9-*cis* Retinoic acid induces the expression of the uncoupling protein-2 gene in brown adipocytes

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Received 10 November 1998

Abstract The expression of uncoupling protein-2 (UCP2) mRNA is up-regulated during the differentiation of brown adipocytes in primary culture. When differentiation of brown adipocytes is impaired, UCP2 mRNA expression is down-regulated. 9-*cis* Retinoic acid causes a dose-dependent induction of UCP2 mRNA levels in brown adipocytes, whereas all-*trans* retinoic acid has no effect. Specific agonists of retinoid X receptors (RXR) induce UCP2 mRNA expression, whereas specific activators of retinoic acid receptors do not. 9-*cis* Retinoic acid, acting through RXR receptors, is identified as a major regulator of the expression of the UCP2 gene in the brown fat cell.

Key words: Brown adipose tissue; Uncoupling protein-2; Uncoupling protein-1; 9-*cis* Retinoic acid; Thermogenesis

1. Introduction

Brown adipose tissue (BAT) is a major site for non-shivering thermogenesis in rodents either in response to cold or in response to overfeeding [1]. It contains the uncoupling protein-1 (UCP1), a tissue-specific mitochondrial protein that uncouples oxidative phosphorylation and leads to the generation of heat [2]. Until 1997, the various aspects of BAT thermogenic function were attributed to UCP1. The discovery of two novel uncoupling protein genes, uncoupling protein-2 (UCP2) and uncoupling protein-3, which are expressed in multiple tissues including brown fat, opened new perspectives [3–6]. These proteins are considered to act as natural uncouplers of oxidative phosphorylation, similarly to UCP1, and their physiological role in mammalian thermogenesis is currently under research. Several lines of evidence indicate that UCP2 expression may be involved in the brown fat thermogenesis elicited by diet-dependent stimuli. Thus, genetic ablation of BAT was reported to cause cold intolerance and impaired diet-induced thermogenesis in mice [7]. However, targeted disruption of the UCP1 gene leads to cold-sensitive animals but does not disturb the normal response to overfeeding [8], in association with up-regulation of UCP2 gene expression in brown fat. Moreover, obesity-prone strains of mice, such as C57BL/6J, are characterized by low basal levels of UCP2 expression in several tissues including brown fat [3].

The mechanisms of regulation of UCP1 gene expression have been widely studied and noradrenaline [9], retinoic acid [10], thyroid hormones [11], insulin and insulin-like growth factor 1 (IGF-1) [12] have been identified as regulators. However, little is known of the molecular signals that mediate changes in UCP2 gene expression. Leptin has been reported to influence UCP2 gene expression in several cell systems including adipocytes [13]. Activators of peroxisome proliferator-activated receptor (PPAR) γ have also been also reported to up-regulate UCP2 mRNA levels in adipose cells [14]. Here we have analyzed the regulation of expression of UCP2 in relation to brown adipocyte differentiation and we have identified 9-*cis* retinoic acid (9-*cis* RA) as a major regulator of UCP2 gene expression in brown adipocytes.

2. Materials and methods

Noradrenaline, insulin, IGF-1, triiodothyronine (T_3), LY171883, all-*trans* retinoic acid (all-*trans* RA) and phytanic acid were from Sigma (USA). Recombinant murine leptin was from PreProtech (USA). WY 14,643 was and 15-deoxy-Δ-12,14-prostaglandin J2 (15d-PGJ2) were from Cayman (USA) and methoprene was from Promochem (Germany). 9-*cis* RA, P-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8,tetramethyl-2-naphthyl)-1-propenyl] benzoic acid (TTNPB) and Am80 were kind gifts of Dr. I. Levine (Hoffman-LaRoche, USA), Dr. E.M. Gutknecht and Dr. P. Weber (Hoffman-LaRoche, Switzerland) and Dr. K. Shudo (University of Tokyo, Japan), respectively.

Brown adipocytes were differentiated in culture from precursor cells as described previously [10]. Three-week-old Swiss mice were killed and interscapular, cervical and axillar depots of BAT were removed. Stromal vascular cells were isolated, plated (5000 cells/cm^2) and grown in Dulbecco's modified Eagle's medium:Ham's F12 medium (1:1, v/v) (DMEM/F12) supplemented with 10% (v/v) FCS, 20 nM insulin, 2 nM T_3 and 100 μM ascorbate. When indicated cells were grown in a hormone-depleted non-differentiating medium in which DMEM/F12 was only supplemented with 10% (v/v) charcoal-treated FCS [10]. The hormonal agonists were added to the regular culture medium on day 9 and cells were harvested 24 h later with the exception of treatments with noradrenaline and 15-dPG-J2, which lasted only 6 h. For exposure to insulin, IGF-1 and T_3, regular culture medium was replaced with the hormone-depleted medium.

RNA from BAT or brown adipocytes was extracted using a guanidine thiocyanate method [15]. 10 μg of RNA was denatured, electrophoresed on 1.5% formaldehyde-agarose gels and transferred to positively charged membranes (N+, Boehringer Mannheim, Germany). 0.2 μg of ethidium bromide was added to RNA samples to check equal loading of gels and transfer efficiency, as reported [16]. Prehybridization and hybridization were performed as reported [17]. Blots were hybridized using a mouse UCP2 probe obtained previously [18], the rat cDNA for UCP1 [19] or the rat cDNA for subunit II of cytochrome *c* oxidase (COII) [20]. The DNA probes were labeled by the random oligonucleotide-primer method using [α-^{32}P]dCTP. Hybridization signals were quantified using a Molecular Image System GS-525 (Bio-Rad). When indicated, statistical comparisons were carried out using Student's *t*-test.

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Abbreviations: BAT, brown adipose tissue; UCP1, uncoupling protein-1; UCP2, uncoupling protein-2; IGF-1, insulin-like growth factor 1; PPAR, peroxisome proliferator-activated receptor; 9-*cis* RA, 9-*cis* retinoic acid; T_3, triiodothyronine; all-*trans* RA, all-*trans* retinoic acid; COII, cytochrome *c* oxidase subunit II; 15d-PGJ2, 15-deoxy-Δ-12,14-prostaglandin J2; RAR, retinoic acid receptor; RXR, retinoid X receptor

PII: S0014-5793(98)01598-1

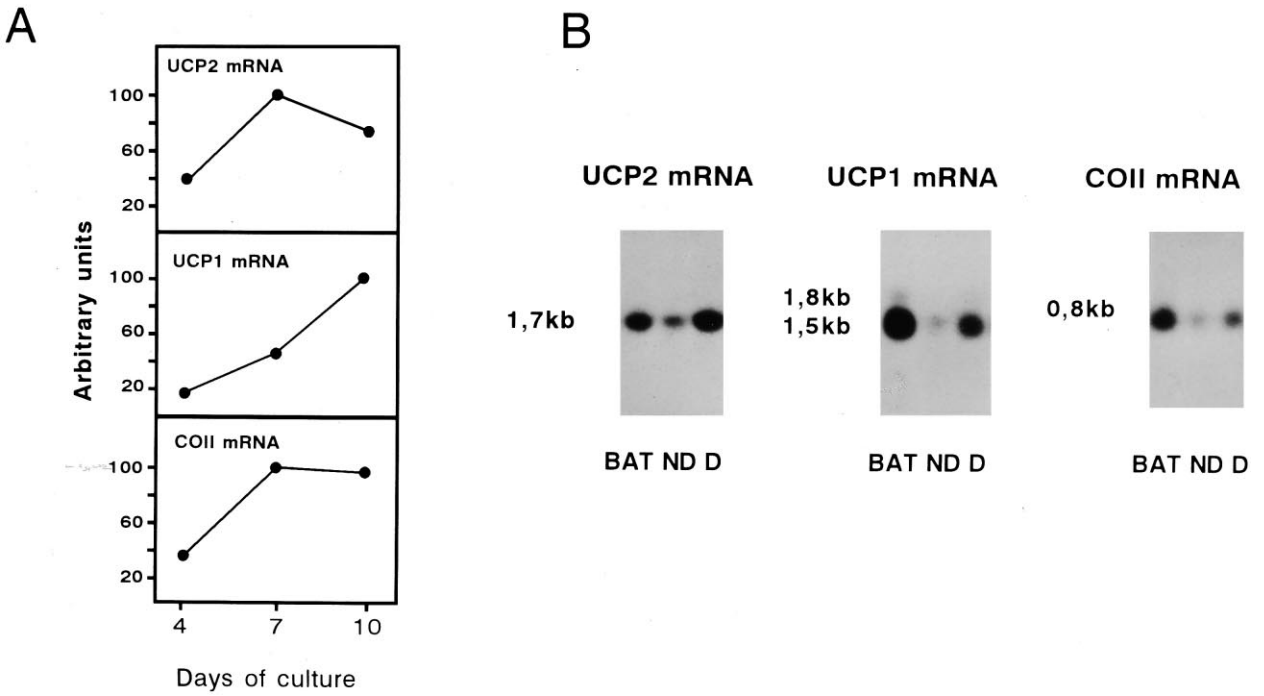

Fig. 1. Changes in the expression of UCP2 mRNA during brown adipocyte differentiation in primary culture. RNA (10 μg) was extracted from cells or tissue and analyzed by Northern blot. A: Points are means of two samples each one from triplicate plates and differing less than 15% for each stage of culture of stromal vascular cells. Data are expressed as percentages relative to the mean value at the developmental time of maximum levels of expression which was set at 100 (arbitrary units). B: Representative Northern blot analysis of equal amounts of RNA (10 μg/lane) from interscapular BAT (BAT), brown adipocytes differentiated in culture (D) (day 10) or cells in which differentiation was blocked (ND) (day 10) because of replacement of regular culture medium with depleted medium (see Section 2 for details).

3. Results

3.1. Differentiation-dependent regulation of UCP2 mRNA expression in brown adipocytes

Fig. 1A shows the profile of expression of UCP2 mRNA in brown adipocytes differentiating from stromal vascular cells obtained from BAT depots of mice. In the conditions of culture used, precursor cells showed non-adipocyte morphology on day 4, they reached confluence and showed the first signals of adipocyte differentiation (accumulation of lipid droplets in cells) on day 7, and they appeared fully differentiated on day 10, when more than 90% of cells showed lipid accumulation. UCP2 mRNA was induced from day 4 (non-differentiated cells) to day 7 (early differentiated cells) of culture, whereas it declined thereafter (day 10, late differentiated cells). When compared with UCP1 mRNA, UCP2 mRNA expression followed an earlier profile of induction. Thus, whereas maximal induction of UCP1 mRNA was observed in terminally differentiated brown adipocytes (day 10), the highest UCP2 mRNA levels were attained earlier, at the beginning of the differentiation process (day 7). The profile of expression of UCP2 mRNA was much more similar to the pattern of induction of COII mRNA, a molecular marker of mitochondrial biogenesis. When precursor cells were cultured for 10 days in a hormone-depleted medium, adipocyte differentiation was blunted and UCP2 mRNA expression was not induced as observed for UCP1 mRNA or COII mRNA (see Fig. 1B). On the other hand, and in contrast to UCP1 mRNA levels, which are lower in brown adipocytes than in BAT, UCP2 mRNA abundance in differentiated brown adipocytes was even higher than that present in the tissue.

3.2. Effects of hormonal agonists on UCP2 mRNA expression in differentiated brown adipocytes: 9-cis RA is a major activator of UCP2 gene expression

Different hormonal agents that are known to influence brown adipocyte thermogenic function or UCP1 gene expression were tested to assess their potential involvement in the regulation of the UCP2 gene. Densitometry analysis of the changes in UCP2 mRNA levels caused by these agents is

Table 1
Effects of hormonal agonists on UCP2 mRNA levels in brown adipocytes in culture

Agonist	Fold induction of UCP2 mRNA levels
Noradrenaline	1.8 ± 0.4
Insulin	1.5 ± 0.2
IGF-1	1.6 ± 0.4
T_3	1.3 ± 0.3
Leptin	2.2 ± 0.2*
PPAR agonists	
LY171883	3.1 ± 0.4*
WY 14,643 10 μM	1.2 ± 0.2
WY 14,643 100 μM	3.4 ± 0.5*
15dPGJ2	2.1 ± 0.2*
All-*trans* RA	1.6 ± 0.4
9-*cis* RA	4.4 ± 0.3**

Differentiated brown adipocytes (day 9 of culture) were exposed to 10 nM insulin, 10 nM IGF-1, 2 nM T_3, 30 nM leptin, 30 μM LY171883, 10 μM or 100 μM WY 14,643, 1 μM all-*trans* RA or 1 μM 9-*cis* RA for 24 h or to 0.5 μM noradrenaline or 10 μM 15d-PGJ2 for 6 h. Changes in UCP2 mRNA levels, expressed as fold induction with respect to untreated controls, are shown as means ± S.E.M. of at least three independent experiments. Statistically significant differences: *$P < 0.05$, **$P < 0.01$.

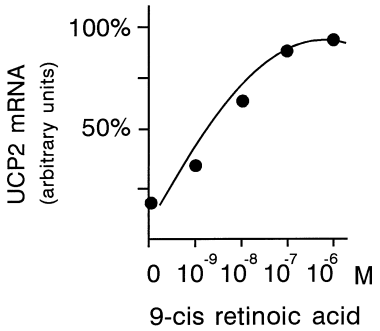

Fig. 2. Dose-response curve for the effect of 9-*cis* RA on UCP2 mRNA expression. Brown adipocytes differentiated in culture from stromal vascular cells (day 9) were exposed to the indicated concentrations of 9-*cis* RA for 24 h. Points are means from at least two independent experiments with triplicate plates.

shown in Table 1. Neither norepinephrine, insulin, IGF-1 nor T_3 significantly modified UCP2 mRNA levels in brown adipocytes. Leptin caused a modest but significant increase. Treatment of cells with several agonists of PPAR had significant effects. LY171883 or 100 μM WY 14,643, non-subtype-specific PPAR activators [21,22], caused a significant induction of UCP2 mRNA levels, whereas 10 μM WY 14,643, a concentration specific for PPARα activation [21], had no effect. 15d-PGJ2, a natural agonist of PPARγ [23], caused a modest but significant induction. Retinoids had very different effects: whereas all-*trans* RA did not affect UCP2 mRNA levels, 9-*cis* RA caused a marked increase and led to the highest effect on UCP2 gene expression in brown adipocytes. Dose-response effects of 9-*cis* RA on UCP2 mRNA expression are depicted in Fig. 2 and show a maximum of activation when brown adipocytes were exposed to 1 μM 9-*cis* RA, similarly to other previously characterized target genes for 9-*cis* RA action [24].

3.3. *Effects of retinoic acid receptor (RAR)- and retinoid X receptor (RXR)-specific agonists on UCP2 mRNA expression in brown adipocytes*

To assess the involvement of the two potential receptors of 9-*cis* RA action, brown adipocytes were exposed to either synthetic retinoids or naturally occurring substances specific for RAR or RXR. Treatment of brown adipocytes with TTNPB or Am80, specific for RAR [25,26], did not affect UCP2 mRNA levels (Table 2). In contrast, synthetic (methoprene) [27] or naturally occurring (phytanic acid) [28] specific activators of RXR caused significant rises in UCP2 mRNA abundance.

Table 2
Effects of RAR-specific or RXR-specific agonists on UCP2 mRNA levels in brown adipocytes in culture

Agonist	Fold induction of UCP2 mRNA levels
RAR-specific	
TTNPB	1.2 ± 0.3
Am80	0.9 ± 0.1
RXR-specific	
Methoprene	2.9 ± 0.4*
Phytanic acid	3.1 ± 0.4*

Differentiated brown adipocytes (day 9 of culture) were exposed for 24 h to 10 μM TTNPB, 10 μM Am80, 100 μM methoprene or 20 μM phytanic acid. Changes in UCP2 mRNA levels, expressed as fold induction with respect to untreated controls, are shown as means ± S.E.M. of at least three independent experiments. Statistically significant differences: $*P < 0.05$.

4. Discussion

The expression of the UCP2 gene shows a differentiation-dependent regulation in the brown fat cell. The profile of expression of the UCP2 gene in differentiating brown adipocytes is clearly dissociated from the pattern of induction of UCP1 gene expression, as the maximal induction of UCP2 gene expression occurs earlier than maximal UCP1 mRNA expression. However, the expression of the UCP2 gene appears to be a component of the differentiated phenotype of the brown adipocyte, as cells that were not allowed to differentiate showed minimum levels of UCP2 gene expression. The parallelism between UCP2 mRNA and COII mRNA profiles of induction strongly suggests that the UCP2 gene may be regulated in coordination with the mitochondrial biogenesis that occurs during the differentiation of the brown adipocyte [29]. Similar conclusions have been reached in previous studies on UCP2 mRNA expression during brown fat prenatal ontogeny [18]. Moreover, the finding that, in contrast to UCP1 mRNA, UCP2 mRNA is not down-regulated in cultured cells with respect to the tissue further indicates a differential regulation of the two genes.

The differential regulation of the UCP2 gene was further established by the analysis of sensitivity of its expression to hormonal stimuli. Powerful activators of UCP1 gene expression such as noradrenaline, insulin or IGF-1 had no effect on UCP2. The present data on lack of stimulus of UCP2 gene expression by noradrenaline are consistent with data in vivo, in which treatment with β-adrenergic agents did not affect UCP2 mRNA expression in brown fat [30]. Moreover, leptin caused a significant but modest increase in UCP2 mRNA levels consistent with previous findings in other cell systems [13]. Activators of PPAR also caused a rise in UCP2 mRNA levels, but only when PPARγ was activated. This indicates that this subtype of PPAR is involved in UCP2 gene regulation, whereas the PPARα subtype is not, even though it is highly expressed in the brown fat cell. However, of all the agents tested, 9-*cis* RA acid was capable of the highest induction of UCP2 gene expression in brown adipocytes.

In 1995, all-*trans* RA was reported to be a powerful inducer of UCP1 gene expression [10], and activation of RAR and RXR receptors was described to mediate this effect [10,31]. The present study identifies another retinoid, 9-*cis* RA, as a major regulator of the expression of UCP2. Retinoids affect gene expression through the activation of RAR or RXR receptors that act as ligand-dependent transcription factors. All-*trans* RA activates RAR receptors when they heterodimerize with RXR. In contrast, 9-*cis* RA can activate RAR but also RXR when it forms homodimers or heterodimers with other members of the nuclear receptor superfamily [32]. The fact that 9-*cis* RA was a strong activator of UCP2 while all-*trans* RA was not suggests that RXR is the receptor mediating the 9-*cis* RA effect and that RAR is not involved in the regulation of UCP2 gene expression. This was confirmed by the use of non-isomerizable, specific agonists of RAR and RXR. The lack of effect of all-*trans* RA on UCP2 may be due to the characteristics of the UCP2 gene regulatory region, since differentiated brown adipocytes express RAR as well as RXR

[31]. On the other hand, the fact that activation of RXR induces UCP2 gene expression and that PPAR activators are also positive effectors suggests that UCP2 gene may be under the control of transcription factors composed of heterodimers of PPAR and RXR. Several genes have been reported to be regulated by these heterodimers in which both partners, PPAR and RXR, retain the sensitivity to activation by their respective ligands [33]. However, the presence of RXR homodimer-dependent gene regulatory elements in the UCP2 gene, irrespective of the presence of PPAR-dependent regulatory elements, cannot be ruled out. Only the analysis of the UCP2 gene regulatory region will allow the identification of the precise molecular mechanisms by which 9-*cis* RA induces the transcription of this gene.

Retinoids exert powerful effects on differentiation and gene expression in adipose tissues [34]. The strong stimulatory action of 9-*cis* RA on UCP2 gene expression, together with previous findings on up-regulation of the UCP1 gene by all-*trans* RA, indicates that vitamin A derivatives may influence energy expenditure in mammals and opens new potential strategies in the development of molecules for the treatment of body weight disturbances.

Acknowledgements: We are grateful to Dr. I. Levine (Hoffman-LaRoche, USA) for 9-*cis* RA, to Dr. E.M. Gutknecht and Dr. P. Weber (Hoffman-LaRoche, Switzerland) for TTNPB and to Dr. K. Shudo (University of Tokyo, Japan) for Am80 as well as to Dr. N. Glaichenhaus (University of Nice, France) and to Dr. D. Ricquier (CEREMOD, CNRS, France) for the COII and UCP1 probes, respectively. This work was supported by DGICYT, Ministerio de Educación y Cultura (Grant PB95-0969) and Generalitat de Catalunya (Grant 97SGR-187).

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