UCP1-independent Thermogenesis in White Adipose Tissue of Cold-acclimated Ucp1−/− Mice*

Received for publication, June 26, 2006, and in revised form, July 27, 2006. Published, JBC Papers in Press, August 16, 2006, DOI 10.1074/jbc.M606114200

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Apart from UCP1-based nonshivering thermogenesis in brown adipocytes, the identity of thermogenic mechanisms that can be activated to reduce a positive energy balance is largely unknown. To identify potentially useful mechanisms, we have analyzed physiological and molecular mechanisms that enable mice, genetically deficient in UCP1 and sensitive to acute exposure to the cold at 4 °C, to adapt to long term exposure at 4 °C. UCP1-deficient mice that can adapt to the cold have increased oxygen consumption and show increased oxidation of both fat and glucose as indicated from serum metabolite levels and liver glycogen content. Enhanced energy metabolism in inguinal fat was also indicated by increased oxygen consumption and fat oxidation in tissue suspensions and increased AMP kinase activity in dissected tissues. Analysis of gene expression in skeletal muscle showed surprisingly little change between cold-adapted Ucp1+/− and Ucp1−/− mice, whereas in inguinal fat a robust induction occurred for type 2 deiodinase, sarcoendoplasmic reticulum Ca2+-ATPase, mitochondrial glycerol 3-phosphate dehydrogenase, PGC1α, CoxII, and mitochondrial DNA content. Western blot analysis showed an induction of total phospholamban and its phosphorylated form in inguinal fat and other white fat depots, but no induction was apparent in muscle. We conclude that alternative thermogenic mechanisms, based in part upon the enhanced capacity for ion and substrate cycling associated with brown adipocytes in white fat depots, are induced in UCP1-deficient mice by gradual cold adaptation.

Specific regulatable thermogenic mechanisms in mammals have two important functions as follows: one is for the control of body temperature, and the other is for the maintenance of body weight. The former can be served through several well defined mechanisms, depending on physiological circumstances, that include shivering, fever, restriction of blood flow to the periphery, and nonshivering thermogenesis in brown adipocytes (1, 2). However, understanding the regulation of body weight by thermogenic mechanisms is problematic. It is probable that in adult humans the primary mechanism for burning off excess calories is through physical activity (3); however, in severely obese individuals where physical activity is not possible finding alternative thermogenic mechanisms that can be activated to burn off excess calories is an important goal. Mice with a targeted inactivation of the Ucp1 gene, which encodes the brown adipocyte-specific mitochondrial uncoupling protein, have phenotypes relating to both body temperature and body weight regulation (4, 5). Because these mice cannot be protected from acute cold exposure by shivering thermogenesis and require a period of slow adaptation to the cold for survival, they can be used to identify UCP1-independent adaptive thermogenic mechanisms (6, 7). We have recently shown that although mice carrying mutations for either Ucp1 or Lep are able to adapt to cold, those mice carrying null mutations for both Ucp1 and the Lep genes cannot survive in the cold, unless they are treated with either leptin or thyroid hormone (8). The increase in muscle sarcoendoplasmic Ca2+-ATPase (SERCA2a) mRNA expression by leptin provides a plausible thermogenic mechanism for survival in the cold.

Although the results with Ucp1−/− and Lep−/− mice suggest that muscle has the potential to contribute thermogenically through SERCA2a mechanisms, definitive evidence showing an induction of SERCA2a in muscle at the protein level has been elusive. Furthermore, Nedergaard and co-workers (7) interpret the induction of cellular components associated with muscle contraction as a consequence of chronic shivering in mice with defective functional brown fat, including a deficiency in UCP1. However, shivering, which is considered to be an effective short term thermogenic mechanism, is unable to generate sufficient heat to maintain body temperature in Ucp1−/− mice acutely exposed to 4 °C (6) nor in mice deficient for dopamine β-hydroxylase (9), acyl-CoA dehydrogenases (10), or β-adrenergic receptors (11). Accordingly, although it is indisputable that shivering is a thermogenic mechanism activated in response to reduced body temperature, additional mechanisms are required to enable mice to adapt to the cold. If perchance shivering is the only mechanism, then we must explain how its capacity can be adaptively induced, how it can be regulated by thyroid hormone and leptin and possibly other hormonal signaling mechanisms, and we must determine the molecular

The abbreviations used are: SERCA, sarcoendoplasmic Ca2+-ATPase; MOPS, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin; RER, respiratory exchange ratio; SR, sarcoendoplasmic reticulum; LTCA, long term cold adaptation; Cox, cytochrome oxidase; AMPK, AMP kinase; PLB, phospholamban.

* This work was supported by National Institutes of Health Grant RO1 HD08431. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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basis for genetic variability that enables Ucp1−/− mice on different genetic backgrounds to exhibit variable sensitivities to the cold.

As a step toward identifying novel thermogenic mechanisms that could be involved in the maintenance of body temperature, we have compared morphological, physiological, and molecular phenotypes of UCP1-deficient mice on two inbred and a hybrid genetic background during gradual adaptation to the cold. At the physiological level, the ability of UCP1-deficient mice to resist the cold is related to their capacity to increase oxygen consumption as indicated by indirect calorimetry. The focus of the molecular analysis was on changes in adipose tissue, because these cannot be associated with muscle-based shivering mechanisms. The results showed an increase of cells within white fat depots with the morphological features of brown adipocytes but without Ucp1 expression. The tissue was characterized by elevated fat oxidation, increased AMP kinase activity, induced levels of total and phosphorylated phospholamban, a regulator of sarcoendoplasmic reticulum Ca2+-ATPase activity, as well as induced levels of other genes that could contribute thermogenically. The results suggest that adipose tissue in cold-adapted UCP1-deficient mice acquires the biochemical machinery necessary for enhanced thermogenesis.

**MATERIALS AND METHODS**

**Animals and Study Design—**Ucp1−/− mice on a 129S1/SvJm (129), C57BL/6J (B6) genetic background were generated as described previously (4, 6). 129 and B6 Ucp1−/− mice were crossed to generate F1 hybrids. 129 wild type mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Two independent studies were performed. In the first study two groups of age-matched 2-month-old male Ucp1−/− and wild type mice (129) were singly housed with corn cob bedding and fed rodent chow (5053, rodent diet 20, LabDiet) *ad libitum*. After 7 days of initial acclimation to 28 °C, one half of the animals from each genotype were acclimated to the cold by reducing the ambient temperature 2 °C per day until it reached 4 °C, whereas the other half remained at 28 °C. Because it was difficult to generate a large number of Ucp1−/− mice at one time and also because of the fact that ~20% of Ucp1−/− mice were hypersensitive to cold, an experiment was initiated when a minimum of six mice of a specific genotype was available and repeated six times to generate sufficient numbers of Ucp1−/− animals. The total number of mice was 45 cold-adapted and 27 warm-adapted Ucp1−/− mice and 43 cold-adapted and 31 warm-adapted Ucp1+/+ mice. Indirect calorimetry measurements were performed twice on two independent groups of cold-acclimated Ucp1−/− (n = 8) and wild type (n = 8) mice. In the second study, Ucp1−/− mice on 129 (n = 9), B6 (n = 9), and F1 hybrid (B6 × 129) (n = 11) backgrounds, together with a 129 wild type group (n = 6) were acclimatized to 4 °C by decreasing the ambient temperature 2 °C per day. Indirect calorimetry was performed. Body weight, body composition (NMR, Bruker), and food intake were measured. Upon completion of the *in vivo* physiological experiments, animals were euthanized by cervical dislocation, and tissue samples were collected and stored at −80 °C. Serum was obtained and stored at −20 °C. All the animal experiments were approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines for the care and use of laboratory animals.

**Phenotypes of Energy Balance—**Body temperature was measured with a rectal thermoprobe (TH-8, Physitemp Instruments Inc., Clifton, NJ), and body composition was analyzed by NMR (Bruker, TX). Energy expenditure was evaluated by indirect calorimetry (Oxymax, Columbus Instruments, OH). Animals with *ad libitum* access to food (specified above) were singly housed 2 days prior to the experiment in air-proof plastic metabolic cages, connected to the oxygen (O2) and carbon dioxide (CO2) sensors, and placed in an incubator enabling precise temperature control. The Oxymax system allowed 16 individually housed animals to be monitored simultaneously. Energy expenditure and respiratory exchange ratio were measured over a 60-s period in 42-min intervals. Physical activity was measured by interruption of infrared beams that sensed movement in Y and Z directions, and data were expressed as counts/h. For a Y count a mouse has to move ~2.0 inches in a horizontal direction, and for a Z count the mouse has to raise its body ~2.0 inches above the chamber floor.

**Quantitative Reverse Transcription-PCR—**TRI reagent (Molecular Research Center Inc., Cincinnati, OH) was used for the RNA and DNA isolation. RNA was further purified by using the RNeasy mini kit and RNase-free DNase (Qiagen, Valencia, CA). Protection of isolated RNA from degradation by RNase was secured with SUPERase-In (Ambion, Austin, TX). The quantity and quality of RNA were determined by spectrophotometry (DU650, Beckman Coulter Inc., Fullerton, CA) and confirmed using the minigel technique or the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Superscript II (Invitrogen) and high capacity cDNA archive kit (Applied Biosystems, Foster City, CA) were used for cDNA synthesis. Quantitative real time PCR was performed using total RNA or cDNA with specific primers and probes designed with Primer-Express™ version 2.0.0 (Applied Biosystems, Foster City, CA). Taqman probes were used for quantification of some gene targets using Taqman Core reagent kit (Applied Biosystems). SYBR Green PCR core reagent kit was used for quantification of other gene targets (Applied Biosystems). All the gene expression data were normalized to the level of cyclophilin b. Specific sets of primers and probes used for gene expression analysis are available on request.

**Western Blot—**Total tissue lysate from adipose tissue and skeletal muscle were prepared in RIPA buffer containing 1% protease and phosphatase inhibitor mixtures (Sigma). Tissues for the study of phosphorylated proteins were clamp-frozen in liquid nitrogen, processed within 2 weeks of collection, and homogenized in 30 mM Tris–HCl (pH 7.6), 2 mM EDTA, and 1% protease and phosphatase inhibitor mixtures (Sigma) (12). Protein content was determined by the Bradford method (Bio-Rad) or the bicinchoninic acid kit (Sigma) (12). Protein content was determined by the Bradford method (Bio-Rad) or the bicinchoninic acid kit (Sigma) (12). Protein content was determined by the Bradford method (Bio-Rad) or the bicinchoninic acid kit (Sigma) (12).
UCP1-independent Thermogenesis

(2D12, mouse IgG2a, Affinity Bioreagents), anti-P-phospholamban (Ser-16) (rabbit anti-phospholamban Ser-16, Upstate Cell Signaling, Charlottesville, VA), anti-AMPK-α antibody (Cell Signaling, Danvers, MA), and anti-phospho-AMPKα1 (Thr-172) (Cell Signaling). Specific antibody-antigen complexes were detected using fluorescent-labeled secondary antibodies (goat anti-rabbit IRDye™ 800, Rockland, Inc., Gilbertsville, PA; goat antiamouse Alexa Fluor® 680, Invitrogen). Bands were visualized and quantified using the Odyssey imaging system (Li-Cor Inc., Lincoln, NE). β-Actin and glyceraldehyde-3-phosphate dehydrogenase (Abcam Inc., Cambridge, MA) were used as internal controls to evaluate the uniformity of protein loading.

Tissue Oxygen Consumption—A Clark-type oxygen electrode (Hansatech Instruments Ltd., Norfolk, UK) was used to measure the oxygen consumption of inguinal adipose tissue. Tissue was finely minced prior to the measurement in the freshly oxygenated (95% O2, 5% CO2) Krebs-Ringer buffer (pH 7.2), containing 1% fatty acid-free BSA. BSA was omitted from the media used for measurement. Oxygen consumption was monitored continuously for ~10 min. The basal respiration rate was recorded for ~2 min, and the succinate (5 mM)-induced increase of the oxygen consumption was measured during the subsequent 5–7 min. Results were normalized to the DNA content and measured spectrophotometrically in the homogenates prepared from the tissue collected after measurement (13).

Palmitate Oxidation Rate—Palmitate oxidation was assessed by measuring production of 14CO2 and 14C-labeled acid-soluble metabolites during incubation of fresh inguinal fat extracts with [1-14C]palmitate (14, 15). Briefly, ~150 mg of inguinal fat was excised, dry-blotted, and immediately placed into ice-cold medium containing 250 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4). The tissue was minced, and the mixture was homogenized in a glass homogenization tube with a motor-driven Teflon pestle. This method has been found to provide intact mitochondria for metabolic studies (16). Tissue homogenate was centrifuged at 6000 rpm for 10 min, and the infranatant, containing mitochondria (upper fat layer was excluded), was collected and used for measurement of palmitate oxidation. Eighty microliters of a 10-fold diluted inguinal fat homogenate were incubated with a 320-μM palmitate oxidase. Eighty microliters of a 10-fold diluted inguinal fat homogenate were incubated with a 320-μM palmitate complexed by 0.3% bovine serum albumin, 0.2 mM palmitic acid, and 0.5% bovine serum albumin, and [1-14C]palmitate (1.0 μCi/ml). After 60 min of incubation at 37°C, 200 μl of 70% perchloric acid were injected to stop the reaction. CO2 produced during the 60-min incubation was trapped with 400 μl of 1 mM sodium hydroxide. The incubation and 14CO2 trapping were performed in a custom made device as described previously (15). The sodium hydroxide was collected and counted for evolved 14CO2 by liquid scintillation. The acidified portion of the incubation media was collected, stored overnight at −4°C, and centrifuged at 15,000 rpm for 15 min, and 0.5 ml of the upper phase was collected and counted by liquid scintillation. Oxidation rate was expressed as nanomoles of CO2 and acid-soluble metabolites per μg of DNA per h.

Endoplasmic Reticulum Vesicle Isolation—Endoplasmic reticulum-enriched microsomes were prepared by a modified protocol of de Meis (17). Briefly, freshly dissected tissues (brown adipose tissue, inguinal fat, and gastrocnemius muscle) were snap-frozen in liquid nitrogen. Tissues were pulverized in liquid nitrogen and then homogenized using a Potter-Elvehjem homogenizer in a buffer containing 10 mM MOPS/Tris buffer (pH 7.0), 1 mM EDTA, 10% sucrose, and a mixture of protease and phosphatase inhibitors (Sigma). Homogenate was centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was collected, and fatty acid-free BSA was added to a final concentration of 2.5 g%. The mixture was centrifuged at 65,000 × g for 40 min at 4°C. The pellet was resuspended and gently homogenized using a Dounce homogenizer in a buffer containing 50 mM MOPS/Tris buffer (pH 7.0), 100 mM KCl, and 2.5 g% fatty acid-free BSA. The homogenate was centrifuged at 10,000 × g for 20 min at 4°C. The pellet was discarded, and supernatant was centrifuged at 65,000 × g for 40 min at 4°C. The pellet endoplasmic reticulum microsomes were gently resuspended using a Dounce homogenizer in buffer containing 50 mM MOPS/Tris buffer (pH 7.0), 0.8 M sucrose, and 5 mM NaN3 and frozen and stored under liquid nitrogen until use. The protein concentration was determined using a bicinchoninic acid assay (Sigma).

Calcium Transport—Calcium transport across the membrane of the sarcoplasmic reticulum was determined spectrofluorometrically as described earlier (18). After thawing sarcoendoplasmic reticulum-enhanced microsomes (50 μg) were immediately added to a cuvette (Sartorius, Germany) containing 50 mM MOPS/Tris (pH 7.0), 100 mM KCl, 10 mM MgCl2, 10 mM sodium azide, and 10 mM potassium oxalate, 5 mM creatine phosphate, 10 μg/ml creatine kinase, and 1.5 μM of the Ca2+-sensitive fluorescent dye Fura 2 (Molecular Probes, Eugene, OR). The cuvette was inserted in a temperature-controlled (37°C) cuvette holder of an LS-55 luminescent spectrofluorometer (PerkinElmer Life Sciences). The reaction was allowed to equilibrate for 3 min, and calcium uptake was stimulated by the addition of 500 μM Mg2+-ATP. After the steady state was reached, CaCl2 was added to a final concentration of 100 μM. At the end of experiment Ca2+ ionophore A23187 (3 μM) or 20 μl of 10% Triton X-100 (0.2%) was added to collapse the calcium gradient across the sarcoendoplasmic reticulum vesicle membrane. Ruthenium red (2 μM), a ryanodine receptor inhibitor, had no effect on the rates of calcium uptake. Thapsigargin (Sigma) inhibitor of Ca2+-ATPase was used to test for specificity of Ca2+ transport.

ATPase Activity—Enzymatic activities were determined on endoplasmic reticulum preparations as described by de Meis (17) with [γ-32P]ATP (PerkinElmer Life Sciences) as the substrate. Reactions in 100-μl volumes were linear for up to 20 min of incubation time and 8 μg of protein. Thapsigargin was obtained from Sigma.

Hormone and Metabolite Determination—Blood glucose levels were determined by the OneTouch Profile blood glucose meter (Lifescan Inc., Milpitas, CA). Serum insulin was measured by sensitive rat insulin RIA kit (Linco, St. Charles, MO),
and β-hydroxybutyrate was determined with Autokit 3HB (WAKO Chemicals, Richmond, VA). Serum T3 and T4 levels were measured with T3 and T4 solid phase RIA kit (Diagnostic Products Corp., Los Angeles, CA). Tissue triglycerides were measured in chloroform/methanol extracts using the L-type TG H kit (WAKO Chemicals). Tissue glycogen was determined by Autokit Glucose CII from WAKO chemicals (Richmond, VA) after digesting the tissues as described previously (19).

Statistical Analysis—All the data are expressed as means ± S.E. Two-way analysis of variance with repeated measures or paired t test assessed the statistical significance of differences between mean values (StatView, version 5.0.1, SAS Institute Inc.). Analysis of variance with Bonferroni post hoc test was used when more than two experimental groups were compared.

RESULTS

Cold Acclimation and Energy Expenditure in Congenic Ucp1−/− Mice—Wild type B6, 129, and (B6 × 129)F1 mice and Ucp1−/− mice congenic on these genetic backgrounds were tested to establish the variability in cold-sensitive phenotypes. All mice were 2-month-old males born and maintained at 27 °C until they were tested for acute sensitivity to the cold (4 °C) or adaptation to the cold with protocols that minimized handling of the mice. All wild type mice, irrespective of their genetic background, were resistant to the cold during an acute test, and except for 1 of 73 (129) mice, they all tolerated long term cold adaptation conducted by lowering the ambient temperature 2 °C per day (Table 1). Accordingly, there is no significant variability in the ability of these wild type strains to tolerate the
cold. In contrast, \textit{Ucp1}^{-/-} congenic lines showed significant genetic background variability in both the acute sensitivity and long term adaptability to cold. With respect to acute cold sensitivity, \textit{129.Ucp1/-} mice were very sensitive (87.5%). \textit{B6.Ucp1/-} mice were modestly tolerant of the cold (32% sensitive), whereas (B6\times129)F1 mice were similar to wild type mice and, with the exception of one mouse, completely resistant to acute cold exposure (Table 1). Adaptation to the cold by gradual reduction of the ambient temperature until it reached 4 °C effectively enabled \textit{B6.Ucp1/-} and \textit{129.Ucp1/-} mice to tolerate the cold, although 16–19% of the mice failed to adapt (Table 1). When hypothermia occurred among the \textit{Ucp1}^{-/-} congenic mice, it generally did so when the ambient temperature dropped below 18 °C.

We have reported previously that at 20 °C \textit{B6.Ucp1}^{+/+} and \textit{B6.Ucp1}^{-/-} mice fed an obesogenic high fat diet had indistinguishable levels of oxygen consumption, but the \textit{Ucp1}^{-/-} mice had a lower respiratory exchange ratio (RER) indicating higher rates of fat oxidation (5). We next established how energy expenditure and the RER varied among \textit{129.Ucp1}^{+/+} and \textit{129.Ucp1}^{-/-} mice during adaptation to the cold when fed a low fat chow diet. We switched from the B6 to 129 strains for these studies because the cold-sensitive phenotype was more penetrant in the 129 strain (Table 1), and chow diet was used because we did not want to introduce an obesity phenotype in a study on the analysis of cold adaptation. Oxygen consumption increased steadily with the expected circadian rhythms and at a similar rate in the two genotypes until the ambient temperature was about 12 °C, at which temperature oxygen consumption became greater in the \textit{Ucp1}^{-/-} mice (Fig. 1). When the ambient temperature reached 4 °C, oxygen consumption was about 30% higher in \textit{Ucp1}^{-/-} mice than in \textit{Ucp1}^{+/+} mice. No differences in the RER, which cycled between 0.8 and 1.0, could be detected between the genotypes. Comparison of body weight, fat mass, and fat-free mass at various temperatures during the adaptation process shows a greater loss of body weight in \textit{Ucp1}^{-/-}, which is mostly associated with fat mass, that is consistent with increased energy expenditure in \textit{Ucp1}^{-/-} mice required to maintain body temperature (Fig. 2). No significant differences in food consumption were measured although both genotypes showed about a 50% increase in food intake. In addition, a gradual reduction in ambulatory physical activity was observed in both groups. Considered together, the data indicate that the adaptability of \textit{Ucp1}^{-/-} mice to the cold depends upon their ability to increase oxygen consumption. This conclusion is consistent with the effects of leptin and thyroid hormone on oxygen consumption in enabling \textit{Ucp1}^{-/-} \textit{Lep}^{-/-} mice to survive in the cold when the ambient temperature falls below 10–12 °C (8).
We next asked whether the variation in cold sensitivity among Ucp1−/− mice on different genetic backgrounds showed any relationship to their levels of oxygen consumption. Comparison of oxygen consumption, RER, and ambulatory activity all give a picture of more robust metabolism and physical activity in the F1 mice than in either B6 or 129 (Fig. 3). Body composition was similar among the three strains, although the hybrid mice weighed about 2 g more than either the B6 or 129 strains (Fig. 4). Food consumption was slightly more in the F1 mice and may account for the higher energy expenditure with less loss of body weight.

**Metabolite Levels in 129.Ucp1+/+ and 129.Ucp1−/− Mice—**

Several informative changes occurred in metabolite levels in serum and tissues during cold adaptation of Ucp1+/+ and Ucp1−/− mice from 27 to 4 °C (Table 2). The differences between genotypes were mostly in magnitude, occurring in the
same direction and sites, but were larger in the Ucp1<sup>−/−</sup> mice. Reduced triglyceride levels in serum together with increased β-hydroxybutyrate levels suggest increased fatty acid oxidation in both genotypes, but particularly in the Ucp1<sup>−/−</sup> mice. Major reductions in liver glycogen levels especially in cold-adapted Ucp1<sup>−/−</sup> mice suggest that glycogen is an important source of fuel for thermogenesis and again more so in Ucp1<sup>−/−</sup> mice than in Ucp1<sup>+/+</sup> mice. The elevated thyroid hormones in serum also suggest a role for these hormones in stimulating thermogenesis, particularly in Ucp1<sup>−/−</sup> mice. Surprisingly, the absence of major changes in either the triglycerides or glycogen of skeletal muscle suggests that muscle metabolism is not a key site for enhanced metabolism to support thermogenesis in either genotype.

**Metabolic Activity of Inguinal Fat in Vitro**—We have reported previously on the robust induction of brown adipocytes in inguinal fat depots of Ucp1<sup>−/−</sup> mice (5). The functional significance of this morphological transformation of the inguinal fat was unclear, because these adipocytes are devoid of UCP1 and, accordingly, incapable of heat production by that mechanism. However, the large difference in morphology between inguinal fat from wild type and Ucp1<sup>−/−</sup> mice encouraged us to evaluate whether metabolic differences, as a function of genotype and/or cold adaptation, could be detected (Fig. 5).

To test this potentiality further, freshly collected adipose tissue, analyzed histologically and shown to have the expected brown adipocyte morphology (Fig. 5A), was finely minced and incubated in a chamber with a Clark-type oxygen electrode to measure oxygen consumption with various substrates. Although these cells did not show measurable increases in oxygen consumption with glucose or fatty acids as substrates, succinate led to a striking increase in oxygen consumption in cells isolated from Ucp1<sup>−/−</sup> mice adapted to the cold (Fig. 5B). Cells from Ucp1<sup>−/−</sup> mice maintained at thermoneutrality or Ucp1<sup>+/+</sup> mice only showed a very slight increase in oxygen consumption. Consistent with an increase in oxygen consumption that is required for active thermogenesis, fatty acid oxidation in explants of inguinal fat were 6–10-fold higher in tissues from cold-adapted Ucp1<sup>−/−</sup> mice compared with Ucp1<sup>−/−</sup> mice maintained at thermoneutrality or wild type mice (Fig. 5C). Furthermore, immunoblot analysis of inguinal fat tissue showed enhanced AMP kinase activity as evidenced by increased (Thr-172) phosphorylation of the AMPK protein in cold-adapted Ucp1<sup>−/−</sup> mice (Fig. 5, D and E), suggesting that the enhanced fatty acid oxidation is occurring as a consequence of enhanced ATP turnover.

**Gene and Protein Expression in Skeletal Muscle and Inguinal Fat**—B6.Lep<sup>−/−</sup>.Ucp1<sup>−/−</sup> mice can adapt to the cold provided they are administered leptin (8). Induction of brown adipocytes was not detected in adipose tissues of these mice; however, the induction of SERCA2a mRNA levels in red gastrocnemius muscle in response to leptin and...
thyroid hormone treatment suggested a potential mechanism based upon Ca\(^{2+}\) cycling. To assess the potential thermogenic mechanisms that might be induced in cold-adapted mice, the levels of several genes associated with energy metabolism were determined in tissues from mice maintained at thermoneutrality and adapted to an ambient temperature of 4 °C (Tables 3–5). The most remarkable finding for gene expression in skeletal muscle was the general absence of significant differences in either the extensor digitalis longus (glycolytic fiber type) or soleus (oxidative fiber type) muscle (Tables 3 and 4). The most consistent difference was in the levels of PDK4 mRNA; this predicts increased lipid oxidation in Ucp1\(^{-/-}\) compared with Ucp1\(^{+/+}\) and higher oxidation in mice at 4 than at 28 °C. PGC1\(\alpha\) mRNA levels were also elevated in the soleus muscle of Ucp1\(^{-/-}\) mice at 4 °C.

Significantly more variation in gene expression was observed in the inguinal fat as shown in Table 5. The large increase in type 2 deiodinase is a strong indication that white adipocytes

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**TABLE 3**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ucp1(^{+/+}) (28 °C) A</th>
<th>Ucp1(^{+/+}) (4 °C) B</th>
<th>Ratio B/A</th>
<th>Ucp1(^{-/-}) (28 °C) C</th>
<th>Ucp1(^{-/-}) (4 °C) D</th>
<th>Ratio D/C</th>
<th>D/B</th>
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<tr>
<td>PDK4</td>
<td>16.0 ± 1.7 (^{a})</td>
<td>44.1 ± 4.7 (^{a})</td>
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<td>40.0 ± 7.03 (^{a})</td>
<td>112.4 ± 18.01 (^{a})</td>
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<td>SERCA2a</td>
<td>88.4 ± 5.1 (^{a})</td>
<td>178.5 ± 22.6 (^{a})</td>
<td>2.0</td>
<td>40.1 ± 4.4 (^{a})</td>
<td>84.5 ± 6.8 (^{a})</td>
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<tr>
<td>RYR1</td>
<td>142.4 ± 25.5 (^{a})</td>
<td>292.1 ± 46.7 (^{a})</td>
<td>2.0</td>
<td>113.0 ± 8.0 (^{a})</td>
<td>211.4 ± 26.1 (^{a})</td>
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<tr>
<td>PHLB</td>
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<td>67.0 ± 9.0 (^{a})</td>
<td>1.8</td>
<td>33.0 ± 4.0 (^{a})</td>
<td>57.0 ± 8.0 (^{a})</td>
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<td>SERCA1</td>
<td>50.4 ± 4.6 (^{a})</td>
<td>73.6 ± 4.2 (^{a})</td>
<td>1.5</td>
<td>45.5 ± 3.8 (^{a})</td>
<td>74.1 ± 3.5 (^{a})</td>
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<tr>
<td>PGCG1(\alpha)</td>
<td>75.0 ± 10.0 (^{a})</td>
<td>93.1 ± 7.9 (^{a})</td>
<td>1.2</td>
<td>85.0 ± 8.0 (^{a})</td>
<td>104.0 ± 4.0 (^{a})</td>
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<td>1.1</td>
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<tr>
<td>Mito-DNA</td>
<td>101.5 ± 5.0 (^{a})</td>
<td>119.9 ± 11.3 (^{a})</td>
<td>1.2</td>
<td>114.6 ± 12.8 (^{a})</td>
<td>79.0 ± 5.0 (^{a})</td>
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**FIGURE 5.** Oxidative metabolism in inguinal fat of cold-adapted mice. A, multilocular morphology of adipose tissue as shown by hematoxylin-eosin-stained 6-μm paraffin sections; B, oxygen consumption in cells suspension determined with a Clark-type oxygen electrode; C, palmitic acid oxidation as determined by the sum of CO\(_2\) production and accumulation of acid-soluble metabolites in cell-free mitochondrial enriched preparations; D and E, levels of phosphorylation (activation) of AMP kinase.
were being converted to brown adipocytes, and the increase in gene expression related to mitochondrial function, for example mitochondrial glycerol 3-phosphate dehydrogenase, PGClα, CoxII, and mitochondrial DNA content supports this interpretation. Although these changes were observed in wild type mice following cold exposure, the extent of induction (conversion) was much more robust in Ucp1−/− mice. The other notable induction was in the level of SERCA2a mRNA, especially in Ucp1−/− mice.

We have previously found increases in SERCA2a mRNA in skeletal muscle, but not in the amount of protein when analyzed by immunoblots. To determine whether any protein implicated in thermogenic mechanisms associated with Ca2+ cycling might be up-regulated in Ucp1−/− mice during cold adaptation, immunoblots were performed for SERCA1 and -2, and for total and phosphorylated phospholamban in red and white gastrocnemius muscles and various adipose tissues (Fig. 6). As shown, no differences could be detected in skeletal muscle for any of these proteins by immunoblots with respect to variation in genotye or ambient temperature. Expression of these proteins in fat depots provides a different view. Although expression in brown fat was similar invarient as in muscle, striking and consistent differences were detected in the three white fat depots analyzed (Fig. 6). An increase in the levels of total pentameric phospholamban occurred specifically in all three depots of cold-adapted Ucp1−/− mice. In addition, using antibodies against phosphorylated (Ser-16) phospholamban, we detected a change in the location and amount of the band, suggesting a shift in the phosphorylation pattern of PLB oligomers. This elevated content of the pentameric phospholamban and increased phosphorylation of the different phospholamban oligomers on Ser-16 is of potential significance as the pattern of expression of phosphorylated phospholambans in inguinal fat of cold-adapted Ucp1−/− mice begins to resemble the pattern found in skeletal muscle (Fig. 6). No change could be detected in expression of SERCA1 and SERCA2 in fat depots as a function of either genotype or ambient temperature.

**Chronic Cold Exposure following Cold Adaptation**—The absence of an increase in SERCA protein by immunoblots when increased levels of mRNA could be detected by quantitative reverse transcription-PCR suggested that possibly the adaptation period was too short relative to protein turnover for increases in major proteins to become evident. According, wild type and Ucp1−/− mice were slowly adapted to 4 °C and then maintained at 4 °C for 1 month (long term cold adaptation (LTCa)) to enhance the deposition of these proteins in skeletal muscle and adipose tissue by providing sustained stimulation to meet the increased thermogenic needs. No differences in body weight, fat mass, or lean mass could be detected between Ucp1−/+ and Ucp1−/− mice following LTCa (data not shown). Similar to the short term cold adaptation, no significant increase in SERCA proteins could be detected in quadriceps femoris or inguinal fat by immunoblot (Fig. 7). No change in phospholamban expression could be detected in muscle; however, in inguinal fat the same increase in total phospholamban was found, as well as a shift in the pattern of proteins detected.

### TABLE 4

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ucp1−/+ (28°C) A (28°C) B</th>
<th>Ucp1+/− (28°C) B</th>
<th>Ratio A/B</th>
<th>Ucp1−/+ (28°C) C</th>
<th>Ucp1+/− (28°C) D</th>
<th>Ratio D/C</th>
<th>Ratio D/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIO2</td>
<td>1.89 ± 0.30</td>
<td>29.82 ± 8.92</td>
<td>15.8</td>
<td>7.01 ± 4.18</td>
<td>221.7 ± 16.1</td>
<td>31.6</td>
<td>7.4</td>
</tr>
<tr>
<td>Ucp1</td>
<td>4.27 ± 0.85</td>
<td>159.38 ± 45.51</td>
<td>38.19</td>
<td>0.17 ± 0.17</td>
<td>0.53 ± 0.46</td>
<td>3.1</td>
<td>300.1</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>4.99 ± 0.77</td>
<td>120.05 ± 13.22</td>
<td>2.4</td>
<td>4.22 ± 0.41</td>
<td>29.68 ± 7.22</td>
<td>7.0</td>
<td>2.5</td>
</tr>
<tr>
<td>PCG1α</td>
<td>18.2 ± 1.49</td>
<td>38.75 ± 3.23</td>
<td>2.1</td>
<td>17.39 ± 2.80</td>
<td>90.67 ± 12.15</td>
<td>5.5</td>
<td>2.5</td>
</tr>
<tr>
<td>mGPD</td>
<td>40.1 ± 3.17</td>
<td>121.7 ± 39.35</td>
<td>3.0</td>
<td>50.32 ± 14.2</td>
<td>243.9 ± 34.5</td>
<td>4.8</td>
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<tr>
<td>COX II</td>
<td>7.26 ± 2.6</td>
<td>140.46 ± 23.80</td>
<td>1.9</td>
<td>36.18 ± 7.08</td>
<td>164.7 ± 16.3</td>
<td>4.6</td>
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<tr>
<td>Hspd1</td>
<td>5.49 ± 4.3</td>
<td>85.91 ± 17.15</td>
<td>1.5</td>
<td>68.9 ± 11.6</td>
<td>271.4 ± 31.7</td>
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<tr>
<td>Mito-DNA</td>
<td>387.7 ± 7.2</td>
<td>662.12 ± 8.54</td>
<td>1.7</td>
<td>114.63 ± 11.9</td>
<td>133.8 ± 18.5</td>
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<td>ACS1</td>
<td>5.81 ± 8.9</td>
<td>100.9 ± 7.7</td>
<td>1.7</td>
<td>39.23 ± 2.42</td>
<td>90.35 ± 2.42</td>
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<tr>
<td>ANT2</td>
<td>66.0 ± 10.6</td>
<td>120.6 ± 15.8</td>
<td>1.8</td>
<td>98.27 ± 21.2</td>
<td>217.3 ± 53.9</td>
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<tr>
<td>FAS</td>
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<td>1901.4 ± 226.1</td>
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<td>685.3 ± 114.7</td>
<td>1465.6 ± 148.4</td>
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<td>HSL</td>
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<td>71.1 ± 24.4</td>
<td>1.3</td>
<td>35.81 ± 2.96</td>
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<td>BSA7</td>
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<td>1.2</td>
<td>45.19 ± 6.88</td>
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<td>116.4 ± 14.6</td>
<td>131.6 ± 13.5</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>ANT1</td>
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<td>1259.5 ± 58.6</td>
<td>1.5</td>
<td>1454.2 ± 24.7</td>
<td>1655 ± 16.9</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Ryr1</td>
<td>86.6 ± 4.14</td>
<td>227.7 ± 30.99</td>
<td>2.6</td>
<td>39.38 ± 3.21</td>
<td>39.39 ± 3.49</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>CPT1</td>
<td>68.1 ± 5.9</td>
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<td>0.9</td>
<td>49.0 ± 11.5</td>
<td>42.74 ± 2.75</td>
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</tr>
<tr>
<td>SERCA2b</td>
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<td>22.0 ± 0.84</td>
<td>0.7</td>
<td>34.62 ± 4.9</td>
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<tr>
<td>NRF1</td>
<td>48.3 ± 4.09</td>
<td>63.1 ± 10.7</td>
<td>1.3</td>
<td>48.84 ± 8.21</td>
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<tr>
<td>ATPaseP1</td>
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<td>78.26 ± 1.70</td>
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<td>0.6</td>
<td>0.75</td>
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</tbody>
</table>
with an antibody specific for the phosphorylated phospholamban (Fig. 7). Increases in both total AMP kinase and in phosphorylated AMP kinase also occurred in LTCA of Ucp1\textsuperscript{−/−} mice. It is apparent that complex interactions are occurring between SERCA and phospholamban that include shifting patterns in the isoforms of phospholamban and the binding of phospholamban antibodies to proteins that migrate in the region of SERCAs (Fig. 8). As described by Suzuki and Wang (20), incubation of samples in Laemmli buffer at 30 °C prior to electrophoresis maintains associations between phospholamban and SERCA, and these are dissociated when the samples are boiled for 10 min (compare Fig. 8, A and B). The fact that these patterns of interaction are different in cold-adapted Ucp1\textsuperscript{−/−} mice suggests that they may be associated with cellular and molecular changes that enhance the thermogenic capability of the mouse. Additional experimentation will be required to elucidate the nature and significance of these molecular interactions to the regulation of thermogenesis in white fat.

Sarcoplasmatic vesicles (SR) were prepared to assay for SERCA enzymatic activity. Thapsigargin-inhibitable SERCA activity was measured in the SR fraction of gastrocnemius muscle, interscapular brown fat, and inguinal fat of cold-acclimated Ucp1\textsuperscript{−/−} and Ucp1\textsuperscript{+/+} mice (Table 6). The SERCA activity (Ca\textsuperscript{2+}-dependent ATPase activity) was 70–80% of total ATPase activity in skeletal muscle and 30–40% of ATPase activity in brown fat. A slightly higher level of Ca\textsuperscript{2+}-dependent ATPase activity was measured in both skeletal muscle and brown fat of Ucp1\textsuperscript{−/−} mice when compared with wild type mice (Table 6). Interestingly, although almost 100% of the Ca\textsuperscript{2+}-dependent ATPase activity in brown fat was inhibited by thapsigargin, 11.4 and 43.8% of the total ATPase activity in muscle of Ucp1\textsuperscript{+/+} and Ucp1\textsuperscript{−/−} mice, respectively, was calcium-dependent and resistant to inhibition by thapsigargin. This suggests that a significant portion of the ATP utilizing processes in muscle of Ucp1\textsuperscript{−/−} mice is a Ca\textsuperscript{2+}-dependent process that cannot be inhibited by the SERCA-specific inhibitor, thapsigargin.
This ATPase activity would most likely be uncoupled from Ca\(^{2+}\) transport as thapsigargin fully inhibited the transport of Ca\(^{2+}\) across the membrane of SR vesicles (Fig. 9). In addition, Mg\(^{2+}\)-dependent ATPase activity is significantly higher in \(\text{Ucp1}^{1/-}\) mice. This was observed in all three tissues studied (Table 6). Therefore, it needs to be noted that despite the fact that inguinal fat SR of either wild type or \(\text{Ucp1}^{1/-}\) mice shows neither Ca\(^{2+}\)-dependent ATPase activity (Table 6) nor ATP-dependent Ca\(^{2+}\) transport across the membrane of isolated SR (Fig. 9), Mg\(^{2+}\)-dependent ATPase activity is highly increased (Table 6). Because this activity was measured in the presence of sodium azide, it should not reflect or be derived from the \(F_{\text{ATP}}-F_{\text{ADP}}\) ATPase activity of the mitochondria, which is increased in these tissues.

Additional genes that could be contributing to thermogenesis in ingunal fat include heat shock protein 1 (Hspd1) and mitochondrial glycerol-3-phosphate dehydrogenase (Table 5). Up-regulation of the adenine nucleotide translocator (ANT2) also indicates that inguinal fat has an enhanced turnover of ATP as expected for a more thermogenically active tissue (Table 5).

DISCUSSION

Mice with a defective \(\text{Ucp1}\) gene are highly sensitive to an acute exposure to the cold at 4 °C but are able to tolerate this temperature when the ambient temperature is slowly reduced. This adaptive response provides an animal model to discover alternative mechanisms of thermogenesis that are independent of \(\text{UCP1}\)-based nonshivering thermogenesis in brown adipocytes. Furthermore, the inability of shivering to protect body temperature in mice upon acute exposure indicates that although shivering may be necessary for the maintenance of body temperature, and even this is an assumption that has not been tested experimentally, it is not sufficient. The most consistent physiological response that predicts the capacity of \(\text{UCP1}\)-deficient mice to adapt to the cold is their ability to increase oxygen consumption. 129.\(\text{Ucp1}^{1+}\) mice approximately double their oxygen consumption during cold adaptation; 129.\(\text{Ucp1}^{1/-}\) mice show a 2.5-fold increase in oxygen consumption, and \((\text{B6 } \times 129)\)F1.\(\text{Ucp1}^{1/-}\), which are completely resistant to the cold, show about a 3-fold increase (Figs. 1 and 3). At the other end of the spectrum, \(\text{B6.Ucp1}^{1/-}\).\(\text{Lep}^{-/-}\) mice, which cannot adapt to the cold, are unable to increase oxygen consumption (8).

We have proposed that the unavailability of brown fat \(\text{UCP1}\)-thermogenesis forces the mouse to use alternative thermogenic mechanisms that were not designed for the efficient regulation and distribution of heat and thus consume more substrate to maintain body temperature (5). The basic observation was that \(\text{Ucp}^{1/-}\) mice gained less weight than \(\text{Ucp}^{1+}\) mice when the ambient temperature was 20 °C but that the difference disappeared when the ambient temperature was raised to 27 °C. If this was the mechanism, then increased oxygen consumption should have been detected in \(\text{Ucp}^{1/-}\) mice at 20 °C. However, although indirect calorimetry showed the expected increase in oxygen consumption when the ambient temperature was reduced from 27 to 20 °C, there was no difference in oxygen consumption between the \(\text{Ucp}^{1+}\) and \(\text{Ucp}^{1/-}\) mice at either temperature. In this study, we now show that differences in oxygen consumption between \(\text{Ucp}^{1+}\) and \(\text{Ucp}^{1/-}\) mice do indeed exist, but they cannot be detected until the ambient temperature is about 10–12 °C, and the differences between genotypes become progressively larger as the ambient temperature progressively falls to 4 °C. This divergence in energy expenditure between these genotypes at 10–12 °C coincides with the crucial temperature when \(\text{Ucp}^{1/-}\).\(\text{Lep}^{-/-}\) double mutant mice can no longer adapt to decreasing ambient temperature unless they receive exogenous leptin or thyroid hormone (8). Thus, the resistance to diet-induced obesity in B6.\(\text{Ucp1}^{1/-}\) mice may be due to increased energy expenditure...
UCP1-independent Thermogenesis

Historically, skeletal muscle has been the primary candidate for UCP1-independent thermogenesis because of its mass and large potential metabolic capacity (21–25). A robust thermogenic mechanism originally described in the heater organ of pelagic fish (22, 26, 27) was recently proposed to supplement the UCP1 thermogenesis in brown adipose tissue (17, 28, 29). These authors proposed that Ca\(^{2+}\)-ATPase, normally transporting calcium back to its storage in endoplasmic reticulum, may be a source of heat production, when calcium transport is “uncoupled” from the ATP hydrolysis, thus rendering the functional activity of Ca\(^{2+}\)-ATPase less efficient (28, 30). Accordingly, we conducted an analysis of the levels of expression of mRNA and protein for components of a Ca\(^{2+}\) transport mechanism in cold-adapted 129.Ucp1\(^{+/−}\) mice. Surprisingly, no significant changes were detected in the muscle for components of this thermogenic mechanism in skeletal muscle; however, we show for the first time the induction of phospholamban, a regulatory component of Ca\(^{2+}\) cycling, in inguinal adipose tissue at the protein level. The elevated levels of the pentameric phospholamban and its phosphorylated form generate a pattern of expression that begins to resemble skeletal muscle rather than adipose tissue (Fig. 6). In addition, induction in expression of mRNA for SERCA2a, PGC-1\(
\text{α}\), CoxII, and mitochondrial DNA point to enhanced mitochondrial biogenesis, and the induction of type 2 deiodinase indicates that brown adipocytes were being induced.

Phospholamban is the principal regulator of SR-Ca\(^{2+}\)-ATPase activity in the heart and in oxidative muscle. The levels of calcium transport have been shown to be commensurate with the degree of phospholamban phosphorylation (31, 32). Dephosphorylated phospholamban acts as an inhibitor of SR-Ca\(^{2+}\)-ATPase activity, and phosphorylation releases the inhibition (20, 33). Phospholamban might also be regulated at the transcriptional level by the cAMP-response element-binding protein and cAMP-response element modulator (34). In addition, cAMP-activated protein kinase directly regulates phospholamban function by modulating its phosphorylation status (35). Furthermore, a phosphorylation-mediated inactivation of phospholamban inhibitory action on Ca\(^{2+}\)-ATPase activity may also be achieved by intracellular calcium-stimulated calmodulin-dependent protein kinase activity (36, 37). Although increases in SERCA2a mRNA levels are 2.5-fold elevated, increased SERCA2a protein levels in the inguinal fat were not evident, and increases in a thapsigargin-inhibitable SERCA activity could not be measured in SR preparations. There remains the observation that increased Mg\(^{2+}\)-ATPase was measured in both skeletal muscle and inguinal fat of cold-

**FIGURE 8.** Western blot analysis of calcium cycling proteins in the heart, quadriceps femoris muscle (Quadriceps m.), and inguinal adipose tissue (AT) from C57BL/6J (wild type, WT) and Ucp1\(^{+/−}\) mice on the B6 background. Cold-adapted wild type and Ucp1\(^{+/−}\) mice were acclimatized to cold by decreasing ambient temperature 2 °C per day. LTCA mice were kept at 4 °C for 54 days. Control mice (TN, thermoneutrality) were kept at 28 °C for the same period of time. Samples were solubilized in Laemmli buffer at 30 °C for 30 min (A, C, and D) or boiled for 10 min (B), prior to loading to SDS-polyacrylamide gel. A, the amount of phospholamban bound to SERCA is increased in inguinal fat of LTCA Ucp1\(^{+/−}\) mice. B, binding between SERCA-PLB was disrupted by boiling. C, membrane A was probed with anti-phosphorylated PLB antibody and showed that the amount of total phosphorylated PLB is increased in inguinal fat of LTCA Ucp1\(^{+/−}\) mice. A small amount of phosphorylated PLB monomer was also detected in LTCA Ucp1\(^{+/−}\) mice. D, composite image of nonphosphorylated (A) and phosphorylated (C) PLB signal. p-PLB, phosphorylated PLB.
UCP1-independent Thermogenesis

### TABLE 6
ATPase activity in tissues of cold-adapted wild-type (Ucp1+/+ ) and Ucp1−/− mice

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Gastrocnemius muscle</th>
<th>Interscapular brown adipose tissue</th>
<th>Inguinal fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO4+</td>
<td>Ucp1+/+</td>
<td>1.08 ± 0.06</td>
<td>2.56 ± 0.04a</td>
</tr>
<tr>
<td>MgSO4+ - TGb</td>
<td>Ucp1−/−</td>
<td>0.88 ± 0.11</td>
<td>2.13 ± 0.05a</td>
</tr>
<tr>
<td>MgSO4+ + CaCl2</td>
<td>Ucp1+/+</td>
<td>6.30 ± 0.02</td>
<td>8.08 ± 0.30b</td>
</tr>
<tr>
<td>MgSO4+ + CaCl2 - TG</td>
<td>Ucp1−/−</td>
<td>1.80 ± 0.13</td>
<td>6.11 ± 0.28b</td>
</tr>
<tr>
<td>CaCl2-sensitive</td>
<td>Ucp1+/+</td>
<td>71.4%</td>
<td>24.5%</td>
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<tr>
<td>CaCl2-sensitive</td>
<td>Ucp1−/−</td>
<td>11.4%</td>
<td>43.8%</td>
</tr>
<tr>
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<td>Ucp1+/+</td>
<td>56.4%</td>
<td>72.5%</td>
</tr>
<tr>
<td>CaCl2-insensitive</td>
<td>Ucp1−/−</td>
<td>31.7%</td>
<td>100%</td>
</tr>
</tbody>
</table>

* ATPase activity levels are significantly different between genotypes (Ucp1+/+ versus Ucp1−/−) at p < 0.01. Each determination was performed in triplicate on an endoplasmic reticulum fraction isolated from a pool of 3 to 6 mice.

** TG is 100 nM thapsigargin.

Figure 9. ATP-dependent Ca2+ transport into the vesicles of sarcoplasmic reticulum isolated from muscle (quadriceps femoris) (A) and inguinal adipose tissue (B) of Ucp1+/+ mice acclimatized to the cold (4°C). Ca2+ transport was determined spectrofluorometrically with the aid of the Ca2+-binding fluorescent dye Fura 2. Binding of the Ca2+ lowers absorption maximum wavelength of Fura 2 from 380 nm (zero Ca2+) to 340 nm (with Ca2+). Lowering the 340/380 ratio therefore represents the disappearance of Ca2+ from the system and their accumulation inside the SR vesicles. ATP was used to stimulate Ca2+-ATPase-dependent Ca2+ transport. In addition, we disrupted the membranes at the end of the test with Triton X-100 to show that Ca2+ was trapped inside the vesicles, as it is released after membrane disintegration. Thapsigargin (TG) completely inhibited the Ca2+ transport into the SR vesicles of skeletal muscle (A, dashed line), suggesting that Ca2+-ATPase-specific Ca2+ transport was occurring.

adapted mice, and although it could also be thermogenic, the exact identity of this ATPase activity is unknown.

These molecular changes in components of Ca2+ cycling together with increased oxygen consumption and fatty acid oxidation of inguinal fat from Ucp1−/− tissue ex vivo indicate that traditional white fat depots have the potential to contribute to the thermogenic capacity of cold-adapted Ucp1−/− mice. In this study we showed increased succinate-induced respiration rate of the adipose tissue from cold-acclimatized Ucp1−/− mice. Succinate was used as a universal metabolic currency and was added to the system containing finely minced adipose tissue. The metabolic activity reflects the sum of the succinate utilization in both adipocytes and in released mitochondria. Elevated metabolic capacity was further documented by an increased palmitate oxidation rate, which was measured in fresh tissue homogenates of adipose tissue. Increased AMPK phosphorylation further supported the idea of higher metabolic capacity of the tissue associated with higher fatty acid utilization. Hepatic and skeletal muscle triglycerides were not affected by cold acclimation; however, cold exposure lowered circulating triglyceride concentrations and elevated β-hydroxybutyrate levels in both Ucp1+/+ and Ucp1−/− mice adapted to the cold. Triglyceride decrease and β-hydroxybutyrate increase were particularly robust in cold-adapted Ucp1−/− mice indicating that increased fat oxidation occurs during cold adaptation. In addition, depletion of hepatic glycogen levels and lowered circulating glucose concentration in cold-acclimated Ucp1+/− mice point to enhanced glucose oxidation as an important fuel for the support of thermogenesis during cold adaptation. The increase in AMP kinase activity observed in this study is consistent with recent reports showing the stimulation of AMP kinase activity during enhanced glucose and fat oxidation in response to increased demands for energy expenditure in adipose tissue (38, 39) and skeletal muscle (40, 41). Accordingly, energy metabolism involving both glucose and fat oxidation is enhanced in cold-adapted mice, but it seems to involve metabolite changes in liver more so than in skeletal muscle.

It is important to understand the type of cell contributing to enhanced thermogenesis in white fat. There are physiological conditions when white adipose tissue has the morphological and biochemical characteristics of brown adipose tissue. These characteristics include the multilocular histological profile, high mitochondrial content, high metabolic capacity, and the presence of UCP1. Cells resembling brown adipocytes were first observed to appear in parametrical adipose tissue of mice (42) and in rats adapted to cold (43). In addition to the induction of brown adipocytes in white fat depots by cold, others have observed an induction of multilocular adipocytes in white adipose tissue of rats and mice following chronic treatment with β3-selective adrenergic receptor agonists (44, 45). The analysis of these tissues from β3 agonist-treated rodents has led to the conclusion by some investigators...
that most of the multilocular adipocytes in white fat did not express UCP1 and that these multilocular cells expressed Ucp3 instead (46). A pattern of immunostaining for UCP1, called harlequin, caused by multilocular adipocytes without UCP1 protein has been described in interscapular brown fat of rats (47). More recently, Granneman and co-workers (48–50) published papers in which multilocular cells were induced in the epididymal fat of rats by CL316,243; however, Ucp1 mRNA was not significantly induced. The molecular analysis by Granneman and co-workers (48–50) concluded that agonist treatment induced a proinflammatory response that was followed by cell proliferation, increases in mitochondrial biogenesis and oxidative metabolism, but no Ucp1 induction. A major question emerging from the studies with β3-adrenergic receptor agonists is whether these pharmaceutical agonists are toxic when chronically administered.

In an extensive morphological and molecular analysis of cold induction of multilocular brown adipocytes in inguinal and retroperitoneal fat depots from four different recombinant inbred strains derived, immunoreactive UCP1 in multilocular adipocytes was always observed (51). The antibody used in this study detected UCP1 and no other proteins as evident from the immunoblot analysis of brown fat tissue from Ucp1−/−/− mice compared with Ucp1+/−/− mice (4). The range of multilocular brown adipocytes in these preparations varied over 65-fold and correlated significantly with levels of Ucp1 mRNA. Thus in our experience, as with others studying brown adipocyte induction with cold exposure (43), exposure of mice to the cold induces multilocular brown adipocytes to form in white fat depots. Our molecular analysis of the inguinal fat in this study showed that a 38-fold induction of Ucp1 mRNA occurred in 129.Ucp1+/−/− following cold adaptation, and this represented 13% of the amount of Ucp1 mRNA that was present in the interscapular fat of cold-adapted Ucp1+/−/+. This level of mRNA in the inguinal fat of Ucp1+/−/− mice was 300-fold higher than that of the weak background signal produced in Ucp1−/−/− mice. These data on Ucp1 expression showing the high potency for induction of Ucp1 in this experimental paradigm, together with the induction of deiodinase (Table 5), strongly suggest that the multilocular cells in inguinal fat depots of Ucp1+/−/− mice are brown adipocytes.

We propose that these brown adipocytes in Ucp1+/−/− mice have acquired properties that enable them to be thermogenic during cold adaptation, independent of the presence of UCP1. It is important to underscore the fact that UCP1-independent thermogenesis will not depend on only one mechanism, but in addition to Ca2+ cycling, other mechanisms such as glycero-phosphatase cycling could be important. It is also possible that SERCA activity in the mitochondria of interscapular brown adipocytes could be thermogenic as recently proposed by de Meis et al. (29).

In summary, we recently showed that mice doubly deficient for both leptin and UCP1 could not adapt for survival to temperatures below 12 °C, unless they were administered either leptin or thyroid hormone during the cold adaptation protocol (8). These mice did not show an adaptive morphological change that increases brown adipocytes in inguinal fat, possibly because a critical contribution from leptin in this process was absent (52). Thus, exogenous leptin and thyroid hormone induced thermogenesis in the double mutant mice, possibly through Ca2+ cycling in the skeletal muscle that enabled them to survive in the cold (8). In this study UCP1-deficient mice showed virtually no change in gene expression in muscle but profound changes in white adipose tissues at the morphological, metabolic, and molecular levels. Although the contribution by white fat metabolism to the thermogenic needs of the mouse cannot be quantified by these studies, it is clear that the strategies available to the animal for thermogenesis are rich and varied.

Because an efficient thermogenic system is crucial for survival of the animal, the selection of effective thermogenic mechanisms will be governed by the genetic constitution of the mouse, as evident from the effects of a hybrid genetic background on the phenotype of Ucp1−/−/− mice and the presence of mutant genes like ob/ob, as well as the nutritional and hormonal milieu.

Acknowledgments—We thank Christie Bearden, Michael Morris, Megan Morris, and Ryan Jackson for excellent technical assistance and Dr. Robert A. Koza for consultation and experimental support on several aspects of this project.

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UCP1-independent Thermogenesis

UCP1-independent Thermogenesis in White Adipose Tissue of Cold-acclimated $Ucp1^{-/-}$ Mice

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doi: 10.1074/jbc.M606114200 originally published online August 16, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M606114200

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