EXPERIMENTAL STUDY

Oestradiol and progesterone change β_3 -adrenergic receptor affinity and density in brown adipocytes

Aurelio Malo and Marisa Puerta

*Department of Animal Biology II (Physiology), Faculty of Biological Sciences, Complutense University, Madrid, Spain**(Correspondence should be addressed to M Puerta, Department of Animal Biology II (Physiology), Faculty of Biological Sciences, Complutense University, 28040 Madrid, Spain; Email: mpuerta@eucmax.sim.ucm.es)*

Abstract

Objective: To check if the oestradiol- and progesterone-driven reduction in noradrenaline responsiveness of brown adipocytes is due to a reduction in either the density or the affinity of β_3 -adrenoceptors (β_3 -AR). β_1/β_2 -AR were also studied.

Design: Four groups of animals were considered. (i) control rats at thermoneutrality, (ii) cold-acclimated rats, to determine β -AR under continuous sympathetic stimulation, which is known to decrease noradrenaline responsiveness, (iii) oestradiol- and (iv) progesterone-treated cold-acclimated rats to determine hormonal effects on β -AR populations in thermogenically active brown adipocytes. **Methods:** Oestradiol and progesterone were chronically elevated by means of s.c. Silastic implants. Densities and affinities of β -AR populations were determined by binding studies using [3 H]CGP-12177 as radioligand.

Results: Two populations of low and high binding affinities (K_d 1.6 and 27.3 nmol/l) corresponding to β_3 - and β_1/β_2 -AR respectively were found at thermoneutrality. β_3 -AR density was higher than that of β_1/β_2 -AR (B_{max} 419 and 143 fmol/mg protein respectively). Cold-acclimated rats showed a reduction of β_3 -AR binding capacity (B_{max} 308 fmol/mg protein). Oestradiol and progesterone reduced the density of β_3 -AR to 167 and 185 fmol/mg protein respectively, while increasing their affinity for [3 H]CGP-12177 (K_d 9.5 and 4.0 nmol/l vs 16 nmol/l in cold-acclimated untreated rats). The density of β_1/β_2 -AR was also reduced after oestradiol treatment (B_{max} 51 fmol/mg protein).

Conclusions: Both oestradiol and progesterone reduce the density of β_3 -AR in brown adipose tissue (BAT) while increasing their affinity for [3 H]CGP-12177. Oestradiol also reduces the density of β_1/β_2 -AR whereas cold-acclimation reduces the density of β_3 -AR.

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Introduction

Oestradiol and progesterone inhibit brown adipose tissue (BAT) thermogenesis (1, 2). This inhibitory action is carried out through a direct interaction with the brown adipocyte, as revealed by the unchanged activity of the sympathetic nervous system in rats with high plasma levels of either oestradiol or progesterone (3, 4). The inhibitory hormonal action appears to take place somewhere downstream of noradrenaline release; thus, brown adipocytes isolated from rats treated with either oestradiol or progesterone show a poor thermogenic response to noradrenaline (5, 6). Since in order to elicit thermogenesis, noradrenaline binds to β_3 -adrenoceptors (β_3 -AR) in plasma membranes, these receptors are the first step in the chain of events leading to thermogenesis and as such are a possible target for female sex steroids. The aim of this work was to examine this possibility; the density and affinity of β_3 -AR in the plasma membranes of BAT were measured in rats treated with either oestradiol or progesterone.

Materials and methods

Female Wistar rats, 200–220 g at the beginning of the experiment, were divided into four groups. Control group: rats maintained for 2 weeks at thermoneutrality (28 °C); they lacked any sympathetic stimulation in their BAT. Cold-acclimated group: rats maintained in the cold (6 °C) for 2 weeks; they had an intense sympathetic stimulation in their BAT and were the model where the inhibitory effects of oestradiol and progesterone were described. Oestradiol-treated group: rats maintained for 4 weeks in the cold and receiving oestradiol during the last 2 weeks. Progesterone-treated group: rats maintained for 4 weeks in the cold and receiving progesterone during the last 2 weeks. Animals were maintained in individual cages with food and water freely available and a schedule of 12 h light:12 h darkness, with lights on at 0800 h. The food provided was a commercial diet containing 66.7% carbohydrate, 19.3% protein, 3.4% fat, 4.9% cellulose and 5.7% mineral (w/w) (Panlab, Barcelona, Spain).

Oestradiol and progesterone were administered by implanting Silastic capsules (1.5 and 3.2 mm internal and external diameter respectively) filled with the corresponding hormone under ether anaesthesia (17 β -oestradiol, one capsule 0.5 cm long containing about 5 mg; progesterone, two capsules, 4 cm long, each containing about 25 mg).

Animals were killed by decapitation. Interscapular BAT (IBAT) was removed and cleaned of adherent tissues in 250 mmol/l sucrose, 10 mmol/l Tris-HCl and 1 mmol/l EDTA adjusted to pH 7.4 (STE) at 4 °C. Purified plasma membranes were obtained as described by Muzzin *et al.* (7). Briefly, about 1.25 g IBAT (pooled from four to ten rats) were minced with scissors into pieces of about 1 mm³. After homogenizing (Potter-Elvehjem, Cole Palmer International, USA) the pooled tissue at 1700 r.p.m. for 1 min, nuclei and cellular debris were removed by a low speed centrifugation (1075 *g* for 10 min). The supernatant was centrifuged at 34 500 *g* for 35 min and the pellet resuspended in STE, homogenized and transferred to a centrifuge tube filled with 18.5% metrizamide. After centrifugation at 156 000 *g* for 60 min in a floating rotor, membranes remained over the metrizamide layer. They were collected and either used immediately or stored frozen at -80 °C.

For radioligand binding studies, membranes (30 μ g protein) were incubated at 37 °C for 30 min in 50 mmol/l Tris-HCl and 10 mmol/l MgCl₂ at pH 7.4 containing GTP 50 μ mol/l with increasing concentrations of [³H]CGP-12177 (44 Ci/mmol, Amersham Pharmacia, UK) in the presence or absence of 100 μ mol/l (-)-propranolol. Once incubated, membranes were collected using a Brandel M-24 harvester (Brandel, MD, USA) equipped with Whatman GF/C paper. Radioactivity was measured after incubating every sample at 60 °C for 20 min in 10 ml scintillation mixture containing 500 μ l Soluene-350 (Packard, Groningen, The Netherlands). Specific binding was defined as the difference between the total binding obtained in the absence of competing ligand and the non-specific binding obtained in the presence of (-)-propranolol.

Three different plasma membrane pools were obtained from each experimental group. Each pool (four to ten rats) was only used to make one binding study. Each binding study was made up of a series of 9–12 tubes in duplicate. The quantitative parameters K_d and B_{max} were determined by Scatchard analysis. One-way ANOVA was used to determine differences between groups, except for β_3 K_d comparisons, where Kruskal–Wallis analyses were made due to the absence of a Gaussian distribution. The Newman–Keuls test was used as *post hoc* analysis. $P < 0.05$ was considered statistically significant.

Results

Muzzin *et al.* (8) showed that [³H]CGP-12177 binds two populations of β -AR on rat IBAT plasma membranes, a

high affinity binding site (0.64 ± 0.017 nmol/l) made up of β_1/β_2 -AR subtypes, representing 30% of the total binding (330 ± 44 fmol/mg protein), and a low affinity binding site (31 ± 9 nmol/l) made up of β_3 -AR, representing 70% of the total binding (770 ± 85 fmol/mg protein). The identity of the low affinity binding site as β_3 -AR was confirmed in the same work by using CHO-K1 β_3 -transfected cells, which also gave a similar low affinity binding site (44 ± 4 nmol/l) for [³H]CGP-12177.

Using [³H]CGP-12177 as AR radioligand as described by Muzzin *et al.* (8) we also found two

Figure 1 Specific binding of [³H]CGP-12177 to IBAT plasma membranes in (A) control rats at thermoneutrality (28 °C) and (B) in cold-acclimated rats (6 °C). Scatchard analysis of the data is represented in the corresponding insert. Each point represents the mean of three different binding studies. Standard errors have been omitted for clarity.

populations of binding sites in control rats (Fig. 1A), a high affinity one having a K_d value of 1.6 ± 0.6 nmol/l and a B_{max} value of 143.3 ± 37.9 fmol/mg protein, and a low affinity one having a K_d value of 27.3 ± 6.9 nmol/l and a B_{max} value of 419.4 ± 11.2 fmol/mg protein (Table 1, first row). Since these values were in excellent agreement with those found by Muzzin *et al.*, we considered the high and the low binding affinity sites in our study as the β_1/β_2 -AR subtypes and the β_3 -AR subtype respectively.

Both β_1/β_2 -AR and β_3 -AR were also found in BAT membranes after cold-acclimation (Fig. 1B). However, IBAT membranes of cold-acclimated rats had a lower density of β_3 -AR than those of control rats (308.6 ± 38.9 vs 419.4 ± 11.2 fmol/protein respectively) (Table 1, second row). They also showed a reduction in the number of β_1/β_2 -AR (95.0 ± 1.0 vs 143.3 ± 37.9 fmol/mg protein respectively) but the difference did not attain statistical significance (Table 1, second row). No effects were detected in binding affinity.

Figure 2 shows that oestradiol and progesterone modified both the binding affinity and the capacity of β_3 -AR. Thus the B_{max} changed from a value of 308.6 ± 38.9 fmol/mg protein in untreated rats to 167.7 ± 15.4 and 185.6 ± 20.1 fmol/mg protein after oestradiol and progesterone treatment respectively (Table 1). Simultaneously, the K_d changed from a value of 16.0 ± 1.5 nmol/l in untreated rats to 9.5 ± 2.0 and 4.0 ± 0.9 nmol/l after oestradiol or progesterone treatment respectively (Table 1).

Only oestradiol produced a change in the β_1/β_2 -AR, which reduced their number from a B_{max} of 95.0 ± 1.0 fmol/mg protein in untreated rats to 51.0 ± 14.1 in oestradiol-treated animals, leaving unchanged their affinity for [3 H]CGP-12177. Progesterone had no effect on either binding affinity or capacity of β_1/β_2 -AR (Table 1).

Discussion

Muzzin *et al.* (8) showed that the low affinity β_3 -AR can be quantified in plasma membranes from rat brown adipocytes and from CHO β_3 -transfected cells with the radioligand [3 H]CGP-12177. The high affinity

β_1/β_2 -AR are also quantified. Using the same radioligand, we also found two different receptor populations with similar relative proportions to those reported by Muzzin *et al.* (8). Therefore, for our study we defined the low affinity binding site as β_3 -AR and the high affinity site as β_1/β_2 -AR. The receptor density for both β_3 - and β_1/β_2 -AR was slightly lower in this study than in the work of Muzzin *et al.* (8); these differences are most likely due to the strain of rat used (Wistar in our experiment but Sprague–Dawley in theirs) or sex (females in our experiments but males in theirs).

After rats had been acclimated to the cold for 15 days, we found a significant reduction in the density of β_3 -AR, from 419.4 ± 11.2 fmol/mg protein in controls to 308.6 ± 38.9 fmol/mg protein in the cold-acclimated animals (Table 1). This result indicated that chronic noradrenaline stimulation diminishes the density of β_3 -AR, which agrees not only with Revelli *et al.* (9), who found that the actual number of β_3 -AR was diminished in rat BAT after 72 h of cold-exposure, but also explains the reduced response to both noradrenaline and to other adrenergic agonists of brown adipocytes isolated from cold-acclimated hamsters (10, 11) and rats (12). Therefore, our results indicate that chronic sympathetic stimulation desensitizes β_3 -AR by reducing β_3 -AR density, and this desensitization would explain the reduced responsiveness to noradrenaline in BAT of cold-acclimated rodents.

We found no significant change in β_1/β_2 -AR density in BAT membranes after cold-acclimation of rats (Table 1). Therefore, our results suggest that chronic noradrenaline stimulation does not change the β_1/β_2 -AR binding capacity of BAT membranes. It could be argued, however, that whereas β_3 -AR are only present in mature brown adipocytes, β_1/β_2 -AR are also present in preadipocytes (13) and that preadipocyte number might have increased during cold-acclimation, thus masking a reduction in binding density. However, Bukowiecki *et al.* (14) showed that in the rat at room temperature the ratio of mature adipocytes to preadipocytes is 40:2, and, most importantly, those proportions are unchanged after 15 days in the cold. It therefore seems more logical to conclude that chronic noradrenaline stimulation, as present in

Table 1 Density and affinity of β_3 - and β_1/β_2 -AR in IBAT plasma membranes from rats at thermoneutrality and from cold-acclimated rats treated with either oestradiol or progesterone. Values are means $\pm$ S.E. of three different binding studies.

	β_3 -AR		β_1/β_2 -AR		$\beta_3:\beta_1/\beta_2$
	B_{max} (fmol/mg protein)	K_d (nmol/l)	B_{max} (fmol/mg protein)	K_d (nmol/l)	
28 °C	419.4 $\pm$ 11.2	27.3 $\pm$ 6.9	143.3 $\pm$ 37.9	1.6 $\pm$ 0.6	75:25
6 °C	308.6 $\pm$ 38.9*	16.0 $\pm$ 1.5	95.0 $\pm$ 1.0	0.7 $\pm$ 0.3	76:24
6 °C+oestradiol	167.7 $\pm$ 15.4**	9.5 $\pm$ 2.0**	51.0 $\pm$ 14.1**	0.5 $\pm$ 0.4	76:24
6 °C+progesterone	185.6 $\pm$ 20.1**	4.0 $\pm$ 0.9**	97.9 $\pm$ 10.4	0.6 $\pm$ 0.1	65:35

* $P < 0.05$ when compared with controls at 28 °C.
** $P < 0.05$ when compared with untreated cold-acclimated rats.

cold-acclimated rats, does not alter density or affinity of β_1/β_2 -AR.

To study the effect of oestradiol and progesterone on β -AR we chose cold-acclimated rats as the experimental model for three reasons. Firstly, oestradiol and progesterone inhibition of BAT thermogenesis was found in cold-acclimated rats (1, 2). Secondly, the direct effect of oestradiol and progesterone on brown adipocytes reducing their responsiveness to noradrenaline was also found in adipocytes isolated from rats at room temperature; this is below thermoneutrality (5, 6). Thirdly, cold-acclimation itself is known to lower noradrenaline sensitivity (10, 11), an effect this study has revealed to be mediated by a reduction in the density of β_3 -AR. We found that oestradiol and progesterone brought about a further reduction in β_3 -AR density, that went from 308.6 ± 38.9 fmol/mg protein in untreated rats down to 167.7 ± 15.4 fmol/mg protein in those receiving oestradiol and to 185.6 ± 20.1 fmol/mg protein in those receiving progesterone (Table 1). However, oestradiol and progesterone not only decreased β_3 -AR density but also increased the affinity of the β_3 -AR for [^3H]CGP-12177 at the same time, as revealed by the diminished K_d found in treated animals (16.0 ± 1.5 nmol/l in untreated rats down to 9.5 ± 2.0 nmol/l in oestradiol-treated rats and to 4.0 ± 0.9 nmol/l in progesterone-treated animals). Therefore, female sex steroids had a dual effect on β_3 -AR; on the one hand, they diminished their density and, on the other hand, they increased their affinity for [^3H]CGP-12177. It is tempting to speculate that this dual effect could be related to the dual mechanism of action of steroid hormones, and thus, besides the well-known genomic effects controlling protein synthesis, there are non-genomic actions of steroids that include allosteric interactions of the hormones with neurotransmitter receptors, cation exchangers, ion channels and G proteins (15–17). According to this dual model, the reduced density of β_3 -AR that we found after either oestradiol or progesterone treatment could probably be the result of a genomic action, since cytoplasmic oestradiol receptors have been identified in BAT (18). However, it seems plausible that the increased affinity of β_3 -AR for CGP-12177 in treated animals could be due to a direct interaction of the steroids with the β_3 -AR. In our system both effects, despite being opposite, do not compensate for each other, since brown adipocytes isolated from rats receiving the steroids showed a

Figure 2 Effect of oestradiol and progesterone on the specific binding of [^3H]CGP-12177 to IBAT plasma membranes. Scatchard analysis of the data is represented in the corresponding insert. Each point represents the mean of three different binding studies. Standard errors have been omitted for clarity. (A) Cold-acclimated rats; (B) cold-acclimated oestradiol-treated rats; (C) cold-acclimated progesterone-treated rats.

reduced response to noradrenaline (5, 6), implying that the effect of reducing density predominates.

Oestradiol but not progesterone also reduced the density of β_1/β_2 -AR from 95.0 ± 1.0 down to 51.0 ± 14.1 fmol/mg protein leaving unchanged their affinity for [3 H]CGP-12177 (Table 1). Again, a genomic mechanism is suggested to explain this effect.

In summary, our study reveals that β_3 -AR density is reduced by chronic noradrenaline stimulation, and by oestradiol and progesterone. Both steroids also increase β_3 -AR affinity for [3 H]CGP-12177. However, only oestradiol reduced β_1/β_2 -AR density leaving the binding affinity unchanged.

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References

- 1 Nava MP, Abelenda M & Puerta M. Cold-induced and diet-induced thermogenesis in progesterone treated rats. *Pflügers Archiv. European Journal of Physiology* 1990 **415** 747–750.
- 2 Puerta M, Nava MP, Abelenda M & Fernández A. Inactivation of brown adipose tissue thermogenesis by oestradiol treatment in cold-acclimated rats. *Pflügers Archiv. European Journal of Physiology* 1990 **416** 659–662.
- 3 Puerta M, Venero C, Castro C & Abelenda M. Progesterone does not alter sympathetic activity in tissues involved in energy balance. *European Journal of Endocrinology* 1996 **134** 508–512.
- 4 Nava MP, Fernández A, Abelenda M & Puerta M. Dissociation between brown adipose tissue thermogenesis and sympathetic activity in rats with high plasma levels of oestradiol. *Pflügers Archiv. European Journal of Physiology* 1994 **426** 40–43.
- 5 Abelenda M, Castro C, Venero C & Puerta M. Reduced oxygen consumption of brown adipocytes isolated from progesterone-treated rats. *Canadian Journal of Physiology and Pharmacology* 1994 **72** 1226–1230.
- 6 Puerta M, Abelenda M, Nava MP & Fernández A. Reduced noradrenaline responsiveness of brown adipocytes isolated from estradiol-treated rats. *Canadian Journal of Physiology and Pharmacology* 1993 **71** 858–861.
- 7 Muzzin P, Colomb C, Giacobino JP, Venter JC & Fraser C. Biochemical characterization of brown adipose tissue β -adrenergic receptor. *Journal of Receptor Research* 1988 **8** 713–729.
- 8 Muzzin P, Revelli JP, Fraser CM & Giacobino JP. Radioligand binding studies of the atypical β_3 -adrenergic receptor in rat brown adipose tissue using [3 H]CGP 12177. *FEBS Letters* 1992 **298** 162–164.
- 9 Revelli JP, Muzzin P & Giacobino JP. Modulation *in vivo* of β -adrenergic-receptor subtypes in rat brown adipose tissue by the thermogenic agonist Ro 16-8714. *Biochemical Journal* 1992 **286** 743–746.
- 10 Cannon B, Bengtsson T, Bronnikov G, Svoboda P, Zhao J & Nedergaard J. Nonshivering thermogenesis in a hibernator, the golden hamster: mediation exclusively through β_3 -adrenergic receptors which are desensitized due to cold acclimation. In *Adaptations to the Cold: Tenth International Hibernation Symposium*, pp 271–279. Eds F Geiser, AS Hulbert & SC Nicol. Armidale: University of New England Press, 1996.
- 11 Nedergaard J. Catecholamine sensitivity in brown fat cells from cold-acclimated hamsters and rats. *American Journal of Physiology* 1982 **243** C250–C257.
- 12 Zhao J, Cannon B & Nedergaard J. Thermogenesis is β_3 - but not β_1 -adrenergically mediated in rat brown fat cells, even after cold acclimation. *American Journal of Physiology* 1998 **275** R2002–R2011.
- 13 Cannon B, Bengtsson T, Dicker A, Jacobsson A, Kuusela P, Thonberg H *et al.* The role of adrenergic stimulation in regulation of heat production and recruitment of brown adipose tissue. In *Thermal Balance in Health and Disease*, pp 87–102. Eds E Zeisberg, E Schönbaum & P Lomax. Basel: Birkhäuser Verlag, 1994.
- 14 Bukowiecki LJ, Geloën A & Collet AJ. Proliferation and differentiation of brown adipocytes from interstitial cells during cold acclimation. *American Journal of Physiology* 1986 **250** C880–C887.
- 15 Gekle M, Silbernagl S & Wunsch S. Non-genomic action of the mineralocorticoid aldosterone on cytosolic sodium in cultured kidney cells. *Journal of Physiology* 1998 **511** 255–263.
- 16 Schneider SW, Yano Y, Sumpio BE, Jena BP, Geibel JP, Gekle M *et al.* Rapid aldosterone-induced cell volume increase of endothelial cells measured by the atomic force microscope. *Cell Biology International* 1997 **21** 759–768.
- 17 Wagner PG, Jorgensen MS, Arden WA & Jackson VA. Stimulus-secretion coupling in porcine adrenal chromaffin cells: acute effects of glucocorticoids. *Journal of Neuroscience Research* 1999 **57** 643–650.
- 18 Wade GN & Gray JM. Cytoplasmatic 17β -[3 H]estradiol binding in rat adipose tissues. *Endocrinology* 1978 **103** 1695–1701.

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