β1-Adrenergic receptors increase UCP1 in human MADS brown adipocytes and rescue cold-acclimated β3-adrenergic receptor-knockout mice via nonshivering thermogenesis

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Submitted 22 February 2011; accepted in final form 24 August 2011

BROWN ADIPOSE TISSUE (BAT) is responsible for nonshivering thermogenesis in mammals. The tissue is exceedingly vascularized and rich in mitochondria, displaying characteristics of a highly metabolically active tissue. In rodents, BAT is heavily sympathetically innervated and responds to the sympathetic neurotransmitter norepinephrine (NE), which triggers a program of signaling and gene expression to proliferate and activate nonshivering thermogenesis through uncoupling protein 1 (UCP1) (10). Norepinephrine mediates these effects by activating adrenergic receptors (ARs) in BAT, primarily β3- and to some extent α1-ARs (10). However, in mouse BAT, mRNA of all three β-AR subtypes can be found (5).

Very recently, several independent researchers have shown that active BAT exists in adult man and that the presence of brown fat is negatively correlated to obesity (13, 35, 43, 48–50). Although it is still not clear how much impact brown adipocytes have on the control of body weight and energy expenditure in humans, the above studies indicate that brown adipocytes could play a role in protection against obesity in humans. Thus, understanding how BAT is recruited and activated in humans could be a step in combating obesity, type 2 diabetes, and its complications. The first generation of β3-AR agonists such as BRL-37344 and CL-316243 (4, 14) was identified from studies performed predominantly in rodent tissues (including evidence showing that they activate BAT), and subsequently, further β3-AR agonists were designed on the structure of these agonists. The majority of the β3-AR agonists available have limited effects in humans and primates, with poor efficacy in vivo and in vitro (2, 3, 32), with efficacy generally observed only in cells transfected with high human β3-AR receptor levels. Subsequently, the cloned human β3-AR has been used to identify agonists with increased efficacy at the human β3-AR and less efficacy at β1- and β2-ARs. One such agonist that has been identified is L755507 (37), which increased lipolysis and metabolic rate in rhesus monkey (17). Although these new human β3-AR agonists may have a positive effect in human brown adipocytes, there is also the possibility that a different signaling pathway exists in human BAT. In humans, BAT activation is inhibited by the β3-AR antagonist propranolol (which displays lower efficacy for the β3-AR compared with the β1- and β2-AR, implicating that human BAT thermogenesis could be regulated by β1-ARs (25, 35, 36, 44) and not β3-ARs as in the case of rodents (10). To investigate this possible alternative pathway, we have examined nonshivering thermogenesis in β3-knockout (β3-KO) mice and investigated β-AR signaling in human multipotent adipose-derived stem (hMADS) cells.

Upon acute exposure to cold, a warm-acclimated mammal (mouse) will utilize all of its available capacity for shivering and nonshivering thermogenesis to maintain its body temperature. After ~3 wk in prolonged cold, nonshivering thermogenesis is fully recruited (9, 10), with UCP1 necessary for...
nonshivering thermogenesis (19, 21). However, UCPI-KO mice are able to survive several weeks in the cold if they have been acclimated successively to colder temperatures (21). Thus these mice are able to utilize other mechanisms, such as shivering to survive in the cold. Because nonshivering thermogenesis is mediated primarily via norepinephrine activation of β3-ARs (10), it has been postulated that β3-KO mice could utilize a different mechanism to survive in the prolonged cold. Thus this mechanism could resemble the possible alternative mechanism used in humans. β3-KO mice can survive 3 wk in the cold (46), but the mechanism(s) whereby this has occurred is unknown. Therefore, in this study, we have investigated how β3-KO mice are able to survive in the cold. β3-KO and wild-type (FVB/N) mice were acclimated to prolonged cold for 7 wk, and parameters related to metabolism and BAT were investigated. The β3-KO mice were able to survive in the prolonged cold and increased their oxygen consumption in response to norepinephrine through upregulation of β3-AR signaling in BAT. Thus, in β3-KO mice, β3-ARs are able to couple to Gs and induce UCPI expression and thus elicit nonshivering thermogenesis. To investigate whether human brown fat could be activated via β3-ARs, we investigated the induction of UCPI in hMADS cells differentiated into brown fat cells. We found that β3-(dobutamine) and β3-AR (CL-316243) [but not β2-AR (terbutaline)] agonists were able to induce UCPI expression. These findings suggest that β3-KO mice could be used to resemble the β3-AR signaling situation in hMADS and together with differentiated hMADS cells be used as a model system for investigations of human BAT activation.

MATERIALS AND METHODS

Chemicals. Collagenase (type II), isoprenaline, CL-316243, dobutamine, forskolin, insulin, isobutylmethylxanthine, norepinephrine, terbutaline, transferrin, and triiodothyronine were from Sigma-Aldrich (St. Louis, MO). ICI-89406 was from Zeneca (Wayne, PA), and cAMP kit (TRK 432) was from Amersham Biosciences (Little Chalfont, UK). Rosiglitazone was from Cayman. hFGF2 was from Peprotech. Newborn calf serum was from Life Technologies (Paisley, Scotland, UK), fetal calf serum was from Dutscher, and all cell culture reagents were from HyClone or Lonza.

Animals. The study was approved by the Animal Ethics Committee of the North Stockholm Region. β3-AR-KO (β3-KO) mice [on a FVB/N (sensitive to Friend leukemia virus B strain) background and originally generated by Susucli et al. (46)] were bred and routinely genotyped at the institute and back-crossed to the FVB/N strain for at least seven generations. Prior to the start of the experiment, all mice were housed at 24°C with a 12:12-h light-dark cycle, with free access to food (R70, Lactamin) and water. Genomic DNA analysis was conducted on mouse tail clippings to determine the genotype of the breeding pairs used in the breeding program, as detailed previously (24). Figure 1 shows an example of genotyping bands.

In the acute cold study, 3-mo-old male mice were single-caged for 1 wk at room temperature (22–24°C) and then acutely transferred to cold (4°C), and their body temperature was measured rectally with BAT-12 Microprobe Thermometer (AgnTho’s).

For the long-term acclimation study, 4-mo-old female mice were single-caged and moved to cold (4°C, with an initial 2 wk at 18°C) for a total of 7 wk. Body weight was measured every 7th day for 41 days, and body composition was measured on days 0, 14, 35, and 41 with a magnetic resonance imaging (MRI) technique (EchoMRI-700/100 Body Composition Analyzer; Echo Medical Systems). Food intake was measured during week 5; food was weighed when supplied, and remaining food (including spillage, etc.) was subtracted.

Indirect calorimetry. For indirect calorimetry, the INCA System was used (Somedic, Hörby, Sweden) (1), as described previously (30). Metabolic rate and NE-induced thermogenesis was examined during week 6, with at least 1 day apart. O2 consumption and CO2 production were measured every 2nd min, and the respiratory exchange ratio (RER) was calculated (volume CO2/volume O2). To obtain resting metabolic rates (RMRs), the mice were placed in the 4-liter metabolic chambers at 30°C in their home cages during the daytime (inactive phase of the mice). The RMR was defined as the average of the three lowest 2-min points and the mean metabolic rate (MMR) as the average of the last 60 min of the measurements (i.e., the average oxygen consumption during the last hour). For measurement of NE-induced thermogenesis the animals were anesthetized with pentobarbital sodium (40 mg/kg ip), and after basal values were obtained, a standard dose of NE (1 mg/kg) was injected subcutaneously. The experiment was run at 33°C since a higher ambient temperature is necessary as the thermoregulatory centers become inactive during anesthesia (9, 21). The anesthetic basal metabolic rate was defined as the mean of the last seven determinations before NE injection and the response to NE as the mean of the three highest points after NE injection minus the anesthetic basal metabolic rate.

Tissue and protein analysis. During week 7, animals were euthanized by 10.220.33.5 on October 14, 2017 http://ajpendo.physiology.org/ Downloaded from Fig. 1. PCR products from genotyping of mice. Gel illustrating expected PCR products from genotyping of mice. Lane 1: 100-bp marker. Lanes 2–4: PCR to indicate presence/absence of neomycin disruption to β3-adrenergic receptor (β3-AR) allele. Lanes 5–7: PCR to indicate presence/absence of wild-type β3-AR allele. Wild-type FVB/N mice (+/+); lanes 2 and 5) display only the β3-AR allele. Heterozygote mice (+/−; lanes 3 and 6) display both neomycin and β3-AR alleles. β3-knockout mice (−/−; lanes 4 and 7) display only the neomycin allele.

AJP-Endocrinol Metab • VOL 301 • DECEMBER 2011 • www.ajpendo.org
β3-AR and Cox4; GE Healthcare). The blots were quantified with a charge-coupled device camera (LAS-1000; Fujifilm) and Image Gauge. The quantified values are presented as the means per milligram of protein loaded (as obtained from the measured protein concentration) and as the total amount of each specific protein in the entire tissue depot. For each graph, the wild-type mice were set to 100%.

Brown fat precursor cell isolation and primary cell culture of brown adipocytes. Brown fat precursor cells (obtained from the intrascapular, axillary, and cervical BAT depots) were isolated as described previously (12) from 3-wk-old FVB/N or β3-KO mice of either sex. Cells were cultured in 12-well culture dishes containing DMEM supplemented with 10% newborn calf serum, 2.4 mM insulin, 10 mM HEPES, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 25 μg/ml sodium ascorbate. Media changes were performed on days 1, 3, and 5. After day 5 in culture, the cells spontaneously convert from a fibroblast phenotype to mature brown adipocyte morphology. This conversion initiates at the reaching of confluence and coordinates with the ability of NE to induce the expression of the most specific brown adipocyte differentiation marker, UCP1 (33, 34, 39, 40).

cAMP measurements. In studies on preadipocytes (to yield cultures expressing only β3-ARs), both FVB/N and β3-KO cells were serum starved overnight and experiments performed on day 3. In studies on mature brown adipocytes, both FVB/N (expressing a mixture of β1- and β3-ARs) and β3-KO (expressing only β3-ARs) cells were serum starved overnight and experiments performed on day 7. On the day of the experiment, the cells were treated with drugs as indicated, and cAMP was extracted (22). Levels of cAMP were measured using a commercial kit (TRK 432; Amersham Biosciences, Piscataway, NJ). All experiments were performed in duplicate, with n referring to the number of independent experiments.

Northern blot. Primary brown adipocytes from both wild-type and β3-KO mice were differentiated as described above. On day 7 the cells were treated with drugs as indicated, RNA was isolated, and UCP1 mRNA levels were measured by Northern Blot as described earlier (8, 12, 40).

hMADS cells. hMADS cells (15, 16, 42) were seeded at 4,500 cells/cm² in DMEM supplemented with 10% fetal calf serum, 2.5 ng/ml hFGF2, 60 μg/ml penicillin, and 50 μg/ml streptomycin. The medium was changed every other day and hFGF2 removed when the cells reached confluence and were triggered for differentiation on day 2 postconfluence (designated as day 0). Cells were then maintained in DMEM-Ham’s F-12 medium supplemented with 10 μg/ml transferrin, 0.85 μM insulin, 0.2 nM triiodothyronine, 1 μM dexamethasone, and 500 μM isobutylmethylxanthine. Three days later, the medium was changed (dexamethasone and isobutylmethylxanthine were omitted) and 100 nM rosiglitazone added. Medium was changed every other day, and cells were used at the indicated days.

mRNA and protein levels in differentiated hMADS cells. Extraction of total RNA, reverse transcriptase reactions, and quantitative RT-PCR assays were performed as described previously (15, 42). The expression of selected genes was normalized to that of the TATA-binding protein gene and quantified using the comparative (ΔΔCt) method. Oligonucleotide sequences, designed using Primer Express software (PerkinElmer Life Sciences), are shown in Table 1.

Table 1. Oligonucleotides used as primers

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<thead>
<tr>
<th>Primer</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>TBP</td>
<td>CAGGAGGACTGAGCGAGTGT</td>
<td>TTTTCTGTCGACATCGTGGAC</td>
</tr>
<tr>
<td>UCP1</td>
<td>GGUGGCGGACTGGCGACAGA</td>
<td>TTCGCGGATCCCTGGCACAAAC</td>
</tr>
<tr>
<td>β3-AR</td>
<td>AGCGGCTACGCCAGAAA</td>
<td>TGAGAGAGATGAGAGAGG</td>
</tr>
<tr>
<td>β3-AR</td>
<td>GGGTCTGTCCTCAAAGATG</td>
<td>AGCATCACGAGAAGAGGAAGGT</td>
</tr>
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For protein measurements, total cellular lysates were subjected to immunoblotting, as described previously (15). Primary antibodies were rabbit anti-human UCP1 and anti-β-tubulin (Santa Cruz Biotechnology). Secondary horseradish peroxidase-conjugated antibodies were purchased from Promega (Charbonnières, France). ECL (Millipore, St. Quentin en Yvelines, France) was used for detection.

Statistics. All data are presented as means ± SE. For the graphs and statistical analysis, GraphPad Prism was used. For the statistical analysis, either Student’s two-tailed t-test or a two-way ANOVA with a Bonferroni posttest was used as indicated in the figure legends. If the interaction in the two-way ANOVA is not significant, it is not indicated in the figures.

RESULTS

β3-KO and wild-type mice exposed to acute cold. β3-KO and wild-type mice were transferred from room temperature to cold (4°C) for 12 h, and their body temperature was monitored. As expected (21), the body temperature of the wild-type mice had a small initial drop of nearly 2°C (with a lowest peak ~4–5 h), which then increased to nearly baseline readings and stabilized in the last hours of the experiment (Fig. 2). However, for the β3-KO mice the initial drop of body temperature as observed in the FVB/N mice was not observed, implying that the β3-KO mice were efficient in using all of their available capacity for nonshivering thermogenesis and/or shivering to obtain the requisite additional heat (9).

β3-KO mice survive in prolonged cold. Since β3-KO mice are able to defend their body temperature upon acute exposure to cold, we investigated whether the mice could survive in the prolonged cold (7 wk). At the start of the experiment (day 0), mice from normal room temperature (22°C) were transferred to cold (1st 2 wk at 18°C, thereafter 4°C). As expected (21, 30, 46), all of the wild-type mice survived the cold. Additionally all of the β3-KO mice were also able to survive in the cold, comparable with other results (although these mice were directly moved to 4°C and then kept at this temperature for a period of 3 wk) (46).

In accord with the findings of Susulic et al. (46), there was no difference in body weight at the start of the experiment between wild-type and β3-KO mice (Fig. 3A). In addition, we could not see any difference in the body weight gain between the wild-type and β3-KO mice over the time frame measured (Fig. 3A). During the experiment, MRI was measured on the indicated days and the fat per lean mass ratio calculated (Fig. 2).
There was no difference between the wild-type and \(\beta_3\)-KO mice in the fat per lean mass ratio at the start or throughout the experiment (Fig. 3B). There was also no difference in either the fat mass or the lean mass between the wild-type and the \(\beta_3\)-KO mice throughout the experiment (Fig. 4). In contrast to the MRI data we obtained in the beginning of the experiment, Susulic et al. (46) found that 15-wk-old \(\beta_3\)-KO mice had a higher total body fat compared with the wild-type mice. One explanation for the difference observed could be that our mice have been backcrossed, whereas those of Susulic et al. (46) were not. At the end of the experiment the inguinal white adipose tissue was dissected, and no difference in white adipose tissue weight was observed (Fig. 3C). During week 5, food intake was measured. No difference in food intake was observed between the \(\beta_3\)-KO and wild-type mice (Fig. 3D). Thus, the \(\beta_3\)-KO mice did not have more insulation (as seen by MRI in Figs. 3B and 4) or eat more than the wild-type mice to counteract the cold temperatures.

**Metabolic rates of cold-acclimated \(\beta_3\)-KO mice.** To examine whether there are any differences in the resting metabolism of the \(\beta_3\)-KO mice, cold-acclimated \(\beta_3\)-KO mice and wild-type mice were placed in metabolic chambers for 3 h at 30°C during their inactive phase. Oxygen consumption and carbon dioxide production were measured, and the RER was calculated. In Fig. 5A, the oxygen consumption rate per lean body mass is calculated. There was no difference between \(\beta_3\)-KO and wild-type mice in regard to MMR per lean body mass (Fig. 5B); however, \(\beta_3\)-KO mice had a significantly lower RMR per lean body mass (Fig. 5C). This indicates that in the overall metabolic rate there is no difference between wild-type and \(\beta_3\)-KO mice, but when the mice are not moving the \(\beta_3\)-KO mice have a somewhat lower metabolic rate than the wild-type mice. If the \(\beta_3\)-KO mice had an increased metabolic rate, this could have explained how they are able to survive in cold. Since we could see no difference in MMR, and the RMR was even lower in the \(\beta_3\)-KO mice, this is not the case. The RER was calculated and is shown in Fig. 5D as RER over time and in Fig. 5E as the average RER for the last 60 min. There was no difference in the RER values between \(\beta_3\)-KO and wild-type mice, indicating that they use the same substrate for their resting metabolism.

\(\beta_3\)-KO mice display a slightly diminished thermogenic response to NE. Upon acclimation to cold, a wild-type mouse recruits BAT and replaces shivering thermogenesis with nonshivering thermogenesis (9, 10). The general pathway for nonshivering thermogenesis is via the \(\beta_3\)-AR/adenylate cyclase/cAMP/PKA-pathway, which leads to the uncoupling of UCP1 (10). Since \(\beta_3\)-KO mice lack the \(\beta_3\)-AR, are these mice able to utilize nonshivering thermogenesis, or do they have to use another mechanism to survive in the cold? Since insulation (MRI data seen in Figs. 3B and 4), food intake (Fig. 3D), and resting metabolism (Fig. 5) could not explain the survival of
Fig. 5. Resting metabolism. The basal oxygen consumption of β3-KO and wild-type mice (n = 9) was analyzed with indirect calorimetry at 30°C for 3 h, as described in MATERIALS AND METHODS. A: oxygen consumption calculated per lean body mass in mice acclimated to 4°C. For clarity, only every 4th minute is shown in the graphs. B: mean metabolic rate (MMR) calculated per lean body mass. MMR is defined as the mean oxygen consumption during the last 60 min (t-test: P = 0.24). C: resting metabolic rate (RMR) calculated per lean body mass. RMR is defined as the mean of the 3 lowest points during the 3 h (t-test: P = 0.024). D: the respiratory exchange ratio [RER; volume CO2 /volume O2 (V˙CO2/V˙O2)] for the mice during all 3 h. For clarity, only every 4th minute is shown in the graphs. E: average RER for the last 60 min, showing lowest to highest value and line as the mean (t-test: P = 0.56). *P < 0.05. P > 0.05 (ns).

After NE injection between wild-type and β3-KO mice. Thus, the β3-KO mice were able to combust lipids to the same extent as the wild-type mice.

Although there is reduced NE-induced oxygen consumption in the β3-KO mice compared with the wild-type mice (Fig. 6, A and B), the data from the NE-induced oxygen consumption suggest that BAT in the β3-KO mice is still functional. Therefore, it is probable that the mice can utilize BAT to survive in the cold. To investigate what mechanism/signaling/receptors are involved, we further investigated BAT function and signaling in the β3-KO mice.

BAT in β3-KO mice. Upon cold acclimation a wild-type mouse will increase the activity of its BAT, indicated by higher protein content and UCP1 expression (compared with mice housed at normal room temperatures) (10, 30). To investigate whether there were any differences in BAT protein parameters and whether this could explain the reduced response in NE-induced thermogenesis in the β3-KO mice, total protein levels, UCP1, β1-AR, and Cox4 protein content were measured in BAT from cold-acclimated wild-type and β3-KO mice.

There was no difference in BAT wet weight, tissue protein concentration (mg protein/mg wet wt), or total protein amount in the tissue (Fig. 7, A–C) between wild-type and β3-KO mice, indicating a full recruitment of BAT in β3-KO mice. However, UCP1 protein per milligram of protein was significantly higher in BAT from β3-KO mice compared with wild-type mice (Fig. 7G). Thus, the slightly diminished response in NE-induced thermogenesis observed in the β3-KO mice (Fig. 6) is not attributable to reduced UCP1 levels. In addition, there was no difference in the levels of Cox4 protein in the β3-KO mice in the prolonged cold, we investigated the nonshivering capacity of these mice.

Mice acclimated to cold for 7 wk were injected with a standard dose of NE (1 mg/kg body wt) (20, 30), and oxygen consumption and carbon dioxide production were measured. As expected (21, 30), the wild-type mice increased their oxygen consumption following NE injection (Fig. 6A). The β3-KO mice also increased their oxygen consumption upon NE injection in agreement with β3-KO mice acclimated to room temperature (46). However, NE-induced oxygen consumption was reduced significantly in the β3-KO mice compared with the wild-type mice. (Fig. 6, A and B).

Prior to NE injection, both the wild-type and β3-KO mice had a RER ≈0.9 (Fig. 6C). Following NE injection, the RER levels decreased to ≈0.8 (transiently) for the wild-type mice, which indicates a change to combustion of more lipids. However, the β3-KO mice also changed their combustion transiently, with an RER of ≈0.8 following NE injection. As shown in Fig. 6D, there was no difference in the RER directly after NE injection between wild-type and β3-KO mice. Thus, the β3-KO mice were able to combust lipids to the same extent as the wild-type mice.
per milligram of protein or in the total Cox4 protein in the tissue (Fig. 7, E and H, respectively) [Cox4 is present in complex IV (cytochrome c oxidase) in the respiratory chain and has been shown to be essential for the assembly and the respiratory function of the enzyme complex (26)]. However, both the level of β1-AR protein per milligram of protein and total β1-AR protein amount in the tissue was increased in BAT from β3-KO mice compared with wild-type mice (Fig. 7, F and I). This indicates that β1-ARs could be compensating for the loss of the β3-ARs in cold-acclimated β3-KO mice.

β-AR agonist-stimulated cAMP production in cultured brown adipocytes. To investigate whether the increase in the NE-induced oxygen consumption in β3-KO mice could be accomplished through β1-AR signaling in BAT, we cultured brown preadipocytes and mature adipocytes from β3-KO and wild-type mice and investigated β-AR-mediated cAMP production, the mediator in the nonshivering thermogenesis pathway (10).

To investigate β-AR-mediated cAMP signaling in wild-type and β3-KO adipocytes, we used the nonselective β-AR agonist isoproterenol and measured increases in cAMP levels in both preadipocytes and mature adipocytes. In cultured preadipocytes (day 3 in culture) only β1-ARs are present, whereas in the mature cells (day 7 in culture) both β1- and β3-AR are present, with the β3-AR composing the major part (with no β2-ARs present in either preadipocytes or mature adipocytes) (5, 12). As shown previously, the β3-KO primary brown preadipocyte cultures express neither β2- nor β3-ARs, and compared with the wild-type mice there is no difference in β1-AR levels (12). Thus, preadipocytes from both wild-type and β3-KO mice express only β1-ARs, wild-type mature adipocytes express both β1- and β3-ARs, and the β3-KO mature adipocytes express only β1-ARs.

There was no difference between isoproterenol-mediated increases in cAMP levels between wild-type and β3-KO preadipocytes (Fig. 8A). Both preadipocyte cultures responded quickly to isoproterenol, with cAMP levels peaking following 5 min of treatment, before declining back to basal levels. As expected (8), the mature wild-type adipocytes had higher cAMP levels after stimulation with isoproterenol compared with the preadipocytes (Fig. 8, A and B). However, the peak was slightly delayed (as compared with the preadipocytes) and occurred following 15 min of isoproterenol treatment; thereafter the cAMP levels declined, and after 90 min the levels were near basal levels. This increase in cAMP levels in mature cells compared with the increase in the preadipocytes corresponds to the transition from a β1-AR signaling to a β3-AR signaling, as shown previously (8). Compared with the mature wild-type cells, the β3-KO mature adipocytes had a different appearance, with cAMP levels peaking after 5 min of isoproterenol treatment, and after 30 min the levels were near basal levels (Fig. 8B). Since the mature brown adipocytes cultured from β3-KO mice were able to respond to isoproterenol and these cells express only β1-AR (and not β2- or β3-AR), the β1-AR is responsible for the signaling to cAMP. To confirm that β1-ARs contribute to CAMP production in brown adipocytes isolated from β3-KO mice (i.e., no influence of β2-AR), concentration-response curves to isoproterenol were performed in the presence/absence of the β1-AR antagonist ICI 89406. As shown in Fig. 8C, ICI-89406 antagonized the response to isoproterenol.
In addition, to ensure that the capacity for cAMP production was not different between the two mice strains, both mature brown adipocyte cultures from wild-type and β3-KO mice were treated with the adenylate cyclase activator forskolin. Adenylate cyclase, which is downstream of ARs, is the enzyme responsible for cAMP production. Forskolin increased cAMP levels in both FVB/N and β3-KO mature adipocytes, with levels peaking following 15 min of stimulation, and cAMP levels then declined and reached near-basal levels after 90 min (Fig. 8D). This indicated that the capacity for cAMP production was similar in both wild-type and β3-KO mature adipocytes.

In wild-type mice, NE and isoprenaline are able to induce UCP1 expression via β3-AR signaling (10, 12), and isoprenaline can induce UCP1 mRNA expression in mature β3-KO brown adipocytes (12); thus β3-ARs are able to induce UCP1 mRNA expression. We show in Fig. 8E that the endogenous ligand NE, which was used for the nonshivering thermogenesis experiments, is also able to induce UCP1 mRNA levels in the β3-KO brown adipocytes. Thus, the main effectors of increasing cAMP levels, UCP1 mRNA levels, and activating nonshivering thermogenesis in β3-KO mice are β3-ARs.

β3-AR agonist-stimulated UCP1 induction in differentiated hMADS cells. Since β1-ARs are able to compensate for the lack of β3-ARs with respect to cAMP signaling and nonshivering thermogenesis in β3-KO mice, this indicated that β3-ARs could have a more important role in human BAT than earlier thought. Therefore, we investigated which β-ARs are expressed in hMADS cells differentiated into brown adipocytes and whether stimulation of β-ARs resulted in induction of UCP1. In differentiated hMADS cells, β1-AR, β2-AR, and β3-AR mRNA was detected (Table 2), in agreement with previous data showing expression of β2- and β3-AR mRNA (15). To investigate whether all three β-ARs were able to induce UCP1 mRNA and protein expression, differentiated hMADS cells were stimulated with the general β-AR agonist isoprenaline, the β1-AR agonist dobutamine (which can also act on β2-AR and to some extent on α1-AR), the β2-AR agonist terbutaline, or the β3-AR agonist CL-316243. In accord with previous data (15), both isoprenaline and CL-316243 were able to induce UCP1 mRNA and protein levels (Fig. 9, A–C), and thus β-ARs and specifically β3-ARs can induce UCP1 expression in hMADS cells. Although UCP1 mRNA levels increased to a lower extent than observed previously by Elabd et al. (15), the UCP1 protein levels increased to the same extent. This difference in mRNA levels, but not in protein levels, is probably due to differences in the concentration and time of agonist stimulation [1 µM for 15 h in this study compared with 100 nM for 6 h in the previous study (15)]. Dobutamine, but not terbutaline, was able to induce UCP1 levels (Fig. 9, A–C), and hence, β1-ARs but not β2-ARs are able to signal in differentiated hMADS cells. Thus, in hMADS differentiated into functional

Table 2. Ct values and relative expressions of β-AR in hMADS cells differentiated into brown adipocytes

<table>
<thead>
<tr>
<th>β-AR</th>
<th>Average Ct Values</th>
<th>Average Relative Expression</th>
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<tr>
<td>β1-AR</td>
<td>30.60 ± 0.14</td>
<td>0.028 ± 0.0076</td>
</tr>
<tr>
<td>β2-AR</td>
<td>28.54 ± 0.37</td>
<td>0.111 ± 0.013</td>
</tr>
<tr>
<td>β3-AR</td>
<td>32.07 ± 0.45</td>
<td>0.0095 ± 0.00070</td>
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</table>

Values are means ± SE. Ct, threshold cycle; hMADS, human multipotent adipose-derived stem.
addition, in differentiated hMADS cells both
and thus rescue the signaling to nonshivering thermogenesis. In
mechanism could involve increased insulation, increased food
the prolonged cold, an alternate mechanism must exist. Such a
not properly functional and the mouse is still able to survive in
If the signaling pathway(s) for nonshivering thermogenesis is
thereafter nonshivering thermogenesis is recruited, and after
start to shiver to maintain its body temperature. Gradually
of its available capacity for nonshivering thermogenesis and
moved from normal to colder temperatures will initially use all
A mouse that has been
3-KO mice were
1-AR signaling accomplished this. Thus, in
the absence of 3-ARs, the 1-AR can increase cAMP levels
and thus rescue the signaling to nonshivering thermogenesis. In
addition, in differentiated hMADS cells both 1- and 3-ARs
were able to induce UCP1 expression.

DISCUSSION
In the present study, we found that the 3-KO mice were able
to survive in cold through nonshivering thermogenesis and
that a switch to β1-AR signaling accomplished this. Thus, in
the absence of 3-ARs, the β1-AR can increase cAMP levels
and thus rescue the signaling to nonshivering thermogenesis. In
addition, in differentiated hMADS cells both β1- and β3-ARs
were able to induce UCP1 expression.

Metabolism in mice exposed to cold. A mouse that has been
moved from normal to colder temperatures will initially use all
of its available capacity for nonshivering thermogenesis and
start to shiver to maintain its body temperature. Gradually
thereafter nonshivering thermogenesis is recruited, and after
around 3 wk of acclimation to cold it is fully recruited (9, 10).
If the signaling pathway(s) for nonshivering thermogenesis is
not properly functional and the mouse is still able to survive in
the prolonged cold, an alternate mechanism must exist. Such a
mechanism could involve increased insulation, increased food
consumption, increased metabolic rate, and/or increased shiv-
ering. The UCP1-KO mice, which cannot utilize nonshivering thermogenesis, can survive several weeks in the cold if they have been acclimated successively to colder temperatures and therefore have increased capacity for shivering (19, 21, 47).

Because the β3-AR is the primary receptor involved in
nonshivering thermogenesis (10), 3-KO mice could, as the
UCP1-KO mice do (21), use another mechanism to survive in
the prolonged cold. However, following 7 wk in the cold,
neither fat content, food intake, nor metabolic rate could
explain the survival of the mice (Figs. 3–5). To test the
capacity for nonshivering thermogenesis, we injected 3-KO mice
with a standard dose of NE and measured oxygen consumption. 3-KO mice were able to increase oxygen consumption following NE injection (although a slightly lower increase than the wild-type mice) and use the same substrate composition as the wild-type mice (Fig. 6). This is in contrast to Cav1-KO mice that are not able to change substrate at the standard dose of NE (30), and thus it seemed that BAT from 3-KO mice was functional and able to be recruited following cold exposure [i.e., equivalent BAT wet weight, BAT total protein content, Cox4 content, and even increased β1-AR and UCP1 expression (Fig. 7)]. Previous studies showed an increase in β1-AR expression in BAT from 3-KO mice accli-
matized to room temperature compared with wild-type mice under the same conditions (12, 46). However, in contrast, Revelli et al. (41) found somewhat decreased amounts of β1-AR mRNA, making it plausible that change in β1-AR protein amount per se is not a major mechanism. However, these mice were on a different background strain (C57Bl/6) compared with the other studies and ours, which were on a FVB/N background (12, 41, 46).

One explanation for increased UCP1 expression in BAT
from 3-KO mice could be that 3-KO mice living at tempera-
tures below thermoneutrality could have increased levels of NE to stimulate BAT to compensate for the loss of 3-ARs. This increase in NE would lead to increased UCP1 expression (10). This explanation would also fit with the fact that there is no decrease in body temperature in 3-KO mice exposed to acute cold. Because of the chronic, precold challenge of living at room temperature, the 3-KO mice, compared with the wild-type mice, would already have a higher capacity for nonshivering thermogenesis (and/or shivering) and will never have to experience the same drop in body temperature in 3-KO mice exposed to acute exposure to cold as the wild-type mice. However, there were no data suggesting that BAT in long-term cold-acclimated 3-KO mice should be more active than in the wild-type mice (with the same amount of total protein and Cox4 expression). On the other hand, RMR per lean body mass from long-term cold-acclimated mice was significantly lower in the 3-KO mice; thus when the mice are not moving, the 3-KO mice have a somewhat lower metabolic rate than the wild-type mice. This could be due to β1-AR expression in a number of other tissues such as brain, intestine, WAT, and bladder. It is possible that β1-ARs are not able to fully compensate for the lack of 3-ARs in these tissues, thus leading to a lower RMR per lean body mass [in vitro studies show that β1-ARs compensate for the lack of β3-ARs in gastrointestinal tissue from 3-KO mice (24), whereas β1- and α1-ARs compensate for the lack of β3-ARs in brown adipocytes derived from 3-KO mice (12)].
However, although the β3-KO mice had a lower RMR per lean mass, this did not affect their survival in cold.

β1- and β3-ARs display differences in both kinetics after adrenergic stimuli and desensitization, leading to effects on oxygen consumption in mice. In brown adipocyte cultures from wild-type mice, β1-ARs are expressed both in preadipocytes and mature brown adipocytes, although they are not functionally coupled in mature brown adipocytes (8). Our experiments in brown preadipocytes show that the production of cAMP in response to the nonselective β-AR agonist isoprenaline exhibits the same kinetics in wild-type FVB/N brown preadipocytes as in β3-KO brown preadipocytes, indicating that the cAMP response in these cells must be mediated by β1-ARs (Fig. 8A).

In mature wild-type brown adipocytes, the effects of β-AR agonists are mediated by the β1-AR (8, 10, 11). However, in mature brown adipocytes from β3-KO mice, isoprenaline-mediated increases in cAMP were attributed to the β1-AR (Fig. 8C). There is unlikely to be a β2-AR-mediated component to this response because we have shown previously that β2-ARs are not expressed in mature brown adipocytes (5, 8, 12, 23). Moreover, β1-ARs (along with α1-ARs) have been shown to rescue glucose uptake in cultured brown adipocytes from β3-KO mice (12), thus further implicating that when β3-ARs are not present, β1-ARs are able to rescue cAMP signaling.

There is an apparent difference in the desensitization between the β1- and β3-ARs in mature adipocytes (Fig. 8B). This difference was not due to differences in the overall integrity of the brown adipocytes, since the adenylate cyclase activator forskolin produced equal cAMP responses in wild-type FVB/N and β3-KO cultures (Fig. 8D). Compared with β3-ARs, the β1-ARs desensitize faster. Unlike β1- or β2-ARs, the distal cytoplasmic tail and third intracellular loop of the mouse and human β3-AR contains fewer Ser/Thr residues and no consensus sites for phosphorylation by PKA; phosphorylation of these residues in the cytoplasmic tail of the β2-AR by G protein–coupled receptor kinase-2 and PKA results in rapid agonist-promoted desensitization of the receptor (7, 18, 38). Thus it is thought that the β3-AR is less liable to agonist-promoted desensitization compared with the other receptors since it lacked the structural requirements involved in β1- or β2-AR desensitization (27, 45).

In mature wild-type brown adipocytes, cAMP levels rose quickly (within 5 min), and the maximum peak occurred following 15 min of stimulation (Fig. 8B). This corresponds well to the NE-induced oxygen consumption kinetics, with a peak 20–25 min after NE injection (Fig. 6A). In mature β3-KO brown adipocytes, cAMP levels rose quickly; however, they immediately started declining after 5 min (Fig. 8B). Thus, the high levels of cAMP were not present in the cells for as long as in the wild-type cultures. This could be an explanation to why NE does not increase oxygen consumption to the same levels in the β3-KO mice compared with the wild-type mice (Fig. 6A). Because β1-ARs are desensitized more rapidly than β3-ARs upon receptor stimulation, the rise in cAMP may not be enough to increase oxygen consumption in the β3-KO mice to the same extent as in the wild-type mice. However, compared with the experiment situation, where the mice get only one injection of NE, mice living in the cold have a continuous flow of NE stimulating BAT and are therefore capable of producing enough heat to survive the cold. One would speculate that a prolonged exposure to cold (which increases sympathetic outflow) would decrease β1-AR levels since continued stimulation of β1-ARs in the rat heart results in loss of β1-AR numbers and desensitization of the β1-AR and also adenylate cyclase pathway (29, 31). However, we show in BAT (Fig. 7, F and I) that this is not the case with an actual increase in β1-AR protein levels, consistent with our previous results showing increased β1-AR mRNA levels in mice exposed to the cold (5).

As stated above, the β3-AR is responsible mainly for nonshivering thermogenesis in mammals; however, a small portion is also dependent on α1-ARs (10). In this study, we have focused on the cAMP pathway, and it cannot be excluded that the increase in oxygen consumption to NE in the β3-KO mice may also act via α1-ARs, since glucose uptake in brown adipocyte cultures from β3-KO mice is rescued by signaling from both β1- and α1-ARs (12). However, in this study we have shown that the part of the nonshivering thermogenesis that is dependent on β2-AR/cAMP signaling is rescued by β1-AR/cAMP signaling.

Both β1- and β3-ARs can induce UCP1 expression in differentiated hMADS cells. Since β1-ARs are able to activate nonshivering thermogenesis in the β3-KO mice, we investigated whether stimulation of β1-ARs induce UCP1 expression in hMADS cells differentiated into brown adipocytes. In these cells, mRNA for all three β-AR subtypes was identified, which contrasts to that in mature mouse brown adipocytes where only β1- and β3-AR mRNA/protein is expressed (5, 12). Intriguingly, stimulation of either β1- or β3-ARs (with dobutamine and CL-316243, respectively), along with the nonselective β-AR agonist isoprenaline, induced UCP1 mRNA and protein levels in these cells, thus indicating that human BAT nonshivering thermogenesis could be dependent on both β1- and β3-AR signaling. The β2-AR agonist terbutaline was without effect, showing that although mRNA for the β2-AR was the most abundant β-AR mRNA species identified in these cells, stimulation of these receptors was not involved in β-AR- mediated UCP1 induction. Also surprising was that the β3-AR agonist CL-316243, which is generally considered to have limited actions at the human β3-AR, was still able to induce UCP1 expression. CL-316243 is a highly selective β3-AR agonist (6, 14), and responses to CL-316243 are abolished in β3-KO mice (12, 24). Hence, it is unlikely that the effect of CL-316243 is due to actions at β1- or β2-ARs, and further investigation using more human potent β3-AR agonists such as L755507 would be valuable. The main implication from the studies performed in human brown adipocytes is that stimulation of β1-ARs results in induction of UCP1. This is important, because in normal wild-type mice there is no evidence for any β1-AR function, which is “unmasked” only by the use of β3-KO mice. Hence, the use of β3-KO mice will allow for easier investigation on the role that human β1-ARs may have in nonshivering thermogenesis.

Relevance of β1-AR signaling. In the absence of β3-ARs, β1-ARs can mediate cAMP signaling and thereby rescue nonshivering thermogenesis and the survival of cold-acclimated β3-KO mice. Together with the fact that both β1- and β3-AR can induce UCP1 expression in differentiated hMADS cells, this implies that the β1-AR present in human BAT could play a more important role than previously thought. With the difficulties encountered with the design and efficacy of human β3-AR agonists, investigation of the role of β1-ARs in human
BAT may be of therapeutic benefit (although the use of existing β1-AR agonists is limited because of its side effects on other tissues). However, by finding ways to deliver specifically activate human BAT, one could potentially combat obesity, type 2 diabetes, and its complications. The model system of β3-KO mice and their primary brown adipocytes could (together with differentiated hMACS cells) be used as a more appropriate model system for β1-AR stimulation of human BAT function.

ACKNOWLEDGMENTS

We are grateful for the skilled technical help from Mansour Djejdane and the personnel in the animal facility for maintaining the breeding and offspring.

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GRANTS

These studies were supported by grants from the Swedish Research Council (VR-NV and VR-M), Novo Nordiskfonden, the Magnus Bergvall Foundation, GRANTS 21205.

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