Gene expression of peripheral blood mononuclear cells is affected by cold exposure

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Reynés B, García-Ruiz E, Oliver P, Palou A. Gene expression of peripheral blood mononuclear cells is affected by cold exposure. Am J Physiol Regul Integr Comp Physiol 309: R824–R834, 2015. First published August 5, 2015; doi:10.1152/ajpregu.00221.2015.—Because of the discovery of brown adipose tissue (BAT) in humans, there is increased interest in the study of induction of this thermogenic tissue as a basis to combat obesity and related complications. Cold exposure is one of the strongest stimuli able to activate BAT and to induce the appearance of brown-like (brite) adipocytes in white fat depots (browning process). We analyzed the potential of peripheral blood mononuclear cells (PBMCs) to reflect BAT and retroperitoneal white adipose tissue (rWAT) response to 1-wk cold acclimation (4°C) at different ages of rat development (1, 2, 4, and 6 mo). As expected, cold exposure increased fatty acid β-oxidation capacity in BAT and rWAT (increased Cpt1a expression), explaining increased circulating nonesterified free fatty acids and decreased adiposity. Cold exposure induced the expression of the key thermogenic gene, Ucp1, in BAT and rWAT, but only in 1-mo-old animals. Additionally, other brown/brite markers were affected by cold during the whole developmental period studied in BAT. However, in rWAT, cold exposure increased studied markers only at early age. PBMCs did not express Ucp1, but expressed other brown/brite markers, which were cold regulated. Of particular interest, PBMCs reflected adipose tissue-increased Cpt1a mRNA expression in response to cold (in older animals) and browning induction occurring in rWAT of young animals (1 mo) characterized by increased Cidea expression and by the appearance of a high number of multilocular CIDE-A positive adipocytes. These results provide evidence pointing to PBMCs as an easily obtainable biological material to be considered to perform browning studies with minimum invasiveness.

brown adipose tissue; peripheral blood mononuclear cells; browning; cold

Peripheral blood mononuclear cells (PBMCs) constitute an easily obtainable fraction of blood cells consisting basically in lymphocytes and monocytes. These cells travel around the body and respond to internal or external signals expressing a wide number of genes, including tissue-specific transcripts, reflecting metabolic adaptations (38). PBMCs are increasingly proposed for clinical diagnostic purposes as they can reflect gene expression patterns of different pathologies (1, 12, 56). Moreover, PBMCs have been shown to be useful for nutritional studies as they can reflect the effects of specific diets and feeding conditions (fasting/refeeding) on gene expression, which occurs in key energy homeostatic tissues as liver and adipose tissue (7, 9, 10, 16, 24, 41, 50, 55, 62). In addition, PBMCs have been proposed as a potential source of obesity biomarkers (31, 41) or acquired predisposition to develop obesity (30).

Mammals are provided with two different types of adipose tissue: white (WAT) and brown (BAT) (13). WAT is mainly composed by white adipocytes, which store as fat the excess of energy in a large lipid droplet (unilocular adipocytes). On the contrary, BAT is made up of multilocular adipocytes, which are specialized in burning fat to produce heat in a process known as facultative thermogenesis, in response to stimulus as cold exposure or the intake of hypercaloric diets in a process mediated by β-adrenergic receptors (13, 28, 42). BAT thermogenic activity is due to the presence of the mitochondrial uncoupling protein 1 (UCP1) and represents an important part of energy expenditure critical to overall energy balance (11, 18, 42). The main thermogenic stimulus is cold exposure: stimulation of the sympathetic nervous system by cold or administration of β-adrenergic agonists induce fatty acid oxidation in adipose tissue and thermogenesis in BAT by increasing BAT size, mitochondriogenesis, as well as UCP1 expression or protein activity to maintain body temperature (11, 14, 21, 42, 46, 48). Moreover, cold exposure and β-adrenergic stimulation induce a process known as browning, which consists in the appearance of brown-like adipocytes in typical WAT depots (3, 13, 15, 17, 20, 21, 37, 60). These adipocytes have been named “brite” (from brown-in-white) or “beige” adipocytes, display a peculiar gene expression pattern, and share some characteristics with classical brown adipocytes, as expression of UCP1 mRNA (3, 6, 34, 51, 52, 57–59). In rodents it is known that BAT/browning induction can increase energy expenditure and help to maintain body weight (6, 33, 60). Interest in BAT has reemerged since the discovery of important amounts of inducible tissue in adult humans (40). As occurs in rodents, cold exposure activates human BAT depots and this could be important in the control of body temperature and adiposity (49). Interestingly, based on gene expression signatures, it has been reported that BAT depots in humans are composed by brite cells (58) so that BAT activation could be in reality representing browning induction, which could be the basis of an interesting strategy for obesity treatment in humans.

BAT studies in humans require invasive techniques such as biopsies of adipose tissue or the use of techniques such as positron emission tomography, which implies the use of radioactive isotopes. Thus it would be of interest to dispose of a readily available source of biomarkers useful to perform BAT studies. We have previously reported in rodents that PBMCs are able to express different brown/brite adipocyte markers and can reflect the induction of some browning markers in adipose tissue in response to the intake of hyperlipidic diets (23). Here we aim to establish the usefulness of PBMCs as a tool to
 MATERIALS AND METHODS

Isolation of peripheral blood mononuclear cells. Trunk blood samples of control and cold-exposed rats (since 1 to 6 mo of age) were used to isolate PBMCs by using EDTA 0.5 M as anticoagulant. After blood collection, PBMCs were isolated by OptiPrep gradient separation, according to the instructions indicated by the manufacturer (Sigma-Aldrich Química, Madrid, Spain) with some modifications. Briefly, anticoagulant-treated blood was filled up to 6 ml with solution C (146 mM NaCl and 1 mM HEPES). Afterward, blood was layered carefully to form a density barrier by mixing 2.7 ml of OptiPrep with 9.3 ml of OptiPrep diluent (solution C diluted 1:1.2 in water), without intermixing (3 ml of density barrier for 2 ml of blood mixed with solution C) in a centrifuge tube. Then the tube was centrifuged at 1000 g for 20 min at 20°C in a swinging bucket rotor with acceleration and deceleration adjusted at zero. PBMCs, together with platelets, were harvested from the interface between plasma layer and OptiPrep. This material was then centrifuged in

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adb3</td>
<td>CTTTCAACCGCGCCTCATCTAC</td>
<td>TGGAATGGAAGCCTCAC</td>
<td>189</td>
</tr>
<tr>
<td>Cidea</td>
<td>TTACCGCTAGAGGACACAC</td>
<td>AACATGAAAGCCTCAC</td>
<td>164</td>
</tr>
<tr>
<td>Cpt1a</td>
<td>TGCTGCGATTAGAACAGTCAG</td>
<td>TGAGAAGCACCAGGACATG</td>
<td>250</td>
</tr>
<tr>
<td>Fasn</td>
<td>CGGGGATGCTTATGCGACAT</td>
<td>AAGAGGAGCAGGACAT</td>
<td>222</td>
</tr>
<tr>
<td>Fgf21</td>
<td>ACAGATGAGGACACCAGAC</td>
<td>AGCGTCTTATGACAGAAT</td>
<td>145</td>
</tr>
<tr>
<td>Hox9</td>
<td>CGGGCACGAGCAAAAGA</td>
<td>AGAAATCTCTTCCTCCAAGTCT</td>
<td>138</td>
</tr>
<tr>
<td>Pgc1a</td>
<td>CTATTAGCTAGACCTGACATG</td>
<td>CCGTCAGGTCAGGACAT</td>
<td>131</td>
</tr>
<tr>
<td>Prdm16</td>
<td>ACGAGCCTTCCTCATCTCCTC</td>
<td>TTACCGTCTGTTCCTTGCT</td>
<td>129</td>
</tr>
<tr>
<td>Slc27a1</td>
<td>AGCGGCTGCTTCTCTCTTC</td>
<td>TTACCGTCTGTTCCTTGCT</td>
<td>198</td>
</tr>
</tbody>
</table>

*Effect of cold exposure (cold-exposed rats vs. their respective controls; Student’s t-test, P < 0.05, or indicated when different).

After the experimental period, the animals were killed by decapitation, and different WAT depots (inguinal, retroperitoneal, epididymal, and mesenteric) were removed and weighed to determine the adiposity index, frozen in liquid nitrogen, and stored at −80°C until RNA analysis (analysis was performed in BAT and retroperitoneal WAT). Blood samples from neck (−500 μl) were stored at room temperature for 1 h and were centrifuged at 1,000 g for 10 min at 4°C to collect serum.

**Isolation of peripheral blood mononuclear cells.** Trunk blood samples of control and cold-exposed rats (since 1 to 6 mo of age) were used to isolate PBMCs by using EDTA 0.5 M as anticoagulant. After blood collection, PBMCs were isolated by OptiPrep gradient separation, according to the instructions indicated by the manufacturer (Sigma-Aldrich Química, Madrid, Spain) with some modifications. Briefly, anticoagulant-treated blood was filled up to 6 ml with solution C (146 mM NaCl and 1 mM HEPES). Afterward, blood was layered carefully to form a density barrier by mixing 2.7 ml of OptiPrep with 9.3 ml of OptiPrep diluent (solution C diluted 1:1.2 in water), without intermixing (3 ml of density barrier for 2 ml of blood mixed with solution C) in a centrifuge tube. Then the tube was centrifuged at 1000 g for 20 min at 20°C in a swinging bucket rotor with acceleration and deceleration adjusted at zero. PBMCs, together with platelets, were harvested from the interface between plasma layer and OptiPrep. This material was then centrifuged in

Table 2. Body weight, adiposity, and NEFA levels

<table>
<thead>
<tr>
<th>Body Weight, g</th>
<th>Adiposity Index, %</th>
<th>BAT, g</th>
<th>tWAT, g</th>
<th>rWAT, g</th>
<th>eWAT, g</th>
<th>mWAT, g</th>
<th>NEFA, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>Final</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mo Control</td>
<td>45.1 ± 1.6</td>
<td>86.2 ± 2.2</td>
<td>1.87 ± 0.06</td>
<td>0.22 ± 0.02</td>
<td>0.94 ± 0.07</td>
<td>0.15 ± 0.01</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>Cold</td>
<td>44.2 ± 1.6</td>
<td>72.8 ± 2.3*</td>
<td>1.14 ± 0.08*</td>
<td>0.35 ± 0.01*</td>
<td>0.47 ± 0.06*</td>
<td>0.09 ± 0.00*</td>
<td>0.10 ± 0.02*</td>
</tr>
<tr>
<td>2 mo Control</td>
<td>180 ± 4</td>
<td>199 ± 5</td>
<td>4.42 ± 0.32</td>
<td>0.32 ± 0.04</td>
<td>2.40 ± 0.15</td>
<td>1.85 ± 0.21</td>
<td>3.54 ± 0.46</td>
</tr>
<tr>
<td>Cold</td>
<td>183 ± 4</td>
<td>196 ± 5</td>
<td>2.91 ± 0.30*</td>
<td>0.58 ± 0.03*</td>
<td>2.06 ± 0.16</td>
<td>1.20 ± 0.23*</td>
<td>1.87 ± 0.29*</td>
</tr>
<tr>
<td>4 mo Control</td>
<td>267 ± 8</td>
<td>273 ± 6</td>
<td>5.89 ± 0.18</td>
<td>0.39 ± 0.02</td>
<td>3.24 ± 0.25</td>
<td>3.36 ± 0.11</td>
<td>7.48 ± 0.35</td>
</tr>
<tr>
<td>Cold</td>
<td>254 ± 7</td>
<td>244 ± 7*</td>
<td>4.68 ± 0.46*</td>
<td>0.72 ± 0.10*</td>
<td>2.50 ± 0.34</td>
<td>2.52 ± 0.24*</td>
<td>5.30 ± 0.62*</td>
</tr>
<tr>
<td>6 mo Control</td>
<td>282 ± 8</td>
<td>281 ± 6</td>
<td>7.44 ± 0.24</td>
<td>0.57 ± 0.05</td>
<td>4.76 ± 0.34</td>
<td>4.55 ± 0.50</td>
<td>7.39 ± 0.34</td>
</tr>
<tr>
<td>Cold</td>
<td>281 ± 6</td>
<td>267 ± 6</td>
<td>5.42 ± 0.43*</td>
<td>0.57 ± 0.05</td>
<td>3.44 ± 0.26*</td>
<td>3.07 ± 0.30*</td>
<td>5.89 ± 0.50*</td>
</tr>
</tbody>
</table>

Body weight before and after cold exposure (g), adiposity index (%), size of the adipose tissue depots and circulating nonesterified free fatty acid (NEFA) levels (mM) in control and cold-exposed rats. The adiposity index was computed as the sum of inguinal, retroperitoneal, epididymal, and mesenteric white adipose tissue depots weights and expressed as a percentage of total body weight. Tissue weights of brown adipose tissue (BAT) and different white adipose tissue (WAT) depots, inguinal (iWAT), retroperitoneal (rWAT), mesenteric (mWAT), and epididymal (eWAT) are presented. Results represent mean ± SD (n = 5 to 6). *Effect of cold exposure (cold-exposed rats vs. their respective controls; Student’s t-test, P < 0.05, or indicated when different).
solution C at 400 g for 10 min at 20°C to wash PBMCs and to remove the
platelets.

Adiposity index. Adiposity was determined by an adiposity index
computed for each rat as the sum of the mass of all the white adipose
tissue depots (inguinal, retroperitoneal, epididymal, and mesenteric)
expressed as a percentage of total body weight.

Measurement of circulating glucose and nonesterified free fatty
acid. Blood glucose concentration was measured by using an Accu-
Chek Glucometer (Roche Diagnostics, Barcelona, Spain) in blood
obtained from the neck at the moment of death. Nonesterified free
fatty acid (NEFA) levels were measured in serum by using an
enzymatic colorimetric NEFA-HR2 kit (from WAKO, Neuss, Ger-
many).

Total RNA isolation. Total RNA from PBMC samples was ex-
ttracted by using TriPure reagent (Roche Diagnostics) and then were
purified with E.Z.N.A. MicroElute RNA Clean Up (Omega Bio-Tek,
Winooski, VT). Total RNA from retroperitoneal WAT and from BAT
was extracted by using TriPure reagent (Roche Diagnostics) and was
then purified by using E.Z.N.A. Total RNA Kit I (Omega Bio-Tek);
isolated RNA from adipose tissues was purified by precipitation with
3 M of sodium acetate and absolute ethanol. RNA yield was quantified
on a NanoDrop ND 1000 spectrophotometer (NanoDrop Technolo-
gies, Wilmington, DE) and its integrity was confirmed by agarose gel
electrophoresis.

**RT-PCR analysis.** Expression of genes of interest (browning mark-
ers and lipid metabolism genes) was determined by real-time RT-PCR
in PBMCs, in the retroperitoneal WAT (rWAT) selected as represent-
itive white adipose depot and in interscapular BAT. Fifty nanograms
of total RNA from PBMCs was reverse transcribed to cDNA by using
iScript cDNA synthesis kit (Bio-Rad, Madrid, Spain) at 25°C for 5
min, 42°C for 30 min, and 85°C for 5 min in an Applied Biosystems
2720 Thermal Cycler (Applied Biosystems, Madrid, Spain). Other-
wise, 250 ng of total RNA from adipose tissues was denatured at 60°C
for 10 min and then reverse transcribed to cDNA by using MuLV
reverse transcriptase (Applied Biosystems) at 20°C for 15 min, 42°C
for 30 min, and a final step of 5 min at 95°C (according to Applied
Biosystem’s procedure) in an Applied Biosystems 2720 Thermal
Cycler. Each PCR was performed from diluted (1/5 for PBMCs and
1/10 for adipose tissues) cDNA template, forward and reverse primers
(5 μM), and Power SYBER Green PCR Master Mix (Applied Bio-
systems) in a total volume of 11 μl, with the following profile: 10 min
at 95°C, followed by a total of 40 temperature cycles (15 s at 95°C and
1 min at 60°C), with a final cycle of 15 s at 95°C, 1 min at 60°C, and
15 s at 95°C. To verify the purity of the products, a melting curve was
produced after each run according to the manufacturer’s instructions.
The threshold cycle (Ct) was calculated with the instrument’s soft-
ware (StepOne Software v2.0, from Applied Biosystems), and the
relative expression of each mRNA was calculated as a percentage of

![Graphs showing gene expression](http://ajpregu.physiology.org/)

Fig. 1. Gene expression of genes involved in lipid metabolism and of the β-3 adrenergic receptor gene measured by real-time RT-PCR, in brown adipose tissue
(BAT), retroperitoneal white adipose tissue (rWAT) and peripheral blood mononuclear cells (PBMCs) of rats at different ages (from 1 to 6 mo) acclimatized
to different room temperatures: 22°C (control) or 4°C for 1 wk (cold). Results represent means ± SE (n = 5 to 6) of ratios of specific mRNA levels relative
to Lrp10, expressed as a percentage of the value of 1-mo-old control animals that was set to 100%. Bars not sharing common letter (a, b, c) are significantly
different (one-way ANOVA, P < 0.05). Least significant difference post hoc was used after the ANOVA analysis. *Effect of cold exposure (cold-exposed rats
vs. their respective controls; Student’s t-test, P < 0.05, or indicated when different).
control rats by the Pffafl’s method (44). Lrp10 was chosen as the housekeeping gene because it has been demonstrated to be a suitable reference gene for studies using adipose tissue and cold exposure (8, 22). Primers for the different genes are described in Table 1 and were obtained from Sigma Genosys (Sigma-Aldrich Química).

Histological analysis. Retroperitoneal WAT samples were fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, overnight at 4°C, washed in phosphate buffer, dehydrated in a graded series of ethanol, cleared in xylene, and embedded in paraffin blocks for light microscopy. Five-micrometer-thick sections of tissue were cut with a microtome and mounted in slides. The area of white adipocytes was measured in hematoxylin/eosin stained section. Images from light microscopy were digitalized, and the area of at least 200 cells of each section was determined by using Axion Vision Software (Carl Zeiss, Barcelona, Spain).

Immunohistochemistry analysis of UCP1 and CIDE-A in retroperitoneal WAT. Five-micrometer serial sections of adipose tissue of the different experimental groups were immunostained by means of the avidin-biotin technique (26). Briefly, serial sections were incubated with normal goat serum 2% in PBS pH 7.3 to block unspecific sites and then overnight at 4°C with primary rabbit polyclonal UCP1 antibody (GeneTex International, Irvine, CA) diluted 1:200 in PBS and with CIDE-A primary rabbit polyclonal antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:150 in PBS, overnight at 4°C. Sections were then incubated with the corresponding biotinylated antirabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA), diluted 1:200, and finally with ABC complex (Vectastain ABC kit, Vector Laboratories). Peroxidase activity was revealed with Sigma Fast 3,3’-diaminobenzidine (Sigma-Aldrich) as substrate. Finally, sections were counterstained with hematoxilin and mounted in Eukitt (Kindler, Germany). Images were acquired with a Zeiss Axioskop 2 microscope equipped with an AxioCam Icc3 digital camera and AxioVision 40V 4.6.3.0 software (Carl Zeiss).

Statistical analysis. All data are expressed as means ± SE. Differences between ages were analyzed by using ANOVA and least significant difference post hoc comparisons. The effect of cold exposure was analyzed by Student’s t-test. The analyses were performed with SPSS for windows (SPSS, Chicago, IL). Threshold of significance was defined at $P < 0.05$ and is indicated when different.

RESULTS

Body weight, adiposity and circulating glucose, and NEFA. As seen in Table 2, exposure to 4°C during 1 wk produced decreased adiposity in animals of the different ages studied (1, 2, 4, and 6 mo) compared with controls; the lower adiposity index was due to a generalized decrease in the size of the different WAT depots (inguinal, retroperitoneal, epididymal, and mesenteric). Decreased adiposity was translated into a 10.220.33.3 on August 12, 2017 http://ajpregu.physiology.org/ Downloaded from
significant lower body weight in 1- and 4-mo-old animals. On the other hand, cold exposure produced an important increase in the size of BAT (around 77% increase), which was evident in animals until 4 mo of age. NEFA circulating levels were increased in cold-exposed animals from the age of 2 mo. However, circulating levels of glucose were not affected with cold exposure in any age (data not shown).

**Effect of cold exposure on expression of genes involved in fatty acid mobilization in BAT, retroperitoneal WAT, and PBMCs.** As a general trend, both in BAT and in rWAT, cold exposure induced an increase in the expression of Cpt1a, a gene involved in fatty acid oxidation, and a decrease in the expression of the key fatty acid synthesis gene Fasn. Moreover, mRNA levels of the Adrb3 gene, coding for the β-3 adrenergic receptor, were decreased in adipose tissue of cold-exposed animals, an effect which was evident mainly in BAT (Fig. 1) and suggests a retroregulatory mechanism due to chronic cold stimulation, as previously suggested based on in vitro studies with brown adipocytes under noradrenaline treatment (45). An influence of age on gene expression was observed for Cpt1a: in rWAT, increased Cpt1a expression was only observed in older rats of 4 and 6 mo, whereas in BAT, Cpt1a expression increased at the different ages except in the oldest rats of 6 mo. In PBMCs, we also observed an increase on Cpt1a mRNA levels, precisely at the age of 4 and 6 mo, as in rWAT, while no effect was observed for Fasn expression in PBMCs of cold-exposed animals (Fig. 1); Adrb3 expression was not detectable in PBMCs (not amplified in any of the samples).

Fig. 3. Gene expression of brown/brite markers measured in the rWAT in the same animals described in Fig. 1. Values not sharing a common letter (a, b, c) are significantly different (one-way ANOVA, P < 0.05). *Versus control group (Student’s t-test, P < 0.05).
Effect of cold exposure on the expression of browning markers in BAT and retroperitoneal WAT: Cold exposure affected gene expression of selected brown/brite markers in a depot-specific manner in BAT and rWAT at different ages of rat development (Figs. 2 and 3). In BAT, cold exposure increased the expression of the key thermogenic gene *Ucp1* at the age of 1 mo, but no change was observed thereafter in older rats. The expression of other genes highly expressed in BAT, *Fgf21* and *Pgc1α*, also increased in BAT of cold-exposed animals at different ages. In addition, cold exposure increased the expression of the brite marker *Slc27a1* in all the ages studied. On the contrary, expression of *Cidea*, a well-known brown adipocyte marker, decreased its expression in response to cold exposure in animals of all the ages studied. The same happened for *Prdm16*, related to brown adipocyte differentiation, whose expression decreased in BAT of 4- and 6-mo-old animals. Our results also showed an increase in basal expression of *Cidea*, *Prdm16*, and *Slc27a1* during rat growth.

As happened in BAT, cold exposure produced increased *Ucp1* expression in rWAT in 1-mo-old animals (Fig. 3). This increased expression was of a higher magnitude than that observed in BAT, although we have to take into account that *Ucp1* mRNA basal expression levels were lower in rWAT than in BAT (see below for comparison). The same expression pattern was observed for other brown adipocyte markers, *Cidea*, *Pgc1α*, and *Prdm16*, whose expression increased in cold-exposed animals the first month of life while, surprisingly, expression of *Fgf21* decreased in these animals. We also observed increased expression of the brite marker *Slc27a1* in response to cold, but in this case the increase was observed in adult animals of 4 and 6 mo of age. An effect of age was observed in basal expression of *Slc27a1*, which decreased during rat development. The expression of the other brite marker, *Hoxc9* (which was nondetectable in BAT), was not affected by cold exposure at any of the ages.

Effect of cold exposure on the expression of browning markers in PBMCs. We did not detect *Ucp1* or *Pgc1α* gene expression in PBMCs: *Ucp1* was not amplified in any of the samples, whereas *Pgc1α* was amplified only in a few samples and out of the limit of detection (threshold cycle >35). However, interestingly, these cells expressed other brown adipocyte markers: *Cidea, Fgf21,* and *Prdm16*, as well as the brite markers *Hoxc9* and *Slc27a1* (Fig. 4). Expression of these genes was increased by cold exposure in an age-dependent manner. *Cidea* (*P* = 0.08, due to interindividual variation), *Fgf21,* and *Cidea* (*P* < 0.05, or indicated when different).

**Fig. 4.** Gene expression of brown/brite markers measured in PBMCs in the same animals described in Fig. 1. *Versus control group (Student’s t-test, *P* < 0.05, or indicated when different).
Prdm16 ($P = 0.12$), and Slc27a1 mRNA levels increased in 1-mo-old animals, whereas Hoxc9 expression was increased in older animals from the age of 4 mo. Sc127a1 expression was also increased in 6-mo-old animals.

Comparison of brown/brite marker expression levels between BAT, rWAT, and PBMCs. Basal levels of the different brown/brite markers studied in BAT, rWAT, and PBMCs of control animals of 1 mo of age (those more prone to BAT activation/browning) are represented in Fig. 5. In BAT, as expected (23), highest expression levels were observed for the brown markers Ucp1 and Cidea, followed by Slc27a1, Pgc1a, Prdm16, and, in much lower levels, by Fgf21; whereas Hoxc9, considered a white/brite marker (43) was not detected. Conversely, Hoxc9 was expressed in rWAT, as well as Slc27a1 (at similar levels as in BAT) and Fgf21 (at higher levels than in BAT). The rWAT also expressed other genes considered as brown markers: Ucp1, Cidea, Pgc1a, and Prdm16, although at lower levels than BAT. The brown/brite adipocyte markers studied, with the exception of Ucp1 and Pgc1a, were expressed in PBMCs. The gene with the highest expression level in PBMCs was Slc27a1, followed by Fgf21, Hoxc9, and Cidea; although the expression of all of them was considerably lower than in adipose tissue. Prdm16 was also expressed in blood cells, but at very low levels.

Effect of cold exposure on immunohistochemistry analysis in retroperitoneal WAT. Gene expression changes observed at the molecular level in rWAT suggested that brown adipocytes could have emerged in response to cold, particularly in the youngest animals (1 mo of age). To confirm browning induction we performed a morphological and immunohistochemical analysis in this adipose depot. At the age of 1 mo, we could observe a strong transformation of the rWAT with the appearance of a high number of UCP1 and CIDE-A positive multilocular adipocytes in cold-exposed animals (Fig. 6). This remodeling from white to brown adipose tissue was also observed, although to a lesser extent, in some of the 2-mo-old rats. In response to cold exposure we could also observe paucilocular adipocytes, which have been described as cells with an intermediate morphology between white and brown adipocytes (2). At the age of 4 mo only a small browning effect was observed, while no multilocularity was evident in the oldest animals studied (6 mo). Of note, in young animals (up to the age of 2 mo) we could observe positive CIDE-A staining also in unilocular white adipocytes, which were negative for UCPI. According to the higher browning effect in young animals, morphometrical analysis performed in rWAT revealed that the size of the unilocular adipocytes decreased with cold exposure only in 1-mo-old rats ($733 \pm 311$ vs. $1,840 \pm 147 \mu m^2$ in cold-exposed and control rats, respectively), but not in the other studied ages (data not shown).

**DISCUSSION**

The interest in BAT-focused therapies to combat obesity and related complications has reemerged since the discovery of inducible brown adipose tissue in humans (40, 47). However, BAT studies require invasive techniques, thus it would be convenient to dispose of easily obtainable biological material suitable to contribute to this research. Here, we have analyzed the capacity to activate BAT and to induce remodeling of white into brown adipose tissue after cold exposure at different ages of rat development by analyzing classical as well as newly described brown/brite markers. Moreover, we have shown that gene expression of a subset of blood cells, PBMCs, reflect certain features of adipose tissue cold response.

It is well known that β-adrenergic stimulation, such as cold exposure, induces an increase of fatty acid catabolism, especially in WAT, while it decreases fatty acid synthesis (8, 29). According to this, we observed increased expression of a key gene involved in fatty acid β-oxidation, Cpt1a, and decreased
expression of the fatty acid synthesis gene *Fasn* observed both in BAT and rWAT. As a likely result of these adaptations, we can observe increased circulating NEFA levels and decreased adiposity in our cold-exposed animals. We have previously described that PBMCs can reflect lipid metabolism gene expression which occur in adipose tissue in response to different stimulus, such as feeding/fasting conditions (7). Here we show how PBMCs also reflected increased *Cpt1a* expression in response to cold exposure, which occurs in adipose tissue.

In parallel with fatty acid mobilization, cold exposure activates BAT thermogenesis (27). Circulating NEFA are used as fuel to support thermogenesis mediated by UCP1 and, moreover, β-adrenergic stimulation results in increased UCP1 levels and BAT recruitment (42). It is known that BAT thermogenic capacity decreases with age (39). Our results show increased BAT size in cold-exposed animals at different ages; however, increased *Ucp1* expression was only observed at the age of 1 mo, when BAT tissue is more active, coincident with increased expression of *Pgclα* coding for a transcriptional coactivator necessary for *Ucp1* expression (4). *Fgf21* codes for a hormone involved in *Pgclα* and *Ucp1* expression, and thus it has an important role in adaptive thermogenesis (19, 34, 36). Accordingly, and as expected (25), BAT *Fgf21* mRNA levels were increased as a response to cold acclimation, except in the oldest animals. Another key regulator is *Prdm16*, which codes for a key transcription factor involved in *Ucp1* expression (28, 34). However, although it is involved in BAT development, *Prdm16* mRNA regulation is not always linked to that of *Ucp1* in this tissue. In fact, we observed decreased *Prdm16* expression in BAT of 4- and 6-mo-old animals. Similar results were obtained by Waldén et al. (57), who reported decreased expression of this gene in BAT of cold-exposed mice; they concluded that *Prdm16* expression levels are not directly correlated to the recruitment status of BAT, although our results point to an importance of *Prdm16* in WAT browning (see below). We also analyzed mRNA expression of other brown adipocyte markers, such as *Cidea*, which code for a multifunctional protein that is highly expressed in BAT mitochondria (5, 58). As described by Shimizu et al. (53), large cold exposure induced a downregulation of *Cidea* gene expression. In addition, our data show that *Cidea*-decreased expression can be observed in response to cold at different ages during rat development, and thus it

![UCP1 and CIDE-A immunostaining in rWAT of the same animals described in Fig. 1.](http://ajpregu.physiology.org/)

We can observe a strong browning induction especially in 1-mo-old cold-exposed animals characterized by the appearance of multilocular brite adipocytes positive for UCP1 and CIDE-A. The asterisks in 1- and 2-mo-old animals indicate unilocular white adipocytes which resulted positive for CIDE-A but negative for UCP1 staining. Paucilocular adipocytes, with intermediate morphology between that of white and brown adipocytes are indicated as PA.
appears as a good marker of cold-activated BAT, with independence of age. Also, the BAT expression of Slc27a1, which codes for fatty acid transport protein 1, and is considered a brite marker (58) and, as expected, its expression increased in response to cold exposure (25) and was maintained at the different ages studied.

In addition to BAT activation, cold exposure induces the appearance of brown-like adipocytes (brite cells), with thermogenic activity in WAT depots (3, 6, 52, 58). This process, known as browning, has been suggested to contribute to energy dissipation in WAT (18, 32, 33). Little is known about the effect of age on WAT browning capacity. Our results show that browning induction in response to cold, analyzed in rWAT, was evident during the first month of life but the response was lower or inexistent later on. In fact, cold exposure induced an important statistically significant upregulation of Ucp1 gene expression only in 1-mo-old animals, which was related to increased expression levels of other brown markers: Cidea, Pgc1a, and Prdm16; gene expression of these markers was not affected in older animals. Accordingly, morphological and immunohistochemical analysis evidenced cold-induced browning and reduction in adipocyte size in rWAT at the age of 1 mo. Interestingly, CIDE-A, which has been described to be expressed exclusively by multilocular adipocytes, was evidenced by immunohistochemistry in unilocular white adipocytes of young animals up to the age of 1 mo, those with a higher browning capacity. Thus the presence of this protein in white adipocytes at early ages could be related to an increased propensity for browning. mRNA expression of the brite marker Slc27a1 also increased in cold-exposed animals, but only in 4- and 6-mo-old animals, while the expression of another brite marker, Hoxc9 was not affected at any age, coincident with what has been previously described in adult mice after chronic cold exposure (57).

PBMCs are able to express ~80% of the genome, including tissue-specific transcripts (38). Although, these cells did not express the key BAT thermogenic gene Ucp1 either in control or cold-exposed animals, they were able to express other brown/brite markers such as Cidea, Fgf21, Hoxc9, Prdm16, and Slc27a1. To our knowledge there are no previous reports on the study of the effects of cold exposure on PBMC gene expression. Slc27a1 was the gene with the highest expression in PBMCs but, with independence of the basal expression levels of the analyzed markers, data presented here demonstrate that these cells can reflect some of the cold response changes in gene expression, which occur in adipose tissue, especially at early stages of life. The most relevant data was the important increase in Cidea mRNA expression observed in cold-exposed rats at the age of 1 mo, coincident to what happened in rWAT. At that age we also observed increased Fgf21 and Slc27a1 mRNA expression, coincident with BAT cold response, and increased Prdm16 expression as observed in rWAT. Thus these genes could be considered as suitable brown/brite markers to be analyzed in PBMCs. Apart from gene expression changes present in the youngest animals studied, cold exposure induced a marked increase in mRNA expression of the brite marker Hoxc9 expression in PBMCs of 4- and 6-mo-old animals, which was not observed in rWAT and seems not related to increased browning capacity.

**Perspectives and Significance**

This work, which includes the analysis of recently described BAT/brite markers, demonstrates that cold exposure induced gene expression changes related to BAT activation and WAT browning mainly at an early age (1 mo). Additionally, in BAT, some of the analyzed markers were affected by cold exposure during the whole developmental period studied. Besides, our results manifested that PBMCs not only expressed brown/brite markers, but also were able to respond to cold exposure, reflecting some of the features observed in adipose tissue, particularly rWAT browning. The possibility of using an easily obtainable biological material, such as PBMCs, to perform BAT studies opens new and interesting possibilities for analyzing the relevance of this tissue and of WAT browning on energy homeostasis and body weight control in humans by using noninvasive approaches.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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