***In vitro* and *in vivo* induction of brown adipocyte uncoupling protein (thermogenin) by retinoic acid**

Pere PUIGSERVER, Francisca VÁZQUEZ, María L. BONET, Catalina PICÓ and Andreu PALOU*

Bioquímica i Biologia Molecular, Laboratori de Biologia Molecular, Nutrició i Biotecnologia, Dept. de Biologia Fonamental i Ciències de la Salut, Universitat de les Illes Balears, Ctra Valldemossa Km 7.5, 07071 Palma de Mallorca, Spain

The effects of retinoic acid (RA) isomers (all-*trans*-RA and 9-*cis*-RA) on the appearance of uncoupling protein (UCP; thermogenin), the only unequivocal molecular marker of the brown adipocyte differentiated phenotype, have been investigated in primary cultures of brown adipocytes, in the brown adipocyte cell line HIB 1B and directly in intact mice. The results obtained with cultured cells indicate that retinoids function as inducers of the appearance of UCP and, at the same time, partially inhibit brown adipocyte cell proliferation. The two RA isomers displayed similar effectiveness as UCP inducers, their effect being comparable with that triggered by noradrenaline, so far considered to

be the main modulator of UCP gene expression. The effectiveness of retinoids as UCP inducers was dependent on the stage of brown adipocyte differentiation, being maximal in confluent primary cells and in the medium-late differentiation stage of HIB 1B cells. Corroborating the results obtained *in vitro*, we show that administration of all-*trans*-RA or 9-*cis*-RA to mice leads to an increase in their brown adipose tissue specific UCP content. 9-*cis*-RA treatment also prevented the loss of UCP on cold deacclimation. To our knowledge, this is the first report of a stimulatory effect of retinoid compounds on UCP induction *in vivo*.

INTRODUCTION

Two main classes of adipose tissue depots can be distinguished according to their cellular pattern: white adipose tissue depots and brown adipose tissue (BAT) depots. White adipocytes, and hence white adipose tissue depots, are specialized in energy storage in the form of triacylglycerols. Brown adipocytes, on the other hand, although they are similar to white adipocytes in enzymic machinery and are also able to store triacylglycerols, function to dissipate energy in the form of heat. The molecular basis of BAT thermogenesis is the uncoupling protein (UCP) or thermogenin, a mitochondrial inner membrane protein exclusive to brown adipocyte mitochondria, which allows dissipation of the proton gradient generated by the respiratory chain as heat, thus uncoupling electron transfer from ATP synthesis. UCP is a molecular differentiation marker specific for the brown adipocyte cell type that distinguishes it from other cell types, including the white adipocyte [1].

During the process of BAT recruitment that occurs on cold exposure or adaptation to high-calorie diets, DNA and protein synthesis are induced, particularly of UCP, leading to an increased BAT thermogenic capacity. So far, the main regulator of BAT growth and development appears to be noradrenaline, which is released by the sympathetic terminals that enervate the tissue and acts on different β -adrenergic receptors subtypes present in the brown adipocyte cellular membrane. In fact, in brown fat cell culture systems noradrenaline has been proved competent in stimulating both DNA synthesis [2] and *ucp* mRNA synthesis and translation to give mature UCP [3–6]. However, factors other than noradrenaline, such as insulin, thyroid hormone and glucocorticoids, have been shown to accelerate the acquisition of the terminally differentiated brown adipocyte phenotype [4,7], and indeed substantial levels of UCP gene expression are attained in late fetal life in most mammalian

species, when the sympathetic nervous system is not yet fully developed [8].

Retinoic acid (RA) is a natural vitamin A derivative known to have profound effects on the growth and differentiation of many mammalian cells [9]. In different cell types of epithelial origin, as well as in muscle cells, RA promotes cell differentiation and inhibits cell proliferation [9–11]. In contrast, the adipose conversion of various cell lines capable of differentiating into white adipocytes is blocked when precursor cells are exposed to RA [12–16]. Cellular responses to RA are mediated by two families of nuclear receptors, which are members of the steroid/thyroid hormone receptor superfamily and function as ligand-activated dimeric transcription factors that bind to specific DNA response elements located in the regulatory region of retinoid-responsive genes [17–19]. RA receptors bind all-*trans*-RA (tRA) as well as 9-*cis*-RA, whereas retinoid X receptors (RXRs) only show binding affinity for the 9-*cis* isomer.

The present study was undertaken to gain insight into a possible role for retinoids in controlling brown adipocyte cell proliferation and differentiation, using both *in vivo* and *in vitro* approaches. Recently, it was reported that the *ucp* gene promoter contains a RA response element and that RA alone is able to induce *ucp* mRNA in cultured brown adipocytes [20,21]. Our results provide evidence for a physiological role for both tRA and 9-*cis*-RA in controlling the thermogenic capacity of BAT, not only in *in vitro* systems but also in whole animals.

MATERIALS AND METHODS

Materials

($\pm$)-Arterenol bitartrate salt (noradrenaline), tRA, triiodothyronine, 3-isobutyl-1-methylxanthine, cortisol and indo-

Abbreviations used: UCP, uncoupling protein (thermogenin); RA, retinoic acid; tRA, all-*trans*-RA; RXR, retinoid X receptor; BAT, brown adipose tissue; DMEM, Dulbecco's modified Eagle's medium; BrdU, 5-bromo-2'-deoxyuridine.

* To whom correspondence should be addressed.

methacin were obtained from Sigma. 9-*cis*-RA was generously donated by Dr. Martin Lebrón (Roche S.A., Madrid, Spain). Newborn calf serum and Dulbecco's modified Eagle's medium (DMEM) were obtained from ICN, and human insulin (Actrapid) was from Novo Industries. Other cell culture reagents were supplied by Sigma, and routine chemicals used were from Merck and Panreac.

Cell isolation and culture

BAT precursor cells were isolated from combined cervical, axillar and interscapular brown fat depots of 4-week-old male NMRI mice (obtained from Letica, Barcelona, Spain), as described by Rehnmark et al. [3]. The cells were grown either in wells of 35 mm diameter or in 25 cm² culture flasks. A 0.2 ml portion of pooled final cell suspension was inoculated per well of 35 mm diameter containing 1.8 ml of culture medium, and a 0.5 ml portion was added per 25 cm² flask containing 4.5 ml of culture medium. The culture medium consisted of DMEM supplemented with 10% newborn calf serum, 4 nM insulin, 10 mM Hepes, 50 units of penicillin/ml, 50 µg of streptomycin/ml and 25 µg of sodium ascorbate/ml. The cells were grown at 37 °C in an atmosphere of 8% CO₂ in air. On the first day after inoculation, the culture medium was removed and the cells were rinsed with DMEM before the addition of fresh medium. The culture medium was also changed on the third day after inoculation, and additional changes were made depending on the type of experiment, as indicated in the Figure legends.

HIB 1B cells (from Dr. B. M. Spiegelman, Dana-Farber Cancer Institute, Boston, MA., U.S.A.) were maintained at 37 °C in an atmosphere of 8% CO₂ in air in preadipocyte growth medium (DMEM with 10% heat-inactivated foetal calf serum, 2 mM glutamine and antibiotics). Differentiation was induced by allowing cells to become confluent in preadipocyte growth medium and then changing to induction medium (preadipocyte growth medium plus 20 nM insulin, 1 nM tri-iodothyronine, 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 µM cortisol and 0.125 mM indomethacin); after 3 days, adipocyte medium (preadipocyte growth medium plus 20 nM insulin and 1 nM tri-iodothyronine) was substituted for induction medium, and 7 days later the cells were fully differentiated into adipocytes.

Cells in culture were treated with noradrenaline, tRA or 9-*cis*-RA at the times and concentrations specified in the Figure legends, and were routinely harvested on day 7 after inoculation.

Cell harvesting

Culture medium was removed and the cells were rinsed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer, pH 7.4) and then scraped with a rubber policeman into 1.5 ml of PBS and transferred to an Eppendorf tube. The cell suspensions were pelleted, resuspended in a minimal volume of PBS and stored at -20 °C prior to sonication followed by measurement of protein and UCP.

Cell proliferation assay

Brown adipocyte cell proliferation was measured using the 5-bromo-2'-deoxyuridine (BrdU) labelling and detection kit from Boehringer Mannheim Biochemica, based on the cell ELISA principle, following the manufacturer's instructions. Primary brown preadipocytes were cultured as described above but in a 96-well microtitre plate. The culture medium was changed on days 1, 3 and 6 after inoculation. Retinoid compounds were added at the concentrations indicated in the Figure legends on

days 3 and 6. BrdU (10 µM) was added on day 6, and the cells were further incubated for 20–24 h. Following this, the cells were rinsed with PBS containing 10% foetal calf serum, fixed with ethanol/HCl and incubated with 0.5 µM nucleases to partially digest the DNA so as to improve the accessibility of the antibodies to BrdU. Incorporated BrdU was detected using a monoclonal anti-BrdU-POD antibody, Fab fragments, and the bound conjugate was visualized with the soluble chromogenic substrate 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) and measured using an ELISA reader.

Experiments *in vivo*

Treatment with tRA

Male NMRI mice (5 weeks old; 25–32 g) were used. The mice were randomly assigned to two experimental groups (three to five animals per group): control animals and tRA-treated animals. The latter received a daily oral dose of 5 mg of tRA emulsified in 300 µl of olive oil for 4 days before being killed; control animals received olive oil only. Two separate experiments were performed.

Treatment with 9-*cis*-RA

Male NMRI mice (2 months old; 35–40 g) were used. They were individually housed in wire mesh cages and maintained at 23 °C with a 12 h-light/12 h-dark lighting schedule until being randomly assigned to six experimental groups. Groups 1 and 2 contained control and 9-*cis*-RA-treated animals respectively which remained at 23 °C and received a daily subcutaneous injection of either 100 µl of olive oil or 4 mg of 9-*cis*-RA emulsified in 100 µl of olive oil, starting 4 days before the animals were killed. Groups 3 and 4 contained control and 9-*cis*-RA-treated cold-exposed animals respectively which remained at 4 °C for 3 days and received a daily subcutaneous injection of either 100 µl of olive oil or 4 mg of 9-*cis*-RA emulsified in 100 µl of olive oil from the day before the beginning of cold exposure until they were killed. Groups 5 and 6 contained control and 9-*cis*-RA-treated cold-deacclimated animals respectively which were cold-exposed at 4 °C for 3 days and then reacclimated to 23 °C for a further 3 days, and received a daily subcutaneous injection of either 100 µl of olive oil or 4 mg of 9-*cis*-RA emulsified in 100 µl of olive oil from the day before the beginning of cold deacclimation until they were killed. Three animals were assigned to each experimental group.

In all cases, the animals were killed with CO₂, followed by cervical dislocation. The interscapular, cervical and axillar BAT depots were dissected out, combined, weighed and homogenized in PBS using a Teflon/glass homogenizer (ten strokes). The tissue homogenates were filtered through a nylon filter (250 µm pore size) and stored at -20 °C prior to protein determination and immunoblotting for UCP; the DNA content was also determined in certain cases (see below). A separate sample of tissue was homogenized in chloroform/methanol (2:1, v/v) for lipid determination (see below), when performed.

Immunoblotting for UCP

Protein concentration was measured by the method of Bradford [22]. For immunoblotting, the samples were denatured by addition of SDS/PAGE sample buffer (SDS/protein ratio 4:1, w/w). SDS/PAGE was carried out according to Laemmli [23], routinely with 20–30 µg of cell or homogenate protein per lane. Electrophoresis, blocking and development of the immunoblot were performed according to Herron et al. [5], and the final colour development was either with an alkaline phosphatase-

conjugated substrate kit (Bio-Rad) or with an ECL kit (Amersham). For quantitative analysis, the bands were scanned with a BioImage computing densitometer (Millipore).

Other parameters

Tissue lipids were determined by the method of Folch et al. [24]. Tissue DNA was determined by a fluorimetric method that uses 3,5-diaminobenzoic acid [25].

RESULTS

Retinoid treatment affects the morphology of cultured brown adipocytes

When precursor brown adipocytes were isolated and plated, confluence was reached by day 7 after inoculation; a high percentage of the cells had acquired the typical differentiated adipocyte morphology by this time, i.e. they were rounded-up and showed lipid accumulation in multivacuolar droplets. On treatment with 9-*cis*-RA or tRA, cell numbers at day 7 appeared to be lower and the cells more fibroblast-like than those in control non-treated cultures (Figures 1A and 1B). The percentage

of cells containing lipid droplets, detected by Oil-Red staining, was also diminished in retinoid-treated cultures (results not shown), suggesting that adipogenesis was reduced by retinoid treatment.

Similar results were obtained with HIB 1B cells, a clonal brown adipocyte cell line isolated from transgenic mice bearing a brown fat hybernoma [26]. 9-*cis*-RA treatment of HIB 1B cells growing in preadipocyte growth medium affected cell morphology in such a way that treated cells were more fibroblast-like, more elongated and more uniformly distributed on the flask surface than non-treated cells (Figures 1C and 1D).

Retinoid treatment induces the appearance of UCP in confluent primary brown adipocytes and in differentiating HIB 1B brown adipocytes

Treatment of primary cultures with tRA or 9-*cis*-RA (1–10 μ M) for a period of 48 h prior to harvesting the cells at confluence (day 7 after inoculation) led to an increase in the UCP levels, as shown in the immunoblot in Figure 2 (upper panel). The induction of UCP elicited by the two retinoid compounds was even greater than that triggered by 10 μ M noradrenaline when

Figure 1 Effect of 9-*cis*-RA on the morphology of cultured brown adipocytes

(A, B) Phase-contrast micrographs of primary brown adipocytes after 7 days in culture. The medium was changed and ethanol (20 μ l) or 10 μ M 9-*cis*-RA in ethanol was added on days 3 and 6 after inoculation: (A) control culture; (B) 9-*cis*-RA-treated culture. (C, D) Phase-contrast micrographs of HIB 1B cells after 7 days of culture in preadipocyte growth medium (see the Materials and methods section for details). The medium was changed and ethanol (20 μ l) or 10 μ M 9-*cis*-RA in ethanol was added on days 3 (when cells were about 70% confluent) and 6 after subculture: (C) control culture; (D) 9-*cis*-RA-treated culture. Magnification: A, B $\times$ 330; C, D $\times$ 660).

Figure 2 Induction of UCP by RA in confluent primary cultures of brown adipocytes and in HIB 1B cells

Upper panel: immunoblot showing UCP induction in primary brown adipocytes by noradrenaline (NA; 10 μ M; lane 2), 9-*cis*-RA (10 μ M and 1 μ M; lanes 3 and 4 respectively) and tRA (10 μ M and 1 μ M; lanes 5 and 6 respectively). Lane 1, control culture. Single doses of the indicated agents were added to the cells on days 3 and 6 of culture, after changing the culture medium; the cells were harvested on day 7, and the UCP content of cell lysates determined by immunoblotting. Samples of 30 μ g of cellular protein were run per lane. Lower panel: immunoblot showing UCP induction in HIB 1B cells by noradrenaline (10 μ M) and tRA (10 μ M). Cells were harvested 2, 5, 8 or 15 days after initiation of the differentiation programme by addition of induction medium, and were treated with water (C), noradrenaline (NA) or tRA for a period of 48 h just prior to harvesting. Samples of 100 μ g of cellular protein were run per lane.

tested under the same conditions. The stimulatory action of the 48 h RA treatment on UCP levels was only visible in cultures at the confluent stage; we could not detect any effect on pre-confluent or post-confluent cultures, harvested at day 3 and 15 after inoculation respectively (results not shown).

Figure 3 Dose-response curves showing the effect of 9-*cis*-RA and tRA on UCP induction in confluent primary brown adipocytes

Cells were grown for 3 days in culture wells of 35 mm diameter and then treated with different concentrations of 9-*cis*-RA or tRA. On day 6 the culture medium was changed, and retinoid compounds were added again at the same concentrations. Cells were harvested on day 7, and the UCP content of cell lysates (30 μ g of protein) was determined by immunoblotting as described in the Materials and methods section. UCP levels are expressed as arbitrary units of integrated absorbance, obtained by scanning of the immunoblots. Values represent the means $\pm$ S.E.M. of three different experiments carried out in duplicate.

Table 1 Protein content of primary cultures of brown adipocytes after treatment with noradrenaline, 9-*cis*-RA or tRA at different stages of differentiation

Brown adipocytes in primary culture were harvested at day 3 (pre-confluent cells), day 7 (confluent cells) or day 15 (post-confluent cells) after inoculation. Before harvesting, cells were treated with 10 μ M noradrenaline, 9-*cis*-RA or tRA for a period of 48 h. The protein and UCP contents of cell lysates were measured as indicated in the Materials and methods section. The values represent the total protein amount per well and are the means $\pm$ S.E.M. of the averages of two or three independent experiments carried out in duplicate. Significant differences: * P < 0.1, ** P < 0.01 for 9-*cis*-RA or tRA compared with control untreated cells. Individual differences from mean values were assessed by Student's *t* test.

Treatment	Total protein (μ g/well)		
	Pre-confluent	Confluent	Post-confluent
Control	11.4 $\pm$ 1.1	76.7 $\pm$ 2.4	74.3 $\pm$ 4.4
Noradrenaline	9.8 $\pm$ 0.6	82.5 $\pm$ 11	93.5 $\pm$ 11.8
9- <i>cis</i> -RA	7.5 $\pm$ 1.0*	44.4 $\pm$ 5.3**	52.5 $\pm$ 5.3*
tRA	6.5 $\pm$ 1.5*	48.0 $\pm$ 4.7**	36.8 $\pm$ 2.4**

The effects of tRA on UCP levels were also tested in HIB 1B cells at various stages after the beginning of the differentiation protocol by changing to induction medium (see the Materials and methods section for details). Again, as in primary cultures, tRA stimulated the appearance of UCP; the stimulatory effect was maximal 8 days after starting the differentiation programme, and seemed to decrease thereafter (Figure 2, lower panel).

The stimulatory effects of the two RA isomers on the appearance of UCP were consistent and dose-dependent, as shown in Figure 3. No difference was found between 9-*cis*-RA and tRA with regard to their ability to induce UCP; analysis of the data by reiterative computer curve-fitting analysis (not shown) gave approximately the same EC_{50} value (0.1 μ M) and maximum response (8.7 units of UCP for 9-*cis*-RA and 9.4 units of UCP for tRA) for the two retinoids. It should be noted that *in vivo* isomerization between the two isomers has been described [27].

Primary cultures of brown adipocytes treated with retinoids show decreased protein content and DNA synthesis compared with control cultures

Primary cultures of brown adipocytes treated with 10 μ M tRA or 9-*cis*-RA for a period of 48 h just before harvesting contained approx. 40 % less protein than parallel control cultures (Table 1). The effect of RA treatment on protein content appeared to be independent of the stage of differentiation of the cultures; thus the effect was about the same in pre-confluent cultures (harvested at day 3 after inoculation), confluent cultures (harvested at day 7) and post-confluent cultures (harvested at day 15). Also shown in Table 1 is the protein content of primary cultures treated with noradrenaline instead of retinoids; as expected [2], confluent and post-confluent noradrenaline-treated cultures had a somewhat higher protein content than control non-treated cultures, although in our hands the differences were not significant. tRA treatment of HIB 1B cells also led to a reduced protein content, independently of the culture stage (results not shown).

DNA synthesis in brown adipocytes was studied by measuring BrdU incorporation into cellular DNA after exposure of the primary cells in culture to different concentrations of tRA and 9-*cis*-RA (from 0.01 to 100 μ M) for 4 days (from day 3 to day 7 after inoculation). Higher concentrations of both retinoids proved to be toxic, as revealed by microscopic examination of the cells, and thus were not included in the study. The two

Figure 4 Dose-response curves showing the effect of 9-*cis*-RA and tRA on DNA synthesis in primary brown adipocytes

Cells were grown for 3 days in a 96-well microtitre plate and then treated (or not) with different concentrations of 9-*cis*-RA or tRA. On day 6 the culture medium was changed, and retinoid compounds were added again at the same concentrations together with 10 μ M BrdU labelling solution. After 20–24 h the BrdU incorporated into cellular DNA was measured, as detailed in the Materials and methods section. Data are expressed relative to the BrdU incorporated by control non-treated cells (100% incorporation) and are the means $\pm$ S.E.M. of four independent experiments carried out in triplicate.

Figure 5 Effect of tRA on the specific UCP content of mouse BAT

Immunoblot showing the UCP levels in BAT from control (C) and tRA-treated (tRA) mice. Samples of 20 μ g of protein were run per lane. tRA-treated animals received a daily oral dose of 5 mg of tRA (170 mg/kg body weight) emulsified in 300 μ l of olive oil starting 4 days before they were killed; control animals received olive oil only.

Table 2 Effect of 9-*cis*-RA treatment on BAT protein and UCP content of mice maintained at 23 °C, cold-exposed mice and cold-deacclimated mice

Mice were treated and killed, and protein and UCP analysis performed as detailed in the Materials and methods section. The values are for combined interscapular, cervical and axillary BAT depots, and are the means $\pm$ S.E.M. of three animals per group. Statistical differences were analysed by a two-way ANOVA; differences between means were assessed by the Newman–Keuls test. Significant differences: * $P < 0.1$, ** $P < 0.05$ for RA-treated animals compared with the corresponding control animals; † $P < 0.05$ for cold-exposed or cold-deacclimated control animals compared with control animals maintained at 23 °C; ‡ $P < 0.05$ for cold-exposed control animals compared with cold-deacclimated control animals; § $P < 0.05$ for cold-exposed and cold-deacclimated RA-treated animals compared with RA-treated animals maintained at 23 °C. N.S., not significant.

Animals		BAT total protein (mg)	Total UCP (arbitrary units)	Specific UCP (arbitrary units/mg of protein)
Kept at 23 °C	Controls	10.6 $\pm$ 1.1	22.7 $\pm$ 1.5	2.20 $\pm$ 0.33
	9- <i>cis</i> -RA treated	11.0 $\pm$ 1.0	36.1 $\pm$ 5.3*	3.25 $\pm$ 0.23**
Cold-exposed	Controls	20.5 $\pm$ 2.7†‡	101 $\pm$ 10†‡	4.98 $\pm$ 0.3†‡
	9- <i>cis</i> -RA treated	15.7 $\pm$ 1.4	70.9 $\pm$ 1.3**§	4.57 $\pm$ 0.31
Cold-deacclimated	Controls	12.2 $\pm$ 1.5	35.3 $\pm$ 3.8	2.91 $\pm$ 0.18
	9- <i>cis</i> -RA treated	12.8 $\pm$ 1.1	62.7 $\pm$ 7.1**§	4.96 $\pm$ 0.70**
ANOVA	Cold	$P < 0.05$	$P < 0.05$	$P < 0.05$
	RA treatment	N.S.	N.S.	$P < 0.05$
	Interaction	N.S.	$P < 0.05$	$P < 0.05$

retinoid compounds caused a dose-dependent inhibition of BrdU incorporation into cellular DNA (Figure 4); at the highest concentration used (100 μ M) the degree of inhibition attained was 28% for 9-*cis*-RA and 38% for tRA. Thus there is a correlation between the DNA synthesis and the protein content data, strongly suggesting that retinoid treatment inhibits brown adipocyte cell proliferation in culture.

Treatment of intact mice with tRA and 9-*cis*-RA leads to an increase in the specific UCP content of BAT

Treatment of 5-week-old mice with a daily oral dose of tRA (170 mg/kg body weight) for 4 days resulted in a body weight loss (16.7 $\pm$ 2.23%; $n = 9$), and led to a marked decrease in BAT weight, the tissue representing about 1% of total body weight in control animals (0.90 $\pm$ 0.06%; $n = 8$) and only 0.5% in tRA-treated animals (0.53 $\pm$ 0.03%; $n = 9$). BAT total protein was significantly lower in tRA-treated animals (12.7 $\pm$ 1.5 mg; $n = 8$) than in control animals (20.1 $\pm$ 1.7 mg; $n = 8$); however, protein represented a higher percentage of BAT weight in tRA-treated animals than in control animals (9.5 $\pm$ 0.8% compared with 7.4 $\pm$ 0.7%), suggesting a preferential loss of tissue lipids. This was confirmed in a second experiment in which we evaluated directly the effect of tRA treatment on the lipid and DNA content of BAT. As expected, the treatment led to a marked decrease in tissue lipids, which represented 37.6% ($\pm$ 1.6%; $n = 5$) of BAT weight in control animals and only 24% ($\pm$ 3%; $n = 4$) in tRA-treated animals; the lipid/protein (mg/mg) ratio was decreased by a factor of 2 after tRA treatment. The total BAT DNA content remained unchanged after tRA treatment. The specific UCP content increased consistently by approx. 2-fold on tRA treatment in the two experiments performed; Figure 5 shows immunoblots from one experiment. The total UCP content, on the other hand, remained practically unchanged due to the decrease in BAT total protein. Thus there was a positive correlation between increased UCP content and increased lipid consumption in the BAT of tRA-treated animals.

In another experiment *in vivo* we evaluated the effects of 9-*cis*-RA, injected subcutaneously at a dose of 107 mg/kg body weight during 4 consecutive days, on mice BAT protein and UCP content. Three experimental conditions were studied: maintenance at 23 $\pm$ 1 °C (control), cold exposure and cold deacclimation. As expected, cold exposure resulted in an increase in BAT weight, and the tissue reassumed its control size after cold deacclimation (results not shown). There was no significant effect of 9-*cis*-RA, injected subcutaneously, on total body weight or BAT weight in this experiment. The total BAT protein content of animals maintained at 23 $\pm$ 1 °C remained unchanged after 9-*cis*-RA treatment, but this treatment impaired the normal increase in BAT protein content upon cold exposure, the increase being lower in retinoid-treated cold-exposed animals (5 mg) than in control cold-exposed animals (10 mg). After cold deacclimation, the total BAT protein content recovered to control values in both control and 9-*cis*-RA-treated mice (Table 2). The effects of 9-*cis*-RA treatment on UCP content are also shown in Table 2; the treatment led to a significant increase in the total and specific UCP content in the BAT of mice maintained at 23 $\pm$ 1 °C. However, the increase in total BAT UCP content that typically accompanies cold exposure was somewhat impaired in retinoid-treated animals, probably reflecting the impaired increase in total protein in the tissue. 9-*cis*-RA treatment, on the other hand, prevented the decrease in UCP content that occurs on cold deacclimation, suggesting that the retinoid allows the ongoing synthesis of UCP under these conditions. In summary, cold-exposure- and cold-deacclimation-dependent changes in

total BAT UCP content were more pronounced in control animals than in 9-*cis*-RA-treated animals, and paralleled those of total protein.

DISCUSSION

We show here that two RA isomers, tRA and 9-*cis*-RA, function as inducers of UCP, the only unequivocal molecular marker of the brown adipocyte differentiated phenotype, and have an inhibitory effect on the proliferation of brown adipocytes in culture, leading to decreased BrdU incorporation and decreased protein levels.

The stimulatory effects of the RA isomers on UCP levels have been demonstrated here in three different biological systems: an immortal cell line, a pseudo *in vivo* system (primary cultures) and directly in intact mice, supporting the idea of a physiological role for RA in controlling the thermogenic status of BAT. Thus we have corroborated the *in vitro* results by showing that both 9-*cis*-RA and tRA are capable of specifically stimulating the appearance of UCP in control mice and (in the case of 9-*cis*-RA) preventing the loss of UCP on cold deacclimation.

RA is known to inhibit the acquisition of the mature white adipocyte morphology, the accumulation of triacylglycerols and the accumulation of differentiation-dependent transcripts in various cell lines capable of differentiating into white adipocytes [12–16], without affecting cell proliferation [16]. The effects of RA on white and brown adipocytes appear, then, to be strikingly different, since the overall effects of RA on brown adipocytes more closely resemble its effects in muscle cells and many cell types of epithelial origin, in which RA inhibits cell proliferation and promotes differentiation [9–11]. However, upon retinoid treatment brown preadipocytes tended to retain a fibroblast-like morphology and the percentage of cells containing lipid droplets was reduced, suggesting that adipogenesis was somehow blocked by retinoid treatment, as occurs in white adipocytes, in spite of the observed rise in UCP levels. Thus UCP induction appears to be independent of fat accumulation. Interestingly, it has been reported that treatment with tRA at concentrations in the same range as those employed in the present work increases *ucp* mRNA abundance in mice BAT primary cultures, regardless of the acquisition of the adipocyte phenotype [21].

In any case, the capacity of primary cultures to show induced UCP expression over the control basal level in response to RA treatment was seen only at the confluent stage; the effect could not be detected in pre- or post-confluent cultures. Similarly, the time-frame of the retinoid-mediated induction of UCP levels appeared to be restricted to certain stages of differentiation of HIB 1B brown adipocytes. This picture is similar to that obtained when noradrenaline or different β -agonists are used as UCP inducers [4,28].

Besides the positive effect on UCP levels, we also detected an inhibitory effect of retinoid treatment on the proliferation of primary brown adipocytes in culture. This was suggested by the reduced protein content of the retinoid-treated cultures (Table 1) and the reduced DNA synthesis in pre-confluent cultures treated with retinoids (Figure 4). The effect of RA on cell proliferation, in contrast to its effect on UCP levels, appeared to be independent of the culture stage (see the protein content data in Table 1); thus a reduced protein content was found even in post-confluent cultures treated with retinoid from day 13 to day 15 after inoculation. Taking into account the fact that adipocytes are quiescent cells, and although some proliferation is known to occur after apparent confluence [2], the latter result strongly suggests that a mechanism other than inhibition of cell proliferation, perhaps induction of proteolysis, is involved in the

decreased protein content of post-confluent cultures after RA treatment. This alternative mechanism could also apply to the other steps of differentiation, but it should be noted that the data in Figure 4 indicate a dose-dependent inhibition of DNA synthesis elicited by retinoids in cultures treated at the pre-confluent stage.

The results suggest that different mechanisms may mediate the two effects of RA on cultured brown adipocytes detected in this work, namely induction of UCP levels and inhibition of cell proliferation. At least two different possibilities can be proposed. (i) It could be that the two effects are mediated by different retinoid receptor subtypes with a distinct time-frame of expression during differentiation; in this context, studies in different adipocyte cell lines have shown that RA receptor α and RXR β are expressed in both pre-adipocytes and adipocytes [15,29], whereas the expression of RXR α and RXR γ is linked to the differentiation process [15]. (ii) Another possibility is that the effect of retinoids on UCP levels, but not on cell proliferation, is dependent on the interaction of retinoid receptors with other transcription factor(s) that are expressed only after the onset of differentiation.

The two RA isomers used appeared to have distinct effects on the protein content of BAT in whole mice. Whereas tRA treatment elicited a marked decrease in BAT weight and protein content, these parameters remained practically unchanged after 9-*cis*-RA treatment. It should be noted, however, that the two retinoids were used at different doses (170 mg of tRA/kg body weight and 107 mg of 9-*cis*-RA/kg body weight) and with different methods of administration (oral administration in the case of tRA; subcutaneous injection in the case of 9-*cis*-RA). Moreover, although treatment with 9-*cis*-RA did not change the BAT protein content in mice maintained at 23 °C, it impaired the normal increase in this parameter upon cold exposure (Table 2); thus an effect of 9-*cis*-RA treatment on protein levels became apparent when the animals were under a situation of metabolic pressure. On the other hand, in spite of the impaired cold-induced increase in BAT total protein in 9-*cis*-RA-treated animals and the marked loss of BAT weight in oral tRA-treated animals, an inhibitory effect of retinoids on the proliferation of brown adipocytes *in vivo*, similar to that found in cultured cells, could not be demonstrated from the tissue DNA data. It is more likely that our data indicate that the positive correlation between increased UCP levels and lipid consumption in BAT accounts for the marked BAT weight loss in oral tRA-treated animals.

In summary, we provide evidence that RA is capable of affecting the thermogenic machinery of brown adipocytes, not only in *in vitro* systems but also *in vivo* in whole animals. Understanding the effects of endogenous vitamin A derivatives in the differential regulation of brown and white adipocyte proliferation and differentiation may contribute to clarifying the role(s) that both types of adipose cell play in body weight disorders.

We thank members of the Palou laboratory for helpful advice, and especially Jordi Oliver for help with the statistical analysis. The HIB 1B cells were a gift from Dr. Bruce M. Spiegelman. We also thank Dr. Martín Lebrón for kindly providing 9-*cis*-RA. This work was funded by DGCyT, Ministerio de Educación y Ciencia, Spain (grant PB92-0748 and PB94-1178 to A.P.), and by the European Commission (contract no. ERBCHRX © CT940490).

REFERENCES

- 1 Ricquier, D., Casteilla, L. and Bouillaud, F. (1991) FASEB J. **5**, 2237–2242
- 2 Bronnikov, G., Houstek, J. and Nedergaard, J. (1992) J. Biol. Chem. **267**, 2006–2013
- 3 Rehnmark, S., Kopecky, J., Jacobsson, A., Nchad, M., Herron, D., Nelson, B. D., Obregon, M. J., Nedergaard, J. and Cannon, B. (1989) Exp. Cell Res. **182**, 75–83

- 4 Rehnmark, S., Né Chad, M., Herron, D., Cannon, B. and Nedergaard, J. (1990) *J. Biol. Chem.* **265**, 16464–16471
- 5 Herron, D., Rehnmark, S., Né Chad, M., Loncar, D., Cannon, B. and Nedergaard, J. (1990) *FEBS Lett.* **267**, 296–300
- 6 Puigserver, P., Herron, D., Gianotti, M., Palou, A., Cannon, B. and Nedergaard, J. (1992) *Biochem. J.* **284**, 393–398
- 7 Castella, L., Nougues, J., Reyne, Y. and Ricquier, D. (1991) *Eur. J. Biochem.* **198**, 195–199
- 8 Giralt, M., Martín, I., Iglesias, R., Viñas, O., Villarroya, F. and Mampel, T. (1990) *Eur. J. Biochem.* **193**, 297–302
- 9 Sporn, M. B., Roberts, A. B. and Goodman, W. S. (eds.) (1994) *The retinoids: Biology, Chemistry, and Medicine*, Raven Press, New York
- 10 De Luca, L. M. (1991) *FASEB J.* **5**, 2924–2933
- 11 Albagli-Curiel, O., Carnac, G., Vandrome, M., Vincent, S., Crépieux, P. and Bonniieu, A. (1993) *Differentiation* **52**, 201–210
- 12 Teboul, M., Bismuth, J., Gharbi-Chihi, J., Valette, A., Bonne, J., Ghiringhelli, O. and Torresani, J. (1992) *Endocrinology* (Baltimore) **130**, 1475–1482
- 13 Sato, M., Hiragun, A. and Mitsui, H. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1839–1845
- 14 Kuri-Harcuch, W. (1982) *Differentiation* **23**, 164–169
- 15 Chawla, A. and Lazar, M. A. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1786–1790
- 16 Stone, R. L. and Bernlohr, D. A. (1990) *Differentiation* **45**, 119–127
- 17 Petkovich, M., Brand, N. J., Krust, A. and Chambon, P. (1987) *Nature* (London) **330**, 444–450
- 18 Evans, R. M. (1988) *Science* **240**, 889–895
- 19 Mangelsdorf, D. J., Ong, E. S., Dyck, J. A. and Evans, R. M. (1990) *Nature* (London) **345**, 224–229
- 20 Cassard-Doulcier, A. M., Larose, M., Matamala, J. C., Champigny, O., Bouillaud, F. and Ricquier, D. (1994) *J. Biol. Chem.* **269**, 24335–24342
- 21 Alvarez, R., De Andrés, J., Yubero, P., Viñas, O., Mampel, T., Iglesias, R., Giralt, M. and Villarroya, F. (1995) *J. Biol. Chem.* **270**, 5666–5673
- 22 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 23 Laemmli, U. K. (1970) *Nature* (London) **227**, 680–685
- 24 Folch, J., Lees, N. and Sloane, J. H. (1957) *J. Biol. Chem.* **266**, 497–509
- 25 Kissane, J. M. and Robins, E. (1958) *J. Biol. Chem.* **233**, 184–188
- 26 Ross, S. R., Choy, L., Graves, R. A., Fox, N., Solevjeva, V., Klaus, S., Ricquier, D. and Spiegelman, B. M. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7561–7565
- 27 Kojima, R., Fujimori, T., Kiyota, N., Toriya, Y., Fukuda, T., Ohashi, T., Sato, T., Yoshizawa, Y., Takeyama, K., Mano, H., Masushige, S. and Kato, S. (1994) *J. Biol. Chem.* **269**, 32700–32707
- 28 Puigserver, P., Picó, C., Stock, M. J. and Palou, A. (1996) *Mol. Cell. Endocrinol.* **117**, 7–16
- 29 Zovich, D. C., Orologa, A., Okuno, M., Wong Yen Kong, L., Talmage, D. A., Piantadosi, R., Goodman, D. S. and Blanner, W. S. (1992) *J. Biol. Chem.* **267**, 13884–13889

Received 13 November 1995/20 March 1996; accepted 29 March 1996