Induction of muscle thermogenesis by high-fat diet in mice: association with obesity-resistance

Vladimir Kus,1 Tomas Pražák,1 Petr Brauner,1 Michal Hensler,1 Ondrej Kuda,1 Pavel Flachů,1 Petra Janovská,1 Dasa Medříková,1 Martin Rossmeisl,1 Zuzana Jilková,1 Bohumír Stefl,4† Eva Pastalková,3 Zdeněk Drahota,2 Josef Houštek,2 and Jan Kopecký1

Departments of 1Adipose Tissue Biology, 2Bioenergetics, and 3Neurophysiology of Memory and Computational Neuroscience, Institute of Physiology, Academy of Sciences of the Czech Republic; and 4Department of Physiology and Developmental Biology, Faculty of Science, Charles University, Prague, Czech Republic

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Kus V, Pražák T, Brauner P, Hensler M, Kuda O, Flachů P, Janovská P, Medříková D, Rossmeisl M, Jilková Z, Stefl B,† Pastalková E, Drahota Z, Houštek J, Kopecký J. Induction of muscle thermogenesis by high-fat diet in mice: association with obesity-resistance. Am J Physiol Endocrinol Metab 295: E356–E367, 2008. First published May 20, 2008; doi:10.1152/ajpendo.90256.2008.—The obesity-resistance of A/J mice compared with obesity-prone C57BL/6J (B/6J) mice. Experiments were performed on male mice born and maintained at 30°C. Four-week-old mice were randomly weaned onto a low-fat (LF) or HF diet for 2 wk. In the A/J LF mice, cold exposure (4°C) resulted in hypothermia; whereas the A/J HF, B/6J LF, and B/6J HF mice were cold tolerant. Cold sensitivity of the A/J LF mice was associated with a relatively low whole body energy expenditure under resting conditions, which was normalized by the HF diet. In both strains, the HF diet induced uncoupling protein-1-mediated thermogenesis, with a stronger induction in A/J mice. Only in A/J mice: 1) the HF diet augmented activation of whole body lipid oxidation by cold; and 2) at 30°C, oxygen consumption, total content, and phosphorylation of AMP-activated protein kinase (AMPK), and AICAR-stimulated palmitate oxidation in soleus muscle was increased by the HF diet in parallel with significantly increased leptinemia. Gene expression data in soleus muscle of the A/J HF mice indicated a shift from carbohydrate to fatty acid oxidation. Our results suggest a role for muscle nonshivering thermogenesis and lipid oxidation in the obesity-resistant phenotype of A/J mice and indicate that a HF diet could induce thermogenesis in oxidative muscle, possibly via the leptin-AMPK axis.

nonshivering thermogenesis; leptin; adenosine monophosphate-activated protein kinase

MANY HEALTH PROBLEMS IN AFFLUENT SOCIETIES are linked to the increased incidence of obesity. Especially meals with high content of fat are obesogenic, due to a low energetic cost of nutrient storage and a low potency of fat intake to promote fat oxidation (20). Induction of lipid catabolism is crucial for adaptive thermogenesis, which is regulated by the sympathetic nervous system, thyroid hormones, insulin (reviewed in Refs. 19 and 26), and leptin (see below) and is possibly involved in the regulation of body weight in humans (12, 26, 49). However, the mechanisms and organs contributing to adaptive thermogenesis need to be better characterized.

In small mammalian species, hibernators, and mammalian neonates, adaptive thermogenesis largely depends on uncoupling protein-1 (UCP1), which is located in brown adipose tissue. Thermogenesis in brown fat could be activated in response to both cold exposure and a meal (6), and its capacity is increased by adaptation to cold or intake of high-fat (HF) diets. Diet can even act as a preacclimation to cold (6, 36), and brown fat thermogenesis serves to maintain both body temperature and energy balance. However, the capacity does not exceed 60% of total adaptive nonshivering thermogenesis (reviewed in Ref. 19), suggesting a role for other organs in this process (16, 24, 50). Whether skeletal muscle can mediate adaptive nonshivering thermogenesis in mammals is a matter of a long-lasting controversy. Skeletal muscle is an important site of whole body energy expenditure, which can account for 20–30% of the total oxygen uptake in the resting state (6, 19, 25). Differences in resting muscle metabolism explain part of the variance in resting metabolic rate among adult humans and may play a role in the pathogenesis of obesity (55).

A unique role in the complex control of energy homeostasis and thermogenesis is played by adipocyte hormone leptin, which acts both centrally in the hypothalamus and also directly in the peripheral tissues (reviewed in Ref. 35). The administration of leptin reverts reduced metabolic rate, depression of body temperature, and excessive adiposity in genetically obese ob/ob mice lacking functional leptin (31). Even in normal mice, leptin induces the capacity for UCP1-mediated thermogenesis (38), and it also stimulates lipid oxidation and uptake of glucose in skeletal muscle by activating AMP-activated protein kinase [AMPK (29)]. The direct thermogenic effect of leptin was also demonstrated in murine skeletal muscle, where exogenous leptin stimulated oxygen consumption (41). Leptin’s effects occurred primarily in the oxidative but not glycolytic type of muscle (29, 41).

We hypothesized that muscle nonshivering thermogenesis could be complementary to that mediated by UCP1 in brown fat and could be activated under the conditions of a strong thermogenic response to a HF diet, possibly in association with obesity resistance. Therefore, mice of two different inbred strains were studied: 1) obesity-prone C57BL/6J (B/6J) mice and 2) A/J mice, which are relatively resistant to the obesogenic effect of a high-fat (HF) diet is counterbalanced by stimulation of energy expenditure and lipid oxidation in response to a meal.

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† Deceased. 1 10, 2008.

Address for reprint requests and other correspondence: J. Kopecký, Dept. of Adipose Tissue Biology, Inst. of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague, Czech Republic (e-mail: kopecky@biomed.cas.cz).
genic environment (45, 46, 53). The different propensities to obesity have previously been shown to be associated with a stronger activation of thermogenesis mediated by UCP1 and UCP2 in brown (17, 48, 52) and white (8, 13, 48, 52) adipose tissue of A/J mice. Mice of the A/J strain also showed a much stronger induction of leptin by the HF diet especially during the early postweaning period (47, 52). In our experiments, to eliminate the confounding effect of cold-induced thermogenesis, all mice were born and maintained at 30°C, i.e., close to their thermoneutral temperature (1), weaned onto a low-fat (LF) or HF diet, and studied 2 wk after weaning. In contrast to their thermoneutral temperature (1), weaned onto a low-fat diet, mice of the A/J strain also showed a much higher body weight gain during 2 wk period after weaning; LF, body weight gain during 2 wk period after weaning; FC, mean food consumption measured at days 2, 4, 9, and 13 after weaning; BAT, interscapular brown fat; DL, dorsolumbar white fat; EPI, epididymal white fat; TG, triglyceride; NEFA, nonesterified fatty acid; T₄, thyroxine; T₃, triiodothyronine. Data are means ± SE (n = 11–14). *Significant effect of diet; †significant effect of genotype.

Shivering was monitored in a separate group of mice by means of electromyography (EMG). Before the experiment, mice were transferred to 20°C and anesthetized using diethyl ether, and 0.5-cm Ni-Cr wire (150 µm diameter) electrodes were inserted under the skin. Two recording electrodes were placed above the left and right shoulders of the forelimbs, and a reference electrode was implanted above the rear back. Immediately after recovery from anesthesia, mice were caged singly without bedding, water, or food. Action potentials were amplified (5,000 times), filtered (300–6,000 Hz), and digitized (32-bit resolution, 32 kHz), and data were stored for analysis performed using custom-made software (AcX; Andre A. Fenton, Institute of Physiology, Prague, Czech Republic). After a 20-min period of initial recording at 20°C, the cages were transferred to 4°C, and recording was performed for 50 min. After the experiment, mice were killed by cervical dislocation. During offline analysis of the data, moving artifacts were eliminated on the basis of their high amplitude and time intervals. Voltage values were integrated over 10-min periods.

**Indirect calorimetry.** Energy expenditure was evaluated using the indirect calorimetry system INCA (Somedic, Horby, Sweden) as described before (1). To minimize stress in the animals, measurements were performed in the standard cages, in which animals had been maintained since weaning (see above), and they were transferred into sealed chambers equipped with thermostatically controlled heat exchangers. Calibration of oxygen sensors was performed before each measurement. Measurements proceeded under a constant airflow rate (1,000 ml/min). Oxygen consumption (V̇O₂) and carbon dioxide production (V̇CO₂) were recorded every 2 min. The system allowed for four individually housed mice to be monitored simultaneously. During the measurements, lasting for 46 h and starting at 3:00 PM (12:12-h light-dark cycle; light from 6:00 AM), animals had ad libitum access to food (i.e., LF or HF diet, see above). During the initial 23 h, the temperature was set to 30°C followed by a drop to 15°C during a 30-min interval at the beginning of the second 23-h period and kept at 15°C until the end of the measurement. The level of substrate partitioning was estimated by calculating respiratory exchange ratio (RER; i.e., V̇CO₂/V̇O₂ ratio). To compare subtle differences between subgroups, the percent relative cumulative frequency (PRCF) curves were also drawn (see Fig. 5), based on RER values pooled from all subgroups, the percent relative cumulative frequency (PRCF) curves were also drawn (see Fig. 5), based on RER values pooled from all subgroups, the percent relative cumulative frequency (PRCF) curves were also drawn (see Fig. 5), based on RER values pooled from all subgroups, the percent relative cumulative frequency (PRCF) curves were also drawn (see Fig. 5), based on RER values pooled from all subgroups, the percent relative cumulative frequency (PRCF) curves were also drawn (see Fig. 5), based on RER values pooled from all subgroups, the percent relative cumulative frequency (PRCF) curves were also drawn (see Fig. 5), based on RER values pooled from all subgroups.
Norepinephrine-stimulated metabolic rate (NEMR) was measured in anesthetized mice as described before (42) and calculated as a difference between $V_{O2}$ measured 15–20 min before and after intra-peritoneal injection of L-norepinephrine D-bitartrate (Sigma-Aldrich; St. Louis, MO). Serum triiodothyronine (T3) and thyroxine (T4) levels were determined with total T4 and total T3 RIA kits (Immunotech, Beckman Coulter, Czech Republic). Lipids, metabolites, and hormones in plasma. Triglycerides (TG) were estimated using diagnostic kit no. 320-A (Sigma-Aldrich). The concentration of nonesterified fatty acids (NEFA) was measured enzymatically using a NEFA C kit (Wako Chemicals, Richmond, VA). Serum triiodothyronine (T3) and thyroxine (T4) levels were determined with total T4 and total T3 RIA kits (Immunotech, Beckman Coulter, Czech Republic).

Measurements of $V_{O2}$ in skeletal muscles. Dissected soleus or gastrocnemius muscle (10 mg wet wt) was placed in a respiratory chamber of Oroboros Oxygraph [Paar, Graz, Austria (15)] filled with 2 ml of freshly oxygenated (95% O2, 5% CO2) Krebs-Ringer bicarbonate buffer (pH 7.4), containing 10 mM glucose and warmed to 37°C. $V_{O2}$ was measured continuously and recorded at 1-s intervals between 10 and 35 min after the start of incubation by use of a computer data acquisition system (Datlab; Oroboros, Innsbruck, Austria). Mean values of $V_{O2}$ were calculated for 5-min intervals. Results were normalized to wet weight of the tissue and also to protein content estimated in the tissue homogenate by using the bicinchoninic acid procedure and BSA as a standard (40).

Measurements of fatty acid oxidation in muscles. Soleus and gastrocnemius muscle was incubated as described above, except that the final volume was 3 ml, and the Krebs-Ringer bicarbonate buffer contained 5 mM glucose and fatty acid-free bovine serum albumin (5 mg/ml, ICN Biomedicals, High Wycombe, UK) complexed (1:3) with palmitic acid (Sigma-Aldrich) and [U-14C]palmitate (0.1 μCi/ml; LACOMED, Czech Republic); see online APPENDIX for details. Muscle lysates were prepared as before (27). Total protein concentration was assessed as above (40). Measured amounts of the catalytic subunits of AMPK (AMPKα1, -α2, and -β1) and AKT (AKT1 and -2) were quantified using Western blots (39) in crude cell membranes (100,000 g). Western blots were incubated with a mixture of primary antibodies: 1) sheep antibodies against AMPKα1 and -α2 (1:3,500; kind gift from D. Grahame Hardie, University of Dundee, UK [53]), and 2) phosphospecific rabbit antibodies against the Thr172 of the α-subunits of AMPK (1:1,000; no. 2535, lot no. 6; Cell Signaling Technology, Danvers, MA). As secondary antibodies, a mixture of infrared dye-labeled antibodies was used: 1) donkey anti-sheep IgG conjugated to infrared dye 800 (1:5,000, no. A-21102, Lot no. 93C2-1; Alexa 800 donkey anti-sheep IgG; Molecular Probes, Leiden, The Netherlands), and 2) donkey anti-rabbit IgG conjugated to infrared dye 680 (1:5,000, no. A-21102, Lot no. 93C2-1; Alexa fluor 680 donkey anti-sheep IgG; Molecular Probes, Leiden, The Netherlands), and 2) donkey anti-rabbit IgG conjugated to infrared dye 680 (1:5,000, no. A-21102, Lot no. 93C2-1; Alexa fluor 680 donkey anti-sheep IgG; Molecular Probes, Leiden, The Netherlands). The amount of the α-subunits of AMPK was quantified by Western blotting and specific antibodies as before (3). Prestained protein M, standards (PageRuler Prestained Protein Ladder; Fermentas, Burlington, ON, Canada) were used to locate positions of AMPK or ACC on the blots. Signals on different blots were compared using a standard prepared from the liver of adult LF diet-fed B/6J mice and expressed as a percentage of the amount of AMPK or ACC on the blots.

Statistics. The data were analyzed by a two-way ANOVA, using SigmaStat statistical software. Logarithmic transformation was used when necessary to normalize the distribution of residuals. Data are expressed as means ± SE (n = 7–8). *Significant effect of diet; there was also a significant interaction between diet and genotype (repeated-measures ANOVA).

**Gene expression analysis.** Total RNA was isolated (Tri Reagent; MRC, Cincinnati, OH), and levels of different transcripts were evaluated using real-time quantitative PCR (qRT-PCR), a DyNamo Capillary SYBR Green qPCR kit (Finnzymes, Espoo, Finland), and a LightCycler 2.0 instrument (Roche Diagnostics, Mannheim, Germany) as before (11). Lasergene software (DNA Star, Madison, WI) was used to design oligonucleotide primers (see online APPENDIX, Supplemental Table S1). To correct for intersample variations, the level of each transcript was normalized to elongation factor-1α (EF-1α), used as an internal standard, and expressed in arbitrary units (AU). Similar results were obtained using cyclophilin-β as an internal standard (not shown).

**Evaluation of UCP1 and protein content.** UCP1 was quantified by Western blots (39) in crude cell membranes (100,000 g) prepared from homogenates of adipose tissue, using purified UCP1 as a standard (23). Total protein concentration was assessed as above (40).

**Quantification of AMPK and acetyl-CoA carboxylase (ACC) in soleus muscle.** Muscle lysates were prepared as before (27). Total amount of the α1 and α2 catalytic subunits of AMPK (AMPKα1, AMPKα2) and the phosphorylated form of AMPK (p-AMPK) were determined by Western blotting using a mixture of primary antibodies: 1) sheep antibodies against AMPKα1 and -α2 (1:3,500; kind gift from D. Grahame Hardie, University of Dundee, UK [53]), and 2) phosphospecific rabbit antibodies against the Thr172 of the α-subunits of AMPK (1:1,000; no. 2535, lot no. 6; Cell Signaling Technology, Danvers, MA). As secondary antibodies, a mixture of infrared dye-labeled antibodies was used: 1) donkey anti-sheep IgG conjugated to infrared dye 680 (1:5,000, no. A-21102, Lot no. 93C2-1; Alexa 800 donkey anti-sheep IgG; Molecular Probes, Leiden, The Netherlands), and 2) donkey anti-rabbit IgG conjugated to infrared dye 680 (1:5,000, no. A-21102, Lot no. 13176; infrared dye 800 anti-rabbit IgG donkey; Rockland, Gilbertsville, PA). Both total and p-AMPK were quantified on the same blot using the Odyssey IR Imaging Systems (Li-Cor Biosciences, Lincoln, NE). Total content of ACC and its phosphorylated form were quantified using Western blotting and specific antibodies as before (3). Prestained protein M, standards (PageRuler Prestained Protein Ladder; Fermentas, Burlington, ON, Canada) were used to locate positions of AMPK or ACC on the blots. Signals on different blots were compared using a standard prepared from the liver of adult LF diet-fed B/6J mice and expressed in arbitrary units. The value for each mouse represents the mean of values obtained from the left and right soleus muscles.
Both log EC50 and Hill slope values were compared in a two-way sigmoidal dose-response (variable slope) function using GraphPad mouse were treated separately. The PRCF curves were fitted with sigmoidal dose-response (variable slope) function using GraphPad software. Both log EC50 and Hill slope values were compared in a two-way ANOVA (diet × temperature). Tukey’s test for all pairwise multiple comparisons was used. All values are presented as means ± SE. Comparisons were judged to be significant at P ≤ 0.05.

RESULTS

Gross phenotypes and plasma parameters. B/6J and A/J mice were born and maintained at 30°C, weaned onto LF or HF diet at 4 wk of age, and killed 2 wk later. At weaning, as well as at the time of euthanasia, mice of both strains had similar body weights independent of the type of diet. Accordingly, body weight gains during the 2-wk postweaning period were similar in all animal subgroups (Table 1). Caloric intake was also similar in all the subgroups. Feeding mice of both strains the HF diet resulted in a decrease of interscapular brown fat (with a significant effect only in B/6J mice). In accord with a previous study (52) performed at 20°C and using a HF diet of a similar composition as in our experiments, the weight of white fat depots was increased by HF diet at 2 wk after weaning, with a stronger effect seen in A/J mice (Table 1). However, when the animals were fed the HF diet for up to 4 mo while still maintained at 30°C, B/6J mice gained more weight than A/J mice, and only in the former mice a strong obesogenic effect of the HF diet on body weight was observed (Fig. 1). Thus, in adult mice, the different propensity to dietary obesity, as detected in previous studies in B/6J and A/J mice maintained at 20–22°C (45, 46, 53), became clearly apparent even close to thermoneutrality, indicating that obesity resistance of A/J mice did not depend on the induction of energy expenditure by cold. On the other hand, the induction of adiposity by HF diet in both strains after weaning may reflect relatively low protein intake for actively growing mice.

At 2 wk after weaning, plasma levels of TG and NEFA were not affected by the diet in either genotype, except for a small induction of NEFA by the HF diet in B/6J mice (Table 1). As expected (47, 52), the HF diet strongly increased (~2.8-fold) leptin levels in A/J mice, whereas no significant induction of leptin could be detected in B/6J mice (Table 1). In contrast, plasma levels of both T4 and T3 were not affected by the HF diet in A/J mice, but they were increased in B/6J mice, indicating the stimulatory effect of the HF diet on thyroid function in B/6J but not in A/J mice (Table 1).

Body temperature and shivering during cold exposure. Assuming that the difference between B/6J and A/J mice in their propensity to HF diet-induced obesity reflected different thermogenic capacity of these two strains, sensitivity of the animals to acute cold exposure might be affected by the diet. To verify this hypothesis, the mice adapted to 30°C were exposed to an ambient temperature of 4°C, and their deep body temperature was measured (Fig. 2). Before the cold exposure (time 0), body temperature was similar in all the subgroups, whereas the temperature tended to be increased by HF diet specifically in A/J mice (legend to Fig. 2). During the first hour in the cold, body temperature decreased in all the subgroups, while the most pronounced decrease (~2.5°C) was observed in the A/J LF mice. However, LF diet-fed mice of both strains showed similar activation of shivering in the cold (Fig. 3). During the next 2 h, body temperature remained stable in all subgroups except for the A/J LF mice, which became hypothermic, and this prompted us to terminate the experiment in this subgroup (Fig. 2). In contrast, all the other mice were able to maintain their body temperature relatively stable up to 3 days in the cold (not shown). The strain-specific response to cold was not apparent in mice born and reared at 22°C, since under these conditions even A/J LF mice were able to maintain their body temperature above 36°C during a 3-day period in the cold (not shown). Thus, the adaptation of the A/J LF mice to the temperature close to thermoneutrality was essential for unmasking the relatively low thermogenic potential of these mice.
when exposed to cold and for demonstration of the induction of the thermogenic capacity of the A/J mice by HF diet.

**Whole body energy expenditure in awake animals.** To characterize whole body energy expenditure and its changes in response to diet in mice of both genotypes, indirect calorimetry was used to analyze V\(\dot{O}_2\) and RER in ad libitum-fed mice reared at 30°C. Continuous monitoring in individually caged mice was performed for 23 h at 30°C, followed by a period of 23 h at 15°C (Fig. 4). When measured at 30°C and during the light phase of the day, energy expenditure in the A/J mice was affected by the type of diet, with a significant 38% increase in response to HF diet (Table 2). In fact, this increase was related to a relatively low V\(\dot{O}_2\) during the light phase in the A/J LF mice compared with all other subgroups, and the V\(\dot{O}_2\) values in A/J HF mice were similar to that in B/6J mice (Table 2). No effect of diet on energy expenditure was detected in the B/6J mice. During the dark phase, V\(\dot{O}_2\) was not affected by diet in either strain of mice (Table 2). Upon a decrease of ambient temperature from 30 to 15°C, mean V\(\dot{O}_2\) values increased approximately twofold in all the subgroups and the differences in V\(\dot{O}_2\) between the genotypes and diets disappeared (Table 2).

The HF diet lowered RER in all animal subgroups, especially during the dark phase (Table 2), suggesting a shift toward lipid oxidation. This shift was also illustrated by the PRCF curves of RER values pooled from all the measurements performed within the same ambient temperature (Fig. 5). Since all the PRCF curves represented normally distributed data (not shown), the mean RER values corresponded to the 50th percentile values of the PRCF curves (34). In A/J but not in B/6J mice, RER values were also decreased in response to the lower ambient temperature. This shift was more significant in mice fed the HF compared with the LF diet (Table 2), resulting also in the steepest PRCF curve in the A/J HF mice when measured at 15°C (Fig. 5). The slope of the PRCF curve is a marker of metabolic flexibility, with a bigger slope suggesting a lower metabolic flexibility between oxidation of lipids and carbohydrates (34). Therefore, the results highlighted modulation of the resting energy expenditure in A/J mice by the dietary treatments and high reliance of A/J mice on fatty acid oxidation during cold exposure.

**UCP1-mediated nonshivering thermogenesis.** Capacity for UCP1-mediated thermogenesis was assessed as an increment in metabolic rate after the injection of norepinephrine to anesthetized animals (NEMR; Refs. 16, 19, 42). NEMR was similar in LF diet-fed mice of both genotypes, it was stimulated by HF diet, and the stimulation was higher in A/J than in B/6J mice (Fig. 6). UCP1 protein content in interscapular brown fat was also increased by HF diet, and this increase was significantly higher in A/J mice (~10-fold) compared with B/6J mice (~3-fold), resulting in approximately twofold higher UCP1 levels in brown fat in A/J than in B/6J mice. Expression of UCP1 was also detected in subcutaneous white fat (see online APPENDIX, Table S2) and skeletal muscles (Table 3; see also Ref. 2) of mice of both genotypes, but the levels of UCP1 in these tissues were by at least two orders of magnitude lower than in the interscapular fat, indicating a negligible quantitative importance for thermogenesis. In some of the previous studies, UCP2 transcript levels in white fat have also been correlated with obesity resistance in A/J mice (13, 48, 52). However, in our experiments, UCP2 expression was similar in all fat depots studied, including interscapular brown and subcutaneous and epididymal white fat, and no effect of either genotype or diet on UCP2 expression was observed (see online APPENDIX, Table S2).

Thus, in both genotypes, the stimulation of NEMR by HF diet could be explained by the induction of UCP1 protein in the interscapular brown fat. A relatively strong induction in A/J mice was associated with the development of cold tolerance of these mice. On the other hand, the cold sensitivity of the A/J LF mice could be attributed to their relatively low whole body energy expenditure during the light phase of the day and could not be explained by an insufficient capacity of either UCP1-mediated energy expenditure or shivering thermogenesis, since no differences in this respect were detected between A/J and B/6J mice fed the LF diet.

**Gene expression in muscles.** A marked increase of V\(\dot{O}_2\) in response to the HF diet in A/J mice during the light phase of the day, when the locomotor activity of animals is minimal (9), suggested involvement of an adaptive mechanism of energy expenditure, compatible with a sustained induction of nonshivering thermogenesis in muscle via the leptin-AMPK axis (see introductory remarks). Therefore, to characterize muscle metabolism, expression of several genes was assessed in gastrocnemius (a predominantly fast, glycolytic fiber type) and soleus (a predominantly slow, oxidative fiber type) muscle (Table 3). Strong effects of both diet and genotype were detected in the case of stearoyl-CoA desaturase-1 (SCD-1), a gene that is specifically repressed by leptin, which also stimulates fatty acid oxidation (4, 7). In both strains and in both types of muscles, the expression of SCD-1 was downregulated by the HF diet. The strongest suppression, approximately fourfold, was observed in the soleus muscle of A/J mice. These results were in agreement with the assumption that leptin was in-
volved in the induction of nonshivering thermogenesis in soleus muscle of A/J mice by HF diet.

There was no effect of genotype or diet on genes engaged in the control of mitochondrial biogenesis and oxidative capacity, as assessed by quantification of the transcripts for nuclear respiratory factor-1 and subunit VIa of mitochondrial cytochrome oxidase (21), respectively. The expression of PPARγ coactivator-1α (PGC-1α), which links nuclear receptors to the transcriptional program of mitochondrial biogenesis and oxidative metabolism (33), could not explain the stimulatory

Fig. 4. Time course of V̇O₂ measurements in mice maintained at 30°C and fed either LF or HF diet for 2 wk after weaning. Indirect calorimetry was performed on singly caged mice with free access to water and diet initially at 30°C for a period of 23 h followed by 23 h at 15°C. V̇O₂ data were sampled at 60 s for every 2 min; however, only mean values of single recordings at 1-h intervals are shown. Arrows indicate beginning of a 30-min period during which the temperature was dropped. Gray areas represent the dark phases of diurnal cycle. A biphasic circadian rhythm of V̇O₂ was observed in B/6J mice, with 2 maxima: 1 in the middle of the day and the other at the end of the dark phase. This pattern was only marginally affected by diet. In contrast, in A/J mice, a monophasic rhythm of V̇O₂ was recorded, with maximum around the beginning of the dark phase of the day and lower V̇O₂ values recorded during the light phase. Data are means ± SE (n = 6–8). For statistical analysis of these data, see Table 2.

Table 2. Indirect calorimetry

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Singly caged mice were born and maintained at 30°C and fed LF or HF diet from weaning. Measurements proceeded at 30°C for 23 h, followed by exposure to 15°C for 23 h. V̇O₂, O₂ consumption; RER, respiratory exchange ratio. See also Figs. 4 and 5. Data are means ± SE (n = 6–8, with ~700 data points per mouse per 23 h). *Significant effect of diet; †significant effect of genotype; ‡significant effect of temperature. All V̇O₂ data were significantly affected by ambient temperature.
The HF diet induced expression of mitochondrial acyl-CoA thioesterase, with the most pronounced effect in the soleus muscle of B/6J mice, and only in B/6J mice did it also upregulate UCP3 (Table 3). This was in agreement with the known stimulatory effect of thyroid hormones (30) on the expression of UCP3 and mitochondrial acyl-CoA thioesterase genes and with the B/6J strain-specific stimulation of thyroid status by HF diet (see above). The induction of UCP3 in both muscles of B/6J mice was associated with upregulation of long-chain acyl-CoA synthetase (required for activation and transport of fatty acid into mitochondria), whereas expression of cytosolic acyl-CoA thioesterase was unchanged (Table 3).

These results suggest thyroid hormone-dependent induction of a mechanism of lipid handling by HF diet, which may preserve the CoASH pool in mitochondria, specifically in B/6J mice, independently of thermogenesis (18).

The expression of superoxide dismutase-1, a marker of reactive oxygen species formation, was similar in all the subgroups. Except for muscle type-specific differences in gene expression, relatively small variations in mRNA levels of PPARα, UCP1 (see above and Ref. 2), acyl-CoA oxidase-1, and the “muscle” isoform of carnitine palmitoyltransferase I (CPT IB) were observed in response to the diet. In contrast, strain-specific differences in the expression of CPT IA (“liver” isoform) were observed both in soleus and in gastrocnemius muscles. In this case, the expression was affected more by the genotype than by the diet, with the most pronounced effect in the soleus muscle, resulting in an approximately twofold higher CPT IA expression in A/J than in B/6J mice. An opposite effect of the genotype on the expression of CPT IA was observed in the gastrocnemius muscle of HF diet-fed mice (Table 3). Furthermore, high levels of mRNA for isoenzyme 4 of pyruvate dehydrogenase kinase (PDK-4) were found in soleus muscles of A/J mice, indicative of sustained lipid delivery and oxidation (Table 3; Ref. 44). Expression of PDK-4 mRNA in A/J mice was not affected in response to the HF diet; however, its expression was stimulated in both the soleus and the gastrocnemius muscles of B/6J mice. All together, the results above suggested a strain-specific loss of regulation of glucose/fatty acid partitioning in the soleus muscle of A/J mice, favoring oxidation of fatty acids.

Oxidative metabolism in muscles and AMPK. To analyze a possible induction of oxidative metabolism by HF diet in skeletal muscles of A/J mice, ex vivo measurements were performed. First, the rate of endogenous V˙O₂ in the gastrocnemius and soleus muscles was assessed during a 35-min period (Fig. 7 and Table 4). Feeding mice the HF diet tended to increase the respiratory rate in gastrocnemius muscle of both B/6J and A/J mice, but this effect was not statistically significant. On the contrary, in the soleus muscle of A/J mice, HF diet increased respiration, which indicates an upregulation of oxidative metabolism through AMPK activation (see above).
diet increased the respiration ~1.8-fold, while no effect was observed in B/6J mice.

Second, oxidation of exogenous palmitate in the muscles was measured (Table 5). In either genotype, no significant effect of diet on palmitate oxidation was observed. However, when palmitate oxidation was measured at 30°C during the light phase of the day. Although locomotor activity was not assessed in this study, it could have contributed to the differences in VO2 as well in cold sensitivity (5, 9, 14).

The stronger obesogenic effect of HF diet in B/6J compared with A/J mice has been previously explained by strain-specific differences in the adrenergic control of thermogenesis (8) and sensitivity to leptin (32), with only A/J mice retaining sensitivity of adipose tissue to both stimuli after several weeks of feeding on a HF diet. The present study confirmed the previous results (8, 17, 48, 52), indicating that the differential response to HF diet could be attributed to the relatively high induction of UCP1 in the interscapular brown fat of A/J mice. In addition, our study demonstrated a HF diet-induced increase of VO2 in soleus muscle, which was specific for A/J mice, and phosphylation (p-AMPK) were measured in the soleus muscle (Fig. 8). In B/6J mice, the HF diet had no effect on either total AMPK or p-AMPK muscle content. In contrast, in A/J mice the HF diet caused an ~1.7- and ~1.4-fold increase in the content of AMPK and pAMPK, respectively; however, only the effect on total AMPK content reached statistical significance (two-way ANOVA). The stimulatory effect of HF diet on AMPK in soleus muscle of A/J mice was further confirmed by assessing the level of phosphorylation of ACC, a downstream target of AMPK, which showed an ~1.4-fold increase in response to HF diet, whereas no effect on ACC phosphorylation was found in B/6J mice (not shown). These results indicated a genotypic difference in the effect of HF diet on energy expenditure in soleus muscle, where the inducibility of respiration by HF diet in the A/J mice was associated with a relatively high potency of the AMPK regulatory pathway.

**DISCUSSION**

The principal finding of this study is the stimulation of muscle thermogenesis and lipid oxidation by HF diet in obesity-resistant A/J mice. Our results indicate that an HF diet could induce nonshivering thermogenesis in oxidative muscle, possibly by the leptin-AMPK axis.

We have demonstrated, for the first time, a profound difference between A/J and B/6J mice in their ability to resist acute cold exposure, providing that the animals were first adapted to the temperature close to thermoneutrality and maintained on an LF diet. Nevertheless, a relatively low whole body energy expenditure of the A/J LF mice, which would predispose these animals to obesity, was enhanced in response to the HF diet. This induction was apparently stronger than in B/6J mice, which exhibited higher energy expenditure than A/J mice when fed the LF diet but are prone to HF diet-induced obesity. That the HF diet induced a larger increase in whole body energy expenditure in A/J than in B/6J mice was also in agreement with the tendency of HF diet to increase body temperature in the former mice in this and in the previous study (47).

Without preacclimation to ambient temperatures around 20–22°C, as used in previous studies (8, 17, 48, 52), the capacity of nonshivering thermogenesis in brown adipose tissue was relatively low, but it was similar in both strains of mice, unless it was stimulated by HF diet. Since the ability to activate shivering was also similar in both B/6J and A/J mice fed the LF diet (Fig. 3), the cold sensitivity of the A/J LF mice could be explained, at least in part, by their relatively low VO2 as measured at 30°C during the light phase of the day. Although locomotor activity was not assessed in this study, it could have contributed to the differences in VO2 as well in cold sensitivity (5, 9, 14).

Table 3. Gene expression in glycolytic and oxidative skeletal muscles

<table>
<thead>
<tr>
<th>Muscle</th>
<th>LF</th>
<th>HF</th>
<th>A/J</th>
<th>LF</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrocnemius</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACOT1</td>
<td>0.94</td>
<td>0.13</td>
<td>0.86</td>
<td>0.06</td>
<td>1.00</td>
</tr>
<tr>
<td>ACOT2</td>
<td>1.55</td>
<td>0.31</td>
<td>3.28</td>
<td>0.33</td>
<td>0.88</td>
</tr>
<tr>
<td>ACOT1</td>
<td>1.11</td>
<td>0.22</td>
<td>1.36</td>
<td>0.21</td>
<td>1.55</td>
</tr>
<tr>
<td>COX6a</td>
<td>0.67</td>
<td>0.06</td>
<td>0.06</td>
<td>0.07</td>
<td>0.59</td>
</tr>
<tr>
<td>CPT IA</td>
<td>2.08</td>
<td>0.27</td>
<td>2.59</td>
<td>0.33</td>
<td>1.77</td>
</tr>
<tr>
<td>CPT IB</td>
<td>0.35</td>
<td>0.04</td>
<td>0.05</td>
<td>0.05</td>
<td>0.50</td>
</tr>
<tr>
<td>NRF-1</td>
<td>2.28</td>
<td>0.19</td>
<td>4.00</td>
<td>0.34</td>
<td>1.92</td>
</tr>
<tr>
<td>PDK-4</td>
<td>0.62</td>
<td>0.19</td>
<td>1.11</td>
<td>0.14</td>
<td>1.11</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>0.71</td>
<td>0.01</td>
<td>1.04</td>
<td>0.27</td>
<td>1.71</td>
</tr>
<tr>
<td>SOD1</td>
<td>0.43</td>
<td>0.15</td>
<td>0.33</td>
<td>0.10</td>
<td>0.50</td>
</tr>
<tr>
<td>SCD-1</td>
<td>0.13</td>
<td>0.35</td>
<td>1.80</td>
<td>0.05</td>
<td>1.79</td>
</tr>
<tr>
<td>SERCA1</td>
<td>0.10</td>
<td>0.13</td>
<td>1.16</td>
<td>0.13</td>
<td>1.23</td>
</tr>
<tr>
<td>UCP1</td>
<td>0.30</td>
<td>0.01</td>
<td>0.44</td>
<td>0.03</td>
<td>0.91</td>
</tr>
<tr>
<td>UCP3</td>
<td>2.09</td>
<td>0.44</td>
<td>3.55</td>
<td>0.69</td>
<td>2.73</td>
</tr>
<tr>
<td>LF/HF ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
suggested activation of nonshivering thermogenesis in this muscle. The association with increased leptin levels, and the selective involvement of the oxidative muscle, strongly suggested the involvement of the leptin-AMPK axis (29, 41). AMPK plays a major role in the regulation of fatty acid oxidation in skeletal muscle while inhibiting ACC by phosphorylation, reducing malonyl-CoA levels, and, thus, enhancing activity of CPT I and β-oxidation of fatty acids (7, 29). Direct stimulation of muscle thermogenesis ex vivo required both AMPK and phosphatidylinositol 3-kinase and could be prevented by pharmacological inhibition of AMPK, and activation of the AMPK axis was required for leptin-induced substrate cycling between de novo lipogenesis and lipid oxidation (41).

Table 4. \( \text{Vo}_2 \) in skeletal muscles

<table>
<thead>
<tr>
<th>Muscle</th>
<th>B/6J LF</th>
<th>B/6J HF</th>
<th>A/J LF</th>
<th>A/J HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrocnemius</td>
<td>112±13</td>
<td>136±23</td>
<td>111±15</td>
<td>125±9.0</td>
</tr>
<tr>
<td>Soleus</td>
<td>155±26</td>
<td>174±14</td>
<td>145±27</td>
<td>255±30*†</td>
</tr>
</tbody>
</table>

\( \text{Vo}_2 \) was measured in muscles dissected from mice adapted to 30°C and fed LF of HF diet, as described also in Fig. 4. Data are means ± SE for the 5-min interval, beginning 10 min after insertion of tissue fragments into measuring chambers. Results were normalized to tissue protein (n = 7–9). *Significant effect of diet; †significant effect of genotypes. Similar results were observed when data were normalized to tissue wet weight (not shown).

Table 5. Palmitate oxidation in soleus muscle

<table>
<thead>
<tr>
<th>Palmitate Oxidation, dpm·mg protein(^{-1})·s(^{-1})</th>
<th>B/6J LF</th>
<th>B/6J HF</th>
<th>A/J LF</th>
<th>A/J HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>−AICAR</td>
<td>10.6±1.3</td>
<td>11.1±1.6</td>
<td>10.9±1.8</td>
<td>11.4±1.5</td>
</tr>
<tr>
<td>+AICAR</td>
<td>13.0±2.2</td>
<td>12.1±1.2</td>
<td>14.6±1.3</td>
<td>19.2±1.2*†</td>
</tr>
</tbody>
</table>

Palmitate oxidation was assessed in soleus muscle dissected from mice adapted to 30°C and fed LF or HF diet. Measurements were performed at 37°C using [U-\(^{14}\)C]palmitate in the absence or presence of 2 mM 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR). Data are means ± S.E (n = 8). *Significant effect of diet; †significant effect of genotype.

Fig. 7. Ex vivo muscle \( \text{Vo}_2 \) during 35-min incubation period at 37°C. Muscle samples were obtained from mice adapted to 30°C and fed either LF or HF diet. Fragments of gastrocnemius or whole soleus muscles were incubated in freshly oxygenated (95% \( \text{O}_2\)-5% \( \text{CO}_2\)) Krebs-Ringer bicarbonate buffer (pH 7.4) containing 10 mM glucose, and \( \text{Vo}_2 \) was measured using polarography (see also Table 4). Data are means ± SE for a 5-min interval, beginning 10 min after insertion of tissue fragments into measuring chambers. Results were normalized to tissue protein (n = 7–9). *Significant effect of diet (repeated-measures ANOVA: diet vs. time).
resents the bulk (98\% (10). Oxidation of intramuscular lipid fatty acid provide only a very small fraction (stimulated by AICAR. In resting soleus muscle, exogenous medium, unless the AMPK and CPT I activities are maximally stimulated by AICAR. In resting soleus muscle, exogenous medium, unless the AMPK and CPT I activities are maximally activated. In the study, both SERCA2a and SERCA2b mRNA were quantified together) or with changes of the thyroid hormone status (Tables 1 and 3). The qualitative difference in the thermogenic response between Ucp1−/−.Lep−/− and A/J mice may thus reflect a much higher induction of thermogenic mechanisms required to substitute for defective UCP1-mediated thermogenesis in the transgenic mice. Additional thermogenic mechanisms may function in this transgenic model that do not operate in A/J mice. However, in both Ucp1−/−.Lep−/− and A/J mice, oxidative but not glycolytic muscle was involved in the adaptive thermogenesis, supporting further the importance of lipid oxidation.

The role and mechanism of leptin-mediated adaptive thermogenesis have recently been investigated by Ukopec et al. (51) using ob/ob mice with a targeted inactivation of UCP1 gene (Ucp1−/−.Lep−/− mice). These mice could not adapt to temperatures below 12°C unless they were administered either leptin or T3 (51). Thermogenesis was possibly activated due to the induction of Ca\(^{2+}\) cycling associated with an increased expression of sarcoplasmic reticulum Ca\(^{2+}\) ATPase-2 (SERCA2) in oxidative muscle (51). On the contrary, the stimulation of thermogenesis in the oxidative muscle of the A/J HF mice was not associated either with upregulation of SERCA2 (in fact, in both the experiments on Ucp1−/−.Lep−/− mice and in our study, both SERCA2a and SERCA2b mRNA were quantified together) or with changes of the thyroid hormone status (Tables 1 and 3). The qualitative difference in the thermogenic response between Ucp1−/−.Lep−/− and A/J mice may thus reflect a much higher induction of thermogenic mechanisms required to substitute for defective UCP1-mediated thermogenesis in the transgenic mice. Additional thermogenic mechanisms may function in this transgenic model that do not operate in A/J mice. However, in both Ucp1−/−.Lep−/− and A/J mice, oxidative but not glycolytic muscle was involved in the adaptive thermogenesis, supporting further the importance of lipid oxidation and mitochondria for muscle thermogenesis.
the control and significance of adaptive nonshivering thermogenesis is less defined than in rodent species, an increase of sympathetic activity is known to be critical for resistance to obesity (see other references in Ref. 9).

In summary, our results demonstrate that both the UCP1-mediated thermogenesis in brown fat and muscle thermogenesis are associated with resistance to dietary obesity in mice. Only in the model of obesity-resistant A/J, but not in obesity-prone B/6J mice, stimulation of thermogenesis in oxidative skeletal muscle in response to an HF diet was observed. Our results further suggest the involvement of the leptin-AMPK axis, with a preferential use of lipids as metabolic substrates, in the induction of nonshivering thermogenesis in oxidative muscle by HF diet. Thus, at least in mice, the adaptive stimulation of lipid oxidation and muscle thermogenesis in response to increased fat content in the diet might contribute to cold resistance and obesity resistance.

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