Eur J Anat, 13 (1): 1-6 (2009)

Immunocytochemical evidence for the coexistence of aromatase P450 and estrogen receptor α in the pituitary gland of the adult male rat

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Summary

In previous studies we demonstrated the immunocytochemical expression of aromatase in rat pituitary cells. The aim of the present study is to demonstrate the synthesis of the enzyme through the expression of the mRNA of the enzyme in pituitary cells by in situ hybridization and the relationship between the immunocytochemical expression of aromatase and estrogen receptor α in adult male rats. In situ hybridization revealed that aromatase-mRNA is located in the cytoplasm of 41% of pituitary cells. By immunocytochemistry, different patterns of reaction for aromatase were found. Moreover, using immunocytochemistry and RT-PCR, all aromatase-positive tumors proved be estrogen receptor α-positive tumors. The immunocytochemical coexistence of aromatase and estrogen receptor α was found. These observations demonstrate the synthesis of aromatase in the pituitary gland and its functional ability to develop its estrogenic effects mediated by estrogen receptor α.

Introduction

The presence of estrogen receptors in the pituitary gland has been described in both male and female rats (Shull y Gorski, 1985, 1989), and direct pituitary effects on hormonal release and the size and proliferation of pituitary cells in response to treatment with estradiol have been reported (Pérez et al., 1986; Carretero et al., 1991, 1992, 1995, 1997) in male rats.

Of the two pathways through which androgens are metabolized -reduction and aromatization- the latter depends on the presence of an enzyme -aromatase P450-, which belongs to the family of cytochrome P450. We have previously described the sexual dimorphic immunocytochemical expression of aromatase P450 in the adult rat pituitary gland; this was more evident in male than in female rats, (Carretero et al., 1999). These results suggest that several pituitary effects of gonadal steroids could be a response to the action of estradiol, derived from the aromatization of testosterone, on pituitary estrogen receptors.

The presence of estrogen receptors in the same pituitary cells that express aromatase

Submitted: October 17, 2008
Accepted: January 20, 2009

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P450 is a prerequisite for accepting an autoparacrine effect of estradiol derived from the aromatization of testosterone in the pituitary gland.

In order to determine whether aromatase P450 and estrogen receptor-α coexist in the same pituitary cells, here a double-labeled immunocytochemical study was carried out in the hypophyses of adult male rats.

Material and Methods

Animals used. Ten adult male Wistar rats, weighing 200 g were used. The animals were chosen randomly and were kept under standard laboratory conditions: 20 ± 2ºC; 55 ± 5% RH; light/dark cycle of 8:00-20:00 h light daily. Free access to food (maintenance diet, Panlab®) and water was provided. The animals were handled according to the guidelines of the European Communities Council Directive (86/609/EEC) and current Spanish legislation for the use and care of laboratory animals (BOE 67/8509-12, 1998).

Animal sacrifice and sample processing. Animals were sacrificed by decapitation after anaesthesia by ether inhalation. The hypophyses were carefully dissected out and immediately submerged in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 24 h. Once the hypophyses had been fixed, they were embedded in paraffin after dehydration in ethanol and clearing in xylene to obtain 5 μm serial coronal sections. The sections were placed on gelatin-chrome-alum-treated slides for immunocytochemical study. Sections for study were taken from all regions of the gland in the rostro-caudal direction.

Immunocytochemistry. A double immunocytochemical study was performed using the Strepto-Avidin-Biotin-Peroxidase and PAP methods. Endogenous peroxidase was blocked by incubation in 0.24% hydrogen peroxide in methanol. Non-specific reactions of goat or swine anti-rabbit IgG were inhibited by incubation with the purified immunoglobulin fraction of non-specific swine serum (Dako®, dilution 1:30). Samples were then incubated with monoclonal anti-rat estrogen receptor-α serum (Alexis®, diluted 1:150) at 4ºC for 24 h in a humid chamber; biotinylated goat anti-mouse IgG serum (Caltag®, diluted 1:350) at room temperature for 40 min, and soluble strepto-Avidin-Peroxidase complex (Caltag®, diluted 1:400) for 40 min at room temperature. The immunohistochemical reaction was visualized with 0.25% 3-3'-diaminobenzidine in TRIS buffer (0.05M, pH 7.4) with 0.015 ml of freshly prepared 3% H_2O_2, after which the slides were washed in distilled water. After the first reaction, sections were incubated for 24 h at 4ºC in a humid chamber with rabbit anti-aromatase polyclonal serum (Rb-851, diluted 1:500). The reaction was allowed to progress by incubation for 40 min at room temperature with swine anti-rabbit IgG (Dako®, diluted 1:100), and later with incubation with soluble peroxidase anti-peroxidase complex (Dako® diluted 1:100) for 35 min at room temperature. The reaction was developed with 4,1 chloro-naphthol ($1.7x10^{-3}$M, Sigma®, in 0.05M Tris-HCl buffer, pH 7.4) to which 0.3% H_2O_2 had been added)

As a control of the reaction, the primary serum was replaced by TBS or normal rabbit serum. In all cases, the reaction was completely abolished. The specificity of the antibody was determined by Western blot and preabsorption tests.

For Western blot, pituitary glands were dissected from adult rats and frozen immediately. Tissues were then disrupted by homogenization in lysis buffer (137 mM NaCl, 10 mM Tris, pH 7.4, 10% glycerol and 1% Triton X-100 containing a cocktail of protease inhibitors). Insoluble material was removed from the lysates by centrifugation at 10,000 rpm for 10 min. Protein concentrations were determined using the standard Bradford assay. 50 μg of total protein from each rat sample was separated by 10% SDS-PAGE. Following electrophoresis, proteins were transferred to nitrocellulose and then blocked for 1 h with 5% non-fat dry milk in PBS. The nitrocellulose membranes were then incubated overnight with either preimmune serum or anti-aromatase antibodies diluted 1:500. Blots were subjected to 3 x 15 min washes with PBS and then incubated for 1 hr with HRP-labeled secondary antibodies (1:10,000 in PBS). Following extensive washing, the blots were revealed by ECL (Amersham). The average exposure time was 2 min. Western blotting demonstrated the existence of a protein of around 50 Kda molecular weight in the pituitary gland (Figure 1).

For preabsorption tests, diluted anti-aromatase serum (1:500) was preabsorbed (24h at 4ºC) with the peptide sequence C-EIIF-SPRNSDKYLLQQ corresponding to residues

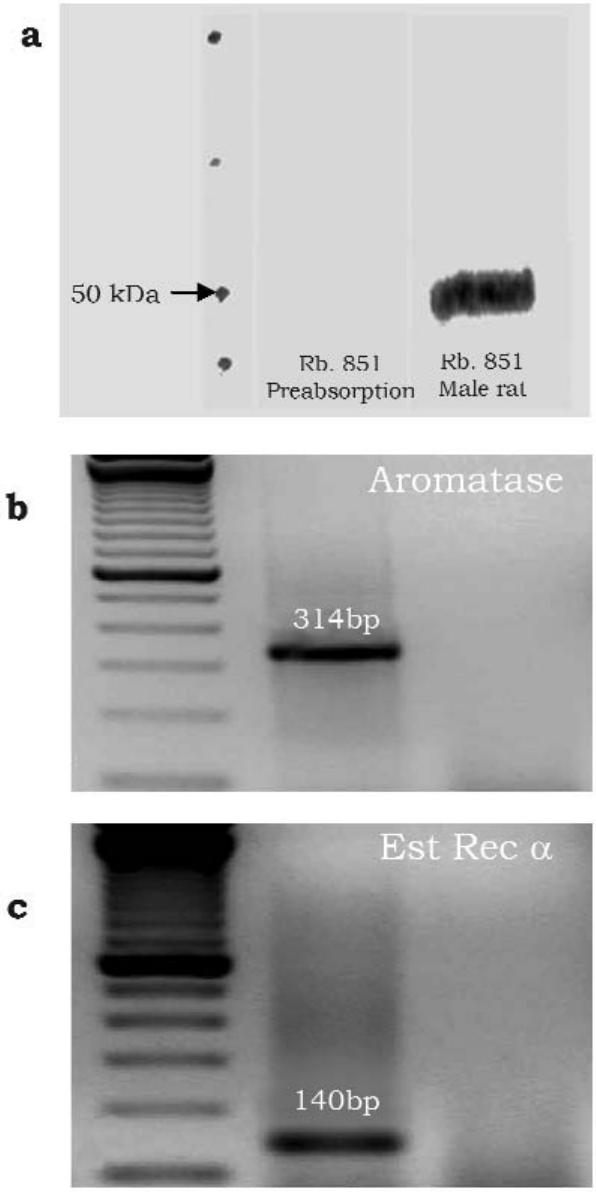

Figure 1. a: Western blot from an adult male pituitary gland using antibodies against Aromatase (Rb-851). After preabsorption, no blots were present. **b**: RT-PCR for aromatase mRNA. **c**: RT-PCR for estrogen receptor α mRNA.

488-502 of mouse cytochrome $P450_{arom}$ -aromatase- (50 µg peptide/ml antibody solution), which was used to obtain the rabbit polyclonal anti-aromatase serum.

In situ hybridization. These studies were performed using a non-isotopic method involving the immunocytochemical detection of biotin using the streptavidin-biotin-peroxidase method. To accomplish in situ hybridization, the biotinylated oligonucleotide 5'BIO-gag gat gac gtg att gac ggc tac ccg gtt aaa aag gga act aac atc att ctg aac atc gga, 100% specific to rat aromatase P450 according to GenBank data base, was used as probe.

Slides were prehybridized in Omnibuffer for 30 minutes at 37ºC. Hybridization with the biotinylated-probe (100 pg/ml in Omnibuffer) was carried out using a Hybaid termocycler overnight at 37ºC. The reaction was stopped by washes in 1xSSC at 54ºC for 20 minutes, 1xSSC at room temperature for 20 minutes, and for 20 minutes in PBS (0.01M, pH 7.4, plus 0.8% NaCl). Biotin was detected using monoclonal anti-biotin antibodies (Roche, 1: 250 in TBS: 0.05M HCl-Trizma, pH 7.4, plus 0.8% NaCl) overnight at 4ºC in a humidity chamber, followed by biotinylated goat anti-mouse (Caltag, 1:250 in TBS). The reaction was amplified using the tyramide amplification kit (Dako) according to the instructions of the manufacturers. The final reaction was developed with 3,3'-diaminobenzidine (0.025M, Sigma®, in 0.05M Tris-HCl buffer, pH 7.4) to which 0.03% H_2O_2 had been added. The slides were counterstained using Mayer's acid haematoxylin.

As controls omission of the probe (figure 1h) and pretreatment with Rnase (figure 1i) were performed, no reaction being observed in any case.

Quantification of aromatase mRNA-expressing cells. The percentage of aromatase mRNA-expressing cells was quantified in each animal following the double-blind procedure. Briefly, eight thousand cells (with intact cell and nuclear profiles) were counted from 20 sections separated from one another by at least 50 µm (400 cells/section) that were chosen randomly from all parts of the gland, then calculating the percentage of reactive cells.

Quantification of immunoreactive cells. The percentage of aromatase- and estrogen receptor α-immunoreactive cells from each animal was quantified following the double-blind procedure. Briefly, four thousand cells (with intact cell and nuclear profiles) were counted from 20 sections separated from one another by at least 50 µm (200 cells/section) chosen randomly from all parts of the gland, after which the percentage of reactive cells was calculated. These percentages were analyzed statistically and the differences among the means were contrasted by ANOVA, $p<0.05$ in the F-Scheffé test being considered significant. The results are expressed as arithmetic mean ± standard errors of the mean.

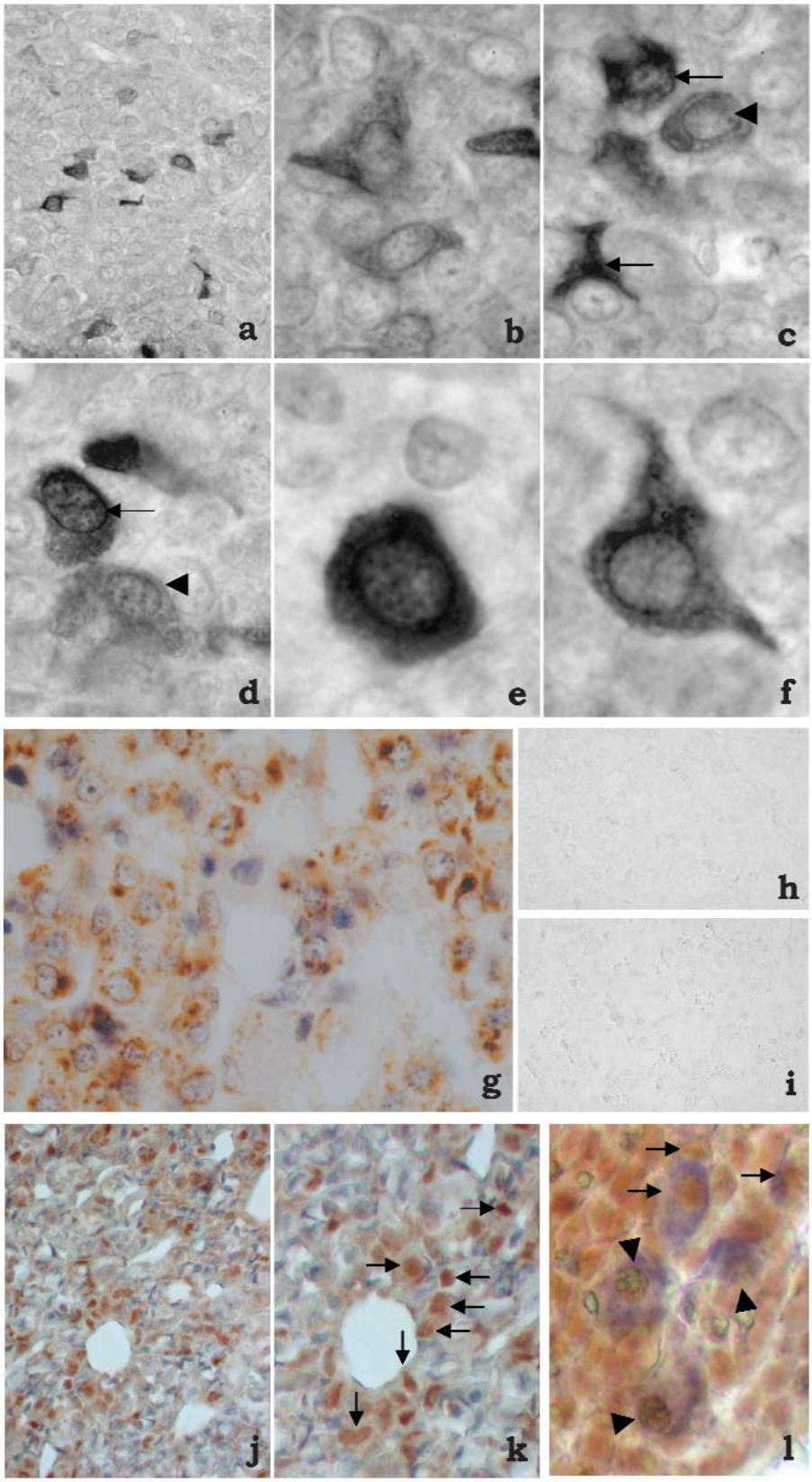

Figure 2. Aromatase-positive cells by immunohistochemistry and in situ hybridization, and estrogen receptor α-positive cells and aromatase- and estrogen-receptor α-positive cells by immunohistochemistry cells from male pituitary glands. **2a:** Isolated aromatase-positive cells in the pituitary gland of male adult rats. x 250. **2b:** Aromatase-positive cells showing the granular pattern of cytoplasmic reaction. x 750. **2c:** Different reaction intensities to aromatase (low intensity, arrowhead) and strong intensity (arrows). x 750. **2d:** Aromatase-positive cells with cytoplasmic reaction (arrowhead) or nuclear and cytoplasmic reaction (arrow). x 750. **2e:** Polygonal aromatase-positive cell. x 1250. **2f:** Irregular aromatase-positive cell with cytoplasmic prolongations. x 1250. **2g:** Granular aspect of the cytoplasmic reaction to detection by in situ hybridization of aromatase mRNA. x 250. **2h:** After substitution of the probe, the reaction for in situ hybridization was abolished. x 75. **2i:** After RNase pretreatment, the reaction for in situ hybridization was abolished. x 75. **2j:** Micrograph showing the distribution of estrogen-receptor α-positive cells. x 125. **2k:** Estrogen-receptor α-positive cells at higher magnification (arrows). x 250. **2l:** Estrogen-receptor α-positive cells (brown) and aromatase-positive cells (blue). Arrowheads point to colocalization of double reaction to the enzyme and receptor in nuclei. Arrow marks the colocalization of the receptor in the nuclei and enzyme only in the cytoplasm. x 400.

Results

Immunocytochemical positivity for aromatase. Pituitary aromatase-positive cells were scarce and appeared mainly isolated in the gland (Figure 2a). The cytoplasm of aromatase-positive cells showed a fine granular aspect (Figure 2b), but the intensity of the reaction was not uniform from one cell to another (Figure 2c). Although the reaction was mainly cytoplasmic (arrow head in Figure 1d), some cells showed a nuclear and cytoplasmic reaction (arrow in Figure 2d). The aromatase-positive cells were polygonal (Figure 2e) or irregular with cytoplasmic prolongations (Figure 2f).

In situ hybridization for aromatase-mRNA. The reaction in in situ hybridization of rat aromatase mRNA was mainly granular and was located in the cytoplasm of pituitary cells (Figure 2g). Isolated and grouped positive cells were found, and the percentage of positive cells was found to be 41.02 + 2.02% of pituitary cells.

Immunocytochemical positivity for estrogen-receptor α. Immunocytochemical analysis revealed that estrogen-receptor α was expressed in a cellular population with a heterogeneous morphology and size (Figure 2j). Positive cells (arrows in Figure 2k) appeared together with negative cells, and very frequently close to blood vessels. Isolated and grouped positive cells were found; 32.89 + 1.64% of pituitary cells were positive.

Immunocytochemical colocalization for aromatase and estrogen-receptor α. The nuclei of aromatase-positive cells (blue) always were estrogen-receptor α-positive (arrows in Figure 2l). In some cases, the nuclei were positive for aromatase and estrogen-receptor α (arrowhead in Figure 2l).

Discussion

As far as we are aware, this is the first study to analyze the presence of aromatase mRNA in the rat pituitary gland by in situ hybridization.

We believe that our results demonstrate the presence of aromatase mRNA in the pituitary gland of adult male rats, confirming previous studies from our laboratory using immunocytochemistry (Carretero et al., 1999, 2001, 2003).

The presence of aromatase in the hypophysis could be functionally related to the responses to estradiol, testosterone or castration that occur in the pituitary cells of the male rat (Carretero et al., 1991, 1992, 1995, 1997, 1998). Different intensities of reaction and different sizes and shapes suggest that different pituitary cells could express the enzyme or different functional states of the cells. This could account for the presence of cytoplasmic or nuclear and cytoplasmic reactions.

Because aromatase P450 is present in the rat pituitary gland, it could be surmised that the presence of estradiol would be the result of testosterone aromatization in the gland. However, confirmation of the presence of estrogen receptors in the same pituitary cells in which aromatase is expressed is a prerequisite for accepting an autoparacrine effect of this estradiol in the pituitary gland, because the effects of gonadal steroids depend on their concentrations and their ability to act on specific receptors (Hutchison and Steimer, 1984; Hutchison et al., 1990, 1991).

Green et al. (1986) and Greene et al. (1986) demonstrated the existence of estrogen receptor α, and Kuiper et al. (1996) and Mosselman et al. (1996) reported the existence of a second estrogen receptor β. These receptors are distributed throughout different organs and regions of the body and their existence would explain the selective actions of estrogens in different tissues (Katzenellenbogen, 1996). The presence of estrogen receptors in pituitary cells has been well documented (Shull and Gorski, 1985, 1989).

Our results also demonstrate that estrogen receptor α is expressed in the pituitary gland of adult male rats and, by double immunolabeling, we have demonstrated that all aromatase-positive pituitary cells are estrogen receptor-expressing cells.

These results suggest that aromatase P450 would exert an important autoparacrine regulation in the pituitary gland because the final product of its action, i.e., estradiol, would act on the specific estrogen receptor α in the same cells where the enzyme is expressed.

The percentage of estrogen-receptor α is high in the male pituitary gland. This suggests that the aromatization of testosterone to estradiol is very important in the autoparacrine regulation of the male pituitary gland, because the serum levels of estradiol are low in males. These results are in accordance with the higher percentage of aromatase-positive cells in adult male than in adult female rats reported in a previous work (Carretero et al., 1999), and they could be related to the expression of aro-

matase in spontaneous prolactinomas in aged rats (Carretero et al., 2002), because prolactinomas are estrogen-induced tumors (Wingrave et al., 1980; Phelps and Hymer, 1983; Gooren et al., 1988; Molitch, 2001; Heany et al., 2002).

In conclusion, our results demonstrate the synthesis of aromatase P450 in the pituitary gland of the rat, the existence of estrogen receptor α in the pituitary gland of adult male rats, and the coexistence of aromatase P450 and estrogen receptor α in the same pituitary cells. These findings suggest that testosterone would be aromatized to estradiol, which would in turn act on specific receptors as an autoparacrine regulator in the gland of adult male rats.

Acknowledgements

This work was supported by the Spanish FIS program No. PI0-21803.

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