Type 2 iodothyronine deiodinase is upregulated in rat slow- and fast-twitch skeletal muscle during cold exposure

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Cold exposure is accompanied by increased oxygen consumption, impaired the decrease of D2 in BAT and soleus after 10 days at 4°C. D2 activities returned to the control levels after 10 days of cold exposure, when an increase of 2.8-fold in D2 activity was detected in white glycolytic gastrocnemius but not in red oxidative gastrocnemius fibers. Propranolol did not prevent muscle D2 induction, but it impaired the decrease of D2 in BAT and soleus after 10 days at 4°C. Cold exposure is accompanied by increased oxygen consumption, UCP3, and PGC-1α genes expression in skeletal muscles, which were partially prevented by propranolol in soleus and gastrocnemius. Serum total and free T3 is increased during cold exposure in rats, even after 10 days, when BAT D2 is already normalized, suggesting that skeletal muscle D2 activity contributes significantly to circulating T3 under this adaptive condition. In conclusion, cold exposure is accompanied by concerted changes in the metabolism of BAT and oxidative and glycolytic skeletal muscles that are paralleled by type 2 deiodinase activation.

BAT function is under direct control of sympathetic nervous system, which is rapidly activated following a decrease in environment temperature. In this circumstance, heat production is accompanied by higher oxygen consumption secondary to energy substrate oxidation and uncoupling protein 1 (UCP1) activation (30). In mammals, thyroid hormones play a major role in the modulation of oxygen consumption and thermogenesis under basal conditions and during cold exposure (30). The expression of UCP1 protein in BAT is dependent on triiodothyronine (T3) action, and its activation is secondary to adrenergic stimulation (14), which also increases the local conversion of thyroxine (T4) into T3 through the acute type 2 iodothyronine deiodinase (D2) stimulation (6, 7). A significant increase in BAT D2 activity is already detected within 30 min after exposure to 4°C (11), highlighting the role of D2 in local T3 production and BAT thermogenic response. The action of T3 in the overall process of heat production was established by the seminal finding that hypothyroid rats do not survive to cold exposure, which parallels impaired BAT recruitment and UCP1 activation (6, 7, 37).

In addition to BAT, skeletal muscle is also relevant for nonshivering thermogenesis, which is related mainly to the uncoupled sarcoenodoplastic reticulum calcium ATPase 1 (SERCA1) activity that is in fact also modulated by thyroid hormones mainly in slow-twitch muscle fibers (1, 9). UCP1-knockout mice survive to cold exposure probably because of the ability of skeletal muscle to overcome the lack of BAT thermogenesis (17). Also, it is well documented that muscle fibers respond to cold exposure through mechanisms such as increased glucose uptake in rats (25). Transition from glycolytic to oxidative muscle fibers in chicken (20), and higher expression of the thermogenic SERCA1 and increased mitochondrial biogenesis in rabbits (2). These skeletal muscle adaptations seem to be peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) dependent (25, 34), which is a transcriptional coactivator that participates in the control of genes involved in energy metabolism and mitochondrial biogenesis (27).

Marsili et al. (24) have demonstrated recently that not only D2 activity constitutively present in skeletal muscle, but its expression is higher in slow-twitch fibers than in fast-twitch ones. Also, D2 activity is upregulated in skeletal muscle of hypothyroid animals (24) and after 4 h of cold exposure in the soleus muscle (28), indicating that local T3 production in skeletal muscle might also play a role during cold exposure.
Since the organism progressively adapts to cold exposure (12), and T₃ plays an essential role during acute BAT recruitment and muscle thermogenesis, our intent was to determine whether skeletal muscle D₂ induction could be implicated in the long-term cold acclimation. We demonstrate that D₂ induction occurs in slow- and fast-twitch muscles at 3 and 10 days after cold exposure, respectively, which is accompanied by increased oxygen consumption as well as T₃ target gene expression in both tissues. Serum T₃ increase is maintained even after 10 days at 4°C, when BAT and slow-twitch muscle D₂ are already normalized, suggesting that fast-twitch skeletal muscle D₂ activity contributes significantly to circulating T₃ under this adaptive condition.

METHODS

Adult male Wistar rats weighing 200–250 g were donated by Instituto Vital Brazil and maintained in an animal facility with controlled light (12:12-h light-dark cycle, lights on at 6 PM) and temperature (23–24°C). The Rio de Janeiro Federal University Institutional Committee for Evaluation of Animal Use in Research approved the study, which was in accordance with the International Guiding Principles for Biomedical Research Involving Animals (Geneva, Switzerland). The rats were divided into three groups: kept at room temperature (23–24°C; control) or exposed to cold (4°C) for 3 or 10 days in individual cages. Food intake and rectal body temperature were determined every day during cold exposure at the same time of the day. To investigate the role of β-adrenergic receptor, DL-propranolol (Sigma-Aldrich) was dissolved in the drinking water at a concentration of 600 mg/l, as described previously (26). After 7–10 days of pretreatment, the rats were exposed to cold (4°C) for 3 or 10 days while receiving propranolol.

After the experimental period, the animals were euthanized by decapitation, and blood was collected from the trunk for serum hormone measurements. Serum was obtained after blood centrifugation at 1,500 g for 20 min and stored at −20°C. Rat tissues were dissected out, snap-frozen in liquid nitrogen, and then kept at −80°C until processing.

Serum hormone measurements. Serum total T₃ and T₄ were determined by specific coated tube radioimmunoassay kits (Diagnostic Systems Laboratories). Intra- and interassay coefficients of variation for T₃ were 5.0–6.5 and 4.2–6.0%, respectively, and the assay sensitivity was 4.3 ng/dl. T₄ intra- and interassay coefficients of variation were 2.9–5.1 and 7.1–7.4%, respectively, and the assay sensitivity was 0.4 µg/dl.

Serum free T₃ (FT₃) and T₄ (FT₄) were measured by an electrochemical luminescence commercial kit (Roche Diagnostics, Mannheim, Germany). Sensitivity varied from 0.023 to 7.77 ng/dl and 26 to 3,255 pg/dl for FT₄ and FT₃, respectively. The inter- and intra-assay coefficients of variation varied from 12.2 to 7.6 and 10.9 to 4.9%, respectively (according the biological variation table; Westgard). All of the procedures were performed, following the manufacturer’s recommendations.

D₂ activity assay. Samples of soleus, red, and white gastrocnemius skeletal muscles were processed as described by Marsili et al. (24). One-hundred milligrams of muscle sample was homogenized in 150 mM sodium phosphate buffer containing 1 mM EDTA, 250 mM sucrose, and 10 mM dithiothreitol (pH 6.9).

BAT (25 mg) was homogenized in 150 mM sodium phosphate buffer containing 1 mM EDTA, 250 mM sucrose, and 10 mM dithiothreitol (pH 6.9).

Twenty micrograms of protein of skeletal muscle microsomes and 20 µg of protein of BAT homogenates were incubated in duplicate for 3 h at 37°C with 125I-labeled T₄ (Perkin-Elmer Life and Analytical Sciences) that was purified previously with Sephadex LH-20, 1 nM T₄, 1 mM propylthiouracil, and 20 mM dithiothreitol (USB) in 100 mM potassium phosphate buffer (pH 6.9) containing 1 mM EDTA and 1 mM T₄ in a final reaction volume of 300 µl, as described previously (13, 19). Specific D₂ activity measurements were confirmed by the inhibition of deiodination reaction in the presence of T₄ in excess (100 nM). Blank incubations were carried out in the absence of protein. The reaction was stopped at 4°C in an ice bath, with the addition of 200 µl of fetal bovine serum (Cultilab) and 100 µl of trichloroacetic acid (50%, vol/vol), followed by vigorous agitation. The samples were centrifuged at 10,000 g for 3 min, and 360 µl of supernatant was collected for the measurement of 125I liberated during the deiodination reaction (γ-Counter Wizard; Perkin-Elmer). Protein concentration was measured by the method of Bradford (10).

D₁ activity assay. One thyroid gland or 15 mg of liver was homogenized in 150 mM sodium phosphate buffer containing 1 mM EDTA, 250 mM sucrose, and 10 mM dithiothreitol (pH 6.9). Homogenates (8 µg of protein for liver and thyroid) were incubated in duplicate for 1 h at 37°C with 125I-labeled rT₃ (Perkin-Elmer Life and Analytical Sciences, Boston, MA) that was previously purified with Sephadex LH-20, 1 µM rT₃, and 10 mM dithiothreitol (USB/Invitrogen) in 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 6.9) in a final reaction volume of 300 µl, as described previously (13, 19). Blank incubations were carried out in the absence of protein. The reaction was stopped at 4°C in an ice bath with the addition of 200 µl of fetal bovine serum (Cultilab, Campinas, Brazil) and 100 µl of trichloroacetic acid (50%, vol/vol), followed by vigorous agitation. The samples were centrifuged at 10,000 g for 3 min, and 360 µl of the supernatant was collected for the measurement of 125I liberated during the deiodination reaction (γ-Counter Wizard; Perkin-Elmer). D₁ activity was related to the protein concentration in the homogenates. Protein concentration was measured by the method of Bradford (10).

Real-time-PCR. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen), following the manufacturer’s instructions. After DNase treatment, reverse transcription was followed by real-time PCR, as described previously (29). Specific oligonucleotides, as described in Table 1, were purchased from Applied Biosystems (Foster City, CA). β-Glucuronidase (GUS) was used as internal control for skeletal muscle data. The relative mRNA expression was calculated with the equation 2⁻ΔΔCT; ΔCT was calculated by the difference between the CT obtained for target mRNA and for GUS mRNA amplification. GUS mRNA expression was not different among the groups.

Oxygen consumption in permeabilized fibers. Mitochondrial respiration was studied in situ using saponin permeabilized fibers, as described previously (2). Muscles were removed and immediately immersed in cold solution BIOPS (10 mM EGTA, 0.1 µM K-Mes, 0.5 mM DTT, 6.56 mM MgCl₂, 5.77 mM ATP, and 15 mM phosphocreatine, pH 7.1). The fibers were separated and permeabilized for 30 min on ice BIOPS solution containing saponin (50 µg/ml). After permeabilization, the fibers were washed for 10 min in a cold mitochondrial respiration solution MIRÖS (0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 80 mM sucrose, and 1 g/l BSA, pH 7.1).

High-resolution respirometry. Skeletal muscle fibers (1.5–2.5 mg of soleus, 3.5–4.5 mg of white gastrocnemius) were dried on filter paper, weighed, and placed on a high-resolution respirometry instrument chamber (Oxigraph-2K; Oroboros) with 2 ml of MIRÖS at 37°C and left for 10 min for acclimatization. The substrate addition protocol to assess O₂ flux was performed sequentially as follows: pyruvate (5
Table 1. Primer sequence in RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>5’ GTGATAAGGAAAGGGAAGGATC 3’</td>
<td>5’ GAAAGGATTAAAGCTAAAAG 3’</td>
</tr>
<tr>
<td>D2</td>
<td>5’ CTGGATAAGGCTCTACAAACG 3’</td>
<td>5’ TGCCAAAGGAGGAGGAGGAC 3’</td>
</tr>
<tr>
<td>D3</td>
<td>5’ ACGGATAGGAAGGAGGACATG 3’</td>
<td>5’ AGCTAGGATGGGAGGATGATC 3’</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>5’ ACCAAACCAAGGAGAAGGAC 3’</td>
<td>5’ GGGTCGTGATGTTCTGCCATG 3’</td>
</tr>
<tr>
<td>UCP1</td>
<td>5’ GCTTGAGCACTCCATCCAAC 3’</td>
<td>5’ ACAGAAAAACGCTAAAGAGG 3’</td>
</tr>
<tr>
<td>UCP3</td>
<td>5’ GCCAAAGATCAGAAGAAAGG 3’</td>
<td>5’ GCCTTCAAGGATACCTTAG 3’</td>
</tr>
<tr>
<td>MHC1</td>
<td>5’ AGGAGAACAGGAAAGGAACTAC 3’</td>
<td>5’ TTAATAGAAAATACATGGGAGG 3’</td>
</tr>
<tr>
<td>MHC2a</td>
<td>5’ TCTGCAAGCTTCAAGGTTG 3’</td>
<td>5’ TGATCAAGATAGAAGATGGAAC 3’</td>
</tr>
<tr>
<td>MHC2x</td>
<td>5’ AGATCGGACTTCAAGGTTG 3’</td>
<td>5’ TGTTGATTTTCCTCTGAACG 3’</td>
</tr>
<tr>
<td>MHC2b</td>
<td>5’ GAGGGACAGGAAAGGACTG 3’</td>
<td>5’ ATCAGGACTGAAATCTGGGAC 3’</td>
</tr>
<tr>
<td>SERCA1</td>
<td>5’ TCTGCAGCTGTCACCTTCTCC 3’</td>
<td>5’ ACCGTTGCTGACTATTACCC 3’</td>
</tr>
<tr>
<td>SERCA2</td>
<td>5’ AATCTGGAGATACACTAGGAC 3’</td>
<td>5’ AGGATGACTGCAGCATGTTG 3’</td>
</tr>
<tr>
<td>SERCA3</td>
<td>5’ AGATCGGACTTCAAGGTTG 3’</td>
<td>5’ CAAAGCTCTGACAGAAGTTG 3’</td>
</tr>
<tr>
<td>PGC-1β</td>
<td>5’ ATATCGCTCCATGGCAGAAG 3’</td>
<td>5’ TGCTCTGCAATACGCTATTG 3’</td>
</tr>
</tbody>
</table>

mM) and malate (5 mM), ADP (3 mM), cytochrome c (10 μM), succinate (10 mM), oligomycin (1 μg/ml), and KCN (10 mM) (2).

Citrate synthase activity. Muscle samples were homogenized in lysis buffer (50 mM sodium phosphate, pH 7.4, 10% octyl-phenol ethoxylate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate) and supplemented with Sigma protease inhibitor mixture (P8340; Sigma). After 30 min on ice, the tissue lysates were centrifuged (13,000 g for 20 min at 4°C), and the resulting supernatants were collected. A reaction mixture containing 20 mM Tris-HCl, pH 8.0, 0.42 mM acetyl-coenzyme A, 0.1 mM DTNB, and 5 μg of total protein was incubated at 37°C for 5 min. The reaction was initiated by the addition of 0.5 mM oxaloacetate. The reduction of 5,5’-dithiobis (2-nitrobenzoic acid) by citrate synthase was measured in a plate reader spectrophotometer (Victor X4; Perkin-Elmer, Norwalk, CT) at 412 nm (extinction coefficient = 13.6 mM/cm corrected by the plate path length = 0.552 cm). The activities are expressed as micromoles of citrate per minute per milligram.

Statistical analysis. Results are expressed as means ± SE, and data were analyzed by one-way analysis of variance, followed by the Bonferroni multiple comparison test, and the level of significance was determined as P < 0.05.

RESULTS

Body temperature, body weight, food intake, and fat pad mass after cold exposure. Rats were able to sustain a constant body temperature during all 10 days of cold exposure (Fig. 1C). Body weight gain was significantly decreased after both 3 and 10 days of cold exposure compared with animals maintained at 23°C for 10 days (Fig. 1B). From the 3rd day onward

Fig. 1. Effect of cold exposure on body temperature, weight gain, food intake, and fat depots in rats kept at room temperature (23–24°C) or exposed to cold (4°C) for 3 and 10 days. A: body temperature of rats that were maintained at 23 or 4°C for 10 days. B: weight gain of control (C) rats that remained at 23°C for 10 days and rats exposed to cold (4°C) for 3 or 10 days. C: food intake was measured during 10 days of cold exposure. D: retroperitoneal fat pad mass was normalized for body weight; n = 15–17 in each group. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. C. Data are shown as means ± SE.
at 4°C, the animals presented a higher food intake when compared with the animals that remained at 23°C (Fig. 1C). Furthermore, we detected a significant decrease in retroperitoneal fat mass after 10 days of cold acclimation (Fig. 1D).

**Serum thyroid hormones.** A significant decrease in serum total and FT₃ levels was observed in the animals that remained for 3 or 10 days at 4°C (Table 2). Moreover, after 10 days of cold exposure, a significant increase in serum total T₄ was also detected, whereas serum free T₃ was already higher at 3 days of cold acclimation (Table 2). In addition, the serum T₃/T₄ ratio increased significantly after 3 and 10 days of cold exposure, corroborating previous findings of higher peripheral conversion of T₄ to T₃ during cold exposure in rats. It is well known that BAT type 2 deiodinase activity and expression increased due to the sympathetic stimulation during cold exposure. However, the possibility of long-term D2 activation in skeletal muscle during cold exposure has never been evaluated thus far.

**Iodothyronine deiodinase activity in liver, thyroid, and BAT.** As shown in Fig. 2, no changes were observed in the activity of type 1 deiodinase in the liver (Fig. 2A) or the thyroid gland (Fig. 2B) at any time point. However, an ~10-fold increase in BAT D2 activity was observed in animals that remained at 4°C for 3 days compared with animals maintained at 23°C (Fig. 2C). On the other hand, BAT D2 activity returned to the control levels after 10 days of cold exposure (Fig. 2C). Thus, the increased peripheral conversion of T₄ into T₃ that remains elevated after 10 days of cold exposure occurs in tissues other than BAT.

**Iodothyronine deiodinase activity and expression in slow- and fast-twitch oxidative and glycolytic skeletal muscle.** In slow-twitch soleus muscle, D2 activity increased 2.3-fold in animals that remained at 4°C for 3 days (Fig. 3A), which was accompanied by significantly higher D2 mRNA expression [relative D2 expression (D2/GUS): 23°C = 0.26, 3 days of cold = 0.51; Fig. 3B]. After 10 days of cold exposure, both the activity and the expression of D2 returned to the baseline levels in the soleus muscle. Conversely, D2 activity in fast-twitch glycolytic white gastrocnemius muscle remained unchanged 3 days after cold exposure, whereas a significant increase of 2.8-fold was observed when the animals remained at 4°C for 10 days (Fig. 3C), accompanied by a higher expression of D2 mRNA [relative D2 expression (D2/GUS): 23°C = 0.012, 10 days of cold = 0.029; Fig. 3D]. D2 activity and mRNA expression in fast-twitch oxidative red gastrocnemius muscle remained unchanged during cold exposure.

No changes in the mRNA expression levels of D1 or D3 were observed during cold exposure in any of the muscle fibers analysed. Of note, in basal conditions, D2 expression is higher in the oxidative fibers (slow-twitch soleus and fast-twitch red gastrocnemius fibers) than in the fast-twitch glycolytic white gastrocnemius fibers but is only modulated during cold exposure in soleus and white gastrocnemius (Table 3).

**Expression of genes involved in energy metabolism and T₃-responsive genes of slow- and fast-twitch oxidative and glycolytic fibers after cold exposure in rats.** From the several genes involved in energy metabolism, we observed a significant increase in the expression levels of PGC1-α after 3 days of cold exposure in soleus and after 10 days of cold exposure in glycolytic gastrocnemius (Fig. 4). We also found a significant increase in the expression levels of UCP3 mRNA in either the soleus or white glycolytic gastrocnemius after both 3 and 10 days of cold exposure, whereas UCP3 mRNA was significantly upregulated only at 3 days after cold exposure in red oxidative gastrocnemius fibers (Fig. 4). Apart from a significant decrease in the expression levels of glycerol-3-phosphate dehydrogenase (GPD)1 after 3 days of cold exposure in soleus fibers, cold exposure did not alter the expression levels of other genes, such as GPD1 and GPD2 in muscle fibers (Fig. 4). Regarding T₃-regulated genes, we did not detect any differ-

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**Table 2. Effect of prolonged cold exposure on serum total T₄ and T₃ in rats**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total T₄, µg/dl</th>
<th>Total T₃, ng/dl</th>
<th>Total T₃/T₄</th>
<th>Free T₄, ng/dl</th>
<th>Free T₃, pg/dl</th>
<th>Free T₃/T₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.12 ± 0.32</td>
<td>62.60 ± 3.23</td>
<td>21.47 ± 2.60</td>
<td>2.76 ± 0.12</td>
<td>273.25 ± 5.98</td>
<td>99.76 ± 3.12</td>
</tr>
<tr>
<td>3 Days at 4°C</td>
<td>1.68 ± 0.17*</td>
<td>73.74 ± 4.75</td>
<td>44.94 ± 2.91*</td>
<td>2.25 ± 0.12*</td>
<td>350.63 ± 11.57*</td>
<td>157.76 ± 6.36*</td>
</tr>
<tr>
<td>10 Days at 4°C</td>
<td>1.35 ± 0.10*</td>
<td>80.29 ± 3.99*</td>
<td>61.71 ± 6.15*</td>
<td>2.15 ± 0.08*</td>
<td>349.50 ± 13.13*</td>
<td>163.53 ± 6.67*</td>
</tr>
</tbody>
</table>

Data shown as means ± SE; n = 8 for each group. T₃, triiodothyronine; T₄, thyroxine. Rats were maintained at 4°C for 3 or 10 days. *P < 0.05 vs. control; #P < 0.05 vs. 3 days.
ences in the mRNA levels of SERCA1 and -2. However, myosin heavy chain (MHC)1 was upregulated by cold exposure only in white glycolytic gastrocnemius muscle, and MHC2a was downregulated in soleus 10 days after cold exposure and highly upregulated in white glycolytic gastrocnemius fibers at both 3 and 10 days of cold exposure but unchanged in the red oxidative gastrocnemius. MHC2x/d was the only MHC isoform upregulated by cold exposure in soleus 3 days after cold exposure, which parallels the increase in D2 activity. It is noteworthy that in the red oxidative gastrocnemius, where no D2 regulation was detected, a significant upregulation of MHC2b occurred during cold exposure (Fig. 4).

Fig. 3. D2 activity and mRNA expression and D1 and D3 mRNA expressions in slow- and fast-twitch oxidative and glycolytic muscles in rats kept at room temperature (23–24°C) or exposed to cold (4°C) for 3 and 10 days. A and D: soleus D1, D2, and D3 mRNA expression and D2 activity. B and E: red fast-twitch oxidative gastrocnemius D1, D2, and D3 mRNA expression and D2 activity. C and F: white fast-twitch glycolytic gastrocnemius D1, D2, and D3 mRNA expression and D2 activity; n = 4–8 in each group. *P < 0.05 vs. C. Data are shown as means ± SE.

Table 3. Effect of propranolol treatment on food intake, weight gain, fat depots, and D2 and UCP1 mRNA expression in BAT after cold exposure in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Food Intake (10 days)</th>
<th>Weight Gain</th>
<th>Retroperitoneal Fat, g</th>
<th>BAT Weight, g</th>
<th>mRNA D2 BAT</th>
<th>mRNA UCP1 BAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control at rt</td>
<td>281.64 ± 7.05</td>
<td>27.14 ± 4.21</td>
<td>4.71 ± 0.52</td>
<td>0.37 ± 0.02</td>
<td>1.00 ± 0.06</td>
<td>1.00 ± 0.13</td>
</tr>
<tr>
<td>Control at rt + Prop</td>
<td>234.93 ± 5.36</td>
<td>−0.42 ± 4.51*</td>
<td>4.55 ± 0.85</td>
<td>0.26 ± 0.01</td>
<td>0.41 ± 0.10</td>
<td>0.33 ± 0.11</td>
</tr>
<tr>
<td>3 days at 4°C</td>
<td>−0.33 ± 5.74**</td>
<td>3.71 ± 0.73</td>
<td>0.49 ± 0.03**</td>
<td>18.46 ± 3.65*</td>
<td>2.35 ± 0.29*</td>
<td></td>
</tr>
<tr>
<td>3 days at 4°C + Prop</td>
<td>−41.62 ± 4.33*</td>
<td>3.24 ± 0.30</td>
<td>0.30 ± 0.02</td>
<td>23.60 ± 4.06*</td>
<td>1.83 ± 0.22*</td>
<td></td>
</tr>
<tr>
<td>10 days at 4°C</td>
<td>−27.60 ± 11.87*</td>
<td>2.10 ± 0.57*</td>
<td>0.57 ± 0.03**</td>
<td>7.67 ± 1.72</td>
<td>1.33 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>10 days at 4°C + Prop</td>
<td>358.31 ± 9.80**</td>
<td>−35.38 ± 9.86*</td>
<td>1.39 ± 0.35*</td>
<td>4.1 ± 0.04*</td>
<td>18.36 ± 4.27*</td>
<td>1.84 ± 0.24*</td>
</tr>
</tbody>
</table>

Data shown as means ± SE; n = 6–10 for each group. BAT, brown adipose tissue; Prop, propranolol; rt, room temperature. *P < 0.05 vs. respective to control; #P < 0.05 vs. Prop in the respective group. Rats were maintained at 4°C for 3 or 10 days.

Citrate synthase activity and oxidative metabolism in slow- and fast-twitch oxidative and glycolytic muscle. We measured the oxygen consumption of permeabilized soleus and white and red gastrocnemius fibers (Fig. 5). As described previously, in all of the conditions tested the white glycolytic gastrocnemius fibers consumed less O2 than soleus and red oxidative gastrocnemius fibers. Cold exposure promoted a significant increase in mitochondrial O2 consumption in soleus fibers after 3 days at 4°C (Fig. 5A), concomitant with D2 stimulation. Interestingly, a significant increase in O2 consumption in the glycolytic gastrocnemius muscle was detected only after 10 days of cold exposure (Fig. 5C), in parallel with D2 and PGC-1α induction.
Interestingly, proton leak in glycolytic gastrocnemius muscle was significantly increased after 10 days of cold exposure together with D2 activation. In fact, at this time point, the oxygen consumption related to ATP synthesis and proton leak in glycolytic gastrocnemius muscle reached the same levels found in soleus and oxidative red gastrocnemius muscles at the basal condition (Fig. 5D). Interestingly, citrate synthase activity was significantly decreased in soleus muscle from cold-exposed animals (Fig. 5E) and significantly increased in white glycolytic gastrocnemius fibers at both 3 and 10 days of cold exposure (Fig. 5F). Also, we demonstrated that the white glycolytic gastrocnemius fibers became redder during cold exposure (Fig. 5H), suggesting an increase in blood supply to this fiber type.

 Altogether, our data demonstrate that local T3 production through D2 induction might be involved in the increased oxygen consumption that occurs in both muscle fibers, albeit at different time points.

**Effects of propranolol administration on body temperature, body weight, food intake, and fat pad mass after cold exposure.**

To investigate the role of β-adrenergic receptors, rats were pretreated with propranolol for 7–10 days before and during cold exposure. The rats treated with propranolol were able to sustain a constant body temperature during the whole cold exposure period (data not show). Body weight gain was significantly decreased after 3 and 10 days of cold exposure in both groups compared with animals maintained at 23°C for 10 days (Table 3). Food intake was decreased by propranolol administration even in cold-exposed animals. Furthermore, the retroperitoneal fat decreased in both groups 10 days after cold exposure compared with rats that remained at 23°C. We observed an increase in BAT weight in control rats when subjected to cold, which was attenuated in propranolol-treated rats. D2 and UCP1 mRNA expressions increased at 3 days of cold exposure in both groups, confirming that BAT D2 induction during cold does not depend on the β-adrenergic receptor.
Interestingly, propranolol administration prevented the normalization of D2 and UCP1 that was detected 10 days after cold exposure, indicating that the β-adrenergic receptor might counteract D2 activation during prolonged cold exposure (Table 3).

Effects of propranolol administration on the expression of genes involved in energy metabolism and T3-responsive genes of slow- and fast-twitch oxidative and glycolytic fibers after cold exposure in rats. Skeletal muscle undergoes important adaptations during exposure to cold. We have shown that during cold exposure, the white portion of the gastrocnemius acquires characteristics of slow fibers, such as the myosin- increased MHC1, MHC2a, type 2 deiodinase, and the mitochondrial content, as assessed by citrate synthase activity. To investigate whether some of these adaptations were mediated by β-adrenergic receptor activation, we analyzed the expression of genes involved in determining the muscle phenotype and citrate synthase activity in rats receiving propranolol (Fig. 6). We observed that D2 induction in the soleus was not impaired by propranolol, as also detected in BAT (Fig. 6 A). In the gastrocnemius, D2 expression did not differ in animals that remained at 4°C for 10 days in the presence or absence of propranolol (Fig. 6 C). Propranolol attenuated the increase in UCP3 mRNA in soleus after 3 days at 4°C (Fig. 6 B), which was not observed in the gastrocnemius (Fig. 6 D). On the other hand, PGC-1α induction and the increase in citrate synthase activity in the gastrocnemius after 10 days at 4°C were blocked by propranolol, showing that the β-adrenergic receptor is involved in white muscle PGC1-α increase and mitochondria biogenesis, regardless of D2 induction (Fig. 6, E and J). MHC1, MHC2a, and MHC2xd mRNA expressions were attenuated in rats that received propranolol (Fig. 6, F–H). Thus, we can conclude that some of the adaptations in skeletal muscle after cold exposure are mediated by the β-adrenergic receptors.

DISCUSSION

We first confirmed that cold-exposed animals showed a significant increase in food intake after both 3 and 10 days at 4°C. As described previously (33), there is an impaired insulin action in the hypothalamus of animals exposed to cold for 4 days, which is combined with decreased serum leptin that could explain why cold-exposed animals are hyperphagic. Our present data also corroborate with previous findings of a large acute increase in BAT D2 that occurs in the first 3 days after cold exposure. Interestingly, however, when rodents are chronically exposed to cold (10 days at 4°C), BAT D2 returns to control levels, and little is known about the adaptations induced by cold exposure in skeletal muscle after the first week of cold adaptation, when shivering is highly decreased.

We have shown, for the first time, that skeletal muscle D2 expression and activity are increased during long-term cold exposure; however, in the soleus slow-twitch muscle, the increase in D2 mRNA and activity occurred 3 days after cold exposure, such as occurred with BAT, returning to control
Previously, we have shown an increased conversion of T4 and T3 after prolonged cold exposure (2), even though the site of T3 production has not been determined. Our present data in rats support the idea that the increase in D2 mRNA expression and activity occurred only 10 days after exposure to cold and did not seem to depend on the β-adrenergic receptor activation. These results are in accord with previous studies demonstrating that treatment with propranolol was unable to prevent the increase of D2 activity in BAT after noradrenaline administration (31). Those authors showed that prazosin, an inhibitor of the α1-adrenergic receptor, indeed prevented the increase in BAT D2 activity after adrenaline administration and cold exposure for 4 h (31). On the other hand, the fast-twitch red oxidative portion of the gastrocnemius muscle expresses high levels of D2, but the enzyme is not modulated by cold exposure.

An intense activation of the thyroid axis has been observed in the early hours of cold exposure, and consequently, increased serum levels of T3 and T4 in rats were described (19, 22). Previously, we have shown an increased conversion of T4 to T3 in rabbits after prolonged cold exposure both at 3 (21) and 10 days (2), although the site of T3 production has not been determined. Our present data in rats support the idea that the peripheral conversion of T4 into T3 can also occur in skeletal muscle secondary to increased D2 activity. Different from muscle and BAT D2, thyroid and liver D1 activities did not change due to cold exposure, although previous findings reported increased thyroid D1 activity after 6 days of cold acclimation. We also show that skeletal muscle D1 and D3 mRNA expression levels do not alter during long-term cold exposure. Taken together, our findings indicate that the changes in serum thyroid hormones and consequently the increase in the T3/T4 ratio detected during prolonged cold exposure are a result of D2 induction not only in BAT but also in skeletal muscles.

The recruitment of different muscle fibers during cold exposure has been observed in chickens (20) and in rabbits (2). Interestingly, a transition from white to red phenotype has been observed in white muscles from these two different species after prolonged cold acclimation. We also show herein that the white glycolytic portion of rat gastrocnemius muscle becomes redder, a change that accompanies the increase in citrate synthase activity and higher oxygen consumption. Also, the white gastrocnemius fibers show significantly higher MCH1 and MCH2a mRNA expressions during prolonged cold acclimation. Although T3 is implicated in the regulation of muscle fiber transition, inducing a slow- to fast-twitch transition phenotype (36), the regulation of type 2 deiodinase in skeletal muscle and its physiological role are largely unknown. Grozovskiy et al. (18) showed that the activity of D2 is increased in response to PPARγ agonists and forskolin administration in cultured myoblasts and myotubes. The possible role of D2 in determining the muscle phenotype has been suggested by the finding that adult D2-knockout animals show differences in the expression of T3-regulated muscle genes such as MyoD, myogenin, and SERCA1 as well as impaired muscle regeneration following an injury (15).

In BAT, D2 is stimulated by the sympathetic nervous system, and its activation leads to increased intracellular T3, one of the mechanisms by which thyroid hormones increase oxygen consumption and thermogenesis in rodents (5, 8). This process is known as BAT recruitment and is paralleled by increases in UCP1 expression (indicator of recruited BAT) and activity, higher numbers of mitochondria, and increased differentiation of preadipocytes into brown adipocytes (12). In addition, a previous study in mice with disruption of D2 gene shows that T3 is important for BAT lipogenesis, which has
already been shown in vitro (4) and in vivo (13) and is fundamental for BAT thermogenesis. The mechanism responsible for D2 induction in skeletal muscle fibers seems not to be dependent on the β-adrenergic receptor activation, since propranolol did not impair D2 induction. However, previous studies described that the sympathetic nervous system is only slightly activated in skeletal muscle during cold acclimation compared with BAT and the heart (16). Thus, future studies focusing on the mechanism involved in skeletal muscle D2 regulation are necessary.

Thus, other genes involved in muscle energy metabolism that are targets of T3 action were also analyzed in the present study. We found an increase in PGC-1α in slow-twitch muscles, which was already shown by Oliveira et al. (25), when rats were exposed to cold for 4 days. However, the increase in PGC-1α expression in fast-twitch muscle fibers was observed only after 10 days of exposure to cold. In our study, UCP3 expression induced by cold exposure was not time dependent and also occurred in the red oxidative gastrocnemius muscle, where D2 is not induced during cold exposure; however, the increase in systemic T3 that already occurs at 3 days of cold, where D2 is not induced during cold exposure; however, the increase in systemic T3 that already occurs at 3 days of cold exposure might play a role in UCP3 regulation. We found an increase of approximately fourfold in the expression of UCP3 in the slow-twitch muscle and a twofold increase in fast-twitch oxidative and glycolytic muscles, as described previously (25). The increase in the expression of UCP3 (34) might be dependent on the expression of PGC-1α, which was also positively regulated. Since PGC-1α and UCP3 induction by cold exposure in soleus and gastrocnemius are all impaired by propranolol, we conclude that the β-adrenergic receptor is involved in white muscle PGC-1α increase and mitochondria biogenesis, regardless of D2 induction. Although the promoter region of both PGC-1α and UCP3 has thyroid hormone-responsive elements (32, 35), during cold exposure the main regulator of these genes seems to be the β-adrenergic stimulus. Therefore, we believe that the local increase in the T3-to-T3 conversion is involved in muscle adaptation processes induced by cold exposure other than mitochondria biogenesis.

In conclusion, cold exposure is accompanied by concerted changes in the metabolism of BAT and slow- and fast-twitch skeletal muscles that are paralleled by type 2 deiodinase activation. During short-term cold exposure, BAT D2 is increased, and shivering thermogenesis is known to occur. After 3 days at 4°C, the increased oxygen consumption occurs both in oxidative slow-twitch muscle fibers and BAT, when D2 is also activated in these tissues. After 10 days at 4°C, when nonshivering thermogenesis is known to take place, BAT and oxidative muscle fibers return to their basal metabolic levels when D2 activity is normalized, whereas white glycolytic fibers are recruited, leading to increased D2 activity and oxygen consumption, which might be implicated in long-term thermogenesis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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SKELETAL MUSCLE D2 INDUCTION BY COLD EXPOSURE  E1029


