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Retinoic acid modulates retinoid X receptor α and retinoic acid receptor α levels of cultured brown adipocytes

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Abstract A novel potential regulatory pathway of brown adipose tissue (BAT) thermogenesis was recently recognized after identifying retinoic acid (RA) as a transcriptional activator of the uncoupling protein (UCP) gene. Here we provide evidence that the UCP responsiveness to RA in primary cultures of brown adipocytes involves RA receptor α (RARα), and show, in the same system and also in CHO cells, that RA down-regulates the steady-state levels of RARα and especially of retinoid X receptor α, suggesting autoregulation of the retinoid pathway and therefore supporting the idea of a physiological role for it in controlling the thermogenic capacity of BAT.

Key words: Uncoupling protein; Retinoic acid receptor α; Retinoid X receptor α; Retinoic acid; Cultured brown adipocytes

1. Introduction

The uncoupling protein (UCP) or thermogenin is an inner mitochondrial membrane protein characteristically expressed in mammalian brown adipocytes that functions as a proton channel, thus uncoupling the activity of the respiratory chain from ATP synthesis [1,2]. It constitutes the molecular machinery for brown adipose tissue (BAT) thermogenesis, which is activated in response to cold exposure or to excessive caloric intake [3]. Norepinephrine, acting via cAMP, appears as the main regulator of UCP synthesis and activity [4,5], as well as of BAT recruitment [6]. Recently, however, a novel potential regulatory pathway of brown fat thermogenesis was recognized involving the natural vitamin A derivative retinoic acid (RA). It was shown that RA stimulates UCP mRNA accumulation in immortalized brown adipocytes [7] and in primary cultures of brown adipocytes [8], as well as the appearance of mature UCP not only in in vitro cell culture systems but also in BAT of whole mice [9], thus suggesting a physiological role of RA in regulating the thermogenic capacity of BAT. The nucleotide sequence conferring RA responsiveness to the UCP gene has been recently identified and characterized as a novel complex RA response element [10,11].

Cellular responses to retinoids are generally mediated by two families of nuclear receptors that belong to the steroid-thyroid hormone receptor superfamily and behave as ligand-activated transcription factors that bind as dimers to the *cis*-acting response elements of target genes (reviewed in [12]). The retinoic acid receptors (RARs) are activated by both all-*trans*-RA (tRA) and 9-*cis*-RA, whereas the retinoid X receptors (RXRs) only show binding affinity for 9-*cis*-RA. So far, three main isotypes of RARs (α, β and γ) and RXRs (α, β and γ) have been recognized in mammalian tissues. RAR/RXR heterodimers appear to preferentially transduce the retinoid signal in vivo [12]; RXRs also form heterodimers with a number of other hormone receptors, such as thyroid hormone receptor, vitamin D_3 receptors, and peroxisome proliferator activated receptors [12].

As happens with most RA-responsive genes, RAR/RXR heterodimers seem to mediate the responsiveness of the UCP gene to RA. This was suggested by transfection experiments showing that ectopic co-expression of both RARα and RXRα maximally enhanced the RA responsiveness of the rat UCP gene promoter when introduced into cells devoid of endogenous RXRα [8], and by electrophoretic mobility shift assay experiments showing that the RA-responsive sequence of the rat UCP gene is able to support the formation of heterodimers between different isotypes of RAR (α, β, and γ) and RXRα, but not of homodimers of these receptors [10].

Here, we provide evidence that the UCP responsiveness to RA in primary cultures of brown adipocytes involves RARα, and show that RA modulates the steady-state levels of both RARα and RXRα.

2. Materials and methods

2.1. Materials

(±)Arterenol bitartrate salt (noradrenaline) and tRA were from Sigma (Spain). RO 41-5253 was a generous gift of Roche S.A. (Spain). Rabbit polyclonal antisera against RARα (F region) and RXRα (A region) were provided by IGBMC (Illkirch, France). Newborn calf serum and Dulbecco's modified Eagle's medium (DMEM) were obtained from ICN (Spain) and human insulin (Actrapid) from Novo Industries (Denmark). F12 medium and fetal bovine serum were from Gibco BRL (Paisley, UK). Other cell culture reagents were supplied by Sigma, and routine chemicals were from Sigma (Spain), Merck (Spain) and Panreac (Spain).

2.2. Primary cell culture

BAT precursor cells from 4-week-old mice were prepared, inoculated and cultured as earlier described [13], in 6-well multidish plates. The culture medium consisted of DMEM supplemented with 10% newborn calf serum, 4 nM insulin, 10 mM HEPES and 126 mM

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Abbreviations: BAT, brown adipose tissue; UCP, uncoupling protein; RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; tRA, all-*trans*-RA; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ECL, enhanced chemiluminescence; CHO cells, Chinese hamster ovary cells

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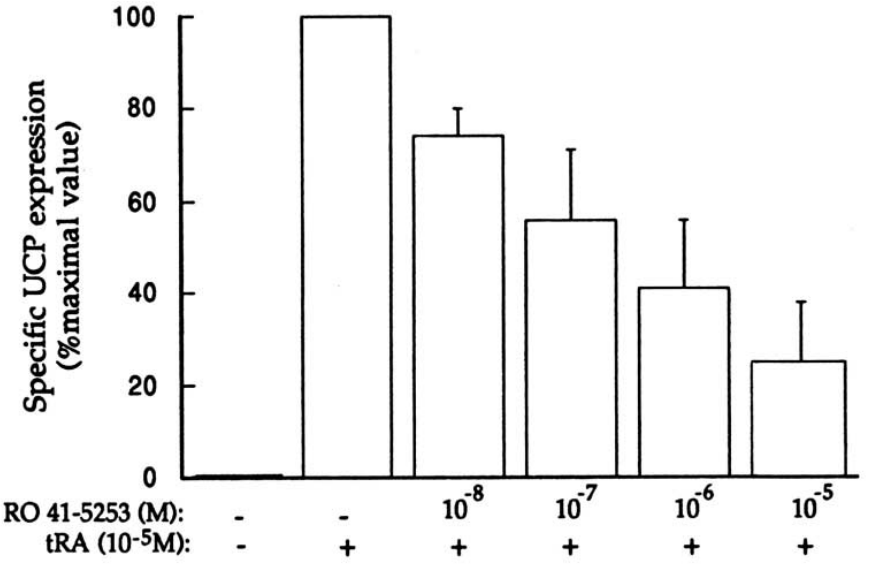

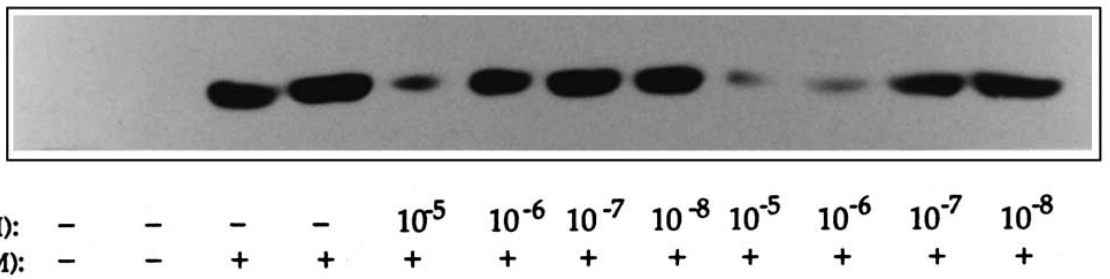

Fig. 1. Effect of RO 41-5253 on the tRA-induced appearance of UCP in cultured primary brown adipocytes. Cells were treated with a single dose of tRA 10^{-5} M alone or with the indicated concentrations of RO 41-5253, for a period of 48 h, from day 5 to day 7 of culture, before being harvested. The UCP content of cell lysates was quantified by densitometric scanning of immunoblots. Results are expressed relative to the specific UCP content of cells treated with tRA only (maximal value) and are the mean ± S.E.M of 4 experimental data. A representative immunoblot is shown (100 μg of cellular lysate protein were run per lane). Control (C), untreated culture.

sodium ascorbate, and with 50 units of penicillin and 50 mg of streptomycin per ml. Cells were grown at 37°C in an atmosphere of 8% CO_2 in air. The culture medium was changed on the first and the third day after inoculation. The cells were treated with tRA, alone or in combination with different concentrations of RO 41-5253, at the times and doses indicated in the figure legends, after changing the culture medium. Cells were harvested at confluence (day 7); the culture medium was removed and the cells rinsed twice with ice-cold phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer, pH 7.4) and then scraped into 1.5 ml of PBS and transferred to an Eppendorf tube. The cell suspensions were pelleted, resuspended in a minimal volume of PBS containing PMSF (0.2 mM final concentration) and stored at −20°C prior to sonication and analysis.

2.3. Cell lines, plasmids and transfections

CHO K1 cells were grown on 100 mm diameter plates in F12 medium supplemented with 10% fetal bovine serum, at 37°C and 8% CO_2. They were treated with a single dose of tRA of 10^{-5} M dissolved in ethanol when 50–70% confluent, and further incubated for a period of 20 h before being harvested; control cells received ethanol only. Transient transfection of 50–70% confluent CHO K1 cells was by the $CaPO_4$ precipitation method [14], with 20 μg of the expression plasmid pSV•sport1hRXRα [15], which contains an *Eco*RI fragment comprising the full translation reading frame of human RXRα cloned into the *Eco*RI site of pSV•sport1. One milliliter of $CaPO_4$ precipitate was added to each plate. After 4 h, the cells were shocked with glycerol, washed twice with PBS, refed with 8 ml of fresh medium supplemented or not with tRA 10^{-5} M, and further incubated for 20 h before harvesting. Harvesting and preparation of cell lysates were as described above. All experiments were performed in triplicate.

2.4. Parameters determined

Protein was determined by the method of Bradford [16]. UCP, RARα and RXRα were determined by immunoblotting. Proteins (70–100 μg) from whole cell lysates were fractionated by SDS-PAGE (10% polyacrylamide) according to Laemmli [17] and electrotransferred onto a nitrocellulose filter as described in [18]. For UCP determination, rabbit polyclonal antisera raised against purified rat UCP obtained in our laboratory [18] was used as primary antibody. Procedures for obtaining the UCP antiserum are described elsewhere [18]; its specificity for UCP (33 kDa) was confirmed by immunoblotting of BAT homogenate proteins, liver homogenate proteins and purified UCP. RARα and RXRα were detected using rabbit polyclonal antibodies raised against synthetic peptides corresponding to cDNA-deduced amino acid sequences of mouse RARα1 (F region) and RXRα (A region), respectively; these antibodies were previously

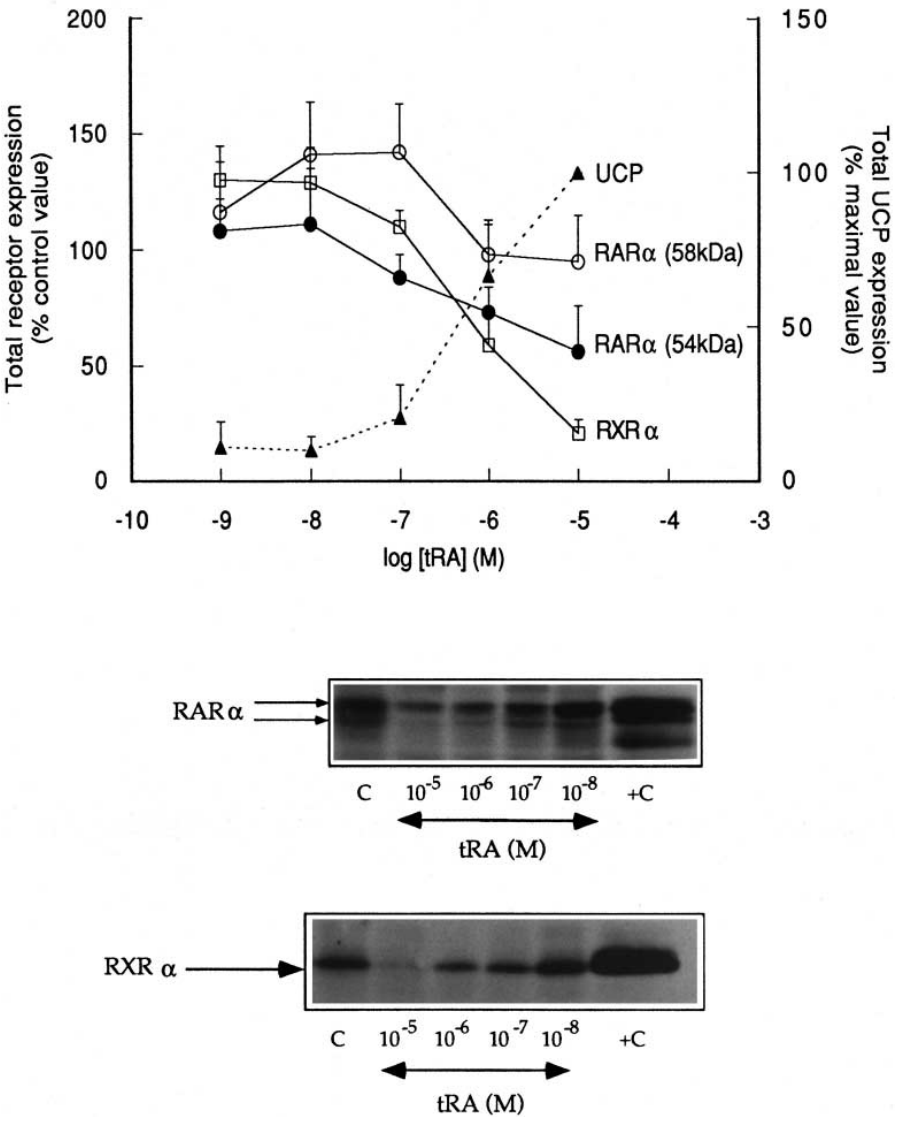

Fig. 2. Effect of tRA on the levels of RXRα, RARα and UCP of cultured primary brown adipocytes. A single dose of tRA (10^{-5}–10^{-9} M) was added to the cultures on day 5. Cells were harvested on day 7 and the RXRα, RARα and UCP contents of cell lysates quantified by densitometric scanning of the corresponding immunoblots. RXRα and RARα data are expressed relative to the corresponding levels in control cultures not treated with tRA; UCP data are expressed relative to the maximal expression attained. The results are the mean ± S.E.M. of 6 (RXRα and RARα results) or 3 (UCP results) independent experiments. Immunoblots for RARα and RXRα: 50 μg of cellular lysate protein were run per lane; C, control untreated culture; +C, liver extract (70 μg protein) that served as positive control.

determined to be specific for various species of RARα (of ~58, ~54 and ~51 kDa) and for a single species of RXRα (~54 kDa), respectively, in different cell systems and tissues [19,20]. Further confirmation of the RXRα band was obtained by transfecting CHO cells with the corresponding gene cloned into an expression plasmid (see Section 2.3). Blocking and development of the immunoblots were performed using an ECL Western blotting analysis system (Amersham), with horseradish peroxidase-coupled donkey anti-rabbit IgG as second antibody, and following the instructions of the manufacturer. The films used were Kodak X-Omat AR films. For quantitative analysis, films were scanned with a BioImage computing densitometer (Millipore).

3. Results and discussion

To further define the RAR involved in the endogenous UCP responsiveness to RA, we evaluated in tRA-stimulated primary cultures of brown adipocytes the effect of the selective RARα antagonist RO 41-5253, which binds to RARα preventing activation by RA [21]. As shown in Fig. 1, RO 41-5253 inhibited the tRA-induced appearance of UCP in a dose-dependent manner, strongly suggesting that in our system the effect of RA on UCP appearance occurs through RARα. In HIB cells – a mouse cell line derived from a brown fat hibernoma – RARγ-RXRα heterodimers have been claimed to mediate UCP responsiveness to RA on the basis of supershift assay experiments showing that anti-RXRα and anti-RARγ antibodies, but not anti-RARα or anti-RARβ, are capable to supershift the RA-responsive sequence of the rat UCP gene retarded by nuclear extracts of these cells [10]; however, the authors did not exclude the possibility that other receptors also participate. Studies at the RNA level have revealed that, in the mouse, RARα is the most ubiquitously distributed RAR isotype, while RARβ and RARγ display a more restricted pattern of distribution [12].

Transcription of some RAR genes themselves as well as of

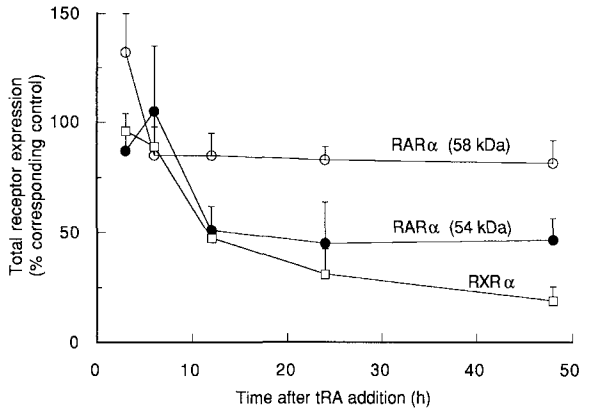

Fig. 3. Time-course of the decrease of RARα and RXRα levels elicited by tRA 10^{-5} M in cultured primary brown adipocytes. A single dose of tRA 10^{-5} M was added to the cells on day 5. Control (untreated) and tRA-treated cells were harvested 3, 6, 12, 24 and 48 h after addition of tRA. RXRα and RARα contents of cell lysates were quantified by densitometric scanning of immunoblots. Data are expressed relative to the total RARα or RXRα content of the corresponding time control cultures (100%), and are the mean ± S.E.M. of 6 independent experiments.

certain proteins involved in the storage, transport and/or metabolism of retinol and RA have been shown to be RA-sensitive [21–23]. Therefore, we investigated in our primary cell culture system the effect of tRA on the relative steady-state levels of RARα and RXRα, by means of immunoblotting (Fig. 2). The two receptors were present in cultured primary brown fat cells, consistent with their endogenous responsiveness to RA. RXRα appeared as a single band corresponding to a protein with an apparent molecular weight of ~54 kDa, in agreement with the predicted molecular weight of mouse RXRα and with previous reports making use of the same antibody preparation [20]; we further identified this band as RXRα by means of transfection experiments in which ectopic expression of RXRα was forced in CHO cells (see Fig. 4). In the case of RARα, the antibodies used were previously reported to specifically detect at least three different RARα proteins species of ~58, ~54 and ~51 kDa in mouse embryos and various adult mouse tissues [19]; in our primary cultures, we always recognized the 58 kDa species and a slightly faster migrating band of about 54 kDa that displayed a characteristic pattern of variation in response to tRA. In most control cultures (not treated with tRA), the latter species was about 50% (±7%, n=8) less abundant than the 58-kDa species, as judged from the relative intensities of the corresponding bands; however, and for unknown reasons, some cultures showed a quite different pattern, with the low molecular weight RARα species being the most abundant.

Treatment of brown adipocytes in primary culture with tRA for a period of 48 h elicited characteristic dose-dependent changes in the steady-state levels of both RXRα and RARα (Fig. 2). Low tRA concentrations (10^{-9}–10^{-7} M) slightly up-regulated the receptor levels. At the highest tRA concentrations tested (10^{-6} and 10^{-5} M), however, both RXRα and the low molecular weight RARα species were consistently down-regulated; down-regulation was especially marked for RXRα, with almost negligible levels at 10^{-5} M tRA. Certain down-regulation of the 58-kDa RARα species at high tRA concentrations was also observed in some individual cultures, but not all (Fig. 2 and immunoblot therein). Fig. 3 shows the time-course of the down-regulation of RXRα and RARα levels elicited by a single dose of tRA of 10^{-5} M added on day 5 of culture. The effect was clear after 24 h, and persisted after 48 h.

The effect of tRA on RARs and RXRs levels is not restricted to brown adipocytes. Thus, we have found that CHO cells express RARα (58 kDa) and RXRα (54 kDa), and that the levels of both receptors are lowered after tRA treatment, especially those of RXRα (Fig. 4). Treatment of confluent 3T3-L1 adipose cells with tRA 10^{-6} M for a period of 24 h was reported to cause a reduction of the RXRα mRNA levels [24]. Those are the kind of results to be expected if down-regulation reflects the existence of negative RA response elements in the corresponding murine gene promoters. However, we have found that ectopic expression of RXRα driven by a constitutive heterologous promoter is reduced in CHO cells upon tRA treatment to an extent that cannot be explained by the mere reduction of the endogenous RXRα levels (Fig. 4), suggesting that down-regulation could as well be the result of a non-transcriptional regulatory mechanism.

Clearly, further studies are needed to elucidate the mechanism(s) underlying the tRA-induced down-regulation of RXRα protein/mRNA levels in the different cell systems in which it has been reported (primary cultures of brown adipocytes, CHO cells, 3T3-L1 cells). As pointed out by Kawada et al. [24], down-regulation of RXRα would have profound implications in the cross-talk of the various signaling pathways

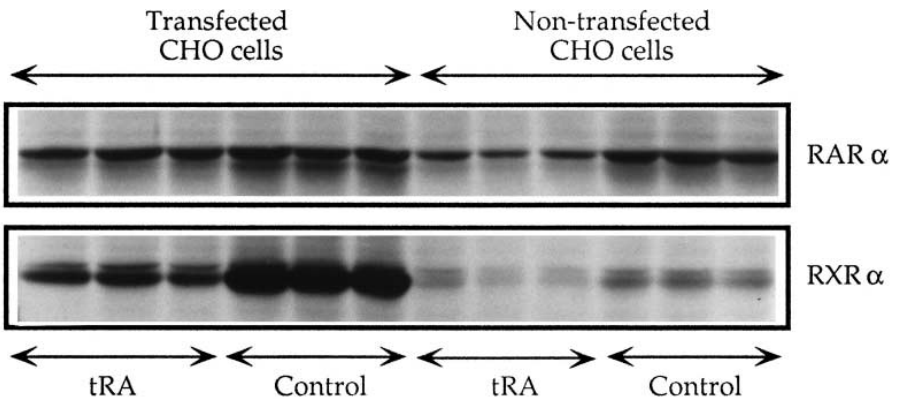

Fig. 4. Effect of tRA on RARα and RXRα levels of control and pSV•sport1hRXRα transiently transfected CHO K1 cells. Cells were cultured, transfected and treated with tRA 10^{-5} M as described in Section 2. 100 μg of cell lysate protein was run per lane.

that use RXRα as a partner in heterodimer formation: lower levels of RXRα would imply competition between the various possible heterodimer partners for the RXRα available.

In our previous report on the effect of RA on brown adipocytes, maximal induction of UCP appearance was attained after treatment of primary cultures with tRA 10^{-6}–10^{-4} M [9], and this result has been reproduced in the present experiments (Fig. 2). Therefore, tRA apparently affects in opposite directions both the activity of pre-existing receptors as transcription factors and the steady-state levels of those receptors. The fact that the same tRA concentrations that effectively induce UCP appearance, presumably through RAR/RXR heterodimers, down-regulate the steady-state levels of two likely heterodimer partners suggests an autoregulated system and thus reinforces the idea of a physiological role of RA in controlling the thermogenic capacity of BAT.

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