CMLS, Cell. Mol. Life Sci. 59 (2002) 1714–1723
1420-682X/02/101714-10

CMLS Cellular and Molecular Life Sciences

Research Article

Opposite actions of testosterone and progesterone on UCP1 mRNA expression in cultured brown adipocytes

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Received 24 June 2002; received after revision 20 August 2002; accepted 26 August 2002

Abstract. The brown adipose tissue (BAT) thermogenic response to diet-induced obesity and cold has been found to be gender dependent. In the present work, we aimed to investigate the effects of the main physiological male and female sex hormones, i.e. testosterone, progesterone and 17-β-estradiol, on the expression of uncoupling protein 1 (UCP1) – the main mediator of BAT thermogenesis – and on UCP2 and lipid accumulation in rodent brown adipocytes differentiated in culture. Testosterone-treated cells showed fewer and smaller lipid droplets than control cells and a dose-dependent inhibition of UCP1 mRNA expression, under adrenergic stimulation by norepinephrine (NE). These effects were reverted by the androgen receptor antagonist flutamide, suggesting they are dependent, at least in part, on the androgen receptor. Progesterone- and 17-β-estradiol-treated cells showed more and larger lipid droplets and progesterone stimulated NE-induced UCP1 mRNA expression at the lower concentration tested, but not at higher concentrations, suggesting that for brown adipocytes, this hormone is dose dependent. 17-β-Estradiol did not have any remarkable effect either on UCP1 or UCP2 mRNA expression. Interestingly, the specific progesterone receptor antagonist RU486 induced UCP1 and UCP2 mRNAs, including UCP1 mRNA expression in non-NE-treated brown adipocytes, suggesting a profound effect of this antiprogestagen on brown adipocyte thermogenic capacity. Thus, are conclude that testosterone, 17-β-estradiol, progesterone and RU486 have distinct actions on brown adipocytes, thus modulating UCP1 and UCP2 mRNA expression and/or lipid accumulation, and that sex hormones are factors that may explain in part the gender-dependent BAT thermogenic response.

Key words. Uncoupling protein; brown adipose tissue; thermogenesis; testosterone; progesterone; estradiol; RU486.

Introduction

Sex-associated differences in rat brown adipose tissue (BAT) thermogenesis in response to diet-induced obesity and cold have been previously described, as have general sex differences in body weight gain and lipolytic activity of white adipose tissue (WAT) [1-4]. Sexual hormones have been proposed as key factors that could account in part for these differences by influencing, among other things, feeding behavior and central nervous responses, although direct actions on BAT could also be of importance.

BAT is of fundamental relevance in the regulation of energy balance and body weight control, at least in small mammals, because it is the main mediator of adaptive thermogenesis, which is highly dependent on the activity of uncoupling protein 1 (UCP1). UCP1 is an inner mitochondrial membrane protein that can dissipate energy as heat by uncoupling oxidative phosphorylation [5], and is

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usually expressed only in BAT. UCP2, which is expressed in other tissues apart from BAT, has been identified more recently [6] and its biological functions are so far poorly understood, although possible functions include control of adaptive thermogenesis in response to cold exposure and diet, control of reactive oxygen species production by mitochondria, regulation of ATP synthesis and regulation of fatty acid oxidation [6]. BAT is densely innervated by the sympathetic nervous system (SNS) and BAT thermogenesis is mainly physiologically regulated by norepinephrine (NE) which acts through adrenergic receptors leading to an increase in intracellular cAMP levels, promoting the activation of thermogenesis [5]. Sympathetic stimulation markedly up-regulates BAT UCP1 expression, but could also up-regulate UCP2 expression [7].

Sex hormones are important factors in determining fat distribution and accumulation and in regulating energy balance [8]. Part of these actions, as classically described, could be mediated by neural actions [8], however, many direct effects of sex steroid hormones on adipose tissue depots have also been described, although there is as yet little information about these effects on BAT. Estrogen and androgen receptors are expressed in preadipocytes and adipocytes in rats [9–11] and humans [12, 13] and progesterone and estradiol binding has been found in different rat adipose tissue depots, including BAT [11, 14].

The direct effects of sex hormones on WAT have been considered in several studies related to their action on the e. g. proliferation/differentiation of adipocytes, lipid uptake and lipoprotein lipase (LPL) activity, lipolysis and leptin production. Results from different studies aimed at investigating the effects of androgens on WAT, testosterone being the most studied, have generally shown that they can be assumed to have anti-adipogenic effects [15]. However, their action remains unclear in BAT. Interestingly, a possible indirect action of progesterone on BAT thermogenesis has been described, since inactivation of UCP1 has been observed during the mid and succeeding stages of pregnancy [16], when progesterone levels rise. On the other hand, estradiol can increase energy expenditure without effects on voluntary exercise and BAT has been proposed as a potential site for estradiol-induced thermogenesis [8, 17]. In this sense, PGC-1, a transcriptional co-activator of the peroxisome proliferator-activated receptor γ (PPARγ) [18] which is important in the differentiation of brown fat cells and UCP1 gene expression in BAT [19], has been reported to be a bona fide estrogen receptor α (ERα) co-activator and may serve as a convergence point between PPARγ and ERα signaling [20].

Considering all these precedents, the objective of the present study was to determine whether possible direct effects of sex steroid hormones on BAT could explain some of the sexual differences in BAT thermogenesis found in previous in vivo studies. To avoid central effects of the gonadal hormones, we chose an in vitro model, mice brown adipocytes differentiated in culture. We designed different experiments to test whether the main typical male and female sexual hormones, i.e. testosterone, progesterone and 17-β-estradiol, have any direct effect on the main mediator of BAT thermogenesis, UCP1, and on the more recently described UCP2, which is also expressed in brown adipocytes differentiated in culture.

Matherials and methods

Chemicals

Testosterone, 17-β-estradiol, progesterone and flutamide were from Sigma (Madrid, Spain), ICI 182.780 was from Tocris (Bristol, UK) and RU486 (Mifepristone) was from Biomol (Plymouth, Pa.). Other cell culture reagents were supplied by Sigma, Cultek (Valencia, Spain) and Gibco BRL (Barcelona, Spain), and routine chemicals were from Merck (Barcelona, Spain) and Panreac (Barcelona, Spain).

Cell isolation, culture and treatments

Brown fat precursor cells were isolated as previously described [21] from 4-week-old male NMRI mice (obtained from CRIFFA, Spain). All animals were treated in accordance with accepted standards of humane animal care. The cervical, interscapular and axillar brown adipose tissue depots were dissected out from each mouse under sterile conditions. The tissue was pooled and incubated in HEPES buffer (pH 7.4, 2 ml/mouse), containing 0.2% (w/v) crude collagenase type II. The tissue was digested for 30 min at 37 °C and vortexed every 5 min. The digest was poured through a 250-µm silk filter into 10-ml sterile tubes. The solution was then cooled at 4 °C for 15–30 min to allow the mature brown fat cells and lipid droplets to float. The infranatant was filtered through a 30-µm silk filter into 10-ml sterile tubes and precursor cells were collected by centrifugation for 10 min at 700 g, washed in Dulbecco's modified Eagle's medium (DMEM), peleted again, and resuspended in 0.5 ml of culture medium per mouse. Pooled final precursor cell suspension (0.5 ml) was inoculated in 25-cm^2 culture flasks containing 4.5 ml culture medium, at an inoculation density corresponding to 1 mouse/flask for most of the assays, except for the Oil red O staining assay, where 0.2 ml of pooled final precursor cell suspension was inoculated per well, using 35-mm-diameter wells containing 1.8 ml of culture medium. This was day 0. From that moment on, cells were incubated at 37 °C in 8% CO_2.

For the first 6 days, the preadipocytes were grown in 5 ml of a medium consisting of DMEM supplemented with 10% newborn calf serum, 4 nM insulin, 4 mM glutamine, antibiotics (50 IU penicillin/ml and 50 µg streptomycin/ml), 10 mM HEPES and 25 µg sodium ascorbate/ml. This medium containing serum was changed on day 1 (cells were washed before with DMEM) and 3. On day 6, the medium

was discarded and a serum-free medium was added, consisting of DMEM-F12 (1:1), free fatty acid bovine serum albumin 0.5%, 4 nM insulin, 4 mM glutamine, antibiotics (50 IU penicillin/ml and 50 µg streptomycin/ml), 10 mM HEPES and 25 µg sodium ascorbate/ml.

The different treatments were carried out on day 6, when cells presented a differentiated morphology and important lipid accumulation, and when they were placed in the serum-free medium. Steroid hormones and inhibitors of steroid hormones were dissolved in ethanol (testosterone, 17-β-estradiol, progesterone or flutamide) or DMSO (ICI 182.780 or RU486), and added to the corresponding flasks/wells, never exceeding a final ethanol or DMSO concentration of 0.01%. An equivalent volume of ethanol or DMSO was added in untreated controls. On day 7, after 24-h treatments and without changing the medium, the cells were exposed to NE for 6 h. After this, the cells were harvested with TriPure (Boehringer Mannheim, Barcelona, Spain) for isolation of RNA.

Oil red O staining

Dishes used for Oil red O staining were washed twice with PBS and fixed by 10% formaldehyde in PBS for 15 min. Fixed cells were PBS rinsed and stained with Oil-red-O-filtered solution (3 mg/ml in isopropyl alcohol) for 1 h. Cells were then washed with water and visualized with a Zeiss phase-contrast microscope (original magnification × 200).

RNA isolation

Total RNA was isolated using TriPure reagent, following the instructions of the manufacturer. RNA was determined using a spectrophotometer set at 260 nm.

Northern blot analysis of UCP1 and UCP2 mRNA and 18S rRNA

RNA (30 µg), denatured with formamide/formaldehyde, was fractionated by agarose gel electrophoresis as described elsewhere [22]. The RNA was then transferred onto a Hybond nylon membrane (Boehringer Mannheim) in 20×SSC (saline sodium citrate buffer: 1×SSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7.0) by capillary blotting for 16 h according to Jacobsson et al. [22], and fixed at 120°C for 30 min.

UCP1 mRNA, UCP2 mRNA and 18S rRNA were analyzed sequentially on the same membrane, in the aforementioned order, by a chemiluminiscence procedure based on the use of anti-sense oligonucleotide probes end-labeled with digoxigenin. We used the following probes obtained from Boehringer Mannheim and labeled both ends: for UCP1 mRNA, 5′-GTTGGTTTTATTC-GTGGTCTCCCAGCATAG-3′; for UCP2 mRNA, 5′-GGCAGAGTTCATGTATCTCGTCTTGACCAC-3′; and for 18S rRNA, 5′-CGCCTGCTGCCTTCCTTGGAT-GTGGTAGCCG-3′.

Fixed membranes were prehybridized at 42°C for 1 hour in DIG-Easy Hyb (Boehringer Mannheim) and then hybridized with the corresponding oligonucleotide probe (used at 35 ng/ml, except the 18S rRNA probe, which was used at 70 pg/ml) in DIG-Easy Hyb at 42°C overnight. Hybridized membranes were submitted to 2×15 min washes in a solution of 2×SSC/0.1% SDS at room temperature, followed by 2× 15 min. washes in 0.1 × SSC/0.1% SDS at 48°C. After blocking, the membranes were incubated first with an anti-digoxigenin-alkaline phosphatase conjugate, and then with the chemiluminescent substrate CDP-Star, essentially as in the protocols provided by Boehringer Mannheim. Finally, membranes were exposed to hyperfilm ECL (Amersham). Bands in films were analyzed by scanner photodensitometry and quantified using the KODAK 1D Image Analysis Software 3.5 (Kodak). The value of integrated optical density (IOD) of each sample and mRNA analyzed was divided by the IOD of the corresponding 18S rRNA band, to check for loading and transfer of RNA during blotting. The mean value of the control NE-treated cell group was set as 100%. Stripping between analysis was performed by exposing the membranes to boiling 0.1% SDS.

Statistics

All data are presented as the mean value ± SE. Differences between groups were assessed by one- and two-way analysis of variance (ANOVA) and Student's t test for post-hoc comparisons, using the program SPSS for Windows.

In the ANOVA analysis, possible effects are: T, effect of testosterone treatment; E, effect of 17-β-estradiol; P, effect of progesterone; NE, effect of norepinephrine, and the interactive effects T×NE, E×NE and P×NE. Results were considered statistically significant at the $p < 0.05$ level.

Results

In the present work, the aim was to study the effects of the main physiological sex hormones on brown adipocytes in vitro, in differentiated cells; thus, we chose brown adipocytes differentiated in primary culture on day 6, the moment when the different hormonal treatments were carried out. On day 6, cells visualized with a phase-contrast microscope showed a differentiated morphology, with important lipid accumulation, in all the experiments. We submitted the cells to 24-h hormonal-specific treatment and, on day 7, a fraction of these cells were submitted to a 6-h NE-treatment, because the main effector of thermogenesis in brown adipocytes, UCP1, is usually only expressed in brown adipocytes in culture under adrenergic stimulation [23].

UCP1 and UCP2 mRNA dose-dependent response to NE in differentiated cells placed in a serum-free medium

To avoid hormonal interference from the serum-containing medium used to grow the cells in primary culture, cells were placed in the serum-free medium on day 6, for 24 h (until day 7). Before the sex hormone experiments were carried out, we tested the capacity of this cell culture system to express UCP1 and UCP2 mRNAs and induce them under NE stimulation. The results of this experiment are presented in figure 1. NE was tested to induce UCP1 and UCP2 mRNAs at the concentrations of 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M during 6 h after 24 h in the serum-free medium. UCP1 mRNA was only induced under NE stimulation in an increasing, dose-dependent manner. 10^{-7} M NE was selected for the subsequent experiments, since it brought about important UCP1 mRNA expression without reaching saturated values. UCP2 mRNA also responded to NE in an increasing, dose-dependent manner; however, unlike UCP1 mRNA, it was expressed in non-NE-treated cells, and differences for UCP2 mRNA induction by the different NE concentrations tested were not as great as for UCP1 mRNA.

Morphological differences of brown adipocytes in culture after hormonal treatment

Representative results from the Oil red O staining assay can be seen in figure 2. In the control conditions, cells presented a differentiated morphology with a high lipid droplet content and had the capacity to respond to a 10^{-7} M NE treatment, showing the characteristic NE-activated morphology of differentiated brown adipocytes in culture due to an adrenergic lipolytic response [24], with much smaller and fewer lipid droplets than the non-NE-treated cells. For the Oil red O staining assay, hormone treatments were carried out only at the higher concentration presented in the rest of the experiments, i.e. 10^{-7} M. Cells treated with progesterone or 17-β-estradiol showed the tendency to have more and larger lipid droplets in all the experiments, leading to an apparently more differentiated morphology, whereas testosterone treatment led to a morphology with fewer and smaller lipid droplets. In all cases (progesterone-, 17-β-estradiol- and testosterone-treated cells), 10^{-7} M NE treatment gave similar images as the corresponding NE-treated control cells, showing much smaller and fewer lipid droplets (data not shown).

Effects of testosterone, 17-β-estradiol and progesterone on UCP1 mRNA in brown adipocytes differentiated in culture

The effects of different concentrations of testosterone, 17-β-estradiol or progesterone, i.e. 10^{-9}, 10^{-8} and 10^{-7} M, on UCP1 levels in differentiated brown adipocytes were tested (see fig. 3). We chose these concentrations because they are approximately physiological [25–28]. For UCP1

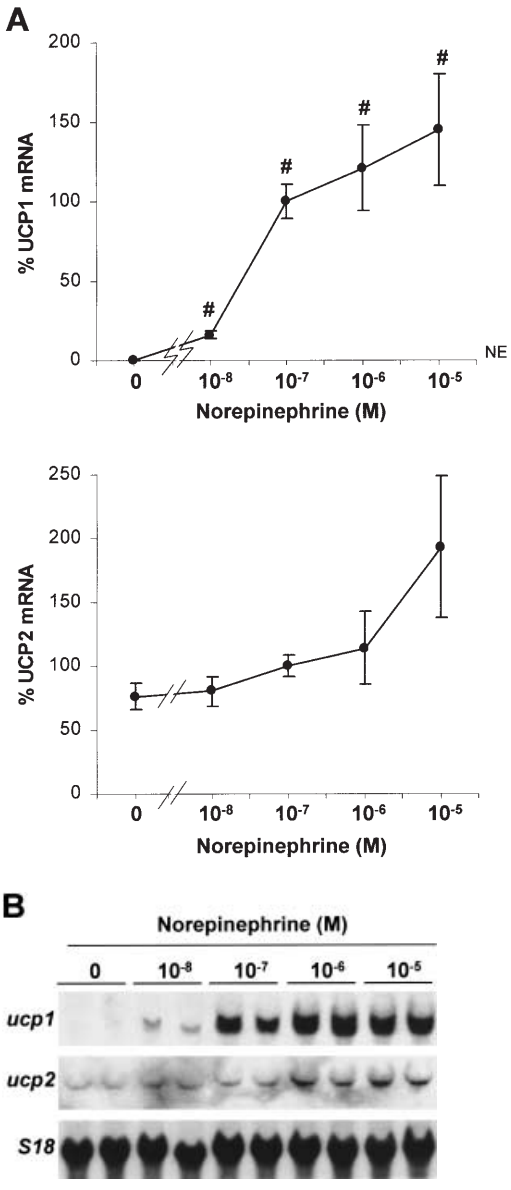

Figure 1. Curves of dose-dependent induction of UCP1 and UCP2 mRNAs by NE in differentiated brown adipocytes placed in a serum-free medium (*A*) and representative Northern blot (*B*) where duplicate lanes for each concentration represent separate experiments (30 µg total RNA per lane). The membranes were probed first for UCP1 mRNA, stripped and re-probed for UCP2 mRNA. 18S rRNA was the last species analyzed, to check loading and transfer. Developed Northern blot membranes were exposed to Hyperfilm ECL (Amersham) and quantified by scanner photodensitometry. Values are expressed as a percentage of the UCP1 or UCP2 mRNA value obtained in the 10^{-7} M NE-treated cell samples, which was set to 100%. Data represent the mean ± SE of three separate experiments performed in duplicate. One-way ANOVA ($p < 0.05$): NE indicates norepinephrine effect. t test ($p < 0.05$): # significant differences of NE-treated versus non treated cells.

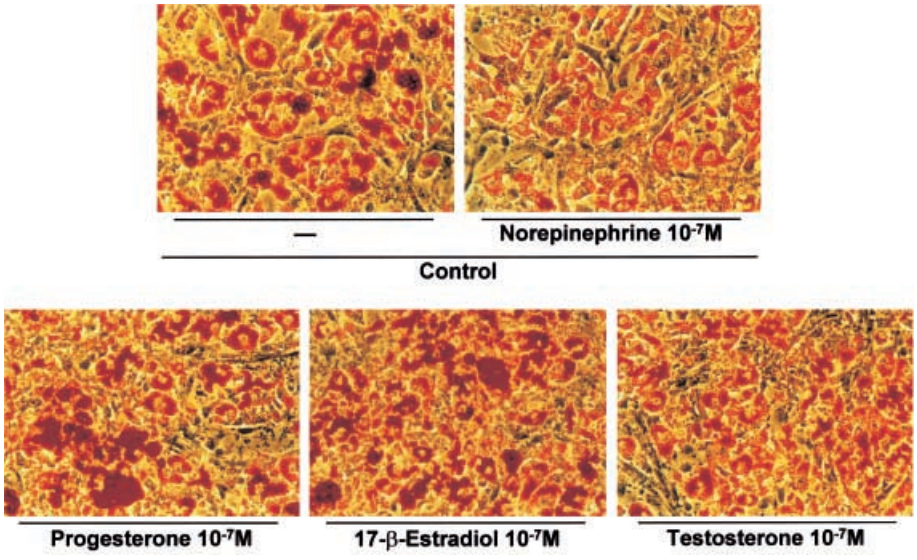

Figure 2. Effect of sex hormones (progesterone, 17-β-estradiol and testosterone) on cell morphology and lipid droplet shape and content in cultured brown adipocytes. Phase-contrast micrographs of Oil-red-O-stained primary brown adipocytes (original magnification × 200) are shown. Cells were grown in culture in a serum-containing medium until day 6; from day 6 to day 7, cells were placed in a serum-free medium and treated with the different sex hormones (10^{-7} M); the sex-hormone solvents used were added to control cells. NE treatment (10^{-7} M) was carried out in half of every group after 24-h hormonal (or control) treatments, for 6 h. After this, cells were Oil red O stained and photographed. In the figure, NE-treated cells are only shown for the control group.

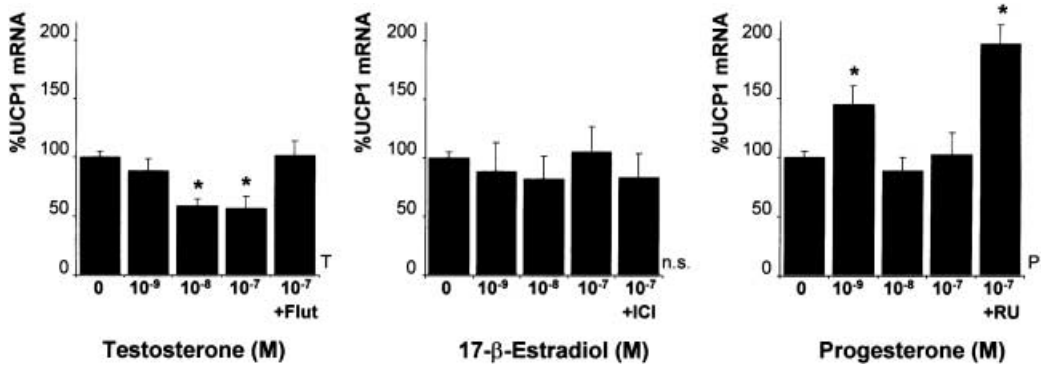

Figure 3. Effects of testosterone, 17-β-estradiol and progesterone on NE-induced UCP1 mRNA in brown adipocytes differentiated in culture. 0 indicates non-hormonal-treated cells (control); in the other groups, each hormone was tested at the concentrations of 10^{-9}, 10^{-8} and 10^{-7} M, while a corresponding receptor antagonist (10^{-6} M) was added at the same time to an additional group of 10^{-7} M hormone-treated cells. The antagonists were flutamide (Flut) for testosterone, ICI 182.780 (ICI) or 17-β-estradiol and RU486 (Mifepristone) (RU) for progesterone. Values are expressed as a percentage of the UCP1 mRNA value obtained for control cell samples, which was set to 100%. Values are means ± SE of three separate experiments performed in duplicate. t-test ($p<0.05$): * significant differences versus control group. One-way ANOVA ($p<0.05$): T, testosterone effect; P, progesterone effect, and n.s., nonsignificant differences.

mRNA, we only present the results obtained in the cells also treated with NE, as UCP1 mRNA is usually only expressed in brown adipocytes in culture under adrenergic stimulation (as seen in fig. 1) and there was no expression with testosterone, 17-β-estradiol or progesterone treatments without addition of NE.

Testosterone reduced UCP1 mRNA NE-induced expression in a dose-dependent manner; UCP1 mRNA levels were significantly lower in brown adipocytes treated with 10^{-8} and 10^{-7} M testosterone compared to control cell levels (this effect was also observed in cells treated with 10^{-6} M of testosterone – data not shown). This inhibitory effect was prevented in 10^{-7} M testosterone-treated cells, when the brown adipocytes were exposed at the same time to the androgen receptor antagonist flutamide (10^{-6} M). Addition to the cells of flutamide (10^{-6} M) alone had no effect per se on UCP1 mRNA levels (not shown). No effects on UCP1 mRNA levels were observed in the 17-β-estradiol-treated cells at any 17-β-estradiol concentration tested, nor did the addition to the cells of ICI 182.780

(10^{-6} M) alone have any effect on UCP1 mRNA levels (not shown). On the other hand, progesterone had a significant stimulatory effect on UCP1 mRNA NE-induced levels, but only at the lowest concentration tested, 10^{-9} M. Surprisingly, the potent antagonist of progesterone, RU486, which binds to progesterone receptors [29], administered (10^{-6} M) to the 10^{-7} M progesterone-treated cells, brought about an important induction of UCP1 mRNA expression in the differentiated brown adipocytes in culture.

Effects of testosterone, 17-β-estradiol and progesterone on UCP2 mRNA in brown adipocytes differentiated in culture

The effects of different concentrations of testosterone, 17-β-estradiol or progesterone, i.e. 10^{-9}, 10^{-8} and 10^{-7} M, on UCP2 levels in differentiated brown adipocytes are compiled in figure 4; both results from NE-treated and non-NE-treated cells are shown.

In non-hormone-treated cells, a low but significant induction of NE treatment on UCP2 mRNA levels was observed. The difference of NE-treated with respect to non-NE-treated cells was more or less maintained in all the testosterone-treated groups of brown adipocytes. Testosterone showed the general tendency to up-regulate NE-induced UCP2 mRNA levels, although it became significant only at the concentration of 10^{-7} M (and also at the concentration of 10^{-6} M – data not shown).

Although 17-β-estradiol brought about some slight down-regulation effects on UCP2 mRNA levels at the concentrations of 10^{-9} M (only for non-NE-treated cells) and 10^{-8} M (only for the NE-treated cells), it showed a varied response in the different groups and did not appear to have any important effect on UCP2 mRNA, as shown for UCP1.

Progesterone did not have any important effect on UCP2 mRNA levels, although it suppressed the differences between NE-treated and nontreated cells. On the other hand, as happened for UCP1 mRNA, there was a surprising effect that the treatment with RU486 up-regulated the UCP2 mRNA levels, in both the NE-treated and non-treated cells.

Effects of RU486 on UCP1 and UCP2 mRNAs in brown adipocytes differentiated in culture

Given the unexpected stimulation of UCP1 and UCP2 mRNAs by the simultaneous treatment of the cells with progesterone (10^{-7} M) and the progesterone receptor antagonist RU486 (10^{-6} M), the action of RU486 was further analyzed on brown adipocytes differentiated in culture. As shown in figure 5, treatment of the cells with RU486 (10^{-6} M) not only increased NE-induced UCP1 and UCP2 expression in the presence of 10^{-7} M progesterone, it also had the same effect in the absence of progesterone. Moreover, RU486-(10^{-6} M) plus progesterone-(10^{-7} M) treated non-NE-stimulated cells showed, apart from a stimulatory effect on UCP2 mRNA levels, an unexpected UCP1 mRNA expression, which is not usually seen by Northern blot in the absence of NE stimulation. Nevertheless, Northern blot analysis showed that RU486 alone (without progesterone and NE) did not cause induction of the UCP1 mRNA levels or of the UCP2 mRNA levels with respect to the control cells (not shown).

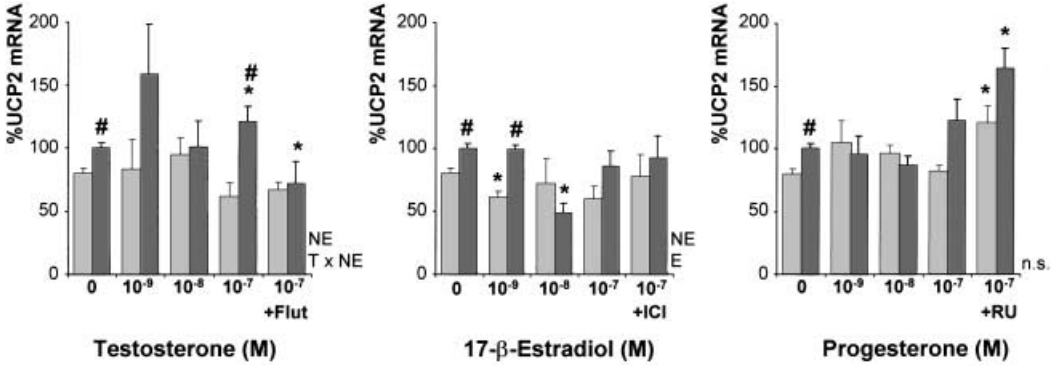

Figure 4. Effects of testosterone, 17-β-estradiol and progesterone on UCP2 mRNA in brown adipocytes differentiated in culture. Results from non-NE-treated cells (light bars) and NE-treated cells (dark bars) are shown. 0 indicates non-hormonal-treated cells (control); in the other groups, each hormone was tested at the concentrations of 10^{-9}, 10^{-8} and 10^{-7} M, while a corresponding receptor antagonist (10^{-6} M) was added at the same time to an additional group of 10^{-7} M hormone-treated cells. The antagonists were: flutamide (Flut) for testosterone, ICI 182.780 (ICI) for 17-β-estradiol and RU486 (Mifepristone) (RU) for progesterone. Values are expressed as a percentage of the UCP2 mRNA value obtained for NE-treated control cell samples, which was set to 100%. Values are means ± SE of three separate experiments performed in duplicate. t test ($p < 0.05$): * significant differences versus control group, # significant differences of NE-treated versus non-NE-treated. Two-way ANOVA ($p < 0.05$): NE, effect of norepinehrine; T × NE, interactive testosterone-norepinephrine effect; E, 17-β-estradiol effect, and n.s., nonsignificant differences.

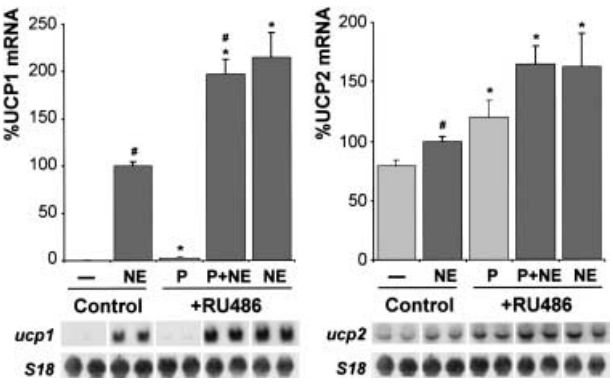

Figure 5. Effect of the progesterone receptor antagonist RU486 on UCP1 and UCP2 mRNA expression in brown adipocytes. Results of control cells are compared to those of RU486 10^{-6} M treated cells, also treated with progesterone (10^{-7} M) (P) or NE (10^{-7} M) (NE) or both. As in previous figures, results from NE-treated cells are presented as dark bars and from non-NE-treated cells as light bars. Representative Northern blot images are also shown; the membranes were probed first for UCP1 mRNA, stripped and re-probed for UCP2 mRNA. 18S rRNA was the last species analyzed, to check loading and transfer. Data are mean values ± SE. t test ($p < 0.05$): * significant differences versus control, # significant differences of NE-treated versus non-NE-treated.

Discussion

The present work demonstrates that testosterone and progesterone have distinct effects on the expression of brown adipocyte uncoupling proteins (UCP1 and UCP2) and lipid accumulation, and thus modulate brown adipocyte capacity for adaptive thermogenesis.

The inhibitory effect shown by testosterone on NE-induced UCP1 mRNA expression in brown adipocytes (fig. 3), which was associated with fewer and smaller lipid droplets in these cells (fig. 2), could have three, perhaps interrelated, explanations. First, in general terms, testosterone has been shown to inhibit male white adipose precursor cell differentiation [15, 30]; thus, a reduced differentiation of possible remaining brown adipocyte precursor cells on day 6 of culture cannot be ruled out as part of the explanation of the effects observed in UCP1 mRNA expression, which has been shown to be dependent on brown adipocyte differentiation [31]. This can also account for the fewer and smaller lipid droplets observed in testosterone-treated brown fat cells. Nevertheless, considering the advanced state of differentiation shown by the cells on day 6 of culture, this explanation could only account for part of the effects observed. Second, testosterone has been shown to stimulate lipolysis in white adipocytes of men and male rats [30], and this could be involved in the lipid droplet state of testosterone-treated cells. Finally, a third possible explanation is an androgen-induced up-regulation of α_2-adrenergic receptor (α_2-AR), and in particular α_{2A}-AR, which has been described in hamster white adipocytes [32–34]: α_2-ARs are expressed in brown adipocytes and decrease the levels of cAMP [35, 36] consequently inhibiting brown fat thermogenesis and UCP1 expression. This latter fact could also be important in the testosterone-dependent inhibition of UCP1 mRNA expression. One should also note that testosterone-observed effects in cultured brown adipocytes on UCP1 mRNA expression were reverted by simultaneous treatment with the androgen receptor antagonist flutamide (it had no effect per se on UCP1 mRNA levels), indicating that these testosterone effects seem, at least in part, to be androgen receptor dependent.

In previous work in which a rodent gender-dependent BAT thermogenic response was shown, changes in UCP2 mRNA levels were coincident with those of UCP1 mRNA [2, 4, 37]. UCP2 mRNA expression showed a distinctly different pattern of response to testosterone than UCP1 mRNA (see fig. 4), with a tendency to be up-regulated when also treated with NE (significant at 10^{-7} M testosterone-treated cells). This would indicate a possible difference in testosterone-dependent regulation of UCP1 and UCP2 mRNAs on brown adipocytes.

Differentiated brown adipocytes treated with 17-β-estradiol tended to have more and larger lipid droplets; actions of estrogens promoting *in vitro* adipose conversion have been described [15] and these observations would agree with the apparently more differentiated morphology of the 17-β-estradiol-treated brown adipocytes. However, we have not seen any important effect of 17-β-estradiol on UCP1 and UCP2 mRNA expression in differentiated brown adipocytes. Thus, further investigation of other possible estrogenic actions on differentiation and development of brown adipocytes would be of interest to explain the effect observed on lipid accumulation.

Progesterone not only brought about an apparently more differentiated state in cultured brown adipocytes, as suggested by the tendency of progesterone-treated cells to have more and bigger lipid droplets (see fig. 2), it also stimulated NE-induced UCP1 mRNA expression, although only at the lower progesterone concentration tested (10^{-9} M). This effect could be due to a stimulatory effect of progesterone on adipose differentiation, which in brown adipocytes leads to a higher capacity to express UCP1 mRNA, as progesterone has been shown to stimulate the expression of ADD1/SREBP1c (adipocyte determination and differentiation 1/sterol regulatory element-binding protein 1c) in rat white adipocytes in vitro [38], a factor which is highly expressed in brown adipocytes and important for their differentiation [39]. However, taking into consideration the advanced differentiated state of the brown adipocytes at progesterone addition (day 6), this can only be a partial explanation. On the other hand, a possible indirect action of progesterone on BAT thermogenesis has been described which could at first appear to contradict the results reported here, since an inactivation

of UCP1 during the mid and succeeding stages of pregnancy (when progesterone levels rise) has been reported [16] (although BAT UCP1 had not been found, until now, to be directly affected by progesterone [40]). As the progesterone effect up-regulating UCP1 mRNA levels in our experiment was only at the lowest concentration administered, perhaps this treatment could be below the threshold concentration of progesterone required for UCP1 mRNA inhibition, and perhaps at the lowest concentration, we can only see the positive effect of progesterone on brown adipocyte differentiation manifested as UCP1 mRNA induction. Thus, progesterone could present a different action on brown adipocytes depending on the levels of this hormone. Another important factor that must be taken into account is that the action of progesterone may be modulated by the presence of estrogens (as occurs in vivo), since most of the effects of progesterone on adipose tissues and also on body weight observed in other work occur only when estrogens are also present, and estrogens have been demonstrated to induce progestin receptor formation [41]. Therefore, further investigation will clarify a possible action of progesterone together with estrogens on brown adipocytes.

The effect of the simultaneous treatment of the cells with progesterone (10^{-7} M) and the progesterone antagonist RU486 (10^{-6} M) stimulating NE-induced UCP1 mRNA expression and UCP2 mRNA expression (in both NE- and non-NE-stimulated cells) was unexpected. As shown in figure 5, RU486 not only brought about an important induction of both NE-induced UCP1 and UCP2 mRNAs in the presence of progesterone, it also had the same effect alone. This fact, added to the lack of effect on these mRNAs in 10^{-7} M progesterone-alone-treated cells (see figs. 3, 4) as compared to controls, suggests the existence of an important stimulatory effect induced by RU486 in the presence of NE. Furthermore, in RU486+progesterone-treated cells (but non-NE-stimulated), apart from a stimulatory effect on UCP2 mRNA, there was unexpected UCP1 mRNA expression. However, RU486 alone (without progesterone and without NE) failed to induce UCP1 mRNA expression (data not shown), thus showing that RU486 has the capacity to induce UCP1 mRNA in the absence of NE, but in the presence of progesterone. Thus, RU486 could be involved in different ways in the regulation of UCP expression. There is little bibliography about RU486 and BAT thermogenesis; nevertheless, RU486 has been reported to stimulate oxygen consumption and BAT activity in vivo in the rat, even though these RU486 actions were suggested to be exerted centrally and not directly on BAT [42]. However, we show here a direct action of RU486 on brown adipocytes. RU486 has been described as acquiring agonist activity in response to stimulation of cAMP signaling pathways and such activity appears to be the result of RU486 activity through the progesterone receptor [29]; this fact could be important in the results observed here, since adrenergic stimulation leads to an increase of cAMP in brown adipocytes, mainly mediated by β_3-adrenergic receptors. Nevertheless, the unexpected UCP1 mRNA expression and the UCP2 mRNA up-regulation in the absence of NE indicate a more profound effect of RU486 on gene expression in brown adipocytes, at least in the presence of progesterone. Moreover, RU486 has been shown to be a potent accelerator of adipose conversion of the 3T3-F442A adipose cells [43]; thus, there could also be an effect on adipocyte differentiation, which could be partially related to higher UCP1 expression.

In summary, we report here direct and opposite effects of the physiological sex hormones testosterone and progesterone on mouse differentiated brown adipocytes, and less marked 17-β-estradiol effects. These effects of sex hormones might contribute to explaining the gender-dependent BAT thermogenic response found in previous work. A possible role of sex hormones on brown adipocyte differentiation could be of relevance. Nevertheless, another possible regulation of UCP1 and UCP2 expression by sex hormones, at the transcriptional level, could be via the modulation of the activity or the expression of α- and β-adrenergic receptors, when taking into consideration the importance of adrenergic stimulation by NE in the physiological regulation of BAT thermogenesis [5]. Furthermore, α- and β-adrenergic receptors have been previously observed to show a sexual dimorphism in BAT in rats [4, 37] and can be influenced by sex hormones in WAT [33, 44, 45]. Altogether, sex hormones can be considered as elements of the varied group of factors regulating BAT activity. Moreover, the reported stimulatory effects of the anti-progestin RU486 on UCP1, the main mediator of BAT thermogenesis, and on UCP2, should be taken into account in future investigations in the realm of thermogenesis function and obesity.

Acknowledgements. This work was supported by the Spanish Government (grants BFI 2000-0988-06 and FIS 01/1379) and by the European Commission (COST Action 918). A. M. Rodríguez was supported by a grant of the Spanish Government, and M. Monjo was supported by a grant of the University of the Balearic Islands.

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