Sex Steroid Receptor Expression Profile in Brown Adipose Tissue. Effects of Hormonal Status

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Key Words

Oestrogen receptor • Androgen receptor • Progesterone receptor • Brown adipose tissue • Gender dimorphism

Abstract

Recent investigations suggest that sex hormones play an important role in the brown adipose tissue (BAT) thermogenic program by acting on several steps of the lipolytic signal cascade and on the UCP1 transcription control. However, the number of studies focusing on steroid receptor status in brown adipose tissue is negligible. In the present study, we analyze steroid receptor mRNA levels in brown adipose tissue in male and female rats and in pregnant and lactating females, all of them models with a different hormonal background. The direct effect of sex hormones on the expression of their receptors was studied in vitro in primary culture of brown adipocytes. Oestrogen receptor (ER α) and androgen receptor (AR) densities were higher in male than in female BAT. PR A+B mRNA expression was downregulated in lactation, suggesting a role of

progesterone signalling in thermogenesis impairment at this stage. In vitro studies showed that progesterone decreased PR A+B mRNA and that testosterone downregulated ER α mRNA. The results highlighted in this study demonstrate the presence of steroid receptor mRNA in BAT and in brown cell cultured adipocytes, supporting the idea that changes in steroid receptor expression would be important for the understanding of sex hormone effects on BAT physiology.

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Introduction

BAT is of fundamental relevance in the regulation of energy balance and body weight control, particularly in rodents [1], as 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 the respiratory chain from ATP synthesis [2]. BAT is densely innervated by the sympathetic nervous system (SNS) and BAT thermogenesis is physiologically regulated mainly by noradrenaline (NA) [3].

Gender dimorphism in energy metabolism has been reported. In particular, some differences between males and females have been found in white adipose tissue metabolism and brown adipose tissue thermogenic capacity in normal housing and feeding conditions and in response to diet-induced obesity and cold exposure [4-7]. Pregnancy and lactation, physiological states characterised by an important rise in sex hormones, are accompanied by an altered white adipose tissue metabolism in order to supply energy to the fetus, and that is also depicted by BAT atrophy of and impaired thermogenesis to save energy for fetus growth.

Thus, sexual hormones have been proposed as key factors that could account, in part, for these differences by influencing feeding behaviour [8,9] and central nervous responses [10,11]. Nevertheless, direct actions of sex hormones on adipose tissue should also be considered as direct effects of sex hormones in determining fat distribution and accumulation [12]. Nevertheless, the available information regarding the direct effects of sex hormones on BAT physiology is scant.

Steroid hormones exert their effects through specific receptors. To date, oestrogen, androgen and progesterone receptors have been reported in white preadipocytes and adipocytes [13-18]. This distribution seems to be depot- and sex- dependent [19] and could be responsible for a depot specific effect of sex hormones on adipose tissue metabolism [20]. As far as BAT is concerned, the presence of oestrogen receptors in BAT was suggested by binding assays [21], although no further studies have been undertaken to date.

The effects of sex hormones on BAT physiology is a matter of controversy. Thus, some studies have reported opposite effects for oestradiol, testosterone or progesterone on BAT thermogenesis capacity and activity. For instance, 17 β -oestradiol has been reported to inactivate BAT in some studies [22,23] or activate it in others [24].

We hypothesise that this could be explained by differences in the receptor signalling, whose characterization in BAT has not been studied. To this extent, we purposed to analyze the androgen receptor (AR), oestrogen receptor α (ER α) and progesterone receptor (PR-A+B) mRNA levels in rats of both genders and in female rats under pregnancy and lactation, all of which are physiological models with a strong hormonal background. Moreover, an *in vitro* model in cultured brown adipocytes

was used to further address the regulation of sex steroid receptors by their own ligands. We also discuss the possible involvement of this receptor profile in brown adipose tissue physiology.

Materials and Methods

Materials

Testosterone, 17- β -oestradiol and progesterone were from Sigma (St Louis, MO, USA). Other cell culture reagents were supplied by Sigma (St Louis, MO, USA), Cultek (Madrid, Spain) and Gibco BRL (Gaithersburg MD, USA); RNA isolation and PCR chemicals were from Roche Diagnostics (Basel, Switzerland) and routine chemicals were from Merck (Darmstadt, Germany) and Panreac (Barcelona, Spain). Microplate spectrophotometer was supplied by Bio-Tek Instruments, Inc (Vermont, USA), Gene Amp 9700 thermal cycler was supplied by Applied Biosystems (California, USA), Lightcycler thermal cycler was supplied by Roche (Basel, Switzerland).

Animals and isolation of brown adipose tissue

Three-month male (n=6) and virgin (n=6), pregnant and lactating female Wistar rats (n=7) were used for the *in vivo* assay (obtained from Charles River Laboratories, Wilmington, MA, USA). Animals were housed at 22°C, with a light-dark cycle of 12 h, and with free access to drinking water and standard chow pellets (Panlab, Barcelona, Spain). Animals were killed by decapitation at the start of the light cycle and interscapular brown adipose tissue was rapidly removed, frozen in liquid N₂ and stored at -70°C until RNA isolation. Animal experiments were performed in accordance with the general guidelines approved by our institutional ethics committee and EU regulations (86/609/EEC).

Primary cultures of mouse brown adipocytes

Brown fat precursor cells were isolated from 4-week-old male NMRI mice (supplied by Charles River Laboratories, Wilmington, MA, USA) as previously described [25]. 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- μ m 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- μ m silk filter into 10-ml sterile tubes and precursor cells were collected by centrifugation for 10 min at 700xg, washed in Dulbecco's modified Eagle's medium (DMEM), pelleted again, and resuspended in 0.5 ml of culture medium per mouse. 0.2 ml of pooled final precursor cell suspension was inoculated in 6-well plates each well containing 1.8 ml of culture medium. This was day 0. From that moment on, cells were incubated at 37°C in 8% CO₂.

For the first 6 days, the preadipocytes were grown in 2 ml of a medium consisting of DMEM supplemented with 10%

Gene	Primer sequence (5'-3') Forward and reverse	Denaturation Temp. °C (time, s)	Annealing Temp. °C (time, s)	Elongation Temp. °C (time, s)	T _m (°C)
AR (r)	5'-ccc atc gac tat tac ttc cc-3' 5'-tta cga gct ccc aga gtc at-3'	95 (3)	60 (10)	72 (18)	84
ER-α (r)	5'-ggc tac gtc aag tcg att cc -3' 5'-atc ttg tcc agg act cgg tg-3'	95 (5)	60 (8)	72 (12)	83
PR A+B (r)	5'-ccc aca gga gtt tgt caa gct c -3' 5'-taa ctt cag aca tca ttt ccg g -3'	95 (5)	64 (10)	72 (15)	83
AR (m)	5'-ggc tcc ctc ttc ctc caa-3' 5'-atg ctt cca cac cca atc c-3'	95 (10)	60 (8)	72 (12)	83
ER-α (m)	5'-cta tcc aca gcc acc caa ga-3' 5'-aac cca cca ttc caa aaa g-3'	95 (10)	63 (8)	72 (12)	82
PR A+B (m)	5'-cag aag cca gcc aga gcc-3' 5'-cac agg taa gca cgc cat agt-3'	95 (5)	64 (10)	72 (15)	86
18S (r, m)	5'-gag gtg aaa ttc ttg gac cgg-3' 5'-cga acc tcc gac ttt cgt tct-3'	95 (5)	60 (8)	72 (12)	82
Cyclophilin (r)	5'-ggc aag acc agc aag aag a-3' 5'-tga agg gga atg agg aaa a-3'	95 (5)	60(8)	72 (12)	85

Table 1. PCR temperature and time conditions (°C, s). r rat, m mouse.

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 previously washed with DMEM) and on day 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. Serum-free medium was used to avoid hormonal interference. Testosterone, 17-β-oestradiol or progesterone (from 10⁻⁹M to 10⁻⁷M) were dissolved in ethanol and added to the corresponding plates, with the final ethanol concentration never exceeding 0.01%. An equivalent volume of ethanol was added in untreated controls. On day 7, after 24 h treatment, the cells were harvested for RNA isolation.

RNA isolation

Total RNA from rat brown adipose tissue (~100mg) as well as from brown adipocytes from mouse primary culture was isolated using Tripure™ reagent following the instructions of the manufacturer. RNA was determined in triplicate using a microplate spectrophotometer set at 260 nm.

Reverse-transcription

For reverse transcription of oestrogen receptor alpha (ERα), androgen receptor (AR) and progesterone receptor (PR-A+B), 1 µg of total RNA was reverse transcribed to cDNA at 42°C for 15 min with 25U MuLV reverse transcriptase in a 10 µl volume of RT reaction mixture containing 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton®X-100, 2.5 mM MgCl₂,

2.5 µM random hexamers, 10 U RNAase Inhibitor and 500 µM of each dNTP in a DNA Gene Amp 9700 thermal cycler.

Real time RT-PCR

The primers used were designed using specific primer analysis software Primer3 (Whitehead Institute for Biomedical Research) and Oligo Analyzer 3.0 (Integrated DNA Technologies, Inc.) and the specificity of the sequences was analyzed by Fasta in the BLAST database (<http://www.ncbi.nlm.nih.gov/BLAST/>) (see Table 1).

Real-time PCR was performed using a LightCycler™ rapid thermal cycler system (Roche Diagnostics, Basel, Switzerland). Reactions were performed in a 10 µL volume containing 3 µL of cDNA sample (diluted 1/10) using 0.5 µM primers, 3 mM of MgCl₂ as well as dNTPs, Taq DNA polymerase and reaction buffer provided in the LightCycler FastStart DNA Master SYBR Green I mix. All real-time conditions are summarised in Table 1. Product specificity was confirmed in initial experiments by agarose gel electrophoresis and routinely by melting curve analysis. For mathematical analysis, the crossing points (CP) values were used for each transcript. CP is defined as the point at which fluorescence rises appreciably above the background fluorescence. 'Fit Point Method' was performed in the LightCycler software 3.3 (Roche Diagnostics), at which CP was measured at a constant fluorescence level. It should be noted that a lower CP means a higher expression rate and that a difference of 1 cycle number theoretically means a 2 fold difference in mRNA levels, although this is dependent on the amplification rate. PCR efficiencies of each amplicon were calculated using the following formula: $E = (F/F_0)^{1/(n-n_0)}$ (values were between 1.7 and 1.9 in our study), where n and n₀ were the crossing point values of F and F₀, fluorescence signals of the linear phase of the logarithmic amplification curve.

Fig. 1. Gender differences in the expression of sex steroid receptors in brown adipose tissue (n=6). Fold changes were calculated by setting male values to 1. Differences between groups were assessed by a Pair Wise Fixed Reallocation Randomisation Test©. The level of probability was set at p<0.05 as statistically significant, where * denotes significant differences vs males.

Fig. 2. Expression of sex steroid receptors in brown adipose tissue during pregnancy and day 5 of lactation (n=7). Fold changes were calculated by setting non pregnant values to 1. Differences between groups were assessed by a Pair Wise Fixed Reallocation Randomisation Test©. The level of probability was set at p<0.05 as statistically significant, where * denotes lactation at day 5 (L5) vs non pregnant (expression was not detected in 6 of 7 samples).

Statistics

Statistical PCR data analysis was performed using the Relative Expression Software Tool (REST). The statistical model used was a Pair Wise Fixed Reallocation Randomisation Test© [26]. Differences in expression between groups were assessed using the means for statistical significance by randomisation tests, a proper model to avoid the normal distribution assumption of the data. The level of probability was set at p<0.05 as

statistically significant. In this study we used two different housekeeping genes, 18S in the case of gender and in vitro studies, and cyclophilin in the case of pregnancy and lactation analysis. For progesterone serum levels, differences between groups were assessed by Student's-t test. The analysis was performed with SPSS 11.0 for Windows. The level of probability was set at p<0.05 as statistically significant.

Fig. 3. Expression of androgen receptor (a), oestrogen receptor- α (b) and progesterone receptor A+B (c) in differentiated brown adipocytes treated with testosterone, 17 β -oestradiol, and progesterone (10^{-9} – 10^{-7} M) for 24 h. (n=6). Fold changes were calculated by setting control group levels to 1. Differences between groups were assessed by a Pair Wise Fixed Reallocation Randomisation Test©. The level of probability was set at $p < 0.05$ as statistically significant, were * denotes significant differences between hormone-treated versus control.

Results

Steroid receptor mRNA expression in brown adipose tissue from male and female rats

Male BAT showed higher AR and ER α mRNA levels than females (Figure 1). The same tendency was observed in the case of PR A+B although it was not statistically significant.

Steroid receptor mRNA expression in brown adipose tissue in pregnant and lactating rats

No significant differences in AR or ER α mRNA expression during the pregnancy days studied or lactation was observed. Interestingly, PR A+B mRNA levels were not detected at day 5 of lactation (Figure 2) indicating a severe downregulation in this stage.

Fig. 4. Progesterone serum levels during pregnancy and lactation. Data represent means $\pm$ SEM of 7 samples per group. Differences between groups were assessed by Student's t test. The level of probability was set at $p < 0.05$ as statistically significant, where * denotes vs control and # vs P13.

Sex hormones modified steroid receptor gene expression in cultured brown adipocytes

Androgen receptor mRNA expression was not modified under oestradiol or progesterone treatment. Nevertheless a tendency to be downregulated by testosterone was observed (Figure 3a).

Oestrogen receptor α mRNA levels were downregulated under testosterone treatment in all the concentrations tested (Figure 3b). On the other hand, neither oestradiol nor progesterone exerted any effect on ER α expression. Of note, ER β 1 and ER β 2 were not detected in vitro and were neither detected in vivo models.

Progesterone receptor mRNA expression was downregulated under the highest concentration of its own ligand (10^{-7} M) (Figure 3c). Neither oestradiol nor testosterone treatment modified the expression of PR A+B mRNA levels.

Progesterone serum levels in pregnancy and lactation

Progesterone serum levels were increased in pregnancy and lactation over control values as expected (Figure 4). A significant downregulation of progesterone levels can be observed from day 13 of pregnancy until day 5 of lactation, although it did not reach control levels.

Discussion

In the present study, we provide evidence for a variation in the content of sex steroid receptor mRNAs in BAT of male and female rats, and in response to pregnancy and lactation. Moreover, we tested in vitro the effect of sex hormones on their receptor mRNA levels in isolated brown adipocytes. The data reported here could help to establish differences in brown adipose tissue sensitivity under hormonal stimulation and to give insight into sex hormone effects in BAT thermogenesis.

The effect of androgen signalling in BAT thermogenesis is a matter of controversy [27]. Recent studies have demonstrated the existence of an androgen response element in UCP1 promoter that suggests a direct effect of androgens on UCP1 expression [28]. Nevertheless, the effects of androgens on UCP1 transcription seem to be contradictory [28-30]. Previous work performed in our group in a similar model reported a decrease in UCP1 mRNA expression under testosterone exposure [29]. These discrepancies could be due to the experimental model used (in vivo vs in vitro) where indirect mechanisms dependent on hormonal action such as adrenergic signalling should be considered [31].

As far as we know this is the first study where AR mRNA expression is demonstrated in BAT. The higher AR mRNA levels in male compared to female BAT

reported here, together with the higher testosterone levels in males [19] leads to a higher androgenic background in males which could, at least in part, be responsible for the lower thermogenic recruitment and lower UCP1 levels of male rats previously reported [6,7]. These results have been recently reinforced by evidence that demonstrate that testosterone decrease lipolysis activity [31], the flux generating step controlling BAT respiration in brown adipocytes [32], and also downregulates PGC1 α mRNA levels [33] a key master of UCP1 transcription. In our study, AR mRNA expression did not change in response to sex hormone treatment or during pregnancy or lactation, conversely to what occurs in white adipose tissue [19], suggesting that there are different factors linked to gender dimorphism in the modulation of androgenic response in BAT and WAT.

In this study, we confirm the presence of ER α mRNA in BAT and brown adipocytes supporting the results of Wade et al. using binding assays [21]. Nevertheless, ER β 1 and ER β 2 were not detected suggesting that the oestrogenic response in brown adipocytes is mainly mediated by ER α .

Interestingly, α ERKO mice shows a decrease in energy expenditure and clear signs of obesity [34] along with a decrease of genes involved in mitochondrial electron chain [35] suggesting BAT function could be compromised due to the lack of ER α . This hypothesis is supported by other studies demonstrating ER can directly modulate the transcriptional activity of PGC1 [36,37], a well established thermogenic coactivator [38], and in vivo studies showing a decrease in UCP1 levels in BAT of ovariectomised animals [39,40]. This prothermogenic effect of ER-oestradiol axis in vivo, is partially supported in vitro where 17 β -oestradiol, although do not exert any effect on UCP1 expression [29], increases adrenergic signalling, an important activator of UCP1, in brown adipocytes [31]. This suggests that adrenergic responsiveness could be one of the mechanisms responsible for the oestrogenic action on BAT function.

We demonstrate that ER α levels are higher in male than in female BAT, as previously reported for white adipose depots [19]. This suggests that oestrogenic action is also of relevance in male BAT. Unfortunately, there are not gender studies addressed to highlighting differences in BAT metabolism under hormonal treatment. On the other hand, the absence of changes in ER α mRNA expression in BAT during pregnancy and lactation suggests that modulation of ER α mRNA in this tissue is not contributing to enhance the oestrogenic signalling observed during these periods due to the increase in

plasma oestradiol levels. To this point, it is worth to point out that a higher variability was observed in the non pregnant female group (not reflected in Figure 2), which could reflect the presence of rats at different phases of the estrous cycle, since it have been demonstrated that oestrogen receptor is modulated during estrous cycle in other tissues [41,42].

The downregulation of ER α mRNA expression elicited by testosterone in vitro, agrees with previous results in mammary gland [43], mouse cerebral cortex [44], rat brain [45], and rat prostate gland [46]. The physiological relevance of this event in BAT is unknown but it could contribute to an impairment of oestrogenic signal (for example, by decreasing the inhibition exerted by oestradiol in α 2/ β 3-adrenergic receptor signalling observed in cell cultured adipocytes [31]). Nevertheless, it seems contradictory that males with a higher androgenic background than females have higher ER α mRNA levels. This suggests that hormones others than testosterone are responsible for the gender differences in ER α mRNA levels in BAT.

In vitro, the absence of modulation of ER α mRNA by oestradiol does not resemble our previous observations in white adipocytes [19] suggesting that the modulation of ER α by its own ligand is depot specific or also probable that there are depot differences in the enzymatic machinery that metabolise sex hormones [47].

To this point, it is worth to mention that changes in the mRNA levels of steroid receptors in our model could be good markers of changes in BAT sex hormone responsiveness modifying its thermogenic features, although posttranslational, traduccional changes as well as activity modulators aimed to change the net hormonal responsiveness should be considered.

Progesterone also seems to exert an important role in BAT physiology, stimulating adrenergic signalling [31] and increasing UCP1 mRNA expression [29]. As expected, PR A+B mRNA expression was observed in female BAT, but more interestingly PR A+B was also found in male BAT. To date, the presence of PR in male adipose tissues has been a matter of controversy [48,49]. Little is known about the role of progesterone in males [50] but the presence of its receptor in BAT is suggestive of a role in the male BAT physiology.

Up to date, the downregulation of UCP1 expression during pregnancy and lactation [51] has been mainly attributed to a fall in sympathetic activity [52,53] explaining, at least partially, the impairment of thermogenesis that takes place at this stage. Nevertheless, the decrease observed in PR A+B mRNA levels during lactation, along

with the clear stimulatory effect of progesterone on UCP1 mRNA levels depicted *in vitro*, suggest that the loss of progesterone responsiveness during lactation could also contribute to the impairment of thermogenesis by acting directly on the brown adipocyte leading to a decrease in UCP1 mRNA levels. Thus, the downregulation of PR A+B receptor would be due to the overstimulation of its own ligand, as demonstrated *in vitro*, or would be dependent to other hormones during lactation. To this extent, prolactin, whose levels are high during lactation, has been shown to downregulate both PR [54] and UCP1 mRNA expression [55], which supports the notion that progesterone axis could act as an effector of the hormonal changes that take place during lactation.

In summary, this study demonstrates that sex steroid receptors are expressed and regulated in BAT and in cell-cultured brown adipocytes, supporting the idea that changes in steroid receptor expression would be important for the understanding of sex hormone effects on BAT physiology. Nevertheless, it is important to take into account that sex hormones also exert a role in BAT physiology by modifying sympathetic nervous system (SNS) activity. Thus, the knowledge of a unified model

integrating both direct and indirect mechanisms of sex hormones could be of relevance in the study of sex hormones on thermogenesis and BAT physiology.

Abbreviations

AR (androgen receptor); ER (oestrogen receptor); PR (progesterone receptor); UCP1 (uncoupling protein 1); BAT (brown adipose tissue), WAT (white adipose tissue).

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