Expression of 3 β -hydroxysteroid dehydrogenase/isomerase in the female rat pituitary

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Abstract

3 β -Hydroxysteroid dehydrogenase/isomerase (3 β -HSD) catalyses an essential step in the biosynthesis of steroid hormones and is widely distributed in peripheral steroid target organs. The present report describes for first time the expression of this enzyme in the pituitary of female rats. Immunohistochemistry at the light microscopic level was performed on pro-oestrous and ovariectomized rat pituitaries. Immunoreactive cells were scattered and randomly distributed throughout the anterior lobe, whereas cells located in the posterior lobe and pars intermedia were

immunonegative. Differences were observed in cell morphology and in the number of 3 β -HSD-immunopositive cells between ovariectomized and pro-oestrous female rat pituitaries, suggesting that steroidogenic activity is affected by ovarian endocrine function. Apart from adenohypophyseal immunoreactive cells, 3 β -HSD immunopositivity was also noted in endothelial cells of almost all pituitary capillaries located in the anterior and posterior lobes.

Journal of Endocrinology (2000) **166**, 95–101

Introduction

Several steroids acting through various steroid receptors influence the differentiation and function of the pituitary (Stefaneanu 1997). The effect of steroids on target cells depends not only on the amounts that can penetrate into the cells, and their interaction with the receptor, but also on the formation of biologically active steroids from inactive precursors at the sites of their actions (Labrie 1991, Labrie *et al.* 1995). Despite numerous studies that identified the presence of steroid receptors in the pituitary of different species (Handa *et al.* 1986, Kimura *et al.* 1993, Sanno *et al.* 1997, Gittoes *et al.* 1998, Shughrue *et al.* 1998, Ozawa *et al.* 1999, Vidal *et al.* 1999b), only a few publications have dealt with the localization of steroidogenic enzymes in adenohypophyseal cells. The scarcity of reports may be due to methodological limitations related to the small amount of these proteins in the pituitary compared with the ovary, testis and adrenal cortex.

Steroidogenesis is complex; it involves several inter-related pathways, indicating that regulation occurs at several points. A key enzyme in the regulation is 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSD) (Conley & Bird 1997), which catalyses the conversion of Δ^5 -3 β -hydroxysteroids to Δ^4 -3-ketosteroids, a reaction that is essential for the biosynthesis of all active steroid hormones. In mammals 3 β -HSD is expressed not only in classical steroid-producing organs (Penning 1997), but also

in several peripheral tissues, including the skin (Dumont *et al.* 1992), breast (Zhao *et al.* 1991), lung (Milewich *et al.* 1977), endometrium (Bonney *et al.* 1985), prostate (El-Alfy *et al.* 1999), liver (Zhao *et al.* 1990, 1991), kidney (Bain *et al.* 1991) and brain (Ukena *et al.* 1999). To our knowledge, no studies have been published that relate to the expression of 3 β -HSD in the pituitary. The aim of the present work was to reveal whether 3 β -HSD is present in the pituitary of the female rat, and to study the potential relationships between its expression and changes in gonadal function.

Materials and Methods

Ten adult female Sprague–Dawley rats (five in the pro-oestrous phase of the cycle and five ovariectomized) from our in-house breeding colony were used in this experiment. They were housed at room temperature with controlled photoperiod, and had a diet of rat pellets and tap water available *ad libitum*. The stage of the cycle was determined by daily vaginal smears.

The animals of both groups were deeply anaesthetized with pentobarbitone and then perfused with 4% paraformaldehyde in PBS on the same day. Ovariectomy was performed 1 week before the animal was killed. The pituitaries were quickly removed and fixed by immersion in 4% paraformaldehyde in PBS at 4 °C overnight. After

fixation the tissues were dehydrated and embedded in paraffin using an automatic processor. Sections of 3 μ m thickness were mounted on poly-L-lysine-coated slides and air-dried overnight at 37 °C.

For immunocytochemical studies the streptavidin–biotin–peroxidase complex technique (Hsu *et al.* 1981) was used, with slight modifications. Before the primary antibody was applied, the sections were pretreated with 1.5% H₂O₂ in absolute methanol for 30 min to inactivate endogenous peroxidase, and then with 10% goat serum for 1 h to block non-specific binding of the secondary antibodies. Subsequently the tissue was incubated overnight at 4 °C with rabbit polyclonal antibody raised against human placental 3 β -HSD (kindly provided by Dr Van Luu-The, Centre de Recherches en Endocrinologie Moléculaire, l'Université Laval, Canada) at a dilution of 1:2000. Slides were washed three times in TBS for 5 min each and incubated for 1 h with biotinylated goat antirabbit IgG (Sigma B-8895, St Louis, MO, USA) diluted 1:100. The buffer rinses were repeated and the slides were incubated for 1 h with streptavidin–peroxidase complex (Boehringer Mannheim No. 1089153, Mannheim, Germany) diluted 1:1000. The final reaction was achieved by incubating the sections for 5 min in a solution of 5 mg diaminobenzidine and 1 ml 1% H₂O₂ in 100 ml TRIS-buffer (pH 7.6). Finally the slides were counterstained with haematoxylin, dehydrated, cleared in xylene, mounted in DePex mounting medium (BDH Laboratory Supplies, Poole, Dorset, UK) and investigated with light microscope.

Immunocytochemical controls

To assure specificity for the immunohistochemical reactions, 3 β -HSD antibody was replaced with PBS. The antiserum applied for immunocytochemical studies was raised by immunizing rabbits with purified human placental 3 β -HSD (Luu-The *et al.* 1989). This antiserum has been widely used to localize the enzyme in tissues of several species, including rat, and its specificity has been tested previously (Pelletier *et al.* 1992). As 3 β -HSD antigen was not available to perform the preabsorption test, the specificity of the 3 β -HSD antibody was verified by immunoblot analysis.

For the immunoblot analysis 50 μ g rat pituitary protein and placental homogenates were electrophoresed separately on a 12% SDS–PAGE gels and transferred to nitrocellulose papers (Sigma N-6016) using a transblot apparatus (Hoefer Pharmacia Biotech Inc., San Francisco, CA, USA). Non-specific binding sites were blocked by incubating the filters in a solution containing 0.1% Tween 20–5% skimmed milk in PBS. The filters were then incubated 1 h at room temperature with rabbit anti-3 β -HSD polyclonal antibody (diluted 1:1000 in 0.1% Tween 20 in PBS). After washing three times (15 min/wash) with 0.10% Tween 20 in PBS, the blots were incubated for 1 h in antirabbit Ig–digoxigenin F(ab)₂ fragment (Boehringer

Figure 1 Immunoblot analysis of rat 3 β -HSD protein. Proteins extracted from rat placenta (A) and female rat pituitaries (B) were separated on a 12% polyacrylamide gel and then transferred to nitrocellulose filters. 3 β -HSD protein was detected by western blot analysis as described in Materials and Methods. The polyclonal antibody recognizes a single 42 kDa protein in the homogenates of the rat tissues examined.

Mannheim No. 1375792), diluted 1:20 in 0.10% Tween 20 in PBS. Subsequently, the filters were washed three times (15 min/wash) with 0.10% Tween 20 in PBS and incubated with antidigoxigenin antibody linked to alkaline phosphatase (Boehringer Mannheim No. 1093274) diluted 1:500 in 0.10% Tween 20 in PBS. The antigen–antibody complexes were visualized using the CDP-star chemiluminescence detection system (Boehringer Mannheim No. 1685627). Chemiluminescence signals were detected in bands with similar molecular masses (approximately 42 kDa) in both pituitary and placenta (Fig. 1).

Morphometric study

After staining, the immunoreactive cells were counted in each pituitary sample and the size of 3 β -HSD-immunopositive cells was estimated by measuring their volume.

The number of 3 β -HSD immunopositive cells were expressed per unit of pituitary volume (N_V). N_V was calculated using the Floderus equation (Floderus 1944):

$$N_V = N_A / (T + D - 2h)$$

where N_A is the total number of nuclei present in ten fields that represent 368 981 μ m², D is the average nuclear diameter (assuming them to be of spherical shape). T is the average thickness of the sections (5 μ m; the sections were always prepared by the same person using the same microtome) and h is the height of the smallest recognizable cap section that was estimated to be $\approx$ 10% of the nuclear diameter.

Figure 2 Pituitary of ovariectomized rat immunostained with 3 β -HSD antibody. Immunopositive cells (arrows) had a round or ovoid shape, occasionally with vacuolated cytoplasm. The endothelial cells of blood vessels were well labelled (arrowheads). Bars represent 10 μ m.

The volume of an individual 3 β -HSD immunopositive cell (V_{ST}) was obtained using the formula:

$$V_{ST} = V_{nu} \times V_{nu/ST}$$

where V_{nu} is the nuclear volume of the 3 β -HSD immunopositive cell and $V_{nu/ST}$ is the volume density of the nucleus in the 3 β -HSD immunopositive cell. The nuclear volume was determined as follows, assuming the nucleus to be of spherical shape:

$$V_{nu} = 4/3\pi r^3$$

Morphometry was performed using a computer image analysis system (Microimage, Media Cybernetics LP, Silver Spring, MD, USA). Each gland was serially sectioned and five sections were randomly selected and analysed. On each section, ten fields were randomly selected at a magnification of $\times 40$ and in each field all immunoreactive cells with recognizable nuclei were counted.

Statistical comparisons of the data were performed by Student's *t*-test. Statistical significance was accepted for $P < 0.05$.

Results

Immunohistochemistry revealed the presence of the enzyme 3 β -HSD in many adenohypophyseal cells. Immunoreactive cells were scattered and were randomly distributed throughout the anterior lobe, whereas immunonegative cells were located in the posterior lobe and pars intermedia. The immunoreaction was moderate to strong, affecting the entire cytoplasm. The cell nuclei were not immunoreactive (Figs 2–4). Differences were

observed in cell morphology and in the number of 3 β -HSD-immunopositive cells between ovariectomized and pro-oestrous female rat pituitaries. In pro-oestrous rats the immunoreactive cells were smaller and mainly polygonal, possessing a narrow cytoplasm (Fig. 3). In the ovariectomized rats 3 β -HSD-immunopositive cells showed a morphology typical of castration or gonadectomy cells: they had a round or ovoid shape, with a voluminous and occasionally vacuolated cytoplasm and eccentric nucleus (Fig. 2). Numerical density and volume of 3 β -HSD-immunoreactive cells were greater in ovariectomized than in pro-oestrous control female rats (Fig. 5). The differences between the two groups were statistically significant ($P < 0.05$).

Apart from in the adenohypophyseal immunoreactive cells, 3 β -HSD immunopositivity was also evident in endothelial cells of almost all pituitary capillaries. Immunopositive capillaries were distributed throughout the entire adenohypophysis, located either far from 3 β -HSD adenohypophyseal cells or in their vicinity (Fig. 4). Several capillaries in the posterior lobe were also immunopositive.

Discussion

Steroid synthesis is regulated principally by the concentrations and tissue-specific composition of steroidogenic enzymes expressed at the cellular level. In mammals, the steroidogenic enzymes are present in several peripheral tissues where they are involved in intracrine formation of steroids that exert their actions locally in paracrine and/or autocrine fashion. According to the classic concept of endocrinology, biologically active hormones are produced and released by the endocrine cells and transported via the circulation to distant sites where they act on their

Figure 3 In the pituitary of pro-oestrous female rats 3 β -HSD-immunopositive cells (arrows) were randomly distributed throughout the anterior lobe. 3 β -HSD immunopositivity was also evident in endothelial cells (arrowheads). Bars represent 10 μ m.

Figure 4 (a) Proestrus female rat pituitary. (b) Ovariectomized female rat pituitary. Arrowheads show endothelial cells expressing 3 β -HSD protein. Occasionally, 3 β -HSD adenohypophyseal cells (arrow) were located in the vicinity of immunopositive capillaries. Bars represent 10 μ m.

target cells. In contrast, intracrinology (Labrie 1991, Labrie *et al.* 1995) postulates the formation of biologically active steroids from inactive precursors in peripheral target tissues.

The pituitary is one of the principal target organs for steroid action: it has been demonstrated that the pituitary contains oestrogen, androgen, gluco- and mineralocorticosteroid, and retinoic acid receptors, suggesting that actions of steroids on the pituitary may be regulated by mechanisms controlling steroid receptor expression (Handa *et al.* 1986, Kimura *et al.* 1993, Sanno *et al.* 1997, Shughrue *et al.* 1998, Gittoes *et al.* 1998, Ozawa *et al.* 1999, Vidal *et al.* 1999b). Little is known about the role played by steroidogenic enzymes in the regulation of pituitary cell function, including hormone synthesis and/or release. Recently, Carretero *et al.* (1999) demonstrated that the enzyme aromatase P450, which is involved

in androgen metabolism, is expressed in the adult rat pituitary, suggesting that this enzyme may play a role in the regulation of adenohypophyseal cell morphology. Green *et al.* (1999) reported that 17 β -hydroxysteroid dehydrogenase (17 β -HSD) isoforms, which reversibly catalyse the final step in the formation of oestradiol from oestrone and that of testosterone from androstenedione, are present in human pituitary adenomas; this is consistent with the view that this enzyme may be involved in the regulation of oestradiol synthesis at local cellular level. These results were in accordance with the finding of 17 β -HSD activity in the anterior pituitary of rats and rhesus monkeys (Resko *et al.* 1979, El & Mahesh 1984). To our knowledge, our study demonstrates for the first time the expression of 3 β -HSD in the female rat anterior pituitary. The enzyme 3 β -HSD is essential for the biosynthesis of all classes of hormonal steroids, such as

Figure 5 (a) Numerical density of adenohipophysial 3 β -HSD immunopositive cells. (b) Volume of adenohipophysial 3 β -HSD immunopositive cells. Each bar represents mean $\pm$ S.E.M. The asterisks denote statistically significant ($P < 0.05$) differences between pro-oestrous (P) and ovariectomized (O) female rats.

progesterone, glucocorticoids, mineralocorticoids, androgens and oestrogens. The presence of 3 β -HSD mRNA in a wide variety of peripheral steroid target organs of the rat, such as breast, uterus and prostate, indicates that these tissues may form active steroids from circulating 3 β -hydroxysteroid precursors. It has also been demonstrated that administration of dehydroepiandrosterone to orchidectomized rats causes a marked increase in prostate weight, accompanied by an increase in prostatic 5 α -dihydrotestosterone- and androgen-dependent mRNA levels (Labrie *et al.* 1989). In the present study 3 β -HSD was localized in individual cells randomly scattered throughout the anterior pituitary in both ovariectomized and pro-oestrous female rats. These results are in complete agreement with those of previous studies showing a scattered cellular distribution of aromatase P450 in female rat pituitaries and 17 β -HSD in non-tumorous and

adenomatous human pituitaries (Carretero *et al.* 1999, Green *et al.* 1999). Our data provide evidence that changes in pituitary steroidogenic activity can be correlated with ovarian endocrine function. A significant increase was observed in the percentage of 3 β -HSD-immunopositive cells in the pituitary of ovariectomized animals compared with pro-oestrous rats. Carretero *et al.* (1999) reported similar results in the expression of aromatase P450 in the rat pituitary. These authors demonstrated a stronger aromatase reaction in dioestrus than in pro-oestrus or oestrus. Stronger expression was also noted in the pituitaries of male rats compared with those of females (Carretero *et al.* 1999). In human pituitary tumours, Green *et al.* (1999) showed that, in contrast to surgically removed pituitary tissue, most of the adenomatous cells *in vitro* were strongly immunopositive for 17 β -HSD, suggesting that culture conditions can stimulate the expression of the enzyme gene.

Our study revealed a strong 3 β -HSD immunopositivity in most of the adenohypophyseal capillaries of pro-oestrous and ovariectomized rats. The presence of 3 β -HSD in pituitary endothelial cells is an intriguing finding because, to our knowledge, steroidogenic activity in pituitary blood vessels has not previously been demonstrated. Earlier studies have reported the expression of steroidogenic intracrine enzymes in the vascular structure of various other organs (Haider & Servos 1998, Pelletier *et al.* 1998, El-Alfy *et al.* 1999, Harada *et al.* 1999). Pelletier *et al.* (1998) described the presence of two different subtypes of 5 α -reductase mRNA in the blood vessel wall of human prostate and skin. Immunocytochemical localization of 17 β -HSD was noted in blood vessel walls of human prostate also (El-Alfy *et al.* 1999). Haider & Servos (1998) showed for first time the presence of 3 β -HSD in the endothelial cells of the rat testis capillaries; El-Alfy *et al.* (1999) demonstrated the presence 3 β -HSD by immunocytochemistry in blood vessel walls of the human prostate. The function of the steroidogenic intracrine enzymes in the blood vessels is unknown. It may well be that the steroids synthesized in the vessels exert a paracrine and/or autocrine action on the vascular walls. Evidence demonstrating that the blood vessels are target structures for steroid action supports this idea. It should also be noted that specific binding proteins for steroids and oestrogen and androgen receptors have been found in vascular tissues (Bayard *et al.* 1995, Venkov *et al.* 1996, Bray *et al.* 1999). It is also well established that oestrogens have a protective effect on arteriosclerotic cardiovascular disease (Mendelsohn & Karas 1999). Elias and Weiner (1984) observed that, in the pituitary, oestrogen treatment induced an increase of vascularity and hyperplasia of lactotrophs. Although it may well be that oestrogens cause a rapid increase in vascularity that precedes proliferation of adenohypophyseal cells, several studies have also demonstrated the expression of angiogenesis-stimulating factors in adenohypophyseal cells, which can stimulate neoformation of vessels (Marin & Boya 1995, Vidal *et al.* 1999a). Further studies are required to elucidate the role of steroids synthesized by cells of capillary endothelium and to identify the pituitary cell types immunoreactive for 3 β -HSD. It remains to be established whether steroid formation in the pituitary plays a role not only in the function of non-tumorous adenohypophyseal cells, but also in the development and/or progression of pituitary tumours.

Acknowledgements

The authors wish to thank Xunta de Galicia (Xuga 26103A96), Research Council of St Michael's Hospital, Mr and Mrs Stephen Jarilowsky and the Lloyd Carr-Harris Foundation for the financial support. We are grateful to Dr Van Luu-The, Centre de Recherches en Endocrinologie

Moléculaire, L' Université Laval, Canada, for the kind donation of the human placental 3 β -HSD antiserum and the staff of St Michael's Health Sciences Library for their contribution to this study.

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Received 27 September 1999

Accepted 10 February 2000