Differential regulation of uncoupling protein-1, -2 and -3 gene expression by sympathetic innervation in brown adipose tissue of thermoneutral or cold-exposed rats

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Abstract The control of uncoupling protein-1, -2 and -3 (UCP-1, UCP-2, UCP-3) mRNA levels by sympathetic innervation in rats was investigated by specific and sensitive RT-PCR assays. In rats reared at thermoneutrality (25°C), unilateral surgical sympathetic denervation of interscapular brown adipose tissue (BAT) markedly reduced the UCP-1 mRNA level (−38%) as compared with the contralateral innervated BAT pad, but was without significant effect on UCP-2 and -3 mRNA levels. Cold exposure (7 days, 4°C) markedly increased UCP-1 (+180%), UCP-2 (+115%) and UCP-3 (+195%) mRNA levels in interscapular BAT. Unilateral sympathetic denervation prevented the cold-induced rise in BAT UCP-1 and UCP-2 mRNAs, but not that in BAT UCP-3 mRNA. Results were confirmed by Northern blot analysis. These data indicate a differential endocrine control of UCP-1, UCP-2 and UCP-3 gene expression in rat BAT both at thermoneutrality and during prolonged cold exposure.

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Key words: Unilateral sympathetic denervation; Semi-quantitative reverse transcriptase polymerase chain reaction; Cold exposure

1. Introduction

Brown adipose tissue (BAT) non-shivering thermogenesis (NST) appears to be the most characteristic adaptive response to cold exhibited by a number of mammalian species. It also plays a crucial role in the energy balance by dissipating excess energy intake as heat [1]. BAT NST is based on an uncoupling of the mitochondrial respiratory chain by the tissue-specific proton translocator uncoupling protein (BAT UCP- or UCP-1) located in the inner membrane of mitochondria [2]. This protein allows the dissipation as heat of the energy that would otherwise be used for ATP synthesis. More recently, it has become clear that other potential uncoupling proteins (UCP-2 and UCP-3) are expressed in several tissues [3–5]. UCP-2 appears to be ubiquitously expressed whereas UCP-3 is preferentially expressed in skeletal muscle and BAT [6]. Expression of these two proteins may also contribute to energy efficiency in cells and thus play an important role in energy balance. The elucidation of the mechanisms controlling UCP-2 and UCP-3 gene expression is therefore of primary importance. The unique co-expression of all three proteins in BAT enables the investigation of the mechanisms controlling the expression of these genes within a single tissue.

BAT-specific UCP-1 gene expression [7] is well known to be markedly up-regulated in the cold under the control of BAT sympathetic innervation [8] through the activation of β-adrenoceptors abundantly expressed on brown adipocytes [9], most of the effect being mediated by β_3-adrenoceptors [10]. Selective adrenergic denervation of interscapular BAT drastically inhibits the cold-induced up-regulation of BAT UCP-1 gene expression [11]. The control of UCP-2 and UCP-3 gene expression has recently received considerable attention but the results obtained so far have been rather contradictory. Cold exposure (5 or 48 h) generally increases BAT UCP-2 mRNA [12,13], although this result is not always found [3]. The BAT UCP-3 gene appears to react more slowly to cold exposure since acute (5 h) exposure of adult mice at 5°C is without effect [13] while longer exposures (2–10 days at 4°C) of rats increase UCP-3 mRNA by 1.5–2-fold [14,15]. The mechanisms underlying the cold-induced up-regulation of UCP-2 are unclear since pharmacological treatment with the β_3-adrenergic agonists CL316,243 for 10 days in mice [3] or BRL 35135 for 3 weeks in rats [16] did not influence BAT UCP-2 mRNA. Similarly, in vitro addition of noradrenaline for 4 h to brown 1B8 adipocytes [17] had no effect. By contrast, preliminary results of Boss et al. [12] indicated a stimulation ($\times 2.1$) of BAT UCP-2 mRNA in response to 32 h of treatment with the β_3-agonist Ro-168714. The cold-induced up-regulation of UCP-3 may more clearly depend on sympathetic innervation because of the marked ($\times 2.1$) increase in BAT UCP-3 mRNA after long-term (3-week) administration of BRL 35135 to rats [16]. However, direct experimental evidence is still lacking and acute injections of the β_3-adrenergic agonist CL214,613 was shown to have no influence on BAT UCP-3 mRNA [18].

The present study was designed to investigate the possible involvement of the sympathetic nervous system in the regulation of BAT UCP-2 and UCP-3 expression as compared with that of UCP-1 both at thermoneutrality and during prolonged cold exposure. These studies required the development of sensitive reverse transcriptase polymerase chain reaction (RT-PCR) assays.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 200–220 g were obtained from a commercial stock-breeder (Iffa Credo, France) and fed a commercial mash (UAR 105) ad libitum. Animals were housed in individual cages and reared at a 25°C ambient temperature under a constant photoperiod

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(12/12 light-dark). Rats underwent unilateral denervation of interscapular BAT pad as described previously [18]. Four days after recovering from the surgery, rats were reared at 4°C (cold-exposed rats, CE) or kept at 25°C (thermoneutral control rats, TN) for 1 week. Rats were then killed on the same day by cervical dislocation. Interscapular BAT (innervated and denervated pads), heart, liver, retroperitoneal white adipose tissue (WAT), soleus (SOL) and extensor digitorum longus (EDL) muscles were rapidly dissected, weighed and frozen in liquid nitrogen. Samples were then stored at −70°C until analysis.

2.2. Total RNA extraction

Total RNA was isolated from 0.2–0.4 g portions of frozen tissue using a guanidinium thiocyanate method [19], quantified by absorbance at 260 nm and its integrity routinely checked by gel electrophoresis.

2.3. Reverse transcription (RT)

RT was performed in a total volume of 25 μl containing 500 ng (BAT) or 1 μg (WAT, heart, liver, SOL and EDL muscles) of total RNA, 25 nmol of dNTPs (Promega), 1 μg polyT primers and 5 μl M-MLV reaction buffer (Promega). Denaturation was at 70°C for 5 min before addition of 25 U RNasin (Promega) and incubation with 200 U M-MLV-RTase (Promega), for 1.5 h at 42°C. RT of the mRNA from each specific tissue was performed simultaneously on all the animals of one experiment.

2.4. Polymerase chain reaction (PCR)

PCR was then performed with 1/10th of the RT reaction in a total volume of 50 μl containing 1 μM of forward and reverse primers, 2.5 U of Taq DNA polymerase (Eurobio), reaction buffer and 1.5 mM $MgCl_2$. Primers for UCP-1 cDNA were forward 5′-GTGAAGGTCAGAATGCAAGC (position 409–428) and reverse 5′-AGGGCCCCCTTCATGAGGTC (position 586–605), chosen according to the rat UCP-1 cDNA sequence (RNUCPG.PE1, EMBL). Primers for UCP-2 were forward 5′-ACAAGACCATTGCACGAGAG (position 788–807) and reverse 5′-CATGGTAAGGGCACAGTGAC (position 1061–1080) (obtained from Gibco), according to the rat UCP-2 cDNA sequence (U69135, GenBank). Primers for UCP-3 cDNA were forward 5′-ATGCATGCCTACAGAACCAT (position 657–676) and reverse 5′-CTGGGCCACCATCCTCAGCA (position 949–968), chosen according to the rat UCP-3 cDNA sequence (U92069, GenBank). To amplify appropriate amounts of the respective cDNAs in the exponential phase of the reaction, 18 cycles of PCR were used for UCP-1 (denaturation at 94°C for 45 s, annealing at 60°C for 60 s, extension at 72°C for 60 s), 27 cycles of PCR were used for UCP-2 or UCP-3 (same parameters as for UCP-1 except at an annealing temperature of 61°C). The length of the amplified fragment was 197 bp for UCP-1, 293 bp for UCP-2 and 312 bp for UCP-3. The amplified products were cloned directly into pGEM-T vector (Promega) and sequenced by the dideoxynucleotide chain-termination method (Sequenase version 2.0 DNA sequencing kit; Amersham).

Non-homologous DNA fragments taken from pNLA vector (mimic), containing the primer templates of UCP-1 and UCP-2 or those of UCP-3, were co-amplified with the target cDNA and used as an internal standard to normalize tube-to-tube variations in amplification efficiencies.

As an invariant gene, we used the nuclear T_3 receptor α1, of which the expression in BAT was found not to vary under the influence of cold or denervation (see Section 3). Primer pairs used for PCR were forward 5′-ATGGCCATGGACCTGGTTC (position 358–376) and reverse 5′-GGGCACTCGACTTTCATGTG (position 1159–1178) according to the rat T_3R-α1 cDNA sequence (M18028, GenBank). 24 cycles of PCR were used (denaturation at 94°C for 45 s, annealing at 66°C for 60 s, extension at 72°C for 60 s). The specificity of amplification was confirmed by sequencing. A standard mimic amplified with the same primers was also introduced into the reaction.

The amplified products were easily separated according to their size on 2% agarose gel stained with ethidium bromide (0.5 mg/ml). Numeric photographs of the gels were acquired using a Kodak Digital Science camera (DC120) and the relative intensity of the bands was quantitated by Kodak Digital Science 1D Image Analysis Software. The target cDNA-to-mimic ratio was then used as a relative estimate of mRNA abundance.

2.5. Northern blots

Northern blots were used to confirm the RT-PCR analysis. Total RNA (20 μg) were transferred to Nylon membranes (Amersham) after gel electrophoresis. Prehybridization (4 h) and hybridization (18 h) were carried out at 42°C. The amplified RT-PCR fragments were used as probes for UCP-1, UCP-2 or UCP-3 transcripts after labeling by random priming with [^{32}P]dCTP (NEN). Blots were washed at 56°C in 1×standard sodium citrate with 0.1% sodium dodecyl sulfate and exposed to Hyperfilm XAR5-Kodak at −70°C.

2.6. Statistical analysis

Values are presented as means ± S.E.M. Statistical significance of observed variations was assessed by one-way analysis of variance (ANOVA). Differences between means were then tested by Scheffé's *F*-test or paired *t*-tests. Statistical significance was accepted at $P < 0.05$.

3. Results and discussion

CE rats (4°C for 1 week) grew less rapidly than the thermoneutral controls and were lighter at the time of killing (277 ± 4 vs. 297 ± 4 g, $P < 0.05$). As expected, interscapular BAT mass was nearly doubled in CE rats (0.43 ± 0.03 vs.

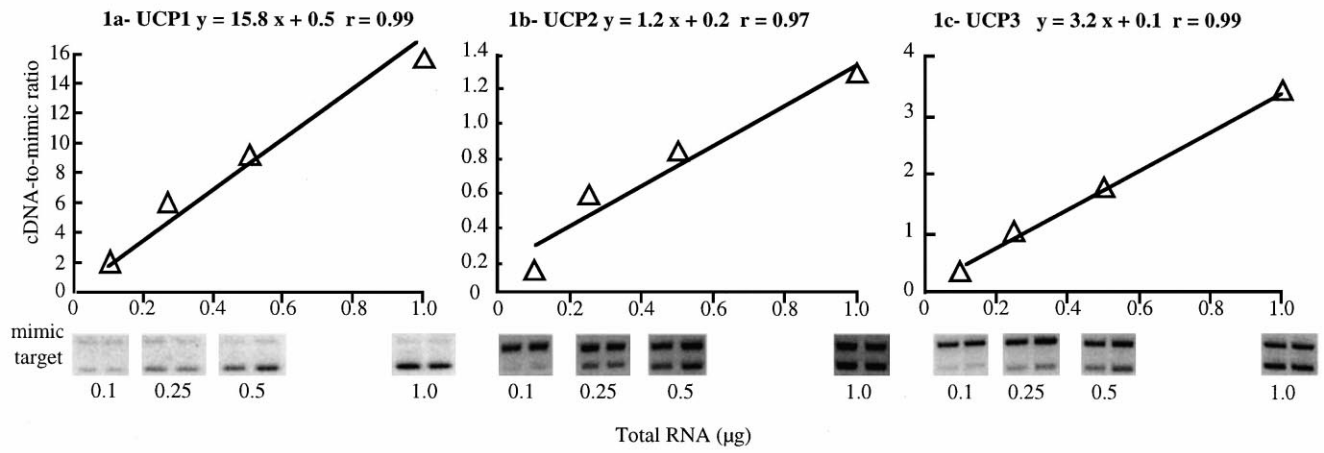

Fig. 1. Relationship between increasing amounts of total RNA (leading to increasing amounts of UCPs mRNA) and cDNA-to-mimic ratio in semi-quantitative RT-PCR assay of relative UCP-1 (a), UCP-2 (b) and UCP-3 (c) mRNA tissue levels. Total RNA from the interscapular BAT of a cold-exposed rat (1 week, 4°C) was used.

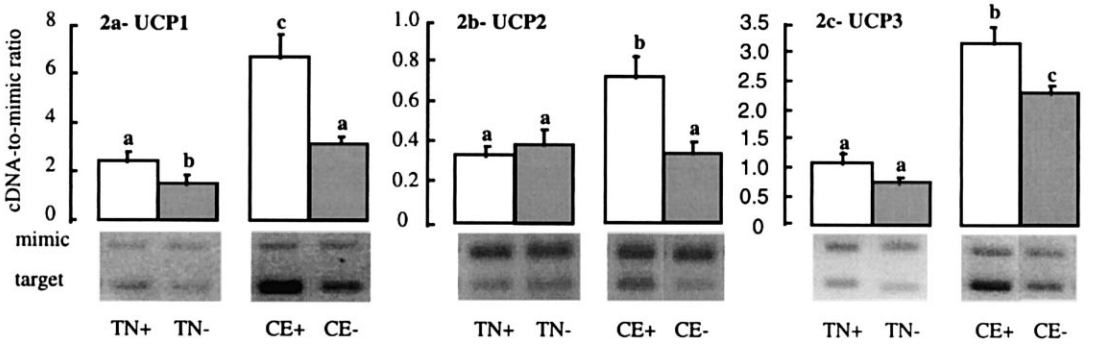

Fig. 2. Effect of cold exposure and unilateral sympathetic denervation on relative levels of UCP-1 (a), UCP-2 (b) and UCP-3 (c) mRNA (expressed as cDNA-to-mimic ratio) in interscapular BAT pads (+, innervated in open bar; −, denervated in shaded bar). TN: control rats reared at thermoneutrality; CE: rats exposed to cold (4°C) for 1 week. Values with different letters are significantly different ($P < 0.05$).

0.26 ± 0.01 g, $P < 0.05$) and the yield of total RNA per unit mass of BAT was increased by 20% in CE rats (1.3 ± 0.1 vs. 1.1 ± 0.1 mg/g, $P < 0.05$).

Validation of the RT-PCR assay was an important prerequisite of the study. It was obtained firstly by sequencing the amplified products, which showed a perfect analogy with the published sequences for UCP-1, UCP-2 and UCP-3 cDNA, respectively. Secondly, the detection of the various UCPs was in accordance with other works [3,6], the expression of UCP-1 being restricted to BAT, that of UCP-2 mRNA being detected in all the tissues investigated and that of UCP-3 mRNA being found mainly in BAT and skeletal muscles (data not shown). The third validation was obtained by establishing the relationship between the cDNA-to-mimic ratio and increasing amounts of target mRNA. This was achieved by increasing the amount of total RNA used in the RT-PCR assay from 0.1 to 1 μg. Fig. 1 shows that for UCP-1, UCP-2 and UCP-3 assays, there was a linear relationship between the two parameters indicating that the cDNA-to-mimic ratio may be used as a good estimate of the relative amount of mRNA of interest in a tissue. We also verified that the amplification efficiencies of target and reference were similar confirming the validity of the method [20]. The semi-quantitative approach used in the present study was preferred to other published methods of competitive PCR using several dilutions of an external standard [21]. Although these methods have the advantage of giving absolute amounts of mRNA in a tissue, they require numerous experimental assays and therefore do not facilitate comparisons of a multitude of animals and tissues. The semi-quantitative method described here allowed us to assess relative changes in tissue levels of UCP-1, UCP-2 and UCP-3 mRNA in different physiological conditions. It is of interest to note that the detection of UCP-2 and UCP-3 mRNA in BAT required 27 cycles of PCR but that of UCP-1 only 18 cycles. Given that we did not observe major differences in the amplification efficiencies between UCP-1 and UCP-2 or -3 (parallelism in cycle-response curves), it follows that the abundance of UCP-2 and UCP-3 transcripts in BAT should be much lower than that of UCP-1 mRNA. Similar observations were also made using Northern blots (data not shown).

As expected, cold exposure (1 week, 4°C) markedly increased (+180%, $P < 0.05$) the UCP-1 cDNA-to-mimic ratio in BAT (Fig. 2a), reflecting a marked up-regulation of the UCP-1 gene. Cold exposure also markedly increased BAT UCP-2 (+115%, $P < 0.05$, Fig. 2b) and UCP-3 (+190%, $P < 0.05$, Fig. 2c) cDNA-to-mimic ratios. These data are therefore consistent with results also showing an up-regulation of UCP-2 and UCP-3 expression in CE animals [12,13]. The amplitudes of the cold-induced rises in BAT UCP-2 or UCP-3 levels were similar to those reported by others for UCP-2 (+140%) [12] or UCP-3 (+100%) [15].

The influence of sympathetic innervation on the expression of UCP-2 and UCP-3 was studied in interscapular BAT because one pad can be denervated leaving the other innervated as an internal control. At thermoneutrality, unilateral sympathectomy slightly reduced the yield of total RNA (−27%, $P < 0.05$) and markedly diminished the UCP-1 (−38%) cDNA-to-mimic ratio ($P < 0.05$). By contrast, sympathectomy was without significant effect on UCP-2 and UCP-3 transcript levels. These results indicate that, at thermoneutrality, BAT levels of UCP-2 and -3 mRNA are less tightly coupled to sympathetic nervous activity than that of UCP-1 mRNA, which appears very sensitive to sympathetic denervation. These results indicate a differential control of the three UCPs by sympathetic innervation at thermoneutrality.

In the cold, unilateral sympathectomy inhibited the increase in BAT mass, and consequently, the cold-induced rise (+98%) in total RNA per BAT pad. The present results demonstrate for the first time that the cold-induced rises in BAT UCP-2 and UCP-3 mRNA levels were differentially controlled by sympathetic innervation (Fig. 2). Indeed, sympathectomy completely abolished the cold-induced rise in UCP-2 mRNA levels (Fig. 2b), as it did for UCP-1 mRNA (Fig. 2a), but did not block the cold-induced increase in UCP-3 mRNA levels (Fig. 2c). Relative levels of UCP-3 transcripts were indeed similarly increased by cold exposure in the innervated or denervated pads. Similar observations were made using Northern blot analysis with specific probes against UCP-2 or UCP-3

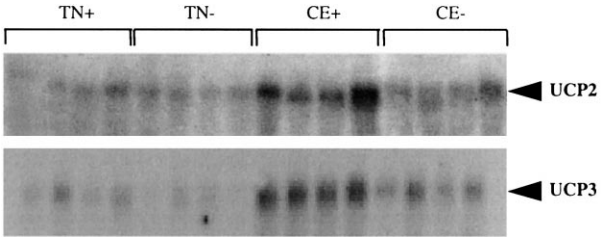

Fig. 3. Northern blot analysis of the effects of cold exposure and unilateral sympathetic denervation on UCP-2 and UCP-3 mRNA relative abundance in interscapular BAT pads (+: innervated; −, denervated). TN: control rats reared at thermoneutrality; CE: rats exposed to cold for 1 week.

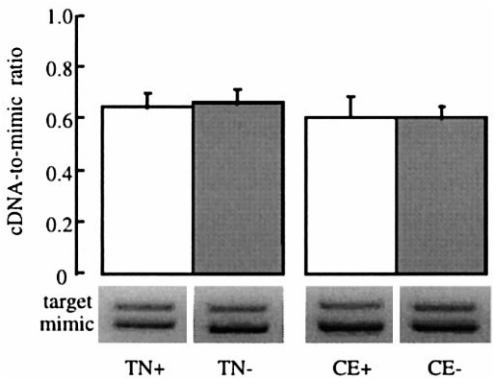

Fig. 4. Effect of cold exposure and unilateral sympathetic denervation on relative levels of nuclear $T_3R\alpha1$ mRNA (expressed as cDNA-to-mimic ratio) in interscapular BAT pads (+, innervated in open bar; −, denervated in shaded bar). TN: control rats reared at thermoneutrality; CE: rats exposed to cold (4°C) for 1 week. There was no significant effect of either cold exposure or sympathetic denervation.

transcripts (Fig. 3). It should however be noted that the relative amount of UCP-3 transcripts was slightly lower (−27%, $P < 0.05$) in the denervated than in the innervated BAT pad of CE rats, indicating a slight influence of sympathetic denervation on UCP-3 expression. The variations in UCP-1, -2 and -3 gene expression were not related to unspecific up- or down-regulation of cellular genes since no effect of cold exposure or denervation was observed for nuclear T_3R-$\alpha1$ gene expression (Fig. 4).

The use of the unilateral sympathectomy model therefore indicates that the sympathetic innervation of BAT plays an essential role in the cold-induced up-regulation of BAT UCP-2, as it does for UCP-1, while other mechanisms are involved in the cold-induced up-regulation of BAT UCP-3 in addition to sympathetic innervation. Control of BAT UCP-2 by sympathetic innervation agrees with results of others [12] indicating a β_3-stimulation of UCP-2 mRNA in BAT and also supports recent data suggesting a potentially sympathetic nerve-mediated central effect of leptin on BAT UCP-1 and UCP-2 [22]. However, such control contradicts other pharmacological studies, which did not show any influence of either β_3-adrenergic agonists in vivo [3,16] or short-term noradrenaline in vitro [17] on UCP-2 gene expression. One possibility is that adrenoceptors other than β_3 are involved in the response in vivo, which could explain why treatments with β_3-adrenergic agonists do not have consistent effects, depending on the specificity of the agonist used. The present results warrant re-evaluating the regulation of the UCP-2 gene by adrenergic agonists. Alternatively, because BAT UCP-2 may play a role in regulating lipids as fuel substrates for UCP-1-mediated thermogenesis in BAT as suggested recently [23], its expression could depend indirectly on BAT thermogenic activation. However, activation of BAT thermogenic activity by long-term β_3-adrenergic agonists was not accompanied by any change in BAT UCP-2 [16].

The present results indicating some control of BAT UCP-3 gene expression by sympathetic innervation agree with the positive effect of long-term administration of the β_3-adrenergic agonist BRL 35135 to rats [16]. However, they clearly indicate that other factors are involved in the effect of cold. Among them, thyroid hormones may play an important role because of the marked up-regulation of UCP-3 by administration of 3,5,3′-triiodothyronine (T_3) to rats [15]. Alternatively, stimulation of lipid mobilization induced by cold exposure may stimulate BAT UCP-3 expression because of the positive effect exerted by high fat feeding on BAT UCP-3 in rats [15]. The present results showing a differential regulation of UCP-2 and UCP-3 during cold exposure are consistent with those obtained after 5 h of cold exposure in adult mice and showing an up-regulation of BAT UCP-1 and UCP-2 but no change in UCP-3 [13]. It can therefore be suggested that the results obtained in mice [13] can be related to an activation of sympathetic nervous activity up-regulating BAT UCP-1 and UCP-2, while other factors are required for the later up-regulation of UCP-3. Therefore, contrary to results obtained by pharmacological treatments with β_3-adrenergic agonists, the present results indicate that BAT UCP-3 expression is not regulated like that of UCP-1 by the sympathetic nervous activity during cold exposure.

It is concluded that there is a differential control of BAT UCP homologue expression by sympathetic innervation both at thermoneutrality and in the cold. In the cold, BAT sympathetic innervation is a major factor involved in the up-regulation of UCP-2 mRNA expression, as it is for UCP-1, but is less involved in the up-regulation of UCP-3 mRNA. At thermoneutrality, by contrast, sympathectomy only affects the expression of UCP-1. The factors involved in such differential regulation now remain to be determined.

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