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ENHANCEMENT OF THE EXPRESSION OF THE α2-ADRENORECEPTOR PROTEIN AND mRNA BY A DIRECT EFFECT OF ANDROGENS IN WHITE ADIPOCYTES

René PECQUERY, Marie-Noëlle DIEUDONNE, Jean-François CLOIX, Marie-Christine LENEVEU, Jean-Pierre DAUSSE and Yves GIUDICELLI #

Department of Biochemistry, CJF INSERM 9402, Faculty of Medicine Paris-Ouest, University René Descartes, 45 Rue des Saint-Pères, 75006 Paris and Centre Hospitalier Intercommunal, 78303 Poissy, France

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In vivo, testosterone-treatment of female hamsters for 4 days promotes a doubling of α2-adrenoreceptor protein in parametrial adipocytes, with a concomitant accumulation of the α2A-adrenoreceptor subtype mRNA. During *in vitro* incubation of minced parametrial fat pads for 6 to 48h with testosterone or dihydrotestosterone (100 nM), α2A-adrenoreceptor protein and mRNA levels were also increased and remained to control levels when an antiandrogen or actinomycin D were added in the medium. It is concluded that in hamster adipocytes, androgens upregulate α2A-adrenoreceptor subtype expression at the mRNA level by an androgen receptor-dependent transcriptional activation.

The importance of the balance between α2 (antilipolytic)- and ß (lipolytic)-adrenoreceptors in the control of lipolysis in adipocytes from several species has been emphasized by numerous studies. Changes in the distribution of these receptors seem to be responsible for some of the site- and sex-related differences in the lipolytic responses to catecholamines in human adipocytes (1-3).

Sex-steroid hormones play an important role by modulating different steps of the lipolytic cascade. Indeed, in a previous study (4), we have shown that changes in the androgenic status modulate the functional α2/ß-adrenergic balance in hamster adipocytes. Testosterone administration , while increasing the ß-adrenergic lipolytic action of adrenaline through enhanced adenylate cyclase activity (5), promoted to a greater extent its α2-adrenoreceptor (α2-AR) mediated antilipolytic potency through a specific and androgen receptor-dependent up-regulation of the adipocyte α2-adrenoreceptors (4).

Androgens are known to exert an important control on gene expression in androgen-target tissues like prostate and skin (6,7). The presence of androgen receptors in hamster adipose tissue (8) strongly suggests that androgens may

To whom correspondence should be addressed. Fax:(33.1)39798240.

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have direct regulatory effects on the expression of some genes in adipocytes. Therefore, the *in vitro* effects of androgens on the expression of α2-AR mRNA and protein were studied using parametrial fat from female hamsters to reduce the interference of endogenous androgens. The present experiments demonstrate that the α2-AR up-regulation seen after *in vivo* testosterone treatment (4) is due to an androgen receptor-dependent transcriptional activation of the α2-AR expression in adipose tissue.

Materials and methods.

Eight week-old male or female Syrian hamsters maintained under constant lighting conditions (light 6 am, dark 8 pm) were killed by decapitation. Epididymal and parametrial adipose tissues were immediately removed under sterile conditions.

For *in vivo* studies, male hamsters were castrated or sham-operated under sodium barbital anesthesia. One week after surgery, half of the castrated animals received daily one subcutaneous injection of testosterone propionate (10mg/kg body weight in 0.1 ml propylene glycol) for 10 days. Testosterone-treated female hamsters received the same daily dose of testosterone-propionate for 4 days. All other animals received the vehicle only. Animals were killed one day after the last injection.

For *in vitro* studies, long-term incubations were performed with parametrial adipose tissue from females. 1 to 2g of minced fat pads were incubated for 6 to 48 h in the presence or absence of androgens at 37°C in Dulbecco's modified Eagle's medium under 5% CO_2 as previously described (9).

[^{3}H]RX821002 binding studies.

Tissue fragments were submitted to collagenase digestion (10). Floating isolated fat cells were used to prepare crude plasma membrane fractions (11) which were used immediately to determine α2-adrenoreceptor binding sites with the specific antagonist [^{3}H]-RX 821002 as previously described (11). Protein concentrations were measured according to Bradford (12) using bovine serum albumine as the standard.

Preparation of an α2A-AR probe by genomic amplification.

The probe used to detect the hamster α2-adrenoreceptor mRNA (α2-AR mRNA) was obtained by amplification (PCR) of hamster genomic DNA. Specific oligonucleotide primers for PCR were designed from the published complementary DNA (cDNA) sequence for rat α2A- and α2D-AR (RG20) subtypes of rat brain (13) and submaxillary gland (14) respectively. A computer algorithm was used to choose the sens (5'-CGTGCTGGTTATTATCGCAGTG) and antisens (5'-AGCCGATGGACGACGAGA) primers framing a 458 base pair long region encoding the peptide from the first membrane domain to the fifth membrane domain. Oligonucleotides obtained commercially (Bioprobe, France) and a thermocycler (Crocodile II, Appligène, France) were employed for PCR. The PCR mixture contained 1μg genomic DNA, 2.5 U Taq polymerase (Promega), 25 pmol of each primers in 100 μl buffer containing Tris 10 mM and $MgCl_2$ 1.5 mM. The PCR was performed under mineral oil for 35 cycles (1 min at 95°C, 1.5 min at 56°C, 1.5 min at 72°C). The amplified product was fractionated on a 1% agarose gel and a 458 bp fragment corresponding to the expected size was visualized after ethidium bromide staining. The 458 bp DNA was extracted by a glass suspension (Sephaglas, Pharmacia). For restriction mapping, the amplified product was digested with BglII enzyme in supplied buffer (New England Biolabs) by incubation at 37°C for 4 h. Restriction products were resolved in 1% agarose gel and detected by Southern blotting with a rat α2A-AR probe. Furthermore, the subtype specificity of the labelled 458 bp DNA probe was checked by hybridization to different full length probes for rat α2B- and α2C-AR subtypes obtained as described in (15).

Northern analysis.

Total RNA was isolated from isolated adipocytes by extraction with guanidium isothiocyanate as described by Chomczynski et al. (16). RNA was analysed by Northern blotting after electrophoresis in 1% agarose/0.66 M formaldehyde gels and alkali-transfer onto nylon membranes (Hybond N+, Amersham). Membranes were baked for 2 h at 80°C and RNA hybridized with the indicated probes, each labeled with [α^{32}P]dCTP by the random primer method (Ready-to-go, Pharmacia). The rat cDNA probe for mouse ß-actin was obtained from Dr M. Buckingham (17).

All results are expressed as means ± SEM. Statistical significance of the data was established using paired Student's *t* test and one way analysis of variance (ANOVA).

Results and discussion.

In a previous report, we have shown that the expression of α2-AR in hamster epididymal adipocytes was androgen-dependent (4). In the present study,, levels of α2-AR in parametrial deposits from female hamsters were significantly lower than those observed in males (285 ± 20 vs 1010 ± 86 fmol/mg protein, n=4, $p < 0.001$) and treatment with testosterone propionate for 4 days resulted in a doubling of the number of binding sites (628 ± 49 fmol/mg protein, n=4, $p < 0.001$ vs control females). Thus, in females like in males, the adipocyte α2-AR is up-regulated *in vivo* by testosterone.

Although androgen receptors are present in hamster adipose tissue (8), these *in vivo* experiments do not establish whether this up-regulatory effect of androgen-treatment is due to a direct (androgen receptor-mediated) or indirect hormonal action on the fat cells.

The effect of testosterone was studied *in vitro* on an homogenous cell population consisting of isolated adipocytes obtained from minced parametrial fat pads, which are more suitable than epididymal ones because of possible interferences by endogenous androgens. The number of α2-AR increased by 90% after 48 h *in vitro* exposure to 100 nM testosterone(Fig.1). Furthermore, 5α-dihydrotestosterone (100 nM), a metabolite of testosterone which cannot be further metabolized to estrogens, was equipotent in upregulating the α2-AR in parametrial adipocytes. To get more information on the *in vitro* modulation of α2-AR by androgens, parametrial adipose tissue was exposed for 48 h with 100 nM testosterone alone or in the presence of either the potent androgen receptor antagonist RU23908 (Anandron)(10 μM) or the transcriptional inhibitor actinomycin D (5μg/ml). As shown in Fig.2, the up-regulatory effect of testosterone on adipocyte α2-AR was no longer observed when either of these agents was present in the incubation medium.

Identification of the hamster α2-AR mRNA was performed by Northern blot analysis using a 458 bp homologous DNA probe obtained by amplification of hamster genomic DNA. Because hamster adipocyte α2-AR exhibit pharmacological characteristics closed to those expected for the α2A- and α2D-AR subtypes (18), we used a set of primers deduced from the published rat α2A- and α2D-AR

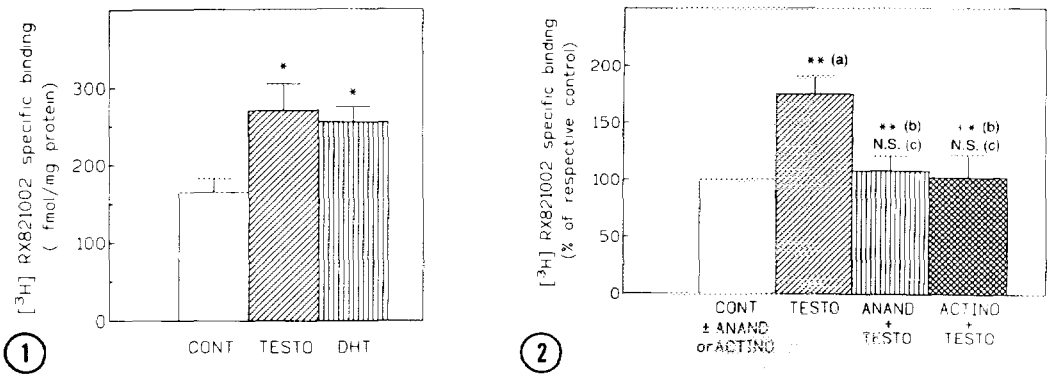

Figure 1. Long-term *in vitro* effects of androgens on fat cell α2-AR levels in parametrial fat from female hamsters. Minced parametrial fat pads were incubated for 48 h in the absence (CONT) or presence of either testosterone (TESTO) or dihydrotestosterone (DHT) at a total concentration of 100nM. α2-AR levels were then measured as described under Materials and Methods. Results are the mean ± SEM of 5 separate experiments. * = $p<0.05$ (Student's t test).

Figure 2. Inhibition of the *in vitro* effects of testosterone on fat cell α2-AR levels by RU 23908 and actinomycine D. Parametrial fat pads were incubated for 48 h in the presence of 10 nM free testosterone alone (TESTO) or combined with either 10 μM RU 23908 (ANAND) or 5 μg/ml actinomycin D (ACTINO). Respective controls (CONT) were incubated in the absence of testosterone. Results are the mean ± SEM of 3-4 separate experiments and are normalized as percentages of their respective control values which were: no inhibitor= 173±18, ANAND= 173±34 and ACTINO= 144±32 fmol/mg protein. Analysis of variance followed by Bonferroni test was used for between -group comparison: TESTO vs CONT (a), inhibitor +TESTO vs TESTO (b) and inhibitor+TESTO vs inhibitor (CONT) (c). ** = Bonferroni p value < 0.001. NS = non significant.

sequences (13,14). Confirmation that the PCR product was suitable to study specifically the α2-AR mRNA expression of hamster adipocyte was obtained by restriction mapping with BglII enzyme yielding fragments of the expected length which hybridized with a full length rat α2A-AR probe already characterized (15). This hamster DNA probe does not hybridized with full length probes for rat α2B- and α2C-AR subtypes (not shown).

Northern analysis with this probe allowed us to detect a single 3.8 kb mRNA band in both male and female adipocytes (Fig. 3). Moreover, testosterone-treatment clearly modified the levels of α2-AR mRNA: in castrated males, mRNA almost disappeared in epididymal adipocytes and was restored to the level observed in adipocytes from control animals by testosterone treatment. The amount of α2-AR mRNA was much lower in adipocytes from control females when compared to control males. When females were testosterone-treated for 4 days the α2-AR mRNA signal raised to the level observed in intact males. Hence, *in vivo*, androgens can induce in hamster adipocytes large variations of α2-AR mRNA abundance which parallel those obtained in binding studies (ref.4 and the present study).

As shown in Fig.4, mRNA levels were already increased after 6 h testosterone exposure and were maintained at a high level at least until 48

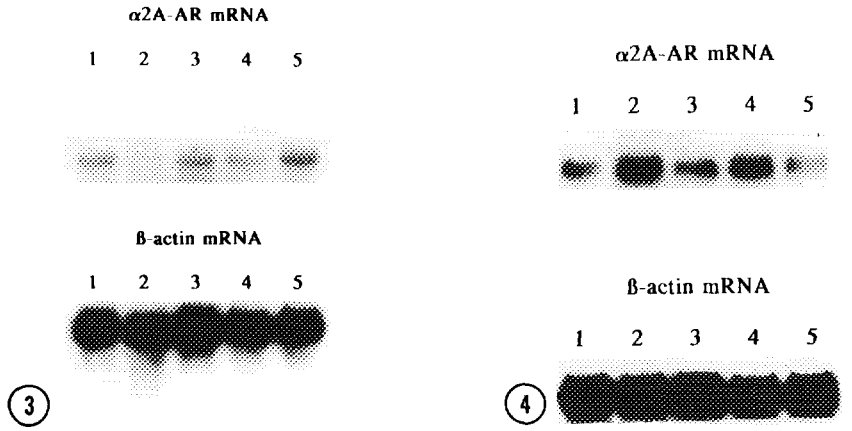

Fig.3. Effects of orchiectomy and androgen treatment on α2A-AR mRNA abundance in adipocytes obtained from male and female hamsters. Northern blot analysis of α2A-AR mRNA and ß-actin mRNA were performed on total RNA (15 μg) extracted from the isolated adipocytes. Lanes: 1, normal male; 2, castrated-male; 3, 1 week testosterone-treated male after orchiectomy; 4, normal female; 5, 4 days testosterone-treated female. Results are from one experiment representative of three.

Fig.4. *In vitro* up-regulatory effect of testosterone (100 nM) on the α2A-mRNA expression in parametrial adipocytes from female hamsters and inhibition by the antiandrogen RU 23908 (10 μM). Northern blot analysis of α2A-AR mRNA and ß-actin mRNA was performed on total RNA (20 μg) extracted from isolated adipocytes. Minced adipose tissue was incubated for 6 h (lanes 1,2) or 48 h (lanes 3-5) without (lanes 1,3) or with testosterone (lanes 2,4 and 5) plus RU 23908 (lane 5).

h. Addition of RU23908 blunted the androgen effect (Fig. 4), indicating that the up-regulation of the α2-AR transcripts is androgen receptor-dependent. Other steroids like 17ß-estradiol and dexamethasone had no modulatory effect on α2-AR mRNA expression in parametrial tissue (data not shown).

In male hamster white adipocytes, we have previously reported that testosterone-treatment following surgical castration results in an up-regulation of α2-AR (4). Here we show that androgens possess an identical upregulatory potency in female hamster adipocytes . Furthermore , using a species specific α2A-AR DNA probe, we were able to demonstrate that both α2A-AR mRNA and protein levels varied similarly under all our experimental conditions. The size of the transcript (3.8 kb) is in accordance with other reports on α2AR mRNA (14,19,20). It must be underlined, however, that androgens had no effect on α2-AR expression and mRNA levels in other hamster tissues like the brain and kidney (unpublished data). Thus, like many other genes expressed in androgen-target tissues such as prostate, skin (6,7) and some specific brain areas (21,22), the hamster fat cell α2A-AR is an additionnal example of an androgen controlled gene.

Our present *in vitro* experiments also demonstrate clearly that testosterone up-regulates the fat cell α2-AR expression and their transcripts

through a direct action at the fat cell level. Our recent characterization of androgen receptors in hamster adipose tissue and adipose precursor cells (8) suggests that this effect occurs at free testosterone concentrations compatible with the androgen receptor affinity in fat cell (Kd= 2-5 nM) (8,23). Moreover, these effects appear to be mediated by adipocyte androgen receptors since RU23908, a potent androgen antagonist (24), abolished the up-regulatory action of androgens on both the α2A-AR gene transcription and expression. Although in fat cells, testosterone may be actively converted into estrogens via aromatase activity (25), a contribution of estrogens to the effects of testosterone seems to be ruled out since α2-AR up-regulation was also induced by the non-aromatizable androgen, 5α- dihydrotestosterone but not by 17β-estradiol. The up-regulating effect of testosterone on α2A-AR mRNA was rapid since it was obvious already 6 hours after testosterone addition. Very similar effects of testosterone have been recently reported for the EGF receptor mRNA in rat liver (26) and for thromboxan A2 receptors in cultured rat aortic smooth muscle cells (27). Enhanced transcription rate or increased transcript stability may account for this modulation. The finding that actinomycine D completely prevents the effects of testosterone *in vitro* strongly suggests that testosterone enhances α2-AR expression through a transcriptional activation.

Previous studies have proposed a transcriptional mechanism to explain the androgenic regulation of adipocyte α2-AR expression in hamsters adapted to a short day photoperiod (20). However, these *in vivo* experiments did not demonstrate that the observed effects were due to a direct action of testosterone *per se* on adipose tissue. Hormone responsive elements specific for androgen receptors (A.R.E.) should be localized in the promoter region of any gene to lead to its transcriptional activation. From the recent literature, testosterone has been reported to up-regulate ß-adrenoreceptors in rat preadipocytes (23), mature adipocytes (28) and rat prostate (29) through androgen receptor-dependent mechanisms. Although steroid hormone responsive elements have been identified in the promoter region of the genes encoding different ß-adrenoreceptor subtypes (30,31), no data exist so far for the α2-AR gene. Whatsoever, the fact that both the ß-(lipolytic) and α2-(antilipolytic) adrenoreceptor expressions are under the control of androgens in adipose tissue , strongly suggests that such a control is physiologically relevant, especially when considering android obesity in male and female subjects (32,33).

In conclusion, our present *in vivo* and *in vitro* studies clearly demonstrate that the up-regulation of the α2A-AR subtype observed after testosterone-treatment in hamster fat cells from both genders, is due to a direct transcriptional activation mediated by the fat cell androgen receptors.

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