Adaptive thermogenesis and thermal conductance in wild-type and UCP1-KO mice

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Adaptive thermogenesis and thermal conductance in wild-type and UCP1-KO mice. Am J Physiol Regul Integr Comp Physiol 299: R1396–R1406, 2010. First published September 8, 2010; doi:10.1152/ajpregu.00021.2009.—We compared maximal cold-induced heat production (HPmax) and cold limits between warm (WA; 27°C), moderate cold (MCA; 18°C), or cold acclimated (CA; 5°C) wild-type and uncoupling-protein 1 knockout (UCP1-KO) mice. In wild-type mice, HPmax was successively increased after MCA and CA, and the cold limit was lowered to −8.3°C and −18.0°C, respectively. UCP1-KO mice also increased HPmax in response to MCA and CA, although to a lesser extent. Direct comparison revealed a maximal cold-induced recruitment of heat production by +473 mW and +227 mW in wild-type and UCP1-KO mice, respectively. The increase in cold tolerance of UCP1-KO mice from −0.9°C in MCA to −10.1°C in CA could not be directly related to changes in HPmax, indicating that UCP1-KO mice used the dissipated heat more efficiently than wild-type mice. As judged from respiratory quotients, acutely cold-challenged UCP1-KO mice showed a delayed transition toward lipid oxidation, and 5-h cold exposure revealed diminished physical activity and less variability in the control of metabolic rate. We conclude that BAT is required for maximal adaptive thermogenesis but also allows metabolic flexibility and a rapid switch toward sustained lipid-fuelled thermogenesis as an acute response to cold. In both CA groups, expression of contractile proteins (myosin heavy-chain isoforms) showed minor training effects in skeletal muscles, while cardiac muscle of UCP1-KO mice had novel expression of beta cardiac isoform. Neither respiration nor basal proton conductance of skeletal muscle mitochondria were different between genotypes. In subcutaneous white adipose tissue of UCP1-KO mice, cold exposure increased cytochrome-c oxidase activity and expression of the cell death-inducing DFFA-like effector A by 3.6-fold and 15-fold, respectively, indicating the recruitment of mitochondria-rich brown adipocyte-like cells. Absence of functional BAT leads to remodeling of white adipose tissue, which may significantly contribute to adaptive thermogenesis during cold acclimation.

nonshivering thermogenesis; cold limit; mitochondrial proton leak; respiratory quotient; myosin heavy chain isoforms

In mammals two forms of facultative heat production exist: shivering and nonshivering thermogenesis. While the former releases heat from involuntary contractions of skeletal muscle fibers, a large proportion of the latter is derived from brown adipose tissue (BAT) and its thermogenic uncoupling protein, UCP1 (5). The ability to produce heat through UCP1-mediated adaptive nonshivering thermogenesis is considered to be a critical evolutionary development that promoted the radiation of eutherian mammals in cold environments (5). Comparative physiological studies of endothermic vertebrates, however, also suggest the presence of UCP1-independent mechanisms of adaptive thermogenesis. Marsupials exhibit increased HPmax in response to cold acclimation, and some species cope with cold winter seasons in their natural habitat (9, 41). Although in these ancient mammals, a gene encoding the UCP1 ortholog is found, the functional significance of marsupial UCP1 for thermogenesis is questionable (21, 22, 31). In birds, despite the lack of UCP1 and BAT, many avian species, even of small body size, inhabit regions where ambient temperatures may decrease below −20°C for prolonged periods of time and are known to exhibit pronounced seasonal changes in HPmax (40). In rats and hamsters, measurements of BAT metabolic activity in vivo suggested that a significant proportion of the cold-induced increase in nonshivering thermogenesis does not originate in BAT (8, 35).

As such, mice with a targeted inactivation of the UCP1 gene (UCP1-KO) provide a mammalian model organism for studying (adaptive) metabolic responses to the absence of functional BAT. UCP1-KO mice cannot replace shivering with nonshivering thermogenesis and exhibit a cold-sensitive phenotype when tested at 4°C (7). However, UCP-1 KO mice are able to survive in the cold (4°C) for prolonged periods when previously acclimated to 18°C (moderate cold). Whereas shivering thermogenesis may be the sole contributor to this increased heat production and cold tolerance in UCP1-KO mice (11), elevated proton leak in skeletal muscle mitochondria (28) and metabolic alterations in white adipose tissue (WAT), including futile ion and substrate cycling and the emergence of brown-like adipocytes, have been suggested (1, 12, 45).

To judge the physiological relevance of any specific biochemical mechanism for adaptive thermogenesis, the magnitude of the cold-induced thermogenic capacity increase must be in accordance with the improvement of the cold limit. Maximal cold-induced thermogenesis and corresponding cold limits have been previously studied in laboratory mice and other small mammals and birds (e.g., Refs. 9, 13, 15, 19, 20, 39), but they have never been determined in genetically modified mouse models with metabolic or thermogenic deficiencies. To further characterize the thermophysiology of UCP1-KO mice, we first studied their metabolic responses to acute cold exposure (5 h at 5°C) in more detail, by simultaneously recording body temperature (Tb), physical activity, metabolic rate (MR), and respiratory quotient (RQ). To assess
the maximal thermogenic capacity, we exposed mice to stepwise decreasing ambient temperatures ($T_a$) in the range of 30°C to −20°C. This protocol enabled us to calculate cold limits, i.e., the threshold ambient temperature at which euthermia could be sustained by maximal heat production at rest (HPmax). The comparison of HPmax and cold limit in warm-(27°C, WA), moderate cold- (18°C, MCA), and cold- (5°C, CA) acclimated mice revealed the extent to which adaptive thermogenesis occurs in the presence and absence of functional BAT. The parallel recordings of $T_a$ and MR enabled us to calculate the thermal conductance and to compare heat production and heat loss of warm- and cold-acclimated wild-type and UCP1-KO mice. By injection of norepinephrine, we determined the fraction of cold-induced HPmax due to nonshivering thermogenesis regulated by the sympathetic nervous system.

In the search for structural or functional adaptations of cold-acclimated UCP1-KO mice outside of BAT, we sampled skeletal muscles, heart muscle, and white adipose tissues. We analyzed the expression of myosin heavy chain (MyHC) isoforms in different skeletal muscles and measured proton leak kinetics in isolated mitochondria from the hind limb skeletal muscles. In white adipose tissues, we measured cytochrome-c oxidase (COX) activity as a surrogate for respiratory capacity and expression of the cell death-inducing DFFA (DNA fragmentation factor alpha)-like effector A (CideA) as a marker for the recruitment of brown adipocyte-like cells. Thereby, we aimed to provide further insights into metabolic alterations and thermoregulatory adjustments, which facilitate cold acclimation in the absence of functional BAT.

MATERIALS AND METHODS

**Mice and maintenance.** Wild-type and UCP1-KO littermates (genetic background C57BL/6J) were derived from heterozygous breeding pairs in our colony. The founder mice for establishing our colony were originally provided by Dr. Leslie Kozak (Pennington Medical Research Center). Mice were born at 27°C and weaned to 24°C at 3–4 wk of age. They were fed Altromin 1314 standard breeding chow (Lage, Germany), had free access to water, and were kept on a 12:12-h light-dark cycle. Mice were genotyped by amplifying a 201-bp (wild-type) and 409 bp (KO) fragment from the UCP1 gene, according to a protocol kindly provided by L. Kozak. After genotyping, the WT and UCP1-KO mice included in our experiments were housed singly throughout the entire study period. Each cage was equipped with sawdust and two to three slices of tissue paper. Except for white adipose tissue sampling, only female mice were used. In all experimental mice, the presence or absence of UCP1 protein was also confirmed post mortem by immunological detection in BAT [as published previously (23)].

**Experimental schedules.** At the age of 2–4 mo, female mice were intraperitoneally implanted with temperature-sensitive transmitters (series 3000; model XM-FH; Mini Mitter, Bend, OR, USA). These transmitters weigh 1.5–1.6 g and are able to register body temperature at ±0.1°C. In addition, they provide a relative measure of gross activity over time, i.e., if the animal is moving relative to a receiver antenna. After 1 wk of recovery from surgery, mice were randomly assigned to warm (WA, 27°C) or to moderate cold (MCA, 18°C) acclimation. Following 3 wk at the respective acclimation temperature, acute cold tolerance to 5°C was investigated, and cold limits were determined 1 wk later. A third group of mice was maintained at 18°C for 3–4 wk, after which ambient temperature was lowered to 5°C for another 3–4 wk (CA, 5°C), until cold limits were determined.

On the molecular level, we determined the expression of MyHC isoforms in various skeletal muscle groups and the heart and measured basal proton leak kinetics in isolated mitochondria from the hind limb skeletal muscles. In white adipose tissues, we measured cytochrome-c oxidase activity as a surrogate for respiratory capacity and CideA expression as a marker for the recruitment of brown adipocyte-like cells. Mitochondrial proton leak kinetics of skeletal muscles, as well as COX activity and CideA expression in white adipose tissues were investigated in separate groups of wild-type and UCP1-KO mice (males and females) acclimated to either 27°C (WA) or 5°C (CA) for 3–4 wk. For CA experiments, mice were kept in climate chambers controlling ambient temperature at a precision of ±0.5°C. Only the mice cold acclimated for mitochondrial studies were kept in a cooling cabinet in which the ambient temperature at the cage level was 10°C during the light phase due to heat production from illumination, while it was 5°C during the dark phase. All experiments and procedures were approved by the German animal welfare authorities (RP Giessen, approval no. MR 17/1–32/2005 and MR 17/1–14/2008).

Indirect calorimetry and body temperature telemetry setup. Mice were transferred to metabolic cages (1.8 l) placed on telemetry receivers (TR 3000, Mini Mitter) in a temperature-controlled (±0.5°C) climate chamber. Body temperature and physical activity were recorded in 1-min epochs using the VitalView software (Mini Mitter). The principal setup of the respirometric system has been published previously (19). In brief, volumes of oxygen consumed ($\Delta$Vol%) and volumes of carbon dioxide ($\Delta$Vol%) produced by each mouse were measured during 60 s every 2 to 4 min using an electrochemical O$_2$ analyzer (S-3AII, Ametek, Pittsburgh, PA) and a CO$_2$ analyzer (UNOR 6N; Sick-Maihak, Hamburg, Germany and Uras 14; Hartmann & Braun, Skyva, Switzerland). The coordination of the different peripheral measuring devices was governed by custom designed VI basic modules developed by G. Heldmaier. Oxygen readings were converted to MR, according to the following equation: $MR$ [ml O$_2$/h] = $\Delta$Vol%O$_2$·flow [l/h]·10. The flow rate was set to 35 l/h. To adjust for differences in flow rates in air leaving and entering the metabolic cages, RQ (volumes of CO$_2$ produced/volumes of O$_2$ consumed) was used. Effective heat production [HP, mW] and thermal conductance C [mW/°C] were calculated according to (17) as Eq. 1: $HP = (4.44 + 1.43·RQ)·MR$ and Eq. 2: $C = HP/(T_b - T_a)^{-1}$, where MR is metabolic rate (ml/h), $T_b$ is body temperature (°C), and $T_a$ is ambient temperature (°C).

**Cold endurance test.** Mice were maintained in cages of 1.8 l with no food or water provided. Following 3–4 h of thermoneutral conditions (30°C; e.g., Refs 16, 27, and 42), ambient temperature ($T_a$) was maintained at 5°C for up to 5 h. In each trial, up to 3 mice and one obligate reference channel were recorded in parallel, yielding a 2–4-min resolution of metabolic readings (i.e., 1 mouse = 2 min resolution, 2 mice = 3 min resolution, 3 mice = 4 min resolution per individual reading). A mouse was immediately rescued from the chamber and classified as cold sensitive if its body temperature dropped below 31°C. All measurements commenced at 0800 CET, and the genotype was blinded to the experimenter. Resting metabolic rates (RMRs) were determined from the lowest mean of three consecutive O$_2$ readings, equivalent to 9–12 min in the climate chamber, which yielded lowest variability (assessed by CV, coefficient of variation).

$HPmax$ and cold limit. For determination of maximal cold-induced heat production (HPmax) at rest and calculation of cold limits, single animals (plus one reference channel) were measured (genotype blinded), which yielded a 2-min resolution of O$_2$ readings. After 2–3 h at 30°C, a mouse was exposed to a series of decreasing $T_a$s for determination of RMRs. In MCA and CA mice, $T_a$ was lowered stepwise from 30, 24, 15, 10, 5, 0°C to −20°C. In WA mice, we were unable to determine RMRs at any $T_a$ ≤ 15°C (mice were continuously active). In this experimental group $T_a$ was lowered stepwise from 30,
27, 24, 20, 18, to −20°C. With the exception of −20°C, each temperature was sustained for at least 20 min and up to 90 min, i.e., until stable readings could be observed over a period of 6–10 min (see Supplemental Fig. S1 in the online version of this article). A mouse was removed from the metabolic chamber when its body temperature clearly dropped despite an increase in heat production (oxygen consumption). During the experiment, none of the animals suffered from frostbite or any other adverse side effects resulting from cold exposure. A whole measurement day lasted up to 8 h, during which time, no food or water was supplied to the mouse.

Maximal resting metabolic rate (RMRmax; ml/h) was defined as the highest MR observed in a nonexercising mouse. These highest values were usually exhibited around the time when mice became hypothermic (see Supplemental Fig. S1 in the online version of this article). RMRmax was converted to maximal resting heat production (HPmax, mW) using Eq. 1. The point of intersection of HPmax with the regression of resting HP with Ta was used to calculate the effective cold limit (see Supplemental Fig. S2 in the online version of this article).

Norepinephrine-stimulated thermogenesis. For determination of maximal norepinephrine-stimulated heat production (NEmax), single mice were maintained in the calorimetry system for 2–3 h at 30°C. Mice were briefly removed from the system and received a subcutaneous injection of norepinephrine (1 mg/kg). The changes in oxygen consumption and carbon dioxide production before and after NA stimulation were measured as outlined above, except that the resolution of readings was set to 20 s. NEmax was calculated from the highest mean, resting metabolic rate at 30°C [= resting metabolic rate at thermoneutrality (RMR)] and was calculated from the lowest mean of three consecutive O2 readings, equivalent to 1 min in the climate chamber. Data were converted to HP according to Eq. 1.

SDS-PAGE electrophoresis of myosin heavy-chain isoforms. Selected skeletal muscle biopsies (M. gastrocnemius, M. tibialis anterior, and M. masseter) were quickly dissected and shock frozen in liquid nitrogen. In addition, heart muscle was obtained from the mice kept at 5°C. Muscle samples from 5 to 20 mg were homogenized on ice in 19 or 38 vol of buffer (in mM): 250 sucrose, 100 KCl, and 5 EDTA, for protein analyses. Electrophoretic separation of murine MyHC isoforms was adapted from a method used in rats and applicable to other rodents (44). SDS-PAGE was run on <1 μg total protein and identified four skeletal muscle isoforms, types MyHC 1, 2a, 2x, and 2b. Although heart-specific isoforms can be separated with native conformation gels, our technique also resolved cardiac isoforms clearly, types MyHC alpha and beta (identical to MyHC 1).

Isolation of skeletal muscle mitochondria. Hindlimb skeletal muscle tissue was quickly dissected and immediately placed in ice-cold isolation CP1-medium containing 100 mM KCl, 50 mM Tris-HCl and 2 mM EGTA, pH 7.4, at 4°C. Muscle tissue was cleansed of adipose tissue patches, minced with scissors, and repeatedly rinsed with CP-1 medium, stirred for 3 min in CP-2 medium (CP-1 plus 1 mM ATP, 5 mM MgCl2, 0.5% (wt/vol) fatty acid free BSA (Sigma no. A3803), 2.1 U/ml protease (Subtilisin A, Sigma-Aldrich), pH 7.4, at 4°C) and homogenized in CP-2 medium using a polytron tissue homogenizer (KineticA, Franklin, OH). The homogenate was stirred in CP-2 for 3 min, and then, mitochondria were isolated using differential centrifugation and resuspended in CP-1 medium. Protein concentration was determined using the Biuret method with fatty acid-free BSA as a standard.

Measurement of mitochondrial oxygen consumption and proton conductance in skeletal muscle. Oxygen consumption was measured using a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) maintained at 37°C and calibrated with air-saturated medium [120 mM KCl, 5 mM KH₂PO₄, 3 mM HEPES, 1 mM EGTA, and 0.3% (wt/vol) defatted BSA, pH 7.2], which was assumed to contain 406 nmol O₂/ml (37). Skeletal muscle mitochondria were suspended at 0.35 mg/ml in 2.5 ml medium and incubated with 8 μM rotenone (to inhibit complex I) and energized with 6 mM succinate. State 3 respiration was established by addition of 600 μM ADP and shifted to state 4 by the addition of 1 μM oligomycin to inhibit the ATP synthase. Finally, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was titrated in 0.3-μM steps to completely uncouple the mitochondria and stimulate maximum respiratory activity. For the measurement of mitochondrial proton leak, mitochondria were incubated with oligomycin, rotenone, and 150 nM nigericin (to convert the pH gradient to membrane potential). The respiratory rate of mitochondria, in the presence of oligomycin (state 4), is proportional to the rate at which protons leak across the inner membrane. The kinetic response of the proton conductance to its driving force (proton motive force) can, therefore, be measured as the relationship between respiration rate and membrane potential when the potential is varied by titration with electron transport chain inhibitors (3, 30). Respiration rate and membrane potential were determined simultaneously using electrodes sensitive to oxygen and to the potential-dependent probe methyltriphenylphosphonium (TPMP⁺) (4). The TPMP⁺ electrode was calibrated with sequential additions of up to 2.5 μM TPMP⁺. Then 6 mM succinate was added to start the reaction, and the respiratory chain was progressively inhibited with up to 1.2 mM malonate. Finally, 0.3 μM FCCP was added to release all TPMP⁺ from the mitochondrial matrix to correct for the drift of the electrodes. TPMP⁺ correction factor was assumed to be 0.35 for skeletal muscle, as described previously (38).

Cytochrome-c oxidase activity assay in white adipose tissues. Mice were killed by means of a CO₂ overdose. Snap-frozen white adipose tissue was homogenized in liquid nitrogen and further homogenized in tissue buffer [10 mM HEPES, 40 mM KCl, 2 mM EGTA, 1% (vol/vol) Tween 20, 2 μM PMSF, 2 μM oligomycin, 10 mM KF] with a Potter-type homogenizer. The homogenate was sonicated with several short bursts, centrifuged (16,000 g, 2 min), and the supernatant was employed for protein quantification by the Biuret method. Activity assays were performed with cleared homogenate equivalent to 300 μg protein in assay buffer [50 mM KH₂PO₄, 2 mM EGTA, 1% (vol/vol) Tween 20, 20 mM ascorbic acid, 5 mM ADP, 2 μM oligomycin] and started by the addition of cytochrome c (40 μM final concentration). COX activity was determined by recording oxygen consumption with a Clark-type electrode (model no. 10; Rank Brothers, Cambridge, UK). For every sample, we calculated the mean of two independent measurements on different days.

Quantitative PCR. Flash-frozen white adipose tissue depots were grinded in liquid nitrogen. A portion of the resulting powder was homogenized with a turrax-type instrument. Total RNA was isolated with a column-based kit (SV Total RNA Isolation System; Promega, Madison, WI) and quantified photometrically. We retrotranscribed 500 ng total RNA into cDNA (QuantitTect reverse transcription kit; Qiagen, Germantown, MD). For quantitative PCR, the SensiMix SYBR reagent (Bioline) was employed with an Eppendorf MasterCycler instrument. For all primer pairs, template dilution series were included to determine and correct for PCR efficiency. Every sample was measured in technical triplicates. CideA expression was normalized to the mRNA abundance of the housekeeping gene Hsp90. Primer sequences: Hsp90 forward: AGGAGGGTCAAGGAAGTTCT, Hsp90 reverse: CTGTTTCTGTATCGCCCAGT, and CideA reverse: GCAGCGGTGTTAAGGAATCTCCTGCT.

Statistical analysis. Unless otherwise indicated, data in figures, tables, and text are expressed as means ± SD. To assess the statistical significance of differences between mean values, a two-way ANOVA (factors genotype, acclimation temperature, and their interaction) was performed. Because of heterogeneity of variances, significant differences in the response to NE injection were assessed using Kruskal-Wallis ANOVA on ranks (H-test), followed by multiple comparisons. Summed activity counts before and after lowering Ta to 5°C (cold
endurance test) were compared using Wilcoxon test. RQ and conductance time courses at 5°C were analyzed by repeated-measures ANOVA. For these statistical analyses, SigmaStat 3.5 (Systat Software, Chicago, IL) was used. A linear mixed-effects model fit by maximum likelihood (random factor: individual mouse, fixed factors: body mass × Ta × genotype) using the statistical software package R (36) was employed to reveal significant differences between slopes for HP vs. Tb between experimental groups. A threshold of $P < 0.05$ was considered statistically significant.

RESULTS

To compare the cold-induced changes in metabolic rate, body temperature, RQ, and thermal conductance, we challenged wild-type and UCP1-KO mice with two paradigms of acute cold exposure: a cold endurance test (5 h at 5°C) and a cold limit test (determination of HPmax).

In the cold endurance test, the metabolic responses in the WA and MCA mice of both genotypes were compared. Body temperatures at 30°C were similar in the four groups of mice, with minimal values observed within the last hour before ambient temperature ($T_a$) was lowered to 5°C (Fig. 1A). Upon cold exposure, body temperature in all groups dropped by 1–2°C within 30 min. Whereas mice from the WA groups were unable to sustain normal body temperature at 5°C ($T_b > 31°C$), the MCA mice of both genotypes were able to maintain euthermia throughout the entire 5-h cold period, albeit at a reduced body temperature set-point compared with 30°C $T_a$. These results confirmed previous published findings that a 3-wk acclimation period to 18°C was sufficient to increase cold tolerance in wild-type and UCP1-KO mice (11).

The MCA mice were heavier than the WA mice, and RMRs were significantly higher. Within WA and MCA acclimation groups, however, body mass and RMRs were not different between genotypes (Table 1), which enabled us to directly compare the metabolic rates of wild-type and UCP1-KO mice. In response to the lowering of ambient temperature from 30 to 5°C (Fig. 1B), all mice increased their metabolic rate. In the WA mice of both genotypes, the metabolic rate at 5°C was almost constantly maintained between 90 and 100 ml O$_2$/h without pronounced fluctuations (Figs. 1B and 2). In contrast, metabolic rates of the MCA mice were consistently higher. Furthermore, we observed a distinct phenotypic difference in the temporal pattern of thermoregulatory heat dissipation between genotypes: MCA wild-type mice displayed intermittent thrusts of oxygen consumption, resulting in a large range of readings, whereas metabolic rates of the MCA UCP1-KO mice appeared much more constant (Fig. 1B). The ability to display metabolic excursions was mirrored in behavior at 5°C (Fig. 2): 5 out of 6 of the MCA wild-type mice showed highly variable metabolic rates and displayed clear bouts of activity every 1.5–2.0 h, as assessed by autoregression analysis and sine curve fitting. In contrast, the MCA KO and also the WA mice of either genotype were much less active during the 5-h period at 5°C and did not display rhythmicity in either MR or $T_b$. A comparison of summed activity counts from the initial 3 h (at 30°C) with the first 3 h in the cold (5°C) revealed that despite substantial interindividual variation in activity levels, all MCA wild-type mice had increased their activity upon cold exposure ($P = 0.028$), while all MCA KO individuals had decreased activity ($P = 0.018$).

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Fig. 1. Body temperature (A), metabolic rate (B), thermal conductance (C), and respiratory quotient (RQ) (D) of warm (WA; 27°C) and moderate cold (MCA; 18°C) acclimated wild-type (solid symbols) and uncoupling protein 1-knockout (UCP1-KO) mice (open symbols) maintained at 30°C and 5°C (cold endurance test). Each data point represents the means ± SE of 10–15 readings collected during 30 min in the metabolic cage. Mice (n = 5) in each group initially entered the experiment, but numbers in the WA groups progressively decreased (A) because mice became hypothermic and had to be removed from the metabolic cage.
In the thermoneutral environment, thermal conductance was higher in MCA compared with WA mice, irrespective of genotype (Fig. 1C, Table 1). Upon cold exposure thermal conductance was immediately reduced in all acclimation groups. On average, thermal conductance at 5°C was not significantly different between genotypes (genotype: P = 0.07; time: P < 0.001; genotype × time: P = 0.43). Mirroring the larger range of metabolic rates seen at 5°C, however, the thermal conductance in the MCA wild-type mice was more variable than in the MCA KO mice.

Throughout the entire experimental period, RQs of the MCA wild-type mice were distinctly lower than in the other three groups (Fig. 1D). In all groups of mice, exposure to 5°C induced a lowering of RQ, reflecting increased lipid oxidation in the cold. This cold-induced decrease in RQ was most pronounced in the MCA wild-type mice. In contrast, it took >2 h until RQs of the MCA KO mice had attained wild-type levels (time: P < 0.001; genotype: P = 0.033, time × genotype: P < 0.001).

The cold limit test was performed to compare the metabolic responses of WA, MCA, and CA mice exposed to stepwise decreasing ambient temperatures, and to determine HPmax from which we could calculate the cold limit for each group. This experiment did not reveal any consistent genotype differences in mean RMRs, body temperature, and, hence, thermal conductance, either within WA or MCA mice (Fig. 3). In contrast, in the CA state, RMRs of the UCP1-KO mice (and also their heat production, graph not shown) were lower compared with wild-type mice. This lowered heat production was frequently accompanied by reduced body temperature and consistently lower thermal conductance.

HPmax progressively increased with cold acclimation (WA < MCA < CA), attaining a maximal recruitment of 473 mW adaptive thermogenic capacity in CA wild-type mice (Table 2). In UCP1-KO mice, a similar recruitment of adaptive thermogenic capacity, though to a lesser extent (227 mW), was also found in MCA and CA. Effective cold limits were obtained by intersecting mean HPmax from each group with the respective linear regressions of mean HP vs. Ta (see Supplemental Fig. S2 in the online version of this article). We did not analyze the WA mice because of the comparatively narrow Ta range available. Comparison of wild-type mice in the MCA and CA state revealed that the improvement of the cold limit by ~10°C

Table 1. Metabolic characteristics of resting warm acclimated (27°C) and moderately cold acclimated (18°C) wild-type and UCP1-KO mice measured at 30°C and 5°C

<table>
<thead>
<tr>
<th></th>
<th>At 30°C</th>
<th>At 5°C</th>
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<tr>
<td></td>
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<td>UCP1-KO</td>
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<tr>
<td></td>
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<td>MCA</td>
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<tr>
<td>Body mass, g</td>
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<td>Conductance, mW/°C</td>
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<td>30.3±4.0</td>
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Data are expressed as means ± SD from n = 5–7 individuals per group. In each parameter, there was a significant effect of acclimation temperature (P < 0.05) but not of genotype. WA, warm acclimated; MCA, moderately cold acclimated; UCP1, uncoupling protein 1; KO, knockout; RMR, resting metabolic rate; Ta, body temperature. a,b Differences between mean values at 30°C and 5°C, respectively, are indicated by superscripted letters that differ.

Fig. 2. Representative original tracings of activity readings (bars), metabolic rate (line and scatterplot) and body temperature (line plot) in individual wild-type and UCP1-KO mice during a cold endurance test (~4 h at 30°C, up to 5 h at 5°C, temperature switch indicated with an arrow). Previously WA mice of either genotype had to be removed from the experiment because of hypothermia (final body temperature <31°C). In contrast, the mice acclimated to MCA (18°C) remained normothermic. Note the reduced activity and the less variable metabolic rates of the WA wild-type (WT) mouse and the two UCP1-KO mice.
in CA mice was well in accord with a further increase in HPmax (+216 mW). Notably, MCA KO mice significantly increased HPmax relative to WA, while the further marginal elevation of HPmax in CA KO mice was not significant. The slope for the regression of HPmax vs. Ta in the CA KO mice was significantly lower than in the other three groups (P ≤ 0.014). Our calculations, therefore, predict an improved cold limit of CA KO mice by about the same magnitude as in the wild-type mice (9°C), even though HPmax had only increased by 69 mW (8%).

In cold-acclimated wild-type mice (MCA and CA), NE produced pronounced elevations in heat production, whereas only minor increases in NE-stimulated heat production were observed in the UCP1-KO mice, which did not attain statistical significance. Subtraction of NE-stimulated maximal thermogenesis (NEmax) and RMrt from HPmax revealed the major components of heat production and their relative contribution to maximal cold-induced heat production within each genotype and acclimation group (Fig. 4).

Finally, we dissected distinct skeletal muscle groups, heart muscle, and white adipose tissue depots to identify structural and functional adaptations in CA UCP1-KO mice. MyHC skeletal muscle isoforms (see Supplemental Fig. S3 in the online version of this article) showed relatively minor but significant alterations in the masseter (increase in type 2x with CA, P ≤ 0.022, decrease in type 2b, P ≤ 0.015) but no genotype differences. Furthermore, CA mice demonstrated prominent shifts toward slower MyHC 2a and 2x isoforms via reductions of MyHC 2b in the gastrocnemius (P ≤ 0.001 for each). The minor changes in tibialis anterior did not reach statistical significance (all P > 0.05). In cardiac tissues, UCP1-KO mice (CA) displayed novel expression of the MyHC beta isoform, but without a significant overall decrease in MyHC alpha.

In isolated skeletal muscle mitochondria, mitochondrial oxygen consumption in state 2 (succinate respiration), state 3 (ADP-induced), state 4 (oligomycin-inhibited leak respiration), and FCCP-induced oxygen consumption showed no differ-

Fig. 3. Means ± SD resting metabolic rate (RMR), body temperature (Tb), thermal conductance, and RQ of wild-type and UCP1-KO mice (n = 5–7 mice per group) acutely exposed to stepwise decreasing ambient temperatures. The mice had been previously acclimated to either 27°C (warm, WA), 18°C (moderate cold; MCA), or 5°C (cold, CA) for at least 3 wk.
enches between acclimation temperatures (here: WA and CA) and genotypes. The state 2–4 values of CA animals, however, tended to be higher than in WA mice (see Supplementary Fig. S4 in the online version of this article). We next compared the full kinetic response of the basal proton leak (monitored as oxygen consumption rate) at 37°C to stepwise changes in its driving force, membrane potential, in skeletal muscle mitochondria of CA and WA mice (Fig. 5). There was no apparent difference in the proton leak curves between WA and CA groups, as well as wild-type and UCP1-KO mitochondria.

We determined the specific activity of COX as a surrogate marker for maximal mitochondrial respiratory capacity in several WAT depots of wild-type and KO mice, both WA and CA (Fig. 6, A and B). In wild-type mice, COX activity was not influenced by ambient temperature in epididymal WAT (eWAT) or inguinal WAT (iWAT). In CA KO mice, however, COX activity was significantly increased compared with WA KO mice in eWAT and in iWAT (P < 0.001), as well as in periovarian WAT of a further group of female mice (data not shown). The cold-induced difference was greatest in iWAT with an approximate 3.6-fold increase (Fig. 6B). CideA expression was assessed as a marker for the emergence of brown adipocyte-like cells in white adipose tissue. In the CA state, CideA mRNA levels were ~15-fold increased in UCP1-KO compared with wild-type mice, whereas in the WA state CideA expression was low and not affected by genotype (P < 0.001, Fig. 6C).

**DISCUSSION**

During cold acclimation, the recruitment of thermogenic capacity in BAT contributes to an overall increase of cold-induced HPmax and improved cold tolerance. In wild-type mice, the increased thermogenic capacity in BAT is represented by a corresponding rise in norepinephrine-stimulated nonshivering thermogenesis capacity (5). In our study, CA wild-type mice showed the expected increase in norepinephrine-stimulated nonshivering thermogenesis relative to WA, and accordingly, an increase in HPmax in the cold by +473 mW. So far, it was known that UCP1 KO mice, lacking functional BAT, can also survive in the cold, but their maximal capacity for adaptive thermogenesis had not been determined. We, here, demonstrate that cold acclimation of UCP1-KO mice increases HPmax by +227 mW. The comparison of the adaptive increase in HPmax demonstrates that in the absence of UCP1-mediated nonshivering thermogenesis, the UCP1-KO mice are still capable of exhibiting almost 50% of the wild-type increase in maximal cold-induced heat production, i.e., 227/473 mW.

There is a therapeutic interest in identifying thermogenic mechanisms in mammals, which may be exploited to combat dysregulations in body composition in humans (6). As the UCP1-KO mice cannot recruit BAT thermogenesis for adaptive heat production in the cold, they may provide models to identify other principal thermogenic processes. In our study UCP1-KO mice in the CA state showed a 2–4-fold increase in COX activity in several WAT depots. This strong recruitment of respiratory capacity was not observed in wild-type mice. CideA, which is highly expressed in murine BAT compared

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**Table 2. Maximal cold induced resting metabolic rate (RMRmax) and maximal heat production of WA (27°C), MCA (18°C), and cold acclimated (5°C) wild-type and UCP1-KO mice**

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type</th>
<th>UCP1-KO</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WA (n = 5)</td>
<td>MCA (n = 6)</td>
<td>CA (n = 4)</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>22.7 ± 0.9</td>
<td>24.5 ± 1.3</td>
<td>26.8 ± 0.7</td>
</tr>
<tr>
<td>RMRmax, ml O₂/h</td>
<td>133.5 ± 11.0</td>
<td>180.2 ± 21.5</td>
<td>218.3 ± 11.4</td>
</tr>
<tr>
<td>HPmax, mW</td>
<td>756.5 ± 65.0</td>
<td>1012.9 ± 121.1</td>
<td>1229.5 ± 61.3</td>
</tr>
<tr>
<td>Cold limit, °C</td>
<td>&gt;5°C</td>
<td>-8.3</td>
<td>-18.0</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD. For the MCA and CA groups, mean heat production (HPmax) was converted into cold limit numbers (°C) from the point of intersection of HPmax with the respective regression equation for heat production (HP) vs. ambient temperature (T\_a; Supplemental Fig. S2). RMRmax, maximum resting metabolic rate; CA, cold acclimated; P\_gen, probability among genotypes; P\_accl, probability among acclimation groups; P\_gen\_accl, probability among genotype × acclimated groups. For clarity, superscripted letters indicating significant differences between groups (derived from multiple pairwise comparisons by Tukey post hoc test) have been omitted.

Fig. 4. Summarizing figure depicting RMRt (resting metabolic rate at thermoneutrality) and maximal norepinephrine-induced heat production (NEmax) of WT and UCP1-KO mice acclimated to different ambient temperatures: WA: 27°C, MCA: 18°C; cold acclimated (CA): 5°C, and in relation to maximal cold-induced heat production (HPmax). The corresponding cold limits for each group of mice are indicated. Data are indicated as means ± SD; n = 4–7 individuals per group. Note that HPmax and NEmax were determined in two different sets of mice for each genotype. RMRt was obtained prior to the injection of norepinephrine (for details, see text).
oxidative phosphorylation capacities of skeletal muscle mito-
thermogenesis (11). The latter may be related to improved
leading to enhanced endurance and capacity for shivering
shivering should have a training effect in skeletal muscle
ferred to the cold, and it has been suggested that sustained
transitions toward slower 2a and 2x isoforms. Our results,
therefore, generally support a specific “training” adaptation
mirrored in skeletal muscle fiber composition of CA mice
irrespective of genotype, but the response is muscle specific, as
we observed no major changes in tibialis. The novel expression
of slower MyHC beta cardiac isoforms is intriguing, as this is
more typically seen in response to hypoxia, fasting, or thyroid
disease but not during exercise (14).

We determined the possible contribution of skeletal muscle
basal proton leak to enhanced heat production, for which
conflicting evidence has been reported (compare Ref. 28 with
Ref. 43). In skeletal muscle mitochondria isolated from wild-
type and UCP1-KO mice, we neither found alterations in
basal proton leak to enhanced heat production, for which
might be anticipated from improved lipid-fuelled oxidation
capacities
might be anticipated from improved lipid-fuelled oxidation
capacities
with WAT (49), was also strongly up-regulated in inguinal
WAT of CA UCP1-KO mice. These observations provide
evidence that a remodeling of WAT occurs, which could
contribute adaptive thermogenesis in UCP1-KO mice.

It has been previously demonstrated that multilocular mito-
chondria-rich adipocytes expressing UCP1 emerge in different
WAT depots of mice following cold acclimation or chronic
β-adrenergic stimulation (29, 48), which is mediated by a
cyclooxygenase-dependent mechanism (25, 46). In UCP1-KO
mice histologically identical brown adipocyte-like cells, which
do not express UCP1, can be induced at even higher abundance
(1, 12). Expression profiling and respiration of inguinal WAT
tissue explants suggest that the mitochondrial content and
respiratory activity is increased in WAT of cold-acclimated
UCP1-KO mice (45). As the mitochondria in these brown
adipocyte-like cells are UCP1 deficient, an alternative futile
mechanism must exist that utilizes the increased COX activity
per mass unit of inguinal WAT for heat dissipation. Futile
cycling of Ca\(^{2+}\) between the endoplasmic reticulum and cyto-
solic compartments could serve as such an ATP sink (45).
According to our results, CA UCP1-KO mice recruited 227
mW adaptive thermogenic capacity, of which 30% (80 mW)
were attributable to increased RMRt occurring in both geno-
types, although the underlying mechanism is not understood.
The remaining increase of \(\sim 150\) mW could be contributed by
the UCP1-KO-specific increases in WAT oxidative capacity.
The contribution of WAT to total metabolic rate has been
estimated to be \(\sim 2.6\)% or 0.89 ml O\(_2\cdot g^{-1}\cdot h^{-1}\) (\(\sim 4.9\) mW/g;
Ref. 26), making up to a total heat dissipation of \(\sim 10\) mW in
a mouse of 20 g with 2 g total fat mass. In the light of the
observed \(\sim 2–4\)-fold increase in respiratory capacity, WAT
could, therefore, contribute significantly to adaptive thermo-
genesis in UCP1-KO mice.

One straightforward conclusion is that mice without func-
tional BAT predominantly depend on sustained shivering ther-
mosgenesis to maintain normothermia. UCP1-KO mice can
only recruit thermogenic capacity when successively trans-
ferred to the cold, and it has been suggested that sustained
shivering should have a training effect in skeletal muscle
leading to enhanced endurance and capacity for shivering
thermosgenesis (11). The latter may be related to improved
oxidative phosphorylation capacities of skeletal muscle mito-
chondria on fatty acid-derived substrates in UCP1-KO mice
(43). In vivo, the proportion of NE-insensitive thermogenesis is
often equated with shivering thermogenesis (18), but we could
not observe major alterations in the thermogenic capacity of
this component (white areas in stacked bars; see Fig. 4).
Furthermore, the fiber composition of the gastrocnemius, mas-
seter, and tibialis muscle did not mirror structural changes that
might be anticipated from improved lipid-fuelled oxidation
capacities. In contrast, we observed modest increases in slower
MyHC isoforms in the gastrocnemius and masseter muscle of
both the wild-type and the UCP1-ablated mice during CA
(5°C). The detectable changes in the gastrocnemius are most
similar to exercise-training responses, which might predict
transitions toward slower 2a and 2x isoforms. Our results,
therefore, generally support a specific “training” adaptation
mirrored in skeletal muscle fiber composition of CA mice
irrespective of genotype, but the response is muscle specific, as
we observed no major changes in tibialis. The novel expression
of slower MyHC beta cardiac isoforms is intriguing, as this is
more typically seen in response to hypoxia, fasting, or thyroid
disease but not during exercise (14).

We determined the possible contribution of skeletal muscle
basal proton leak to enhanced heat production, for which
conflicting evidence has been reported (compare Ref. 28 with
Ref. 43). In skeletal muscle mitochondria isolated from wild-
type and UCP1-KO mice, we neither found alterations in
mitochondrial respiration rates (states 2–4) nor in the basal
proton leak using succinate as a complex II substrate. To assess
basal proton leak in isolated mitochondria, the control for the
concentration of free fatty acids is of particular importance, as
fatty acids increase proton leakage either by a flip-flop mech-
anism or by activation of mitochondrial transporter proteins,
e.g., UCP3 or ANT1. In mitochondrial preparations from
skeletal muscle of wild-type and UCP1-KO mice, the concentra-
tion of free fatty acids may vary either due to different
carryover of fatty acids during the isolation procedure or due to
systematic genotype differences in the fatty acid level. To
measure basal proton leak, it is, therefore, essential to buffer
fatty acid at comparatively low levels by the addition of albumin.
Previously published genotype differences in basal proton leak
kinetics between skeletal muscle mitochondria from wild-type
and UCP1-KO mice (28) may have been caused by differences
in free fatty acid levels in mitochondrial preparations and thus may reflect an increased fatty acid-sensitive proton leak.

In wild-type mice, increased cold tolerance with cold acclimation corresponded to the pronounced recruitment of adrenergically stimulated nonshivering thermogenesis capacity. In contrast, the increase in cold tolerance of KO mice could not be directly related to major changes in HPmax (Fig. 4). This is particularly evident from the comparison of cold limits in the CA UCP1-KO knockout mice with the MCA wild-type mice displaying similar values for HPmax. As judged from our metabolic readings, an increase in acute cold tolerance of UCP1-KO mice may also be supported by lowered body temperature and metabolic rates leading to lower thermal conductance (reduced heat loss) in the cold (Fig. 3). This conclusion is in line with a previous report by Wang et al. (47), who demonstrated that UCP1-KO mice use sustained vasoconstriction in response to acute changes of ambient temperature to reduce heat loss. We recently demonstrated that loss of recruitable thermogenic power from BAT increases the arousal duration from torpor in UCP1-KO mice and renders this process energetically more costly (32). Thus, in the cold, UCP1-KO mice are forced to use the dissipated heat more efficiently than wild-type mice.

Challenging mice with a stepwise reduction of ambient temperature revealed consistently higher RQs in MCA and CA KO as opposed to MCA and CA wild-type mice. In MCA mice, this difference is definitely maintained for several hours in the cold (Fig. 1D). To our best knowledge, our study is the first demonstration of clear differences in RQ between wild-type and UCP1-KO mice (compare Refs. 24 and 1). At first sight, higher RQs in UCP1-KO mice in vivo may contradict the finding that oxidative phosphorylation capacities of skeletal muscle mitochondria on fatty acid-derived substrates are improved (43). The delayed or diminished lowering of RQs in acutely cold exposed UCP1-KO mice may, however, be interpreted as a lag in the activation of lipid oxidation for thermogenesis in the cold. Most likely, the inability of UCP1-KO mice to recruit uncoupled respiration upon cold-induced norepinephrine release in BAT provokes the activation of thermogenic pathways, which can less readily switch to lipid utilization.

Despite significant alterations in the thermophysiology of the UCP1-KO mice leading to elevated HPmax, the underlying mechanism(s) must be detrimental to the animal’s well being, as the life span of UCP1-KO mice in the cold is markedly reduced (10). As shown from our cold endurance test, acute cold exposure abolished thermoregulatory flexibility in the UCP1-KO mice, as well as in the WA mice (Fig. 2). WA mice and MCA KO mice, furthermore, displayed significantly lower physical activity in the cold. Periodic BAT activity has been associated with rhythmic elevations in brain temperature, and low or diminished levels of UCP1 may interfere with the ability to fully express normal ultradian rhythmicity of thermogenesis (34). Sustained cold-induced shivering thermogenesis in UCP1-KO mice was further associated with severe dysfunction of (red) soleus muscle (2), and recruitment of other tissues for heat production with less mitochondrial uncoupling capacity might also increase systemic radical damage affecting lifespan (33).

In summary, CA UCP1-deficient mice can exhibit a substantial increase in thermogenesis and an improved cold tolerance as low as \(-10^\circ\text{C}\). Absence of functional BAT leads to remodeling of white adipose tissue, which could significantly contribute to adaptive thermogenesis with cold acclimation. It is, however, likely that the adaptive increase in HPmax of UCP1-KO mice is not due to enhanced oxidative capacity from a single thermogenic tissue but reflects multiple thermogenic pathways by which the lack of functional BAT can be compensated, provided heat loss does not exceed the rate of heat production (i.e., acclimation to the cold occurs successively). In vivo, heat loss may further be substantially reduced by
vasoconstriction and decreased thermal conductance, thereby facilitating body temperature set-point maintenance. We further conclude that small mammals may not only benefit from net increase in heat production supported by BAT nonshivering thermogenesis but also UCP1-mediated uncoupling allows metabolic flexibility (short-term thermoregulation and physical activity) and a rapid switch toward sustained lipid-fuelled thermogenesis in response to acute cold stress. In their natural environment, small dwelling mammals are frequently exposed to immediate changes in ambient temperature when leaving or entering their nest or burrow and will thus profit from the instantaneous availability of BAT nonshivering thermogenesis to maintain constant body temperature and alertness.

**Perspectives and Significance**

Exploring the metabolic consequences of BAT ablation is of interest since this tissue has been associated with the evolution of endothermy, but it is absent in birds and in adult marsupials and monotremes. Furthermore, the significance of BAT in adult body weight regulation is currently under investigation. The UCP1-knockout mouse line provides a mammalian model to directly compare endogenous heat production independent of UCP-1-mediated nonshivering thermogenesis. Any search exploring thermogenic mechanisms in mammals will benefit from knowledge on the magnitude of thermogenic capacity recruitment in the presence and absence of functional BAT and the metabolic characterization of any UCP1-independent thermogenesis. Reminiscent of classical studies measuring tissue-specific metabolic rates in rats and hamsters, modern less-invasive functional magnetic resonance imaging technologies, e.g., PET/CT of 18-fluor-deoxyglucose uptake, need to be applied to directly assess the contribution of selected tissues to the total heat production of the body in vivo.

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**DISCLOSURES**

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