Lack of Obesity and Normal Response to Fasting and Thyroid Hormone in Mice Lacking Uncoupling Protein-3

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Un coupling protein-3 (UCP3) is a mitochondrial protein that can diminish the mitochondrial membrane potential. Levels of muscle Ucp3 mRNA are increased by thyroid hormone and fasting. Ucp3 has been proposed to influence metabolic efficiency and is a candidate obesity gene. We have produced a Ucp3 knockout mouse to test these hypotheses. The Ucp3 (−/−) mice had no detectable immunoreactive UCP3 by Western blotting. In mitochondria from the knockout mice, proton leak was greatly reduced in muscle, minimally reduced in brown fat, and not reduced at all in liver. These data suggest that UCP3 accounts for much of the proton leak in skeletal muscle. Despite the lack of UCP3, no consistent phenotypic abnormality was observed. The knockout mice were not obese and had normal serum insulin, triglyceride, and leptin levels, with a tendency toward reduced free fatty acids and glucose. Knockout mice showed a normal circadian rhythm in body temperature and motor activity and had normal body temperature responses to fasting, stress, thyroid hormone, and cold exposure. The base-line metabolic rate and respiratory exchange ratio were the same in knockout and control mice, as were the effects of fasting, a β3-adrenergic agonist (CL316243), and thyroid hormone on these parameters. The phenotype of Ucp1/−/Ucp3 double knockout mice was indistinguishable from Ucp1 single knockout mice. These data suggest that Ucp3 is not a major determinant of metabolic rate but, rather, has other functions.

Human obesity is the result of energy intake greater than metabolic expenditure and is increasing in incidence (1). On an evolutionary time scale, obesity is a recent development, attributed to the interaction of predisposing genetic backgrounds with a sedentary lifestyle and an abundance of food (2, 3). Little is known about the molecular mechanisms and genes that contribute to the regulation of metabolic rate. For example, metabolic efficiency decreases with increased food intake, and it increases with lowered food intake (4), but the mechanistic details are unknown.

The discovery of uncoupling protein (UCP, 1 now named UCP1) illustrated one way to regulate metabolic efficiency. UCP1 uncouples oxidative phosphorylation by allowing leakage of protons into the mitochondrial matrix without the phosphorylation of ADP (5). Heat is released because UCP1 degrades the proton gradient energy without storing it chemically or using it to perform physical work. At the whole-body level, this shows up as metabolic inefficiency. UCP1 is expressed only in brown adipose tissue (BAT), which is a major heat-producing tissue in small mammals. In addition to cold-induced thermogenesis, BAT and UCP1 have been implicated in diet-induced thermogenesis, the increased energy expenditure that accompanies increased food intake (6). Activation of BAT and increased expression of Ucp1 cause reduced adiposity (7–9). However, BAT is present in only small amounts in large mammals, so its role in regulating energy homeostasis in adult humans is problematic (10).

Interest in UCPs increased with the discovery of proteins similar to UCP1, including UCP2 (11, 12), UCP3 (13–15), BMCP1 (16), and UCP4 (17). These proteins are expressed in tissues besides BAT and, thus, are candidates to influence energy efficiency and expenditure. UCP3 is particularly interesting since muscle Ucp3 mRNA levels increase in response to thyroid hormone, as expected for a mediator of thyroid thermogenesis (13, 18, 19). UCP3 has also been shown to decrease mitochondrial membrane potential (13, 20) and to be capable of electrophotorectic flux of protons and alkylsulfonates (13, 20), consistent with UCP3 functioning as an uncoupling protein. A confounding observation is that muscle Ucp3 mRNA levels increase with fasting (13, 21, 22), which is not expected since energy expenditure is reduced with fasting.

Since metabolic rate, metabolic efficiency, and obesity are integrated properties of the whole animal, we have produced mice lacking UCP3. The knockout mice have less muscle mitochondrial proton leak, demonstrating that UCP3 contributes to leak. Surprisingly, the Ucp3 (−/−) mice are not obese and have normal thyroid thermogenesis.

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¶¶ The abbreviations used are: UCP, uncoupling protein; BAT, brown adipose tissue; FFA, free fatty acids; ERR, respiratory exchange ratio; Tb, core body temperature; Δp, protonmotive force; ROS, reactive oxygen species; kb, kilobase(s); PCR, polymerase chain reaction; nt, nucleotide(s); TPMP+, methyltriphenylphosphonium.
Ucp3 Knockout Mice

**Materials and Methods***

**Targeting Construct—** A Ucp3 genomic clone was isolated from a bacterial artificial chromosome 129/SvJ library (23). The targeting vector (p2199) was constructed by subcloning (with appropriate modification of DNA ends) a 4.5-kb HindIII fragment including Ucp3 exon 1 into the NotI/XhoI sites of the pLOXneo vector (24) and a 1.4-kb EcoRI fragment downstream of exon 2 into the SalI/BamHI sites.

**Generation of Recombinant Mice—** The targeting vector was linearized with NotI and electroporated into TC1 (129/Sv/c57) embryonic stem cells (25). The transformants were selected with G418 and 1-2-deoxy-2-fluoro-D-glucose-arabinofuranosyluracil)-5-idouracil. To identify homologous recombinants, genomic embryonic stem cell DNA was Southern-blotted and probed with a 3′-flanking probe and an internal probe. The 3′ probe is a 6.5-kb EcoRI/BamHI fragment containing intron 2. PCR was performed at 94 °C, 30 s; 56 °C, 30 s; and 72 °C, 30 s for 30 cycles, followed by extension at 72 °C, 7 min. The intron 2 probe was PCR amplified using primers x615 (5′-TTGGCGGT-TCTCAGCAACTC-3′) and x658 5′-TAAGGG-3′ (sense Ucp3 exon 2), and x658 5′-CTATAGCGCAAGGAAC-3′ (sense Ucp3 exon 2), and x658 5′-CTATAGCGCAAGGAAC-3′ (antisense Ucp 3 intron 2). PCR was performed at 94 °C, 30 s; 56 °C, 30 s; and 72 °C, 30 s for 30 cycles, followed by extension at 72 °C, 7 min. The product size from the wild type allele is 460 base pairs and from the recombinant is 490 base pairs. Mice were maintained on a 12-hour light/dark cycle (6:00 p.m. and 6:00 a.m.) and standard pellet diet (NIEH-47; 55% fat by weight) and housed at 21–24 °C (unless noted otherwise). Ucp1 knockout mice have been described previously (26).

**RNA Analysis—** RNA was extracted using TRIzol (Life Technologies, Inc). Northern blots (maximum strength Nyttran Plus; Schleicher & Schuell) were hybridized using Radio-hyb (Amersham Pharmacia Biotech) and quantitated with a PhosphorImager (Molecular Dynamics). Quantity of PCR products was confirmed by gel electrophoresis. Probes were used rat Ucp1 (at 81–1154 in GenBank™ M11814), mouse Ucp2 (EcoRI/NofI fragment of IMAGE ID 570531 (at 238–1491 in U69135)), mouse Ucp3 probe (at 1–723 in AF053352) (13), and mouse Ucp3 exon 2-specific probe (at 95–283 in AF053352 PCR with primers 7975 5′-AGCAAAGAATGTCAGGGCAGG-3′ and 627 5′-TTGGCGGT-TCTCAGCAACTC-3′). RT-PCR, first-strand cDNA was made with murine mammary tumor virus reverse transcriptase (CLONTECH), then amplified with primer 622 (5′-CCCTAGGGTTGGGCGCCCTGCA-3′, exon 1) and primer 615 (5′-TCCTCTGCTGTAATGCGCAAC-3′, exon 5). The PCR product from primer 622 to 615 was sequenced by fluorescent dye terminator cycle sequencing.

**Western Blotting—** Leg muscle mitochondria were isolated (27) from mice that were fasted for 24 h. A rabbit antibody to the C terminus of human UCP3 (ABS0464, Enzo International, Temecula, CA) was used at 1:1000 dilution. The secondary antibody was peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch), diluted 1:20,000. Blots were detected by Enhanced Chemiluminescence (ECL, Amersham Pharmacia Biotech).

**Preparation of Mitochondria—** Skeletal muscle mitochondria were isolated from hind limb and forelimb skeletal muscles using the method of Bhattacharya (28). BAT mitochondria were isolated from the interscapular depot as described (29), except that the defatted bovine serum albumin was removed from the homogenate during centrifugation steps. Liver mitochondria were isolated through homogenization and isolation in 250 mM sucrose, 5 mM Tris-HCl, and 1 mM EGTA (pH 7.4), and the final pellet was resuspended in 120 mM KCl, 2 mM KH2PO4, 2 mM MOPS, 1 mM EGTA, and 5 mM HEPES (pH 7.2). Protein concentrations were determined by the Bieure method using bovine serum albumin as the standard.

**Measurement of Mitochondrial Oxygen Consumption—** Mitochondrial respiration was measured using a Hansatech Clark-type oxygen electrode whose incubation chamber was maintained at 37 °C and magnetically stirred. Rates were measured in 1.0 ml of medium (120 mM KCl, 20 mM N-acetyl-cysteine, 10 mM KH2PO4, 1 mM EGTA, and 5 mM HEPES, pH 7.2) using 0.5 mg of mitochondrial protein/ml. All respiration rates were determined simultaneously and in parallel with measurements of protonmotive force (Δp). The complex II substrate, succinate (10 mM), was added as the fuel source, and rotenone (5.0 μM, a complex I inhibitor) was added to prevent oxidation of any endogenous NAD-linked substrates. Nigericin (80 ng/ml) was added to convert −ΔpH to voltage units; Δp is thus expressed in mV.

**Oxygen consumption under state 4 conditions (saturating succinate with no ATP synthesis) was determined in the presence of experimentally determined maximal amounts of the ATP synthase inhibitor, oligomycin (muscle, 0.34 μg/ml protein; BAT, 0.06 μg/ml protein; and liver, 0.2 μg/ml protein).**

**Measurement of Mitochondrial Δp and Top-down Elasticity Analysis—** Δp was determined using a methyltriphosphosphosphonium (TPMP+)-sensitive electrode that was constructed using the methods of Kamo (30). Outputs from the TPMP+ and oxygen electrodes were routed to two voltmeters whose reference sockets were connected together; data were then fed into a data analysis software package (Duo-World, Precision Instruments, Sarasota,FL). Calibration of the TPMP+ electrode, determination of mitochondrial matrix volumes, and calculation of Δp from TPMP+ electrode data were carried out as described (31). Briefly, Δp is calculated using the Nernst equation,

\[
\Delta p = 61.5 \cdot \log(a_{\text{TPMP}}/a_{\text{TPMP+}}),
\]

where TPMP+ /TPMP+ is the uncorrected ratio of the accumulation of the cation inside and external to the mitochondria (and includes non-specific binding of TPMP+). The \(a_p\) correction factor was measured using the volume matrix-independent method that adjusts the TPMP+ accumulation ratio to the accumulation ratio for 31Rh, a K+ congener that does not bind, over a range of membrane potentials (31). The \(a_p\) correction factor, which serves as a baseline control at +60 mV, +3.1 and 0.36; BAT, 0.25 and 0.20; and liver, 0.22 and 0.29 (mean values of duplicates). Average mitochondrial matrix volumes (used in calculation of the TPMP+ accumulation ratio) were determined in triplicate by subtracting the excluded (\(\text{[14C]}\text{ sucrose}\)) volume from the total (\(\text{H}2\text{O}\)) volume. These values were, for Ucp3(-/-) mice and controls, respectively: muscle, 0.52 and 0.48 μl/mg of protein; BAT: 0.85 and 0.45 μl/mg of protein; and liver, 0.44 and 0.46 μl/mg of protein. Mitochondrial protein leak activity (29, 32) was assessed in the presence of the ATP synthase inhibitor, oligomycin, with incremental additions of the respiratory chain inhibitor, malonate (0, 0.33, 0.66, 1.0, 2.0, 3.0, and 5.0 mM).

**Monitoring Body Temperature—** Core body temperature (\(T_b\)) was continuously monitored in conscious, unrestrained animals using a VitalView system (MiniMitter, Sunriver, OR) (33). Briefly, each animal was anesthetized with halothane, and a temperature-sensitive transmitter (PDT-4000) was implanted intra-abdominally. The signal emitted by the transmitter is received and then converted into a temperature by the VitalView software. Mice were allowed to recover >7 days after implantation; typically a normal \(T_b\) circadian rhythm was reestablished 3 days after implantation. \(T_b\) values were measured each second and collected as 5-min averages. During temperature measurement, mice were individually housed in standard barrier plastic cages at room temperature or at 30 ± 1 °C, as indicated. Thyroid hormone (T3) was dissolved in 4 mM NaOH and given intraperitoneally.

**Indirect Calorimetry—** Oxygen consumption and carbon dioxide production were measured using a 4-channel Oxymax system (Columbus Instruments, Columbus, OH). The animal chamber was wire mesh floors, using a 1 liter/min flow rate, 75-s purge, and 60-s measure), with one mouse per chamber and testing knockout mice simultaneously with controls. Motor activity (total and ambulating) was determined by infrared beam interruption (Opto-Varimex mini, Columbus Instruments). Mice had free access to food and water except during fasting, when only water was available. Resting oxygen consumption was calculated as the average of the points with less than 6 ambulating beam breaks/min, omitting the first hour of the experiment. The respiratory exchange ratio (RER), the ratio of carbon dioxide produced to oxygen consumed, was calculated using the same data points. Oxidation of carbohydrate produces a RER of 1.00, whereas fatty acid oxidation results in a RER of 0.70–0.84. Oxygen consumption data were normalized to (body weight)^0.75 (35–37), although mice of similar weights were used within each experiment.

The effect of the β3-specific adrenergic agonist, CL316243 (38), was measured as follows, with each mouse serving as its own control. At ~9 a.m., mice were placed into the calorimeter chambers (prewarmed to 30 °C), and base-line data were collected. Three hours later, CL316243 (1 mg/kg intraperitoneally from a 0.1 mg/ml stock in saline) and after a 1-h delay, data were collected for 2 h.

**Biochemical Assays—** Glucose was measured using a Glucometer Elite (Bayer). Insulin (Linco, St. Charles, MO, SRI-13K) and leptin (Linco, ML-82K) were measured by radioimmunoassay. Triglycerides and glycerol (Sigma, 359–11), FFA (Roche Molecular Biochemicals, 1383175), and β-hydroxybutyrate (Roche Molecular Biochemicals, 907979) were assayed using the indicated kits. AniLytics (Gathers-
RESULTS

Production of Ucp3-ablated Mice—The Ucp3 knockout mice were generated by deletion of a 4-kb region, including the first coding exon (exon 2), with insertion of a 2-kb PGKneo selection cassette (Fig. 1A). On Southern blots, the mutated DNA yields an 8.5-kb fragment when assayed as detailed in Fig. 1B. The targeted deletion removes exon 2 (219 nt), which includes the translation initiation codon. Northern analysis detected no mRNA-containing Ucp3 exon 2 in muscle of Ucp3 (−/−) mice and reduced levels in +/+ mice (Fig. 1C). A slightly shorter Ucp3 transcript was detected in knockout mice with a probe to exons 1–5. Sequencing of reverse transcriptase-PCR products demonstrated that this mutated Ucp3 mRNA contains exon 1 spliced directly to exon 3. Like normal Ucp3 mRNA, levels of the mutant Ucp3 mRNA were increased in muscle and decreased in BAT by fasting (not shown). Western blotting was used to determine if UCP3 protein is present (Fig. 1D). A ∼35-kDa UCP3 band was observed in mitochondria from wild type muscle and but not knockout mice (calculated $M_r = 33,910$). The exon 2-deleted transcript, if translated, would produce an amino-truncated protein missing 64 of 308 amino acids; no such band (calculated $M_r = 27,241$) was detected. These data demonstrate that we have produced a UCP3-deficient mouse.

Theoretically, up-regulation of UCP2 in muscle could compensate for the lack of UCP3. However, Ucp2 mRNA levels in muscle were unchanged in Ucp3 (−/−) as compared with wild type mice (levels in Ucp3 (−/−) were $98 ± 12\%$ that of the level in wild type, $n = 3$) [3/group], indicating the absence of transcriptional compensation for the lack of UCP3 function. BAT Ucp2 levels, which appeared to be slightly elevated in the knockout mice (levels in Ucp3 (−/−), were $184 ± 4\%$ that of the level in wild type, $n = 3$) [3/group; Fig. 1E]. Ucp1 mRNA levels in BAT from Ucp3 (−/−) mice were unchanged at $84 ± 18\%$ of the wild type level ($n = 3$) [3/group].

Mitochondrial Proton Leak—To examine the effects of the Ucp3 knockout on mitochondrial proton leak, we used the top-down elasticity approach (29, 32). The system consists of the reactions that produce $\Delta\psi$ (i.e. substrate oxidation) and the two sets of reactions that consume $\Delta\psi$ (i.e. ATP synthesis and proton leak). Mitochondrial membrane $\Delta\psi$ was measured under conditions preventing ATP synthesis, so that the energy produced by substrate oxidation goes solely to balance the proton leak. By measuring the oxygen consumption and $\Delta\psi$ under different rates of substrate oxidation, leak can be quantitated.

Fig. 2 shows the characteristics of proton leak reactions in mitochondria isolated from skeletal muscle, liver, and BAT. Muscle expresses Ucp3 at high levels, liver does not express Ucp3, and BAT expresses low levels of Ucp3. All of these tissues express Ucp2, whereas only BAT makes UCP1. In skeletal muscle mitochondria from Ucp3 (−/−) mice, $\Delta\psi$ is significantly higher than in wild type mitochondria and, for any given value of $\Delta\psi$, the corresponding oxygen consumption is much lower in the Ucp3 (−/−) mice (Fig. 2A). Thus, there is decreased proton leak in the Ucp3 (−/−) mitochondria over the complete range of metabolic rates studied. As expected, liver mitochondria from Ucp3 (−/−) and control mice showed no difference in state 4 oxygen consumption or in the overall characteristics of proton leak (Fig. 2B).

In BAT mitochondria from Ucp3 (−/−) mice, there was no significant difference in $\Delta\psi$ or in the overall kinetics of the proton leak reactions, but there was a slightly lower state 4 respiration rate (Fig. 2C). These results are from mitochondria incubated in the absence of GDP, which inhibits UCP1 function (39). We and others have shown that UCP2 and/or UCP3 are not inhibited by concentrations of GDP that inhibit UCP1 (29, 40–42). Experiments with Ucp3 (−/−) mitochondria from BAT and muscle confirmed these observations (not shown). These results support the conclusions 1) that UCP3 functions as an uncoupling protein, 2) that Ucp3 (−/−) mice lack functional UCP3, and 3) that there is little or no up-regulation of leak in muscle to compensate for the loss of UCP3.

General Phenotype—Homozygous knockout mice were born at the expected frequency, with no observed signs of abnormality, illness, or increased mortality at up to 1 year of age. No anatomic abnormalities were observed on gross or light microscopic examination of 6-month-old mice. Body weight and fat pad weights were similar in Ucp3 knockout and control mice (Table I), although in some experiments, there was a tendency toward increased body weight in younger male mice (data not shown). On a high fat diet, Ucp3 (−/−) and control mice gained weight to a similar extent (data not shown). Serum biochemical
Ucp3 Knockout Mice

Fig. 2. Proton leak in mitochondria isolated from skeletal muscle (A), liver (B), and BAT (C). Graphs show the relationship between Δp and leak-dependent respiration in mitochondria from Ucp3 (−/−) (△) and wild type control (●) mice. Oxygen consumption was varied by titration of state 4 (maximal, non-phosphorylating) respiration with increasing amounts of a complex II inhibitor, malonate, as described under "Materials and Methods." Each point represents the mean ± S.E. of duplicate determinations using separate mitochondrial preparations from 6 to 8 male mice, aged 18 to 26 weeks. The wild type and Ucp3 (−/−) BAT state 4 data are different at p = 0.01.

screening panels (electrolytes, routine enzymes) were unremarkable (data not shown). Insulin, triglycerides, and leptin were not different from sex-matched control mice, whereas FFA and glucose sometimes was slightly lower (Table I).

Body Temperature Regulation—Core body temperature (Tb) and physical activity were monitored continuously via telemetry. Male Ucp3 knockout mice housed at −23 °C showed no difference from controls in Tb or its circadian rhythm or in the stress-induced fever response to a novel environment (i.e. cage switch; maximum fever height, 2.2 °C in controls and 2.8 °C in knockout mice). Knockout and control mice housed at 30 °C also showed no difference in Tb or its circadian rhythm and developed similar fevers upon treatment with lipopolysaccharide (2.5 mg/kg, intraperitoneally; 1.3 °C increased in controls and 1.1 °C increased in knockout mice).

Ucp3 (−/−) mice showed normal adaptation to 4 °C. In an attempt to maximize the possible role of UCP3 in maintaining Tb with cold exposure, mice were adapted to 30 °C. Response of the warm-adapted mice to 4 °C for 24 h was no different between wild type and Ucp3 (−/−) animals in terms of survival (17 of 19 controls and 14 of 17 knockout mice) or in Tb (36.0 ± 0.11 °C in controls and 35.72 ± 0.13 °C in knockout mice). Motor activity was not different between Ucp3 knockout and control mice in all Tb experiments. Thus, no significant abnormality in thermoregulation was found in the Ucp3 (−/−) mice.

Metabolic Rate—Oxygen consumption was measured to see if the UCP3 proton leak is a significant contributor to total body metabolic rate. Resting oxygen consumption in Ucp3 knockout mice was the same as controls (8.3 ± 0.6 ml/h/g0.75 in knockout mice and 9.0 ± 0.4 ml/h/g0.75 in controls, n = 4/group, 8-week-old females, measured at room temperature). The RER was also the same (0.824 ± 0.018 in knockout mice and 0.809 ± 0.016 in controls). The resting metabolic rate in these mice was reduced equally when measured at thermoneutrality (32 °C, to 6.1 ± 0.4 ml/h/g0.75 in knockout mice and 5.8 ± 0.4 ml/h/g0.75 in controls). Control and Ucp3 (−/−) mice showed the same increase in metabolic rate in response to CL316243, a β3-adrenergic-specific agonist (fold stimulation: 2.00 ± 0.15 in knockout mice and 1.85 ± 0.13 in controls, n = 8/group). In conclusion, the Ucp3 (−/−) mice show no difference in regulation of metabolic rate.

Ucp1/Ucp3 Double Knockouts—We bred Ucp1/Ucp3 double knockout mice to see if the Ucp1 knockout phenotype was made more severe by the additional loss of UCP3 function. Mice lacking UCP1 have a reduced metabolic response to a β3-adrenergic-specific agonist and are cold-sensitive (26). Ucp1/Ucp3 double knockout mice did not show lower β3-stimulated oxygen consumption than the Ucp1 single knockout (Fig. 3). Similar data were obtained with the non-selective β agonist, isoproterenol (0.3 mg/kg, intraperitoneally; not shown). Additionally, Ucp1/Ucp3 double knockout mice were no more sensitive to cold than Ucp1 knockout animals (not shown). These data suggest that Ucp3 is not a modifier gene of the Ucp1 knockout phenotype.

Thyroid Thermogenesis—Thyroid hormone is a major regulator of metabolic rate, and treatment with thyroid hormone causes an increase in Ucp3 mRNA levels. However, during 4 days of T3 treatment (1 mg/kg/day), Ucp3 knockout mice also showed the same increase in resting oxygen consumption as controls (1.72-fold stimulation in knockout mice and 1.89 in controls, Fig. 4A). Tb increased similarly in control and Ucp3 knockout mice (e.g. −1 °C increase in the light-cycle minimum Tb, not shown). Tb increased similarly after a single maximal (1 mg/kg) or half-maximal (0.1 mg/kg) T3 dose in control and knockout mice (Fig. 4B). These studies do not preclude a subtle role for UCP3 in the thyroid-induced increase in body temperature and whole-body metabolic rate but do demonstrate that UCP3 is not the sole mediator of these effects.

Response to Fasting in Knockout Mice—A large increase in Ucp3 mRNA levels occurs with fasting (13, 21, 22), so the response of Ucp3 knockout mice to a 24-hour fast was examined. Similar reductions with fasting were observed in body weight (11.9 ± 3.6% in controls versus 14.3 ± 2.3% in Ucp3 knockout mice) and Tb (2.52 ± 0.47 °C in controls versus 2.04 ± 0.41 °C in Ucp3 knockout mice, comparing the last 8 h of the fast to the same time of day in the fed state). The metabolic rate
We have produced a mouse lacking UCP3. Proton leak in mitochondria isolated from Ucp3 (-/-) skeletal muscle is reduced. This result supports the hypothesis that UCP3 functions as an uncoupling protein. Previous evidence that UCP3 is an uncoupling protein included its sequence similarity to UCP1 and the demonstration that expression of UCP3 reduced the mitochondrial membrane potential (13, 20, 43, 44). The measurable reduction in leak suggests

**DISCUSSION**

**UCP3 Is an Uncoupling Protein**—We have produced a mouse lacking UCP3. Proton leak in mitochondria isolated from Ucp3 (-/-) skeletal muscle is reduced. This result supports the hypothesis that UCP3 functions as an uncoupling protein. Previous evidence that UCP3 is an uncoupling protein included its sequence similarity to UCP1 and the demonstration that expression of UCP3 reduced the mitochondrial membrane potential (13, 20, 43, 44). The measurable reduction in leak suggests
that UCP3 is a significant contributor to proton leak in muscle and that other muscle proteins do not compensate for the loss of UCP3. The small reduction in BAT mitochondrial state 4 and that other muscle proteins do not compensate for the loss that UCP3 is a significant contributor to proton leak in muscle.

\[ \text{UCP3 Knockout Mice} \]

Table II

Effect of 24-h fast on 8-month-old male mice

|                      | Wild type | Fed       | Fasted    | Ucp3 (−/−) | Fed       | Fasted    |
|----------------------|-----------|-----------|-----------|------------|-----------|-----------|
| Body weight (g)      |           | 40.4 ± 1.6| 37.4 ± 1.6| 40.3 ± 1.5 | 37.7 ± 1.6|
| Glucose (mg/dl)      | 227 ± 15  | 104 ± 13  | 154 ± 17  | 154 ± 30   |
| Triglyceride (mg/dl) | 220 ± 33  | 159 ± 8   | 254 ± 12  | 161 ± 38   |
| FFA (μM)             | 679 ± 52  | 935 ± 36  | 781 ± 73  | 605 ± 77   |
| β-Hydroxybutyrate (mg/dl) | 3.1 ± 0.4 | 12.4 ± 0.8 | 2.4 ± 0.7 | 4.1 ± 1.9 |
| BUN (mg/dl)          | 29.4 ± 1.7| 31.5 ± 0.7| 24.7 ± 1.5| 35.1 ± 5.5|

* Different from fed wild type, \( p = 0.002 \).

* Different from fasted wild type, \( p \leq 0.003 \).

After obtaining base-line serum, the mice were fasted for 24 h and then sacrificed. Data are the mean ± S.E., \( n = 5–6 \). BUN is blood urea nitrogen.

Our results show that in the absence of UCP3, the muscle mitochondria protonmotive force is increased. In addition, the proton leak-dependent oxygen consumption is dramatically lower in the absence of UCP3. Thus, we have demonstrated lower proton leak in the absence of UCP3. However, since the catalytic mechanism used by UCP3 is unknown, we do not know if UCP3 protein is the actual proton translocator. In fact, even the uncoupling mechanism used by UCP1 remains the subject of debate, with two schools of thought (45). One is that UCP1 allows fatty acid anions to traverse the membrane, with a net flux out from the matrix. Coupled with spontaneous flip-flop of protonated fatty acids, the net result is that the fatty acids function as cycling protonophores (46, 47). The other proposal is that UCP1 directly catalyzes proton translocation, with the fatty acids acting as cofactors (48). These proposed mechanisms are complicated by the observation that fatty acids can stimulate uncoupling in the absence of UCP1 (42). UCP3 could use the same mechanism as UCP1 or might electrophoretically transport other metabolites, such as those related to fatty acid oxidation. The Ucp3 knockout mice will likely prove useful in addressing these important mechanistic aspects of UCP3 function.

**Lack of an Obvious Ucp3 (−/−) Phenotype**—We have not detected striking phenotypic abnormalities in the Ucp3 (−/−) mice. In particular they show no obesity, normal thermoregulation, an intact thermogenic response to thyroid hormone, and a normal response to fasting. The lack of an observable phenotype is probably not due to up-regulation of other muscle genes, since muscle Ucp2 mRNA is not increased and since we measure a large decrease in leak in muscle mitochondria. It is possible that under in vivo conditions, muscle compensates for the lack of UCP3. Other tissues may also contribute. However, we believe that phenotypic differences will eventually be identified upon testing the Ucp3 (−/−) mice under the appropriate conditions.

Ucp3 was first identified in searches for obesity candidate genes, and sequence variants have been identified in obese humans (49–51). Notably, an obese and diabetic compound heterozygote carrying alleles R143X and an intra 6 splice mutation was shown to have an elevated respiratory quotient (indicating decreased fractional fat oxidation) (49). However, a study of homozygotes for the intron 6 splice mutation detected no alteration of mitochondrial coupling or respiratory enzyme activity or systemic oxygen consumption or respiratory quotient (51).

The Ucp3 knockout mice are not obese, casting doubt on the role of UCP3 in single gene obesity. The Ucp3 (−/−) mice do show increased coupling, unlike the humans homozygous for the intron 6 splice mutation. The difference between the Ucp3 (−/−) mice and human intron 6 homozygotes may be due to differences between mice and humans; it is also possible that the intron 6 splice mutation encodes a functional protein. In summary, the knockout mice suggest that UCP3 null mutations are not a cause of monogenic obesity, but more needs to be done on the role of other UCP3 mutations and the role of UCP3 in polygenic forms of obesity.

**What is the function of UCP3?**—Proposed functions for UCP3 have focused on heat generation in response to a cold environment, to thyroid hormone, and to increased dietary intake (53). However, the idea that UCP3 contributes to regulated heat production needs to be reconciled with the fact that fasting greatly increases Ucp3 mRNA levels. During fasting there is increased, not decreased, energy efficiency and, particularly in smaller mammals, a reduction, not increase, in heat production and body temperature (33). This suggests that the uncoupling activity of UCP3 is used for purposes other than heat generation.

If the purpose of UCP3 is not facultative thermogenesis, what is its physiologic function? There is a very strong correlation between Ucp3 expression and free fatty acid levels. Each of the conditions identified as increasing Ucp3 mRNA levels is characterized by increased free fatty acid levels or oxidation rates. Examples include acute fasting (13), lipid/heparin infusion (22), acute thyroid stimulation (13), and acute exercise (52). Similarly, examples of decreased UCP3 correlating with decreased free fatty acid levels/oxidation include hypothyroidism (15) and chronic hypocaloric diet (43). However, correlation does not establish cause; do increased fatty acids induce Ucp3, and/or, is Ucp3 induced in order to handle increased free fatty acids? Does UCP3 transport molecules that are generated as a by-product of fatty acid oxidation?

Another proposed role for UCPs is in minimizing the effects of reactive oxygen species (ROS). ROS, such as the superoxide radical anion and H2O2, are produced as a by-product of substrate oxidation. The high reactivity of ROS causes damage to proteins, lipids, and DNA. Mitochondrial state 4-like conditions in the cell, with ample fatty acid substrate and little ATP production, will produce an increased redox pressure, presumably increasing the probability of ROS production. A proton leak would counter this and should decrease ROS production. There is some evidence in favor of a scenario in which UCP2 decreases H2O2 production (54) and some correlative data suggesting the hepatic UCP2 increases in response to oxidant...
production (55). Data supporting a role for UCP3 in protecting from ROS is reported in Vidal-Puig et al. (56); further investigation
is needed.

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