–4-fold higher in the skeletal muscle of UCP3KO mice compared with wild-type mice, this increase was significantly lower in UCP3KO mice compared with normal mice (21.1 ± 4.6; UCP3KO, 18.7 ± 2.4, p = NS) and concentrations were reached within 5 min. The mean basal plasma glutamate 13C enrichments (controls, 0.006 mM; UCP3KO, 0.087 ± 0.006 mM). During the infusion of [2-13C]acetate, acetate concentrations rose to 0.109 ± 0.001 mM in the controls (p = 0.02 compared with basal) and to 0.124 ± 0.015 mM (p = NS compared with basal). Steady-state plasma acetate concentrations were not significantly different in the controls compared with the UCP3KO mice. Glutamate 13C enrichments reached a plateau of 8% within 30 min. From the time course of glutamate C3 and C4 enrichment, we calculate similar TCA cycle flux rates in both groups of mice (normal mice, 0.74 ± 0.04 μmol/g/min; UCP3KO, 0.71 ± 0.03 μmol/g/min).

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The qualitative difference in the regulation of mitochondrial coupling due to these perturbations may indicate that the regulation of mitochondrial function is dependent on other factors in addition to the demand for ATP. An increase in UCP3 content (such as those caused by T3 treatment (9), fasting (10), or transgenic overexpression of UCP3 (7) or UC1 (16) in muscle, etc.) would tend to decrease the membrane potential and subsequently the rates of ATP synthesis. However, because cellular energy demands are not decreased, substrate oxidation must increase to maintain the membrane potential necessary for adequate ATP synthesize proton flux. In contrast, ablation of UCP3 leads to an increase in membrane potential while not inhibiting substrate oxidation. If the mitochondrial response of the UCP3KO mice was to simply reduce substrate oxidation to maintain constant ATP synthesis rates, then the NADH/NAD ratio could increase to abnormally high levels and profoundly disrupt rates of numerous cellular oxidation-reduction reactions.

Under the steady-state conditions of our experiment, the increased rate of ATP synthesis must be balanced by an equivalent rate of ATP hydrolysis. Metabolic control theory provides one of the best tools currently available to describe the complex interactions that multiple pathways exert on each other to regulate metabolite concentrations and enzymatic flux rates, supporting the possibility that the rate of ATP hydrolysis is determined by the rate of ATP synthesis (17). Analysis of the factors responsible for regulating the rate of ATP consumption reveals that the rate of ATP consumption is not controlled exclusively by the reactions of ATP hydrolysis but that control is shared to a similar degree by the reactions of ATP production (i.e. mitochondrial phosphorylation and oxidation) and ATP hydrolysis (17). It is likely that the UCP3KO mice maintain ATP concentrations constant by increasing the rate of one of the ATP-consuming futile cycles in the muscle, the major ones being Na+-K+-ATPase, Ca2+-ATPase, and protein turnover (18). Whether increased ATP utilization is occurring in all reactions that utilize ATP via a mass action effect, or is occurring in a subset of reactions involving systems that are designed to sense ATP (e.g. KATP channels, membrane depolarization, or ion pumping), remains to be determined.

In conclusion, the results presented here provide the first direct evidence for alterations of energy metabolism in UCP3KO mice in vivo. UCP3 disruption in skeletal muscle resulted in a doubling of the ATP synthesis rate without any increase in TCA cycle flux rate, under fasting conditions, implying an increased degree of mitochondrial energy coupling. Therefore, these data suggest an important role for UCP3 in the regulation of skeletal muscle mitochondrial energy metabolism in vivo.

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