Both retinoic-acid-receptor- and retinoid-X-receptor-dependent signalling pathways mediate the induction of the brown-adipose-tissue-uncoupling-protein-1 gene by retinoids

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The intracellular pathways and receptors mediating the effects of retinoic acid (RA) on the brown-fat-uncoupling-protein-1 gene (*ucp-1*) have been analysed. RA activates transcription of *ucp-1* and the RA receptor (RAR) is known to be involved in this effect. However, co-transfection of an expression vector for retinoid-X receptor (RXR) increases the action of 9-*cis* RA but not the effects of all-*trans* RA on the *ucp-1* promoter in brown adipocytes. Either RAR-specific {*p*-[(*E*)-2-(5,6,7,8,-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid} or RXR-specific [isopropyl-(*E*,*E*)-(*R*,*S*)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate, or methoprene] synthetic compounds increase the expression of UCP-1 mRNA and the activity of chloramphenicol acetyltransferase expression vectors driven by the *ucp-1* promoter. The RXR-mediated action of 9-*cis* RA requires the upstream enhancer region at $-2469/-2318$ in *ucp-1*. During brown-adipocyte differentiation RXRα and RXRγ mRNA expression is induced in parallel with UCP-1 mRNA, whereas the mRNA for the three RAR subtypes, α, β and γ, decreases. Co-transfection of murine expression vectors for the different RAR and RXR subtypes indicates that RARα and RARβ as well as RXRα are the major retinoid-receptor subtypes capable of mediating the responsiveness of *ucp-1* to retinoids. It is concluded that the effects of retinoids on *ucp-1* transcription involve both RAR- and RXR-dependent signalling pathways. The responsiveness of brown adipose tissue to retinoids *in vivo* relies on a complex combination of the capacity of RAR and RXR subtypes to mediate *ucp-1* induction and their distinct expression in the differentiated brown adipocyte.

Key words: adipocyte, energy metabolism, mitochondria, transcription, vitamin A.

INTRODUCTION

Brown adipocytes contain the uncoupling protein 1 (UCP-1), a unique component of the mitochondrial inner membrane that acts as a natural uncoupler of oxidative phosphorylation and provides a molecular mechanism for adaptive thermogenesis [1]. The action of noradrenaline on *ucp-1* transcription is considered to be a major mechanism for adaptive changes in brown-fat thermogenic capacity in response to sympathetic nervous system activity [2]. However, retinoic acid (RA) has been identified as a novel transcriptional activator of *ucp-1* [3]. RA is a vitamin-A derivative with powerful effects on gene expression in different cell types, including adipose cells [4]. The identification of RA as an activator of *ucp-1* transcription may explain changes in brown-fat *ucp-1* expression associated with ontogeny and cell differentiation that are not attributable to adrenergic regulation [5].

The mechanisms by which retinoids affect gene expression are complex. RA acts through two kinds of retinoid receptor, the RA receptor (RAR) and the retinoid-X receptor (RXR), members of the nuclear-hormone-receptor superfamily. Moreover, two active isomers of RA, all-*trans* RA and 9-*cis* RA, influence gene transcription through distinct pathways. All-*trans* RA activates gene expression mainly through RAR/RXR heterodimers that bind to RA response elements and stimulate transcription due to the binding of all-*trans* RA to RAR (for review, see [6]). 9-*cis* RA can also activate the RAR component of RAR/RXR heterodimers but it can also activate gene expression by binding to RXR [7,8]. 9-*cis* RA activates transcription of genes containing regulatory elements capable of binding RXR homodimers or heterodimers of RXR and other receptors of the thyroid/retinoid-receptor family, such as peroxisome proliferator-activated receptors (PPARs) or orphan receptors [9,10]. Most of the cell types studied so far show a remarkable capacity to isomerize all-*trans* RA to 9-*cis* RA [11]. Both all-*trans* RA and 9-*cis* RA have been reported to activate the transcription of *ucp-1* [12,13].

It is considered that the structure and sequence of *cis* elements present in target genes determine their ability to bind a particular receptor dimer containing RAR and/or RXR moieties, and thus respond to a specific pathway of retinoid activation. Thus artificial constructs consisting of direct repeats of variants of the RGGTCA heptad spaced by 5 or 2 bp are characteristic of all-*trans* RA action through RAR/RXR-binding heterodimers [14]. 9-*cis* RA responsiveness is achieved in these constructs through the binding of RXR homodimers to *cis* elements containing single-bp spacing or other alignments that are permissive for the binding of heterodimers of RXR and orphan receptors [9,10]. The region of *ucp-1* responsible for responsiveness to retinoids is in the upstream enhancer of the gene and shows a complex arrangement of putative heptad repeats that, similarly to other

Abbreviations used: UCP-1, uncoupling protein 1; RA, retinoic acid; RAR, RA receptor; RXR, retinoid-X receptor; TTNPB, *p*-[(*E*)-2-(5,6,7,8,-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid; methoprene, isopropyl-(*E*,*E*)-(*R*,*S*)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate; COII, subunit II of cytochrome *c* oxidase; CAT, chloramphenicol acetyltransferase; PPAR, peroxisome proliferator-activated receptor; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium.

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naturally occurring promoters, do not strictly follow these simple rules [3,12,13]. Moreover, the presence of a PPARγ-responsive element in the *ucp-1* enhancer [15] that may be involved in RXR-mediated signalling has been reported.

An additional level of complexity in conveying responsiveness to retinoids occurs *in vivo* as a consequence of the expression of different subtypes of the retinoid receptor. At least three subtypes, α, β and γ, are found for both RAR and RXR [16,17]. Brown adipose tissue shows a differential pattern of expression of RAR and RXR subtypes with respect to other mammalian tissues, including white fat [18]. This suggests a specific role for these receptors in mediating the responsiveness of this tissue to the action of retinoids.

To date, pharmacological strategies to affect *ucp-1* expression have relied on the classical adrenergic regulation of this gene, but the discovery of *ucp-1* as a target of RA has opened a new perspective in this field. Understanding the pathways and receptors that mediate this effect in the brown-fat cell is essential for undertaking a potential pharmacological design involving retinoids to influence *ucp-1* expression. The goal of the present study was to establish the main intracellular pathways by which retinoids activate *ucp-1* transcription in the brown-fat cell. This has been undertaken by a combination of the following approaches: (i) the study of the action of RA isomers and receptor-specific retinoid derivatives on the expression of endogenous *ucp-1* and the transfected *ucp-1* promoter, (ii) the determination of the ability of RAR and RXR subtypes to convey the retinoid responsiveness of the *ucp-1* promoter and (iii) the establishment of the pattern of expression of RAR and RXR subtypes associated with brown-adipocyte differentiation and *ucp-1* expression.

EXPERIMENTAL PROCEDURES

Materials

All-*trans* RA, tri-iodothyronine and insulin were obtained from Sigma. 9-*cis* RA was a gift from Hoffman–LaRoche (Nutley, NJ, U.S.A.). Isopropyl-(*E*,*E*)-(*R*,*S*)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate (methoprene) was from Promochem (Wesel, Germany). *p*-[(*E*)-2-(5,6,7,8,-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) was a gift from Hoffman-LaRoche (Basel, Switzerland). [α-^{32}P]dCTP and D-threo-[1,2-^{14}C]chloramphenicol were from Amersham. Cell-culture media and fetal calf serum (FCS) were obtained from Whittaker.

Plasmids

The plasmid (-4551)UCP1-CAT, in which the region -4551 to $+110$ of rat *ucp-1* drives the promoterless chloramphenicol acetyltransferase (*CAT*) gene was a kind gift from D. Ricquier (CEREMOD, CNRS, Meudon, France). The derived plasmid ($\Delta-2469/-2283$)UCP1-CAT, in which the $-2469/-2283$ region of (-4551)UCP1-CAT had been deleted, and the plasmid ($-2469/-2318$)UCP1-CAT, in which the $-2469/-2318$ region had been placed upstream in (-172)UCP1-CAT, were described previously [3]. (-172)UCP1-CAT is the CAT expression vector driven by the region -172 to $+110$ in the *ucp-1* gene. The plasmid ($-2494/-2318$)UCP1-CAT was obtained by cloning the $-2494/-2318$ region (*Aat*II–*Xba*I fragment) upstream in (-172)UCP1-CAT. pRSV-RARα and pRSV-RXRα were expression vectors for the α-subtypes of human RAR [19] and RXR [20], respectively. Mammalian expression vectors that contained the α, β and γ subtypes of murine RAR and RXR, driven by the simian-virus-40 promoter, were provided kindly by P. Chambon (IGBMC, Illkirch, France) [21–24].

Cell culture

Primary culture of differentiated brown adipocytes was performed as described previously [3]. Precursor cells obtained from the interscapular, cervical and axillary depots of brown adipose tissue from Swiss mice were plated on 60-mm Petri dishes (7500 cells/cm^2) and grown in 5 ml of Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (1:1) supplemented with 10% FCS, 20 nM insulin, 2 nM tri-iodothyronine and 100 μM ascorbate. On day 7 of culture, 80–90% of the cells were considered to be differentiated on the basis of lipid accumulation and acquisition of brown-adipocyte morphology. CV-1 cells were cultured in DMEM supplemented with 10% FCS.

Transfection assays

Murine primary brown adipocytes differentiated in culture were transfected by the calcium phosphate-precipitation method on day 7 of culture [25]. Each transfection contained 7 μg of UCP1-CAT vector with or without 0.25 μg of the expression vector pRSV-RARα and/or pRSV-RXRα. RSV-β-galactosidase (2 μg) was included in all the experiments to assess the efficiency of separate transfections. After transfection, the cells were incubated for 24 h with or without the addition of retinoids or retinoid analogues at the concentrations indicated in each experiment. For each condition, at least three plates were pooled. The experiments were performed at least three times and independent plasmid-DNA preparations were tested for each experiment. CV-1 cells were transfected by calcium phosphate precipitation. Each transfection contained 5 μg of ($-2469/-2318$)UCP1-CAT, 0.05 μg of each expression vector for the α, β or γ subtypes of RAR and RXR and 2 μg of RSV-β-galactosidase. The cells were incubated for 38 h in DMEM without added serum. Analysis of CAT activity was performed as described in [26] and acetylation of [^{14}C]chloramphenicol was determined by TLC and quantified by radioactivity counting (AMBIS). The CAT activity was normalized using β-galactosidase activity as a standard.

RNA isolation and Northern-blot analysis

Total RNA was extracted using a guanidinium hydrochloride-based method [27]. For Northern-blot analysis, 20 μg of total RNA was denatured, electrophoresed on 1.5% formaldehyde/agarose gels and transferred to positively charged nylon membranes (N$^+$, Boehringer Mannheim). Ethidium bromide (0.2 μg/ml) was added to RNA samples in order to check equal loading of gels and transfer efficiency. Prehybridization and hybridization were carried out at 65 °C using a solution of 0.25 M Na_2HPO_4 (pH 7.2)/1 mM EDTA/20% SDS/0.5% blocking reagent (Boehringer Mannheim) [28]. Blots were hybridized to DNA probes corresponding to the cDNA for rat *ucp-1* [29], 0.5 kb of the cDNA for subunit II of cytochrome *c* oxidase (COII) [30] or the full-length cDNAs for murine RARα, RARβ, RARγ, RXRα, RXRβ and RXRγ [21–24]. The cDNA probes were labelled with [α-^{32}P]dCTP using the random oligonucleotide-primer method. Filters were washed under stringent conditions [20 mM Na_2HPO_4 (pH 7.2)/1 mM EDTA/1% SDS for 90 min at 55 °C] and exposed for autoradiography [28]. Blots were stripped and rehybridized sequentially as needed in each case. Autoradiographs were quantified by densitometric scanning (Pharmacia Biotech).

RESULTS

RXR potentiates 9-*cis* RA stimulation of the *ucp-1* promoter

The comparative effects of 9-*cis* RA and all-*trans* RA on *ucp-1* promoter were determined by transient transfections into brown-fat cells of (−4551)UCP1-CAT, the plasmid construct in which CAT is driven by the −4551/+110-bp non-coding region of the rat *ucp-1*. As shown in Table 1, results indicated that both 9-*cis* RA and all-*trans* RA activated (−4551)UCP1-CAT to a similar extent. However, a substantial difference was detected in the ability of co-transfected RAR and RXR to potentiate these effects. RAR co-transfection increased the responsiveness of the *ucp-1* promoter to all-*trans* RA and 9-*cis* RA. Conversely, co-transfection of the RXR expression vector did not modify the responsiveness of (−4551)UCP1-CAT to all-*trans* RA, whereas it enhanced the stimulatory action of 9-*cis* RA. The effects of co-transfection of equal amounts of RAR and RXR expression vectors were indistinguishable from the individual action of RAR and RXR in the presence of all-*trans* RA and 9-*cis* RA action.

Effects of the RAR-specific and RXR-specific agonists TTNPB and methoprene on UCP-1 mRNA expression and *ucp-1* promoter activity in brown adipocytes

In order to establish the involvement of ligand activation of RAR or RXR on the effects of retinoids on *ucp-1* expression, two synthetic ligands were used. TTNPB is a RAR-specific ligand for all RAR subtypes that, at micromolar concentrations, mimics the action of all-*trans* RA or 9-*cis* RA on RAR-mediated stimulation of gene expression [31]. Methoprene is a synthetic analogue of insect juvenile hormone that binds and activates RXR but not RAR [32] and has been used to assess the role of ligand activation of RXR in gene expression [33]. Brown adipocytes in culture were exposed to different concentrations of these compounds for 24 h and the effects on UCP-1 mRNA abundance were determined and compared with the effects of all-*trans* RA and 9-*cis* RA. TTNPB increased UCP-1 mRNA abundance in a dose-dependent manner (see Table 2). Methoprene was less effective, but at 100 μM, the concentration known to activate RXR [32], it caused a significant rise in UCP-1 mRNA expression. The effects of TTNPB and methoprene were specific for UCP-1 mRNA because the mitochondrially encoded COII mRNA was unaffected (see Figure 1).

The (−4551)UCP1-CAT plasmid was transfected into brown adipocytes and the effects of TTNPB or methoprene were determined, either in the absence of co-transfected receptors or in the presence of the co-transfected expression vectors for RAR or RXR. As shown in Table 3, a very different behaviour was observed when RAR or RXR was co-transfected. Co-transfection of the expression vector for RAR enhanced the response to

Table 1 Effects of all-*trans* RA and 9-*cis* RA on (−4551)UCP1-CAT expression in transiently transfected brown adipocytes: influence of RAR and RXR co-transfection

Brown adipocytes differentiated in culture (day 7) were transfected with 7 μg/plate of the (−4551)UCP1-CAT plasmid. When indicated, 0.25 μg of either the expression vector pRSV-hRARα (RAR) or pRSV-hRXRα (RXR) or an equivalent mixture of pRSV-RARα plus RXRα (RAR + RXR) was co-transfected. After transfection, cells were exposed or not to 1 μM all-*trans* RA or 1 μM 9-*cis* RA. Results are expressed as CAT activity relative to control, which is set to 1, and are means ± S.E.M. of three independent experiments, each performed in triplicate. Statistically significant differences ($P < 0.05$) between treated groups and controls are shown as *, and between 9-*cis* RA and all-*trans* RA as †.

Co-transfected receptor	Treatment	Relative CAT activity
–	–	1.0 ± 0.2
	All-*trans* RA	3.2 ± 0.5*
	9-*cis* RA	3.0 ± 0.2*
RAR	–	1.3 ± 0.2
	All-*trans* RA	9.6 ± 1.1*
	9-*cis* RA	5.8 ± 0.9*†
RXR	–	1.7 ± 0.2
	All-*trans* RA	2.9 ± 0.4
	9-*cis* RA	9.1 ± 0.7*†
RAR + RXR	–	2.7 ± 0.4
	All-*trans* RA	9.8 ± 1.3*
	9-*cis* RA	9.0 ± 1.0*

Table 2 Effect of TTNPB or methoprene on UCP-1 mRNA levels in brown adipocytes

Brown adipocytes differentiated in culture (day 7) were exposed to 1 μM all-*trans*-RA, 1 μM 9-*cis* RA or to the indicated concentrations of TTNPB or methoprene for 24 h. Untreated cells were used as a control. RNA (20 μg) was analysed by Northern-blot hybridization using the UCP-1 cDNA probe. Results were analysed by densitometric scanning and normalized using the hybridization signal obtained with the COII cDNA probe. Data are expressed relative to the untreated control (set to 1) and are means ± S.E.M. of three independent experiments. Statistically significant differences of treated cells versus control ($P < 0.05$) are shown as *.

Treatment	UCP-1 mRNA (arbitrary units)
–	1.0 ± 0.3
All-*trans* RA	7.8 ± 0.7*
9-*cis* RA	8.9 ± 1.1*
TTNPB (10 μM)	5.5 ± 0.6*
TTNPB (1 μM)	1.9 ± 0.4
Methoprene (100 μM)	4.7 ± 0.5*
Methoprene (10 μM)	1.2 ± 0.2

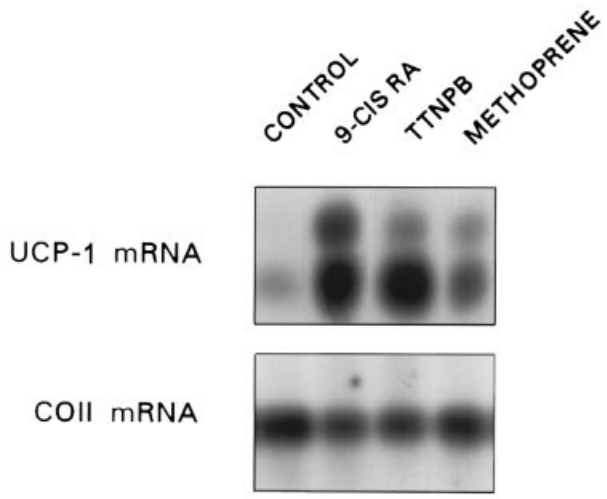

Figure 1 Representative Northern-blot analysis of the effects of TTNPB and methoprene on UCP-1 mRNA expression in brown adipocytes

Brown adipocytes differentiated in culture (day 7) were exposed to 1 μM 9-*cis* RA, 10 μM TTNPB or 100 μM methoprene for 24 h. Untreated cells were used as control. Three plates were pooled for each treatment and 20 μg of total RNA was analysed as described in the Experimental procedures section. The filters were hybridized first with the UCP-1 cDNA probe and thereafter with the COII cDNA probe.

Table 3 Effect of TTNPB or methoprene on (−4551)UCP1-CAT expression in transiently transfected brown adipocytes

Brown adipocytes differentiated in culture (day 7) were transfected with 7 μg/plate of (−4551)UCP1-CAT. When indicated, 0.25 μg of either the expression vector for pRSV-hRARα (RAR) or pRSV-RXRα (RXR) was co-transfected. After transfection, cells were exposed or not exposed to 10 μM TTNPB or 100 μM methoprene. Results are expressed as CAT activity relative to control, set to 1, and are means ± S.E.M. of three independent experiments, each done in triplicate. Statistically significant differences ($P < 0.05$) were shown as: *, treated groups versus the respective untreated controls; †, methoprene versus TTNPB.

Co-transfected receptor	Treatment	Relative CAT activity
–	–	1.0 ± 0.3
	TTNPB	1.8 ± 0.3
	Methoprene	1.9 ± 0.4
RAR	–	1.1 ± 0.3
	TTNPB	4.5 ± 0.6*
	Methoprene	2.0 ± 0.3†
RXR	–	2.0 ± 0.4
	TTNPB	2.4 ± 0.3
	Methoprene	6.1 ± 0.9*†

TTNPB, whereas the action of methoprene was unaffected. Conversely, co-transfection of the RXR expression vector did not cause any enhancement of TTNPB action but increased the effects of methoprene. These findings indicate that the effects of retinoids on *ucp-1* involve ligand activation of not only RAR but also RXR.

The −2469/−2318 region of *ucp-1* is required for the RXR-dependent stimulation of *ucp-1* promoter activity

Figure 2 shows the effects of 9-*cis* RA or methoprene on the expression of deletion mutants of the 5′ region of *ucp-1* driving the *CAT* reporter gene when co-transfected with the RXR expression vector. Results using the (Δ−2469/−2283)UCP1-CAT construct showed that the lack of upstream enhancer in *ucp-1* suppressed RXR-mediated activation by 9-*cis* RA or methoprene. A construct in which only the −2494/−2318 region was placed upstream of the proximal region of *ucp-1* was responsive to 9-*cis* RA and methoprene in the presence of co-transfected RXR. The shorter construct (−2469/−2318)UCP1-CAT, in which the PPARγ regulatory element at −2494 [15] had been deleted and which was insensitive to the thiazolidinedione PPARγ activators (results not shown), retained the RXR-mediated ligand activation (see Figure 2). This indicates that the RXR-mediated ligand activation of *ucp-1* does not require the PPARγ responsive element. Thus the −2469/−2318 region, previously found to contain the elements for RAR-mediated activation by RA [3], also contains the elements responsible for RXR-dependent ligand activation.

Expression of RAR and RXR receptors during brown-fat-cell differentiation

Considering that there are three subtypes, α, β and γ, for each of these receptor types, two different approaches were followed to examine the intracellular mechanisms that mediate the action of retinoids on *ucp-1* expression. First, expression of the α, β and γ subtypes of RAR and RXR was determined during the differentiation of brown adipocytes in culture. When stromal

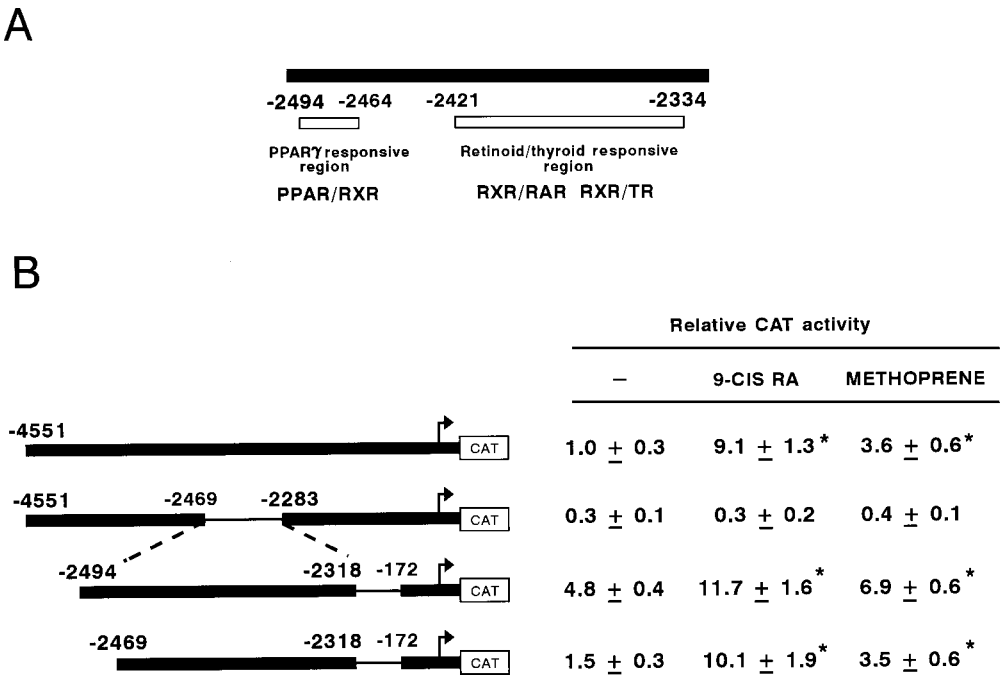

Figure 2 Effects of 9-*cis* RA and methoprene on the activity of UCP1-CAT deletion mutants in brown adipocytes co-transfected with RXR

(**A**) Schematic representation of the enhancer of *ucp-1* showing the two major regions of RXR binding: the PPAR-responsive region and the retinoid/thyroid-responsive region (data from [3,12,13,15]). (**B**) Brown adipocytes differentiated in culture (day 7) were transfected with 7 μg/plate of (−4551)UCP1-CAT or with corresponding amounts of the derived deletion mutations shown on the left. The expression vector pRSV-RXRα (0.25 μg) was co-transfected. After transfection cells were untreated or exposed to 1 μM 9-*cis* RA or 100 μM methoprene. Results are expressed as CAT activity relative to (−4551)UCP1-CAT, which is set at 1, and are means ± S.E.M. of three independent experiments, each one done in triplicate. Statistically significant differences ($P < 0.05$) with respect to untreated controls are shown by *.

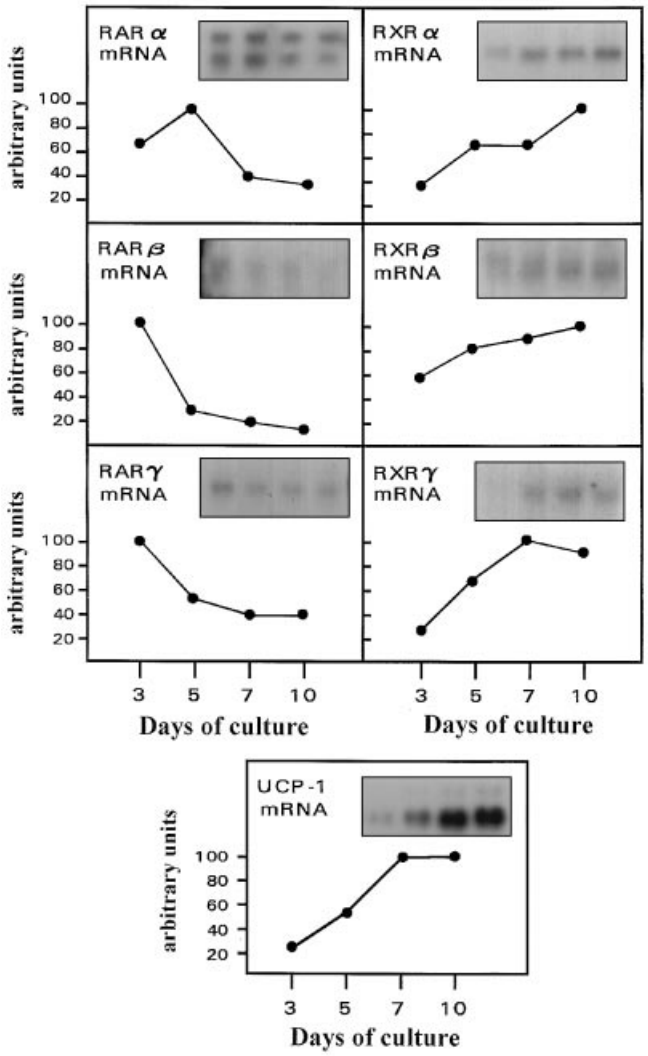

Figure 3 Expression of RAR and RXR subtypes during brown-adipocyte differentiation

Brown-adipocyte precursor cells were isolated and grown in culture for 3, 5, 7 or 10 days. Total RNA (20 μg) from pooled plates on each day of culture was analysed by the Northern-blot hybridization procedure described in the Experimental procedures section. Hybridizations were performed using the murine cDNAs for RARα, RARβ, RARγ, RXRα, RXRβ and RXRγ and rat UCP-1. Points are means from at least two independent experiments. Examples of the Northern-blot analyses are depicted in the inserts. Lanes correspond, from left to right, to RNA from cells on days 3, 5, 7 and 10 of culture.

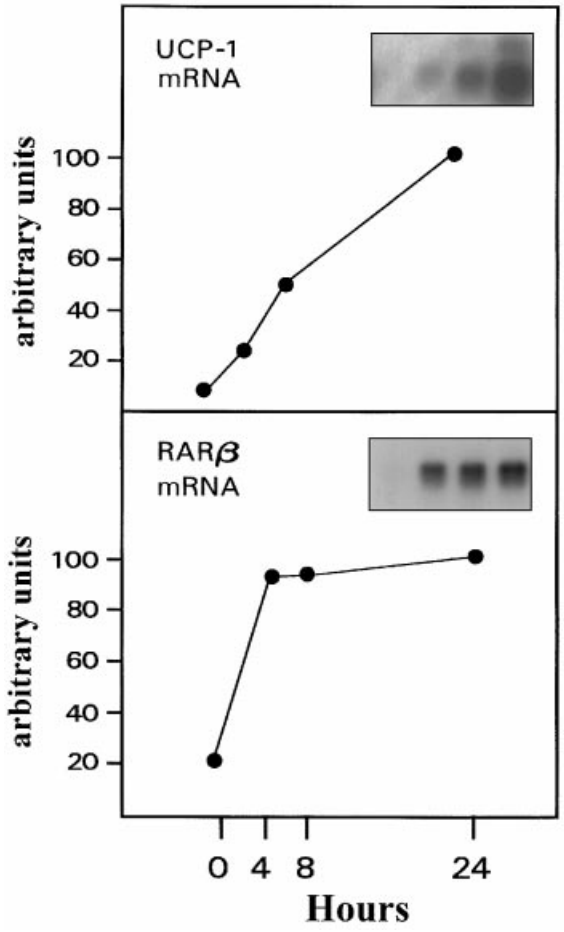

Figure 4 Time course of the effects of all-*trans* RA on RARβ mRNA and UCP-1 mRNA levels

Brown adipocytes differentiated in culture (day 7) were exposed to 1 μM all-*trans* RA for 4, 8 or 24 h. Total RNA (20 μg) from pooled plates at each time point of exposure was analysed by Northern-blot hybridization using the RARβ and UCP-1 cDNA probes. Points are means from at least two independent experiments. Examples of the Northern-blot analyses are depicted in the inserts. Lanes correspond, from left to right, to untreated cells (0 h) or cells treated during 4, 8 or 24 h.

vascular precursor cells from brown-fat depots were placed in culture they followed a complex process of differentiation. At day 3 cells showed a fibroblast-like appearance whereas at day 5 small lipid droplets appeared and at days 7 and 10 more than 90 % of the cells showed multiple lipid droplets characteristic of the brown adipocyte. The expression of UCP-1 mRNA, the marker of brown adipocytes, strictly followed the pattern of brown-adipocyte differentiation (see Figure 3, bottom panel). All the RAR- and RXR-subtype mRNAs were detected in the cells but dramatic changes in their relative abundances were observed throughout differentiation (Figure 3). The levels of the three mRNAs for RAR subtypes decreased as differentiation proceeded. For RXR subtypes, a weak change was observed for RXRβ mRNA expression whereas RXRα and RXRγ followed a marked increase in parallel with the acquisition of adipocyte morphology and the rise in UCP-1 mRNA.

RARβ expression is highly sensitive to all-*trans* RA in different cell types [23,34]. We determined the effects of all-*trans* RA on RARβ mRNA expression in brown adipocytes and we compared the time course of these effects with that for the action of all-*trans* RA on UCP-1 mRNA. All-*trans* RA caused a marked increase in RARβ mRNA that reached a maximum at 4 h of exposure and remained high for the following 24 h (Figure 4). In contrast, all-*trans* RA caused a progressive increase in UCP-1 mRNA levels and maximum levels were attained only after 24 h exposure to all-*trans* RA.

The capacity of RAR and RXR subtypes to mediate the retinoid-dependent stimulation of *ucp-1*

In the second approach we identified the receptor subtypes that mediated the action of retinoids on *ucp-1*. For this purpose CV-1 cells, which are devoid of retinoid receptors, were used. In these cells retinoid action is highly dependent on co-transfected re-

Table 4 Effects of RAR and RXR subtypes on the action of all-*trans* RA, 9-*cis* RA, TTNPB or methoprene on the retinoid-responsive region of the *ucp-1* gene

CV1 cells were transfected with 5 μg/plate of (−2469/−2318)UCP1-CAT and, when indicated, co-transfected with 0.05 μg/plate of one of the expression vectors for murine RARα, RARβ, RARγ, RXRα, RXRβ or RXRγ. Cells were exposed to 1 μM all-*trans* RA, 1 μM 9-*cis* RA, 10 μM TTNPB or 100 μM methoprene. Results are expressed as the fold induction of CAT activity relative to non-transfected, non-treated controls, set to 1, and are means ± S.E.M. of three independent transfections performed in duplicate. Statistically significant ($P < 0.05$) induction of CAT activity in co-transfected cells with respect to non-cotransfected cells for each treatment is shown by *.

Co-transfected receptor	Treatment	Fold induction of CAT activity
–	All-*trans* RA	1.4 ± 0.2
	9-*cis* RA	2.1 ± 0.4
	TTNPB	1.4 ± 0.4
	Methoprene	1.9 ± 0.3
RARα	All-*trans* RA	24.5 ± 3.2*
	9-*cis* RA	22.0 ± 4.1*
	TTNPB	24.9 ± 3.1*
RARβ	All-*trans* RA	7.5 ± 1.0*
	9-*cis* RA	10.1 ± 0.8*
	TTNPB	12.6 ± 1.1*
RARγ	All-*trans* RA	1.9 ± 0.5
	9-*cis* RA	2.1 ± 0.4
	TTNPB	2.0 ± 0.4
RXRα	All-*trans* RA	10.0 ± 1.8*
	9-*cis* RA	22.5 ± 3.4*
	Methoprene	20.2 ± 2.8*
RXRβ	All-*trans* RA	1.1 ± 0.3
	9-*cis* RA	1.9 ± 0.4
	Methoprene	1.8 ± 0.4
RXRγ	All-*trans* RA	3.1 ± 0.4*
	9-*cis* RA	5.0 ± 0.6*
	Methoprene	4.3 ± 0.5*

ceptor subtypes [11]. The (−2469/−2318)UCP1-CAT plasmid was transiently transfected into CV-1 cells and the effects of co-transfection of expression vectors for the murine α, β and γ subtypes of RAR and RXR were studied in the presence of all-*trans* RA, 9-*cis* RA and TTNPB or methoprene. Co-transfection of RARα enhanced the expression of the (−2469/−2318)UCP1-CAT construct more than 20-fold in the presence of all-*trans* RA, 9-*cis* RA or TTNPB (see Table 4). A weaker but significant effect was observed for RARβ, whereas RARγ had no effect. Therefore RARα, and to a lesser extent RARβ, appear to be the subtypes that convey the RAR-mediated effects of RA on *ucp-1* expression. When RXR-subtype expression vectors were co-transfected a marked effect was observed for RXRα. It enhanced the expression of (−2469/−2318)UCP1-CAT more than 20-fold in the presence of 9-*cis* RA or methoprene, and to a much lesser extent in the presence of all-*trans* RA. CV-1 can partially isomerize all-*trans* RA to 9-*cis* RA [11], which might explain this effect of all-*trans* RA in the presence of RXRα. No significant effects were observed by co-transfection of RXRβ in the presence of all-*trans* RA, 9-*cis* RA or methoprene. Co-transfection of RXRγ increased (−2469/−2318)UCP1-CAT expression in the presence of 9-*cis* RA and methoprene, but to a much lesser extent than RXRα. This indicated that RXRα is the main subtype to convey the RXR-mediated responsiveness to 9-*cis* RA of *ucp-1*.

DISCUSSION

Here we attempt to establish the intracellular signalling pathways that mediate the action of retinoids on *ucp-1* expression in the brown adipocyte. 9-*cis* RA, an isomer of all-*trans* RA with the distinct property of ligand activation of both RAR and RXR receptors, is at least as effective as all-*trans* RA in increasing UCP-1 mRNA levels in the brown-fat cells ([12,13] and the present results). The action of 9-*cis* RA on *ucp-1* expression could occur through activation of RAR, similarly to all-*trans* RA. However, we observed a specific enhancement of 9-*cis* RA action elicited by co-transfection of an RXR expression vector. This strongly suggested that the stimulatory action of 9-*cis* RA on *ucp-1* expression could involve not only the activation of RAR but also the activation of a RXR-mediated pathway.

The hypothesis that ligand-mediated stimulation of RARs or RXRs increases *ucp-1* expression was confirmed by the positive effects of synthetic compounds (TTNPB and methoprene) that are non-isomerizable and selectively activate RARs or RXRs. The RAR-mediated action of all-*trans* RA on *ucp-1* occurs through the upstream enhancer of this gene [3,12,13]. Sears et al. [15] reported a PPARγ-responsive element in the 5′ region of this enhancer, which could mediate RXR-dependent retinoid effects. Our results indicate that ligand-dependent activation of *ucp-1* by RXR does not require the PPARγ-responsive site. Thus, RXR-dependent activation by retinoids occurs through elements present in the previously characterized retinoid-responsive region of *ucp-1*. This region from −2469 to −2318 contains various sites that bind RAR or RXR *in vitro* [3,12,13]. The identification of the particular nucleotides in the *ucp-1* enhancer involved in responsiveness to RXR or RAR ligand-dependent activation is beyond the scope of this article. However, several authors have reported the identification of multiple sites in the rat retinoid-responsive region of *ucp-1* that mediate most of the RA effects and have nucleotide sequences and spacing alignment that differ from standard retinoid response elements selective for RAR or RXR ligand activation [3,12,13]. This is consistent with the complex pattern of effects of the RA isomers and the involvement of distinct types of retinoid receptor that we report here.

Several clues to the physiological pathways for the action of retinoids on *ucp-1* expression in the brown-fat cell are provided by present results on the expression of RAR and RXR subtypes, and their ability to confer retinoid responsiveness to the *ucp-1* promoter. The expression of RAR subtypes decreases during brown-fat-cell differentiation and low expression levels are present when brown adipocytes attain a differentiated adipocyte morphology and maximal expression of *ucp-1*. However, from co-transfection assays it was evident that both RARα and RARβ mediate all-*trans* RA responsiveness of the *ucp-1* retinoid-responsive region. Although low, RARα expression in differentiated adipocytes may be enough to mediate all-*trans* RA responsiveness of *ucp-1*. Moreover, the high sensitivity of RARβ expression to RA indicates a potential involvement of this RAR subtype in the effects of all-*trans* RA on *ucp-1* expression. This is further supported by the observation that the effects of all-*trans* RA on RARβ mRNA expression in brown adipocytes reach a maximum long before the highest levels of UCP-1 mRNA are attained. Inhibition of protein synthesis did not suppress RA stimulation of *ucp-1* expression but it decreased the extent of this stimulation [3]. The action of RA on the expression of retinoid receptors such as RARβ constitutes a potential mechanism of amplification of RA effects on *ucp-1* expression.

The expression of RXRα mRNA and RXRγ mRNA increased in parallel with brown-adipocyte differentiation and *ucp-1* expression. RXRα showed maximal effectiveness in mediating 9-*cis* RA stimulation of *ucp-1* promoter expression, thus suggesting that this RXR subtype could account for the response of *ucp-1* to 9-*cis* RA *in vivo*. However, the parallel between changes in

RXRγ and UCP-1 mRNA expression during brown-adipocyte differentiation is striking. RXRγ, an RXR subtype with preferential expression in muscle and particular regions of the brain [8], is highly expressed in brown adipose tissue in contrast to white fat [18]. Although transfection assays did not support a major role of RXRγ in mediating the effects of 9-*cis* RA on *ucp-1* expression, RXR receptors can act as non-ligand-dependent factors co-operating with other members of the nuclear-receptor superfamily in the regulation of gene transcription, such as the thyroid hormone receptor [16]. RXRγ may have such a role in *ucp-1* expression or the expression of other genes associated with brown-adipocyte differentiation.

In summary, independent activation of RARs or RXRs can cause stimulation of *ucp-1* expression and the effects of RA on *ucp-1* expression involve ligand binding of RXR. These findings support the notion that *ucp-1* is not exclusively regulated by a single receptor-selective retinoid, in contrast with other genes, which are entirely dependent on whether it is RAR (as for example the *RAR*β gene [35]) or RXR (for example the cellular retinol-binding protein type II or apolipoprotein AI genes [36,37]) that is liganded. The behaviour of *ucp-1* is much more similar to the rat growth hormone or murine Fas-ligand genes, which are responsive to ligand activation of both RAR and RXR [38,39]. Moreover, from the combination of endogenous expression and capacity of ligand-dependent transactivation, RARα and RARβ as well as RXRα appear to be the receptor subtypes that mediate most of the effects of RA on *ucp-1* in the brown adipocyte. All these findings might have important implications for the design and use of receptor-selective retinoids as pharmacological agents capable of affecting *ucp-1*.

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