$^{13}$C/$^{31}$P NMR Assessment of Mitochondrial Energy Coupling in Skeletal Muscle of Awake Fed and Fasted Rats: Relationship with Uncoupling Protein 3 Expression

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Summary

In order to examine the relationship between mitochondrial energy coupling in skeletal muscle with change in uncoupling protein 3 (UCP3) expression during the transition from the fed to fasted state, we used a novel non-invasive $^{31}$P/$^{13}$C NMR spectroscopic approach to measure the degree of mitochondrial energy coupling in the hindlimb muscles of awake rats before and after a 48 hr fast. Compared to fed levels, UCP3 mRNA and protein levels in the gastrocnemius increased 1.7 (p<0.01) and 2.9 (p<0.001) fold respectively following a 48 hr fast. Tri-carboxylic acid (TCA) cycle flux measured using $^{13}$C NMR as an index of mitochondrial substrate oxidation was 212±23 and 173±25 nmol/g/min (p=NS) in the fed and 48 hr fasted groups respectively. Unidirectional ATP synthesis flux measured using $^{31}$P NMR was 79±15 and 57±9 nmol/g/s (p=NS) in the fed and 48 hr fasted groups respectively. Mitochondrial energy coupling as expressed by the ratio of ATP synthesis to TCA cycle flux was not different between the fed and fasted states. To test the hypothesis that UCP3 may be involved in the translocation of long chain free fatty acids (FFA) from the mitochondrial intermembrane space to the matrix under conditions of elevated FFA availability, U-$^{13}$C palmitate/albumin was administered in a separate group of rats with (+) or without (-) etomoxir (an inhibitor of carnitine palmitoyl-transferase I). Palmitate oxidation and/or transport into the mitochondrial matrix was assessed by GC/MS analysis of M+2 glutamate enrichments in the hindlimb muscles. The ratio of glutamate enrichment [(+ etomoxir/(- etomoxir) was the same between groups indicating that UCP3 does not appear to function as a translocator for long chain FFA in skeletal muscle following a 48 hr fast. In summary, these data demonstrate that despite a 2-3 fold increase in UCP3 mRNA and protein expression in skeletal muscle during the transition from the fed to fasted state mitochondrial energy coupling does not change. Furthermore, UCP3 does not appear to have a major role in FFA translocation into the mitochondria. The physiological role of
UCP3 following a 48 hr fast in skeletal muscle remains to be elucidated.

**Introduction**

Mitochondrial uncoupling proteins (UCP) play an integral role in regulating cellular energy consumption via non-shivering thermogenesis (1). This is accomplished by diminishing the proton motive force across the inner mitochondrial membrane which results in uncoupling of respiration from ATP synthesis. Unlike UCP1 which is expressed exclusively in brown adipose tissue (BAT), the recently discovered homolog UCP3 is expressed primarily in muscle (2,3) and is encoded in a chromosomal region linked to hyperinsulinemia and obesity (4). Because quiescent skeletal muscle utilizes approximately 33% of whole body oxygen consumption (5), much attention has been given to the control and function of UCP3 as a means of regulating energy expenditure and body weight.

Increased UCP3 mRNA expression results from a number of physiological perturbations including thyroid hormone (4,6) and leptin (4,7-9) in skeletal muscle and β-adrenoreceptor agonist (4,10) in white adipose tissue (WAT) which have all been implicated in increased regulatory thermogenesis. However, a paradoxical situation occurs in fasting when UCP3 mRNA expression in skeletal muscle has been shown to increase ~2 to 12 fold (4,11-16) at a time when mitochondrial energy coupling efficiency might be expected to remain constant or increase to conserve energy. This paradoxical relationship may be a result of UCP3 mRNA expression not correlating with protein levels or possibly as a result of alterations in the concentration of an unknown allosteric regulator to UCP3. It has also been suggested that UCP3 may play a role in the regulation of lipids as fuel substrate rather than mediators of regulatory thermogenesis (17). Therefore, the UCP3 protein may function specifically as a free fatty acid anion translocator rather than a direct proton shuttle or as part of a proton-FFA cycle under conditions of elevated plasma FFA following a 48 hr
fast. It is now generally accepted that fatty acids are necessary in activating uncoupling proteins via direct or indirect mechanisms (15,18-20).

Although indirect measurements of mitochondrial uncoupling have been made in UCP3 reconstituted vesicles, transfected yeast, or myoblasts coupled with electric potential measurements using an electrode method (18,21) or coupled with fluorescence measurements using flow cytometric techniques (4,11), these measurements have not been made in explicit tissues of interest in situ. Additionally, although electric potential measurements across the inner mitochondrial membrane provide an index for potential energy uncoupling, they do not measure functional energy uncoupling. This is because of the non-ohmic nature of proton conductance which is not linear with the inner mitochondrial membrane potential (22).

We recently reported on the development of a novel NMR spectroscopic method used to assess mitochondrial energy coupling in skeletal muscle, non invasively, by combining $^{13}$C NMR spectroscopy to measure rates of mitochondrial substrate oxidation along with $^{31}$P NMR spectroscopy to assess rates of ATP synthesis in chronic T$_3$ (triiodothyronine) treated rats (a model of increased UCP3 expression) (23). Therefore in this study, we used this non-invasive NMR technique to determine if increased UCP3 mRNA and protein levels correlate with measurements of mitochondrial energy coupling in skeletal muscle of 48 hr fasted rats. Additionally, we tested the hypothesis that UCP3 may function as a fatty acid translocator under fasting conditions during which levels of plasma FFA are increased.
Experimental Procedures

Animals

Sprague-Dawley rats (Charles River, Raleigh, North Carolina) were individually housed in an environmentally controlled room with a 12 h light/dark cycle and maintained on standard rat chow (Ralston Purina Co., St. Louis, Mo). Two groups of rats were used: 1) fed (n=17); 2) 48 hr fasted (n=19). When weighing ~300 g the 48 hr fasted rats had cannulas inserted into both right jugular vein and carotid artery (24) and allowed to recuperate for 5 days prior to fasting. Following fasting, these rats weighed ~270 g. The fed group weight was pair matched to the 48 hr fasted group at the time of the experiment.

In vivo NMR experimental design

On the day of the NMR experiment, rats were placed in a customized restraining tube that allowed their left hindlimb to be secured to the outside of the tube in a manner to limit free movement of the leg for NMR measurements. The rats were transiently anaesthetized (<30 s) with a low dose (2.5-5.0 mg) of thiopental (Sigma) in order to place them in the restraining tube. This protocol was approved by the Yale University Animal Care Committee.

The $^{31}$P NMR measurements preceded the $^{13}$C NMR measurements in each experiment. During the $^{31}$P measurements, unlabeled acetate was administered at a constant infusion rate (138 $\mu$mol/kg/min). During the $^{13}$C measurements, 2-$^{13}$C acetate (sodium salt, 99% enriched, Cambridge Isotope Laboratories, Cambridge, MA.) was administered in order to study the glutamate labeling kinetics for tri-carboxylic acid (TCA) cycle flux measurements (Fig. 1). The 2-$^{13}$C acetate infusion consisted of a bolus (100 mg/kg body weight) for 1 min followed by a 138 $\mu$mol/kg/min continuous infusion for 150 min (sufficient time required to achieve isotopic steady state). Blood samples were
drawn during the baseline NMR measurement, at 7.5 min, 15 min, and every 15 min thereafter to assess the 2-13C acetate enrichment time course. At the end of the in vivo NMR experiment, rats were anaesthetized with thiopental (50 mg/kg). Superficial skin was rapidly removed from the left hindquarter followed by in situ freeze clamping of the gastrocnemius and biceps femoris muscles. Rats were euthanized with a lethal dose of thiopental.

All in vivo NMR experiments were performed on a Bruker Biospec 7.0T system (horizontal/22 cm diameter bore magnet). Both 13C observe/1H decouple and 31P observe NMR spectroscopy were performed using concentric surface coils [the outer 1H coil (30 mm) tuned to 300.54 MHz, and the inner dual frequency 13C or 31P coil (18 mm) tuned to 75.59 and 121.66 MHz respectively. The rat hindlimb was positioned over the 13C/31P coil (vertical in plane) and placed in magnet isocenter. Due to the 13C/31P sensitivity in the hindlimb experiments, it was necessary to measure the bulk signal from the larger tissue beds of mixed fiber type including the gastrocnemius and biceps femoris.

Global 1H shimming was followed by localized shimming using a STEAM sequence over a 1x2x2 cm volume of the leg. Water line widths of 35-60 Hz, as a reflection of the average inhomogeneity caused by the subtle movements of the leg were routinely achieved. The creatine/phosphocreatine peak (54.4 ppm) in the 13C spectrum and ß-ATP (-16.0 ppm) in the 31P spectrum were used as an internal reference standard for movement of the leg. If severe movement compromised these peak integrals, the experiment was terminated.

All spectra was processed off-line using Nuts NMR processing software (Acorn NMR Inc., Fremont, CA) with peak fitting capabilities. Baseline subtracted 13C NMR and 31P NMR spectra were processed using Gaussian filtering and a Gaussian weighted peak fitting algorithm.
**31P NMR saturation transfer study**

The saturation transfer study requires that two sets of spectra be acquired: one with and one without steady state saturation. To measure the kinetics of $P_i\rightarrow$ATP, a continuous wave selective saturation of the $\gamma$-ATP resonance was used. In the spectrum without $\gamma$-ATP saturation, the CW pulse was placed an equal frequency offset to the downfield side of $P_i$. The ratio of the resulting magnetization ($M_z$) to the equilibrium magnetization ($M_0$) in the absence of $\gamma$-ATP saturation is given by:

$$\frac{M_z}{M_0} = 1 / (1 + kT_1) \quad (1)$$

where $k$ is the first order rate constant describing the loss of magnetization from $P_i$, and $T_1$ is the intrinsic spin-lattice relaxation time for the $P_i$ nucleus. A 90° pulse was optimized for the non-selective detection of both spectra (TR=4.4 s, NS=128, SW=20 KHz, 4K data). The spin-lattice relaxation time measured using an inversion recovery pulse sequence in the presence of continuous $\gamma$-ATP saturation is defined as the observed $T_1$ ($T_{1\text{obs}}$) and is related to $T_1$ by the following equation:

$$\frac{1}{T_{1\text{obs}}} = \frac{1}{T_1} + k \quad (2)$$

An adiabatic half passage pulse was used to invert all $P_i$ spins in the inhomogeneous volume of the surface coil (TR=6.0 s, NS=64, SW=20 KHz, 4K data) for the 180° pulse of the inversion recovery sequence. The 6 variable delay lengths used in the inversion recovery experiment will be between 10 ms-6 s. Solving the above two equations simultaneously (where $\Delta M = M_0 - M_z$) yields the following equation:

$$k = \frac{1}{T_{1\text{obs}}} \times \frac{\Delta M}{M_0} \quad (3)$$

Therefore, the rate constant ($k$) may be calculated after acquiring spectra for the two experiments.
described above (saturation transfer and inversion recovery). The unidirectional ATP synthesis flux was calculated as $k \times [\text{Pi}]$. The Pi concentration was extrapolated from the baseline NMR spectrum (comparing peak integrals from Pi and $\gamma$-ATP) and [ATP] measurement using a biochemical assay.

The NMR measured unidirectional ATP synthesis flux results from flux through both the F1F0 ATP synthase enzyme and the coupled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) reactions. Although the net glycolytic contribution to the production of ATP (via GAPDH and PGK) versus that of oxidative phosphorylation is small, these enzymes are at near equilibrium and consequently the unidirectional production of ATP (measured using the $^{31}$P saturation transfer experiment) via these enzymes can be high. We previously described a technique where uniformly labeled glucose (1,2,3,4,5,6,6-D$_7$) was administered to rats to determine whether there are differences in the glycolytic ATP synthesis flux contribution between groups (23). In separate experiments, this technique was applied to both fed (n=3) and 48 hr fasted (n=3) rats. The glyceraldehyde-3-phosphate (GAP) M+2/M+4 (5.8±1.1 vs 5.0±1.3 in fed vs 48 hr fasted respectively) and M+3/M+4 (1.3±0.7 vs 1.6±0.5 in fed vs 48 hr fasted respectively) ratios were similar in both groups suggesting no differences in the glycolytic ATP contribution.

$^{13}$C NMR glutamate turnover study

Observation of the $^{13}$C label turnover in the NMR detectable glutamate pool in skeletal muscle was necessary for the TCA cycle flux calculation. Therefore, $^1$H decoupled $-^{13}$C NMR spectroscopy was performed in the following manner: an initial frequency selective sinc pulse (20 ms) set on the low field side of the methylene carbon of lipids at 30 ppm was immediately followed by a non-selective hard pulse (approximately 70° flip angle, 5 mm from surface coil). The sinc pulse power was adjusted to eliminate most of the signal in that region. This method improved the
baseline subtraction of lipid peaks between 22-30 ppm. Broadband $^1$H Waltz-16 decoupling was applied during acquisition, and additional NOE was achieved using low power decoupling (0.4 W) during the relaxation delay (TR=0.5 s, NS=1800, SW=20 KHz, 4K data). A 15 min baseline spectrum was followed by subsequent 15 min acquisitions throughout the duration of the experiment.

**TCA cycle flux ($V_{tca}$) calculation**

Metabolic steady state equations were derived for isotopic mass flow into the TCA cycle as previously described in brain (25,26), heart (27,28) and liver (29). Yale University CWave software (Dr. Graeme F. Mason) was used to calculate the TCA cycle fluxes. The mathematical modeling was based on non-linear least squares fitting of the calculated parameters (e.g. 4- and 2-$^{13}$C citrate, $\alpha$-ketoglutarate, glutamate, etc.) from the set of isotopic mass balance equations describing the label flow through the TCA cycle to the acquired NMR data using a Runge-Kutta algorithm with an adaptive step size.

From the labeled mass flow schematic shown in Fig. 1, we obtain the following isotopic mass balance equations which were used in the analysis:
\[
\frac{\partial C2AcCoA}{\partial t} = C3Pyr_{pdh} \frac{C2Acet_{acet}}{Acet} - C2AcCoA_{tca} \\
\frac{\partial C4Cit}{\partial t} = \left( \frac{C2AcCoA}{AcCoA} - \frac{C4Cit}{Cit} \right) V_{tca} \\
\frac{\partial C4\alpha-KG}{\partial t} = \frac{C4Cit}{Cit} - \frac{C4\alpha-KG}{\alpha-KG} \left( V_{tca} + V_{gln} \right) \\
\frac{\partial C4Glu}{\partial t} = \frac{C4\alpha-KG}{\alpha-KG} V_{gln} - \frac{C4Glu}{Glu} \left( V_{gln} + V_{gln}^{-1} \right) \\
\frac{\partial C3Oaa}{\partial t} = 0.5 \times \frac{C4\alpha-KG}{\alpha-KG} V_{tca} + 0.5 \times \frac{C3\alpha-KG}{\alpha-KG} V_{tca} + \frac{C3Asp}{Asp} V_{asp}^{-1} + \frac{C3Pyr}{Pyr} V_{pc} - \frac{C3Oaa}{Oaa} \left( V_{asp} + V_{tca} + V_{Oaa} \right) \\
\frac{\partial C2Cit}{\partial t} = \left( \frac{C3Oaa}{Oaa} - \frac{C2Cit}{Cit} \right) V_{tca} \\
\frac{\partial C2\alpha-KG}{\partial t} = \frac{C2Cit}{Cit} V_{tca} - \frac{C2Asp}{Asp} V_{asp}^{-1} - \frac{C2Glu}{Glu} \left( V_{tca} + V_{gln} \right) \\
\frac{\partial C2Glu}{\partial t} = \frac{C2\alpha-KG}{\alpha-KG} V_{gln} - \frac{C2Glu}{Glu} \left( V_{gln} + V_{gln}^{-1} \right)
\]

where:
AcCoA=acetyl CoA; Pyr=pyruvate; Acet=acetate; Cit=citrate; \(\alpha\)-KG=\(\alpha\)-ketoglutarate; Glu=glutamate; Gln=glutamine; Oaa=oxaloacetate; Asp=aspartate; and the C2, C3, or C4 prefix refers to \(^{13}\)C label at the respective carbon isotope positions. \(V_{pdh}\)=pyruvate dehydrogenase flux; \(V_{acet}\)=acetate thiokinase flux; \(V_{tca}\)=tri-carboxylic acid cycle flux; \(V_{gln}\), \(V_{gln}^{-1}\)=aminotransferase and glutamate dehydrogenase flux; \(V_{asp}\)=aspartate aminotransferase flux; \(V_{Oaa}\)=efflux from oxaloacetate (necessary to maintain steady state).

The \(\alpha\)-KG=Glu exchange via glutamate dehydrogenase and/or aminotransferase reaction \((V_{gln}, V_{gln}^{-1})\) is rapid with respect to \(V_{tca}\) in the brain (26), but has been shown to be significantly slower in heart (27,28). Therefore, it was necessary to include both 2- and 4-\(^{13}\)C glutamate turnover data in the mathematical analysis to discriminate between \(V_{tca}\) and \(V_{gln}\). The glutamate pool concentration was determined in tissue extracts for use in the mathematical analysis while all other
intermediate pool concentrations were taken from literature (27,30). In the model, 2-\textsuperscript{13}C acetyl CoA pool turnover was described as rapid and set equal to the 2-\textsuperscript{13}C acetate precursor pool turnover which was measured in plasma and tissue extracts. To test this assumption (i.e. 2-\textsuperscript{13}C acetate turnover=2-\textsuperscript{13}C acetyl CoA turnover), we used a non-steady state isotopic analysis of glutamate labeling derived from 2-\textsuperscript{13}C acetate measured in rats at 15, 30, 60, and 150 min. The rapid turnover of 2-\textsuperscript{13}C acetyl CoA enrichment (58.7±0.3, 59.4±2.6, 53.6±0.6, and 56.3±5.0 at respective time points) suggests that this assumption is valid.

**Determination of The Capacity for UCP3 to Function as a FFA Translocator**

It has been shown that acyl CoA synthetase can function to a low degree in the mitochondrial matrix (31). Therefore we measured the capacity for UCP3 to function as a FFA translocator to increase β-oxidation in a separate group of rats. We measured relative rates of U-\textsuperscript{13}C palmitate oxidation in skeletal muscle in vivo with (+) and without (-) CPT1 inhibition by etomoxir in the fed and 48 hr fasted groups. These measurements were performed in the fed rats to determine the relative decrease in palmitate oxidation resulting from CPT1 inhibition. The control value (% decrease in palmitate oxidation in the fed group) was compared to the values obtained from the 48 hr fasted group to determine whether or not UCP3 functions in part as a FFA translocator (i.e. % decrease is less than control % decrease).

In the basal group [(-) etomoxir], a constant infusion (0.935 \(\mu\)mol/kg/min) of \textsuperscript{13}C palmitate (Cambridge Isotope Laboratories, Andover, MA) bound to 12% bovine albumin (Sigma, St. Louis, MO) was administered for 120 min. In the etomoxir group, etomoxir (10 \(\mu\)mol/kg, gift from Bristol-Myers Squibb) in 100\(\mu\)l sterile water was administered 60 min prior to U-\textsuperscript{13}C palmitate infusion (32). A bolus administration (25 mg/kg) of nicotinic acid (Sigma) was given every hour
to maintain basal FFA concentrations (33). After 120 min, hindlimb muscles were freeze clamped in situ and glutamate enrichments (index of relative palmitate oxidation) were measured in tissue extract using gas chromatography-mass spectrometry (34). The measured M+2 glutamate enrichments (~0.5-2%) were significantly greater than the 0.02% background enrichment.

**Analytical Procedures**

Muscle tissue extracts were prepared for high field NMR analysis by homogenizing approximately 1 g of combined quadriceps and gastrocnemius muscle as previously described (35).

Glutamate (34) and acetate (36) $^{13}$C enrichments in plasma and/or skeletal muscle were determined using a Hewlett-Packard 5890 gas chromatography (HP-1 capillary column, 12 m x 0.2 mm x 0.33 mm film thickness) interfaced to a Hewlett-Packard 5971A mass selective detector operating in the electron impact ionization mode.

The $P_i$ concentration was extrapolated from the baseline NMR spectrum (comparing peak integrals from $P_i$ and ($\gamma$-ATP) and measured ATP concentration (ATP assay kit #366, Sigma-modified for tissue analysis). Plasma free fatty acids were determined using an acyl CoA oxidase based colorimetric kit (WAKO NEFA-C, WAKO Pure Chemical Industries, Osaka, Japan). Tissue extract glutamate concentration was determined using a 2300 STAT PLUS biochemical analyzer (Yellow Springs Instrument Co., Yellow Springs, OH).

**UCP3 mRNA and protein measurements:**

*Mitochondria Isolation*: Skeletal muscles were homogenized in the buffer containing 0.1 M KCl, 0.05 M Tris-HCl pH 7.4, 0.005 M MgCl$_2$, 0.001 M EDTA and freshly added protease inhibitor cocktail (Boehringer Mannhein, Indianapolis, IN). After brief homogenization, samples
were spun at 650g for 10 min. Supernatant was collected and transferred to a new tube and spun at 14,000g for 10 min. Pellets were collected and resuspended in 0.15 M KCl.

Northern Blot Analysis: Total RNA (20 µg) was electrophoresed in a 1.5% agarose gel containing formaldehyde, as described by Lehrach et al. (37) and transferred to hybond nylon membranes (Amershan Life Science) by capillary blotting. Probes were labeled by random priming with [α-32P]dCTP (New England Nuclear Life Science, Boston, MA) to a specific radioactivity of approximately 2x10^8 cpm/µg DNA. RNA blots were hybridized in hybridization buffer containing 50% formamide, 0.25 M Na_2HPO_4, 0.25 M NaCl and 1 mM EDTA at 65°C overnight, then washed in a solution containing 0.25 M Na_2HPO_4, 0.5% SDS and 1 mM EDTA at 65°C for 20 min. Blots were exposed to scientific imaging film (NEN Life Science) at –80°C with intensifying screens. The signals on the membrane were quantified by instant imager (Canberra Company, Meriden, CT).

Western Blot Analysis: Mitochondrial protein (25 µg) was loaded on SDS-PAGE (4-15% Tris-HCl gradient gel, Bio-Rad), and transferred to nitrocellulose (Bio-Rad) at 100 V for 1 hr. Nitrocellulose was blocked in a buffer containing 5% FBS in 1×TBS and 0.2% Tween 20 for 1 hr at room temperature. The membrane was incubated with rabbit anti-human UCP3 antiserum (Alpha Diagnostic International, San Antonio, TX) at room temperature for 1 hr. A 14 amino acid peptide sequence mapping near the C-terminus of human UCP3 was used to generate anti-UCP3. The peptide was coupled with KLH carrier protein and antibodies generated in rabbits. The control peptide has been used to affinity purify the antibodies. This peptide has no significant homology with UCP1 or UCP2, and its sequence shows a homology of 93% with the mouse UCP3. The membrane was washed in 0.2% Tween 20 and 1×TBS for 10 min. After wash, the membrane was
incubated with goat anti-rabbit IgG-HRP conjugate for 1 h at room temperature. The signal was detected by exposing the membrane with ECL on Hyperfilm ECL film (Amersham, Piscataway, NJ) and quantified by densitometry scanning.

**Statistical Analysis**

All data are reported as the mean±SEM. A Student’s $t$ test analysis was performed on data to determine significance at a minimum $p<0.05$ threshold between groups.
Results

UCP3 mRNA expression and protein levels increased approximately 1.7 and 2.9 fold respectively following a 48 hr fast as illustrated in Fig. 2 (p< 0.01 and p<0.001 respectively). The increase in UCP3 was associated with a significant increase in basal plasma FFA in the 48 hr fasted group (1.18±0.11 versus 0.63±0.07 mM in the 48 hr fasted and fed groups respectively, p< 0.001).

A baseline subtracted $^{13}$C NMR spectrum of the awake rat hindlimb at 120 min may be seen in Fig. 3. The $^{13}$C label turnover of 4-$^{13}$C glutamate followed by slower turnover of 2-$^{13}$C glutamate may be seen in the inset. The 4- and 2-$^{13}$C glutamate signals appeared at 34.4 and 55.5 ppm respectively in the first spectrum, and increased until steady state signal intensities were achieved (~120 min). Labeling of 4-$^{13}$C glutamate was the result of 2-$^{13}$C acetyl CoA condensing with oxaloacetate to produce 4-$^{13}$C citrate which in turn labeled $\alpha$-ketoglutarate and glutamate at the C4 position (Fig. 1). The 2-$^{13}$C glutamate peak appears as a result of additional turns of the TCA cycle which allows for scrambling the C4 label of glutamate to 2- and 4-$^{13}$C glutamate (27.9 ppm) (Fig. 1). The 3-$^{13}$C glutamate and 2-$^{13}$C acetate (24.2 ppm) peaks were not observable, because they reside in the frequency bandwidth which was partially suppressed by the aliphatic lipid suppression pulse sequence.

The NMR derived 2- and 4-$^{13}$C glutamate turnover data from the fed and 48 hr fasted groups is shown in Fig. 4. The curves represent the best fits of the data to the mathematical model. The actual flux through the TCA cycle strongly depends on both the time course of the 2-$^{13}$C acetate plasma enrichment (Fig. 4) and on the tissue glutamate pool concentration. There were no
differences in the glutamate pool size following acetate administration in the fed vs 48 hr fasted groups (2.42±0.22 and 2.43±0.34 µmol/g respectively), although there was a slight increase (~0.6 µmol/g) in glutamate pool size in the 48 hr fasted group from basal levels. Although there were no differences in the calculated TCA cycle flux in the fed and 48 hr fasted groups respectively (212±23 and 173±25 nmol/g/min, Fig. 5a), there were differences in metabolism between the groups as reflected by the differences in substrate enrichments (Fig. 4). There was less endogenous acetate production (166±6 vs 218±13 µmol/kg/min, p<0.05) as reflected by a higher 2-13C acetate enrichment in the 48 hr fasted versus control group (Fig. 4). Additionally, there appeared to be increased anaplerosis (0.33±0.03% vs 0.12±0.04% of the TCA cycle flux, p<0.01) as reflected by the lower 2-13C glutamate enrichment with respect to 4-13C glutamate enrichment in the 48 hr fasted versus control group (possibly due to increased amino acid catabolism).

A 31P NMR saturation transfer experiment was performed to determine the kinetics of ATP synthesis. The set of spectra shown in Fig. 6 are the result of a saturation transfer experiment performed in the hindlimb muscles of an awake rat. In the bottom spectrum, a continuous wave (CW) radio frequency pulse was used to saturate the γ-ATP resonance (-2.4 ppm). In the top spectrum (no γ-ATP saturation), the CW pulse frequency was placed symmetrically to the downfield side of Pi (4.9 ppm). The resulting loss in magnetization of Pi (ΔM) is due to the exchange of saturated γ-ATP nuclei with non-saturated Pi nuclei. The fractional change in Pi (ΔM/M₀) and the observed spin-lattice longitudinal relaxation constant (T₁obs) of Pi together were used to calculate the unidirectional ATP synthesis rate constant (k) (Table 1). The unidirectional ATP synthesis flux was
then calculated as \( k \times [\text{Pi}] \) (Table 1). There were no differences in unidirectional ATP synthesis flux in the 48 hr fasted (57±9 nmol/g/s) versus control group (79±15 nmol/g/s) (Fig. 5b).

The TCA cycle generates reducing equivalents (NADH, FADH\(_2\)) which are necessary for mitochondrial respiration. Since the TCA cycle activity is coupled to \( \text{O}_2 \) consumption via a stoichiometric relationship, the flux through the TCA cycle may be used as an index of substrate oxidation at steady state. Therefore, the ratio of the measured unidirectional ATP synthesis flux to TCA cycle flux may be used as a qualitative index of the degree of coupling between mitochondrial substrate oxidation and ATP synthesis. Since the extent of basal mitochondrial uncoupling present as a result of combined proton transport and leaks across the inner mitochondrial membrane is unknown, this ratio was normalized to the fed group. When analyzed in this manner, there were no differences in mitochondrial energy coupling between fed and fasted groups (Fig. 5c).

To assess the potential for UCP3 to function as a FFA translocator, U-\(^{13}\)C palmitate oxidation was measured in skeletal muscle in fed and 48 hr fasted groups with or without etomoxir. The efficacy of etomoxir on inhibiting palmitate oxidation in skeletal muscle was measured in the fed group as a ratio of M+2 glutamate with etomoxir versus without etomoxir \([(+) \text{ etomoxir}/(-) \text{ etomoxir}]\) (Table 2). The degree to which UCP3 may function as a FFA translocator would be manifested as an increase in this ratio. Although the M+2 enrichments were higher in 48 hr fasted versus fed group reflecting increased palmitate oxidation in this group, the ratios were equal (0.79±0.14 vs 0.75±0.08, p=NS) in the fed and fasted groups respectively. These results suggest that UCP3 does not have a major role as a FFA translocator following a 48 hr fast.
Discussion

It has been shown by a number of investigators that UCP3 mRNA expression is upregulated following a fast (4,11-16). This is surprising considering the basal metabolic rate should be reduced during conditions of starvation (38). Despite an ~3 fold increase in UCP3 protein following a 48 hr fast in the present study, there was no detectable change in skeletal muscle mitochondrial energy coupling as assessed by the $^{13}\text{C}^{31}\text{P}$ NMR technique. These results are in contrast with the ~60% and ~80% reduction in skeletal muscle mitochondrial energy coupling we recently observed in T3 and dinitrophenol treated rats respectively (23).

UCP3 mRNA expression, which to date, has been used as an index of mitochondrial uncoupling and thermogenesis in vivo possibly does not correlate with ‘true’ mitochondrial uncoupling. Although UCP3 protein measurements generally have not been documented in the past, we were able to measure a significant increase in UCP3 protein which correlated with an increase in mRNA following a 48 hr fast. Unfortunately, measurements of membrane bound protein content are not necessarily commensurate with function due to the allosteric nature of these proteins. In a study by Boss et al. (11), in vitro heat production was measured in mice soleus muscle following a 24 hr fast using microcalorimetry measurements in perifused muscle preparations. They showed that under basal conditions, there were no differences in heat production between control and fasted groups. However when carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), a chemical uncoupler, was administered, the increase in heat production in the fasted group was blunted versus the control group. These results suggest that the fasted group may function at a lower degree of
mitochondrial coupling. Although more recently, it was shown that mitochondrial proton conductance remained unchanged in rat hindlimb skeletal muscle following a 24 hr fast (16).

There are several lines of evidence which suggest that UCP3 may not function in a regulatory thermogenic capacity. It has been shown by Beinengraeber et al. (39) that simultaneous mutation of histidine residues H145 and H147 results in loss of the proton transport capacity of UCP1, and both of these residues are absent in mouse and rat UCP3. In humans with a splice mutation that results in removal of the terminal coding exon of UCP3, no changes in muscle mitochondrial coupling activity or systemic oxygen consumption was present (40). Additionally, under conditions of thermoneutrality where there is a reduced need for regulatory thermogenesis, UCP3 mRNA expression remained elevated in skeletal muscle of 24 hr (11) or 48 hr (17) fasted versus control rodents. UCP3 mRNA expression in skeletal muscle tends to oppose that of UCP1, a known thermoregulator, in brown adipose tissue (BAT) under certain conditions such as cold exposure (11,41) or fasting (4) during which energy sparing is a necessary physiological response. Recently it was shown that although skeletal muscle mitochondria appeared to be more tightly coupled in UCP3 knockout mice, there were no apparent differences in physiological phenotypes when compared with wildtype mice (42,43).

It has also been hypothesized that circulating FFA is an important mediator for UCP3 expression due to the strong relationship between perturbations where UCP3 expression is increased [i.e. thyroid hormone (4,6), glucocorticoid (4), β-adrenoreceptor agonists (4,10), triglyceride emulsion (15), fasting (4,11-16)] and increases in circulating plasma FFA concentration. Weigle et
al. (15) have shown that an increase in systemic FFA stimulated UCP3 expression in muscle. Additional studies supporting this relationship using either nicotinic acid to reduce or etomoxir to increase plasma FFA concentrations have also demonstrated a decrease (14) or increase (44) in rat soleus UCP3 expression respectively. Under these conditions, the uncoupling protein may function as a FFA carrier protein explicitly which would be necessary to maintain elevated levels of lipid oxidation especially if the acylcarnitine translocator cannot maintain the FFA levels necessary to drive β-oxidation in the mitochondrial matrix. This theory would require that FFA become acylated in the matrix, and there is evidence that acyl CoA synthetase can function to a low degree in the mitochondrial matrix (31). To test this hypothesis we measured relative rates of U-13C palmitate oxidation in skeletal muscle in vivo with (+) and without (-) CPT1 inhibition by etomoxir in the fed and 48 hr fasted groups. These measurements were performed in the fed rats to determine the relative decrease in palmitate oxidation resulting from CPT1 inhibition. The control value (% decrease in palmitate oxidation in the fed group) was compared to the values obtained from the 48 hr fasted group to determine whether or not UCP3 functions in part as a FFA translocator (i.e. % decrease is less than control % decrease). As our data demonstrate (Table 2), there was no difference in palmitate oxidation in the 48 hr fasted versus fed rats when etomoxir was administered suggesting UCP3 in skeletal muscle does not function as a long chain FFA transporter under conditions of fasting. Potentially UCP3 functions directly as an acyl-carnitine translocator. However, due to the low homology (~20% similarity to acyl-carnitine carrier protein) this scenario also remains unlikely.
It is also possible that UCP3 functions as a regulator of mitochondrial reactive oxygen species (ROS) scavenging (43,45). This may be true especially during conditions of elevated concentrations of FFA which have been shown to promote ROS formation in mitochondria as well as in peroxisomes (46). ROS formation is prevalent under conditions of oxidative stress when reduced electron flow down the respiratory chain results in increased $O_2$ reduction. Following a fast, ADP levels are low in the cell (state 4) during which oxidative stress in the cell might be expected to be increased. Therefore, the role of UCP3 may be to increase uncoupling and commensurate $O_2$ consumption even during state 4 conditions thereby minimizing ROS production. Possibly, the UCP3 protein which is an anion transporter functions to directly transport the free radicals out of the matrix and thereby minimize lipid peroxidation in the membrane bilayer. Superoxide anions or hydrogen peroxide formation as an index of ROS production has previously been measured in a variety of tissues and correlated with UCP3 (43) or UCP2 (47,48) expression respectively. These studies which showed an inverse relationship between UCP expression and ROS accumulation provide an intriguing link between uncoupling proteins and a physiological role in ROS scavenging. This may also be a function of the recently cloned UCP4 protein in brain (49).

It must be emphasized that although electrochemical potential measurements have been made on all three mitochondrial uncoupling isoforms via transfecting yeast, plasmids or myotubes these measurements have not been made in explicit tissues of interest in situ. Additionally, although electric potential measurements across the inner mitochondrial membrane provide an index for potential energy uncoupling, they are in no way a measure of functional energy uncoupling. This
is because the inner membrane electric potential is not only controlled by proton conductance, but also by substrate oxidation and ATP synthesis. Currently the best method to assess proton conductance is to measure its kinetics indirectly (22,50). This entails using oligomycin which is a high affinity inhibitor of the F1F0 ATPase. The oxygen consumption measured using oligomycin drives the proton leak, but these measurements must be made in isolated mitochondria. Therefore, a limitation to using this *in vitro* method to measure proton conductance is that it may be difficult to establish an environment similar to that *in vivo* with regards to allosteric effectors and inhibitors.

The novel non-invasive NMR approach we used in the present study circumvents the limitations of these *in vitro* measurements of mitochondrial energy coupling.

In summary, despite a significant increase in both UCP3 mRNA expression and protein content in the hindlimb muscles of 48 hr fasted rats, we did not observe a concomitant decrease in mitochondrial energy coupling as assessed by $^{13}$C/$^{31}$P NMR. These measurements correlate with the lack of an increase in whole body thermogenesis previously reported in fasting rats (51). However, it is possible that the presence or absence of some critical co-regulator is required for UCP3 to function as a mitochondrial uncoupler in the fasted state. It is also possible UCP3 may be functioning as a mitochondrial energy uncoupler while the transient proton leak across the inner mitochondrial membrane is reduced or some other unknown mechanism to increase mitochondrial energy coupling efficiency is present. Additionally, it appears that UCP3 does not play a role in the translocation of long chain fatty acids across the inner mitochondrial membrane following a 48 hr
fast. Therefore, the role of increased UCP3 expression in skeletal muscle under fasting conditions remains to be elucidated.
Acknowledgments

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References


**Abbreviations:**

UCP, uncoupling protein; TCA, tri-carboxylic acid; FFA, free fatty acid; NMR, nuclear magnetic resonance; ATP\textsubscript{ase}, ATP synthase; P\textsubscript{i}, inorganic phosphate; CPT1, carnitine palmitoyl-transferase I; BAT, brown adipose tissue
Figure Legends

1) Intramuscular labeling schematic. 2-13C acetate was used as the precursor. Labeling of 4-13C glutamate/glutamine occurs as a result of label entering the tri-carboxylic acid (TCA) cycle via condensation of 2-13C acetyl CoA with oxaloacetate (Oaa). With multiple turns of the TCA cycle, label will appear at C2, C3, and C4 isotopomer positions of glutamate and glutamine (glu/gln) and C2, C3 isotopomer positions of Oaa and malate (Mal). The labeled intermediates and associated fluxes were used to develop the isotopic steady state mathematical model for calculating the TCA cycle flux ($V_{tca}$).

2) UCP3 mRNA and protein measurements. (A, B), The Northern blot analysis for UCP3 mRNA in gastrocnemius muscle reflects a 1.7 fold increase in expression following a 48 hr fast versus fed rats. (C, D), The Western blot analysis for UCP3 protein in skeletal muscle reflects a 2.9 fold increase in UCP3 content following a 48 hr fast versus fed rats. Each lane contains RNA or protein from a single rat. Data are presented as mean±SEM.

3) A spectrum acquired from the rat hindlimb at 135-150 min. The 2-13C glutamate peak appears at 55.5 ppm on the shoulder of the creatine/phosphocreatine peak (54.4 ppm), and the 4-13C glutamate peak obscured by a co-resonating aliphatic lipid peak appears at 34.4 ppm. Therefore, all spectra were baseline subtracted before peak integration. 3-13C glutamate (27.9 ppm) and 2-13C acetate (24.2 ppm) were not observed as they reside in the frequency bandwidth which was partially suppressed due to the aliphatic lipid suppression pulse sequence used. Turnover of the 2- and 4-13C glutamate peaks in the hindlimb muscles of a fed rat is illustrated in the baseline subtracted spectra shown above. 2-13C acetate label
incorporates rapidly into 4-$^{13}$C glutamate, and more slowly into 2-$^{13}$C glutamate (2$^{\text{nd}}$ turn of the TCA cycle).

4) $^{13}$C enrichment time course of plasma 2-$^{13}$C acetate ($!$), and tissue 4-$^{13}$C ($#$) and 2-$^{13}$C ($Q$) glutamate in the fed (upper panel) and 48 hr fasted (bottom panel) groups. The least squares best fits of the 2- and 4-$^{13}$C glutamate data to the mathematical model are represented by the stippled and solid lines respectively.

5) Mitochondrial energy coupling measurements. (A-C), Data obtained from $^{13}$C and $^{31}$P NMR experiments in fed and 48 hr fasted rats. (A, B), The calculated TCA cycle flux and unidirectional ATP synthesis flux respectively. (C), Degree of mitochondrial energy coupling expressed as a ratio of ATP synthesis flux to TCA cycle flux normalized to the fed group. Data are presented as mean±SEM. NS; not significant.

6) $^{31}$P NMR saturation transfer study. The spectra shown were obtained from a saturation transfer experiment performed in the hindlimb muscles of an awake rat. In the bottom spectrum, a continuous wave (CW) radio frequency pulse was used to saturate the $\gamma$-ATP resonance (-2.4 ppm). In the top spectrum (no $\gamma$-ATP saturation), the CW pulse frequency was placed symmetrically to the downfield side of $P_1$ (4.9 ppm). The resulting loss in magnetization of $P_1$ ($\Delta M$) is due to the exchange of saturated $\gamma$-ATP nuclei with non-saturated $P_1$ nuclei.
Table 1

<table>
<thead>
<tr>
<th></th>
<th>$\Delta M/M_0$</th>
<th>$T_{1\text{obs}}$ (s)</th>
<th>$k$ (s$^{-1}$)</th>
<th>$P_i$ (µmol/g)</th>
<th>ATP synthesis flux (nmol/g/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FED</td>
<td>0.29±0.05</td>
<td>2.27±0.29</td>
<td>0.15±0.02</td>
<td>0.59±0.07</td>
<td>79±9</td>
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<tr>
<td>48 hr FASTED</td>
<td>0.41±0.07</td>
<td>2.49±0.31</td>
<td>0.18±0.04</td>
<td>0.38±0.07$^*$</td>
<td>57±15</td>
</tr>
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</table>

ATP synthesis flux data. $\Delta M/M_0$ is the fractional change in $P_i$ magnetization as a result of saturation transfer. $T_{1\text{obs}}$ is the observed spin-lattice relaxation time of $P_i$ during $\gamma$-ATP saturation in seconds. $k$ is the rate constant (s$^{-1}$). The ATP synthesis flux is calculated as $P_i \times k$. Data are presented as mean±SEM. $^*$ p<0.05 vs fed group.
Table 2

<table>
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<tr>
<th></th>
<th>Glutamate M+2 (+) etomoxir (APE)</th>
<th>Glutamate M+2 (-) etomoxir (APE)</th>
<th>Glutamate M+2 [(+) etomoxir/(-) etomoxir]</th>
</tr>
</thead>
<tbody>
<tr>
<td>FED</td>
<td>0.92±0.12</td>
<td>1.17±0.28</td>
<td>0.79±0.14</td>
</tr>
<tr>
<td>48 hr FASTED</td>
<td>1.46±0.17*</td>
<td>1.95±0.21** †</td>
<td>0.75±0.08</td>
</tr>
</tbody>
</table>

M+2 glutamate enrichments (APE; atom percent enrichment). Fed and 48 hr fasted rats were administered U-\textsuperscript{13}C palmitate with (+) etomoxir or without (-) etomoxir to assess the potential for UCP3 to function as a FFA translocator. Data are presented as mean±SEM. * p<0.01 vs fed group; ** p<0.03 vs fed group; † p<0.05 vs (+) etomoxir group.
Fig. 1

The diagram represents the metabolic pathway involving various intermediates labeled with 13C isotopes. The pathway includes the following steps and intermediates:

1. **2-13C Acetate**
2. **2 or 3-13C Pyr** to **2-13C AcCoA** via **V$_{scf}$** and **V$_{ph}$ (3-13C Pyr)**
3. **2 or 3-13C Asp** to **2 or 3-13C Oaa** via **V$_{asp}$** and **V$_{Daa}$**
4. **4-13C Glu** and **4-13C Gln**
5. **4-13C Cit**
6. **2 or 3-13C Cit**
7. **4-13C Glu** and **4-13C Gln**
8. **2-13C Cit**
9. **2 or 3-13C Glu**

The pathway involves the tricarboxylic acid (TCA) cycle, with the 2nd turn starting from **2 or 3-13C Glu**.
Fig. 2

A. UCP3 mRNA levels in fed and fasted states.

B. Muscle UCP3 mRNA levels in fed and 48 hr fasted states, with a significant difference (P<0.01).

C. UCP3 protein levels in fed and fasted states.

D. Muscle UCP3 protein levels in fed and 48 hr fasted states, with a highly significant difference (P<0.001).
Fig. 5

A

TCA cycle flux

![Bar chart showing TCA cycle flux comparison between FED and 48 hr FASTED conditions.]

B

ATP synthesis flux

![Bar chart showing ATP synthesis flux comparison between FED and 48 hr FASTED conditions.]

C

mitochondrial energy coupling (ATP synthesis flux/TCA cycle flux)

![Bar chart showing mitochondrial energy coupling comparison between FED and 48 hr FASTED conditions.]
Fig. 6
13C/31P NMR Assessment of Mitochondrial Energy Coupling in Skeletal Muscle of Awake Fed and Fasted Rats: Relationship with Uncoupling Protein 3 Expression
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