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Oestradiol Induced Inhibition of Neuroendocrine Marker Expression in Leydig Cells of Adult Rats

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Contents

The objectives of this work were to determine the changes in the expression of neuroendocrine markers in Leydig cell by oestradiol treatment, and to determine whether testosterone is able to recover partially the effects of hormonal suppression induced by oestradiol. Adult male rats were injected daily with either 50 μg of oestradiol or oestradiol plus testosterone propionate (25 mg every 3 days) for 15 days. The animals were sacrificed and testicles were dissected and processed by routine histological protocols. FSH and LH serum levels were determined by radioimmunoassay. The visualization of antigens was achieved by the streptavidin-peroxidase immunohistochemical method. Antibodies against chromogranin A (CrA), S-100 protein (S-100), P substance (PS), synaptofisin (SYN), neurofilament protein (NF), gliofibrillary acidic protein (GFAP) and neuron specific enolase (NSE) were used. The mean LH and FSH serum concentrations were consistently suppressed with hormonal treatments. Intermediate filaments (NF and GFAP) showed no difference in their expression. The expression of S-100, NSE and SYN was significantly lower in both hormone-treated groups. In oestradiol-treated rats, the immunoreactivity of CrA and SP decreased significantly but was restored after testosterone supplementation. Although the nature and functions of many of these substances in Leydig cells remain unknown, these results are consistent with the hypothesis that the expression of some neuroendocrine markers is hormonally controlled.

Introduction

Biochemical, immunological and immunocytochemical studies suggest that in addition to steroid hormones, Leydig cells of various rodents and human produce and possess a high number of substances characteristic of neuroendocrine cells (Davidoff et al. 1993). In this sense, we have recently confirmed the immunoexpression of neuronal and glial markers in Leydig cells of different laboratory rodents (Ortega et al. 2004a). Indeed, neuroendocrine cells contain neurosecretory granules and these are irregularly distributed through different tissues. More than 200 different peptides and many biogenic amines are produced in these cells, including: chromogranin A (CrA), neuron specific enolase (NSE), P substance (PS), calcitonin, somatostatin, neurotensin, bombesin, parathyroid hormone-related protein and thyroid-stimulating hormone-like peptide with B chain homology (di Sant'Agnese and Cockett 1996; Xing et al. 2001). These cells constitute part of a general endocrine regulatory system named APUD by Pearse (1986).

On the other hand, it has been suggested that oestradiol plays a role in the paracrine control of testicular function (Limanowski et al. 1999; Carreau et al. 2003; Goyal et al. 2003; Hess 2003). Also, the oestrogen administration in the male mimics the effect of hypophysectomy on the testis by suppressing gonadotrophin and testosterone secretion (Steinberger 1971; van der Molen et al. 1981). As a consequence, oestradiol treatment has been widely used to study the testicular physiology in the hormone-suppressed model (Blanco-Rodríguez and Martínez-García 1997).

Previously, we analysed changes in the neuroendocrine markers expression in rat Leydig cells, showing that some of these proteins could be affected by the exposure of adults animals to constant light, possibly affecting gonadotrophin levels (Ortega et al. 2004b). In the present study our objectives were to determine changes in the expression of neuroendocrine markers in Leydig cell by oestradiol treatment and to determine whether testosterone is able to recover partially the affects of hormonal suppression induced by oestradiol.

Materials and Methods

Animals and treatment

All the procedures were carried out according to the Guide for the Care and Use of Laboratory Animals [National Research Council (NRC) 1996]. Wistar rats were provided by the Center for Experimental Biology and Laboratory Animals, Faculty of Veterinary Sciences, UNL. Before the experiment, the animals were kept with a controlled cycle of light-darkness (lights on between 6:00 AM and 8:00 PM), and at a temperature of 20–24°C with free access to water and commercial balanced food (Cargill, Argentina).

Adult male Wistar rats ($n = 15$), 12 weeks old, weighing 360 g (± 20) were used. The animals were divided into three groups of five. Animals in each group were treated as follows: group E (oestradiol-treated rats) received 50 μg oestradiol 3-benzoate (Sigma Chemical Co., St Louis, MO, USA), suspended in 0.25 ml of olive oil (vehicle), i.m. daily for 15 days; group E + T (testosterone-supplemented, oestradiol-treated rats) was additionally injected i.m. with 25 mg testosterone propionate (Sigma Chemical Co.) every 3 days; the control group (C) received the vehicle alone in the same way of treated animals.

This kind of oestradiol treatment has previously been shown to reduce significantly the concentration of testosterone as well as gonadotrophins in the plasma and testes (Blanco-Rodríguez and Martínez-García 1997). The dose of testosterone was chosen based on the findings of previous studies so that the testosterone concentration would maintain spermatogenesis

quantitatively (Blanco-Rodríguez and Martínez-García 1997; Sharpe et al. 1998).

Tissue sampling

Control and treated rats were killed by decapitation. Trunk blood was collected and centrifuged, and serum stored at −20°C until used for hormone assays. Testicles were dissected and fixed in 10% buffered formalin for 12 h, washed in buffer saline phosphate (PBS) and processed for inclusion in paraffin. Sections (5 μm) were mounted on slides previously treated with 3-amino-propyltriethoxysilane (Sigma) and stained with haematoxylin–eosin for a previous observation.

Hormone assays

FSH and LH serum levels were determined by radioimmunoassay (RIA) using the kit provided by NIDDK as previously described (Ortega et al. 2004b). Intra and inter assay coefficients of variance for LH and FSH were $<8\%$ and 12% respectively. Minimum detectable concentrations were 0.16 and 1.18 ng/mL of serum for LH and FSH respectively.

Immunohistochemistry

Properties and concentration from used antibodies are reported in Table 1. Each antibody was assayed in at least three sections of each testicle from each individual. Immunostaining was performed as previously described (Ortega et al. 2004a,b). In brief, after deparaffination, microwave pre-treatment (antigen retrieval) was performed. The endogen peroxidase activity was inhibited and non-specific binding was blocked. All sections were incubated with the primary antibodies 18 h at 4°C and then for 30 min at room temperature with rat-pre-absorbed biotinylated secondary antibodies selected specifically to one each of the two types of primary antibodies used (mono or polyclonal) (Table 1). The visualization of antigens was achieved by the streptavidin-peroxidase method (Histostain®, SP Peroxidase Bulk Kits; Zymed, San Francisco, CA, USA) and 3.3-diaminobenzidine (Liquid DAB-Plus Substrate Kit; Zymed) was used as chromogen. Finally, the slides were washed in distilled water and counterstained with Mayer's haematoxylin, dehydrated and mounted.

To verify immunoreaction specificity, adjacent control sections were subjected to the same immunohistochemical method replacing primary antibodies by non-immune serum. Sections of skin, small intestine, pancreas and brain from human were used as positive controls following the indications of the suppliers. Also the same rat's tissues were used, to check the specificity of the reactions.

Table 1. Used antibodies, suppliers and dilutions

Antibodies	Clone	Supplier	Dilution
Primary antibodies			
Neurofilament protein*	DA2,FNP7, RmdO20.11	Zymed	1:100
Glial fibrillary acidic protein	Polyclonal	Zymed	Prediluted
S-100 protein	Polyclonal	Zymed	1:50
Chromogranin A	Polyclonal	Zymed	1 : 50
Substance P	Polyclonal	Zymed	1 : 50
Neuron-specific enolase	NSE-1G4	Zymed	Prediluted
Synaptofisin	Polyclonal	Zymed	Prediluted
Secondary antibodies			
Anti-rabbit IgG	Polyclonal	Zymed	1 : 300
Anti-mouse IgG	Polyclonal	Chemicon	1 : 120

*This antibody reacts with all three major polypeptides of human neurofilament [i.e. low (68 kD), middle (160 kD) and high (200 kD) molecular weight] polypeptides.

Image Analysis

Image analysis was performed using Image Pro-Plus 3.0.1® system (Media Cybernetics, Silver Spring, MD, USA). Images were digitized by a CCD colour video camera (Sony, Montvale, NJ, USA) mounted on top of a conventional light microscope (Olympus BH-2, Olympus, Tokyo, Japan) using ×40 objective lenses. Microscopic fields were digitized and stored in a 24 bits true colour TIFF format. The resolution of the images was set to 640×480 pixels, and the final screen resolution was 0.257 μm/pixel.

Testicular cells were identified on the bases of nuclear shape and histological localization in accordance with descriptions by numerous authors. Particularly, Leydig cells were recognized by their nucleus mostly spherical, with a characteristic distribution of heterochromatin (Kerr and Sharpe 1985; Hardy et al. 1989; Shan et al. 1997; Zhu et al. 2000).

The details of image analysis as a valid method for quantifying expression levels are given elsewhere and the methodological details are previously described (Gimeno et al. 1998; Ortega et al. 2004a,b; Ellis et al. 2005; Witkiewicz et al. 2005). Briefly, to obtain quantitative data regarding immunohistochemical staining in Leydig cells, three sections for each specimen and antibody were evaluated, and 25 representative fields of interstitial area were analysed. Using AutoPro macro language, an automated sequence operation was created to measure the optical density (OD). In this automated analysis process, the images of immunostained slides were converted to an 8-bit grey scale, and the operator calibrated arbitrarily the grey level so that the background staining of the histological slides was regarded as zero (0) and the most intense stained was assigned as ten (10). The OD was measured as a mean grey intensity of each pixel divided by the total number of pixels measured (Ortega et al. 2004b).

Statistics

Results were expressed as the mean ± standard error of mean (SEM). Data were analysed by one-way ANOVA using Duncan's multiple range test by SPSS software (version 11.0 for Windows, SPSS Inc., Chicago, IL, USA). For all test, statistical significance was accepted at $p < 0.05$.

Results

Hormonal concentrations

Hormone data from control and hormone treated animals are summarized in Fig. 1. In E group, the

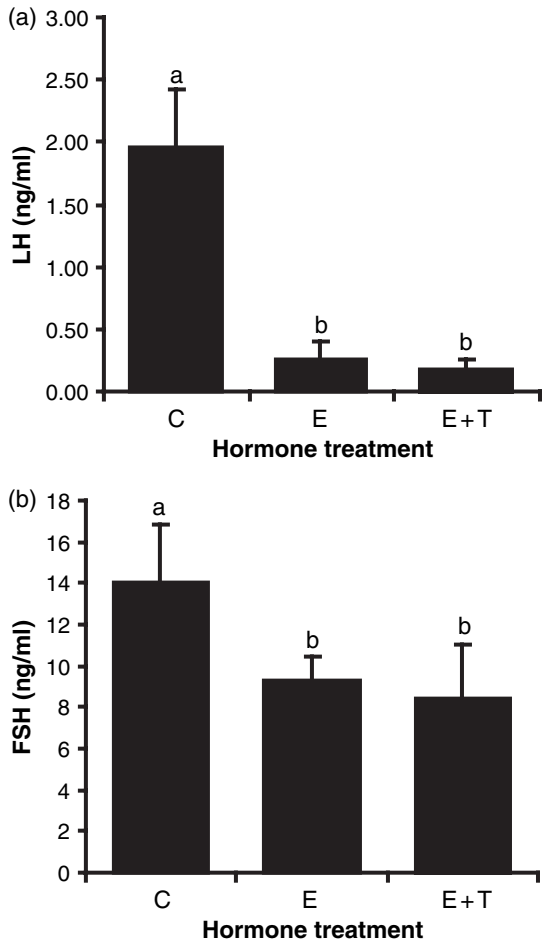

Fig. 1. Comparison of serum concentrations of (a) LH and (b) FSH in control (C), oestradiol-treated (E) and testosterone-supplemented, oestradiol-treated (E + T) rats. Values are mean ± SEM. No common letters are significantly different ($p < 0.05$)

serum LH as well as FSH concentrations were consistently suppressed by greater than 87% and 34%, respectively, compared with the corresponding concentration in C group. In the E + T animals, the serum concentrations of both hormones were similar with respect to rats that received oestradiol alone (group E) and they decreased significantly (LH by 90% and FSH by 39%) compared with control values.

Immunohistochemistry

Immunoreactivity for the antigens under study was obtained after using all antibodies in the positive control tissues. Table 2 presents the results of the image analysis of the immunoreactivity in control and hormone-treated animals, and the stained pattern of each antibody is shown in Fig. 2.

Intermediate filaments [neurofilament (NF) and gliofibrillary acidic protein (GFAP)] showed no difference in their expression between controls and both treated groups. NF was evidenced in the perinuclear region of Leydig cells and in nerve fibre bundles and GFAP was present only in the cytoplasm.

Immunoreactivity for S-100 protein (S-100), CrA, SP, NSE and synaptofisin (SYN) antibodies was found within almost all Leydig cells diffusely distributed throughout the cytoplasm of the cell bodies.

Table 2. Values of optical density of the immunoreactions evaluated by image analysis of interstitial area in control (C), oestradiol-treated (E) and testosterone-supplemented, oestradiol-treated (E + T) rats. Values represent mean ± SEM of arbitrary units

Antibodies	C	E	E + T
Neurofilament protein	4.20 ± 0.961[a]	4.96 ± 0.455[a]	4.66 ± 0.382[a]
Glial fibrillary acidic protein	2.58 ± 0.487[a]	2.52 ± 0.253[a]	2.90 ± 0.367[a]
S-100 protein	2.18 ± 0.097[a]	1.44 ± 0.188[b]	1.24 ± 0.287[b]
Chromogranin A	6.58 ± 0.568[a]	3.78 ± 0.753[b]	5.66 ± 0.609[a]
Substance P	5.54 ± 0.345[a]	4.04 ± 0.180[b]	5.16 ± 0.483[a]
Neuron-specific enolase	1.98 ± 0.124[a]	1.10 ± 0.114[b]	1.14 ± 0.136[b]
Synaptofisin	3.10 ± 0.126[a]	2.02 ± 0.231[b]	2.14 ± 0.280[b]

Within a row, means with no common letters are significantly different ($p < 0.05$).

The expression of S-100, NSE and SYN was significantly lower in both hormonal-treated groups with an average OD reduced by 33%, 45% and 34% in group E and 43%, 41% and 31% in group E + T, respectively. In oestradiol treated rats, the average OD of CrA and SP decreased to 57% and 73% of the control values, respectively and both parameters were restored after testosterone supplementation with similar values to control group.

Discussion

Coinciding with our previous works (Ortega et al. 2004a,b) and other authors (Schulze et al. 1991; Davidoff et al. 1993, 2002; Middendorff et al. 1993), we found that rat Leydig cells express numerous neuroendocrine markers. However, this is the first work conducted to perform a study about the effects of hormonal suppression on the neuroendocrine markers expression in Leydig cells of adult rats.

The suppression of Leydig cell function via a considerable decrease in peripheral gonadotrophins by oestradiol administration has been successfully attempted. In this study oestradiol treatment for 15 days resulted in >85% and 30% suppression of LH and FSH concentrations, respectively. These results are equivalent to other gonadotropin-suppressing treatments (Blanco-Rodríguez and Martínez-García 1997). In relation to neuroendocrine marker immunoexpression, we observed a reduction in the staining of S-100, CrA, SP, NSE and SYN in oestrogen treated animals. No suppression of CrA and SP immunoreactivity was evidenced in the testosterone-supplemented group.

Is widely accepted that oestradiol treatment imitates the effect of hypophysectomy on the testis because of gonadotrophin and testosterone suppression (Steinberger 1971; Russell et al. 1981; van der Molen et al. 1981; McLachlan et al. 1994; Blanco-Rodríguez and Martínez-García 1997). Also, oestrogens play an important role in the growth, differentiation and function of male reproductive tissues although, the role played by this hormone in testicular function regulation has not been totally clarified (Limanowski et al. 1999; Carreau et al. 2003; Goyal et al. 2003; Hess 2003). The oestrogen

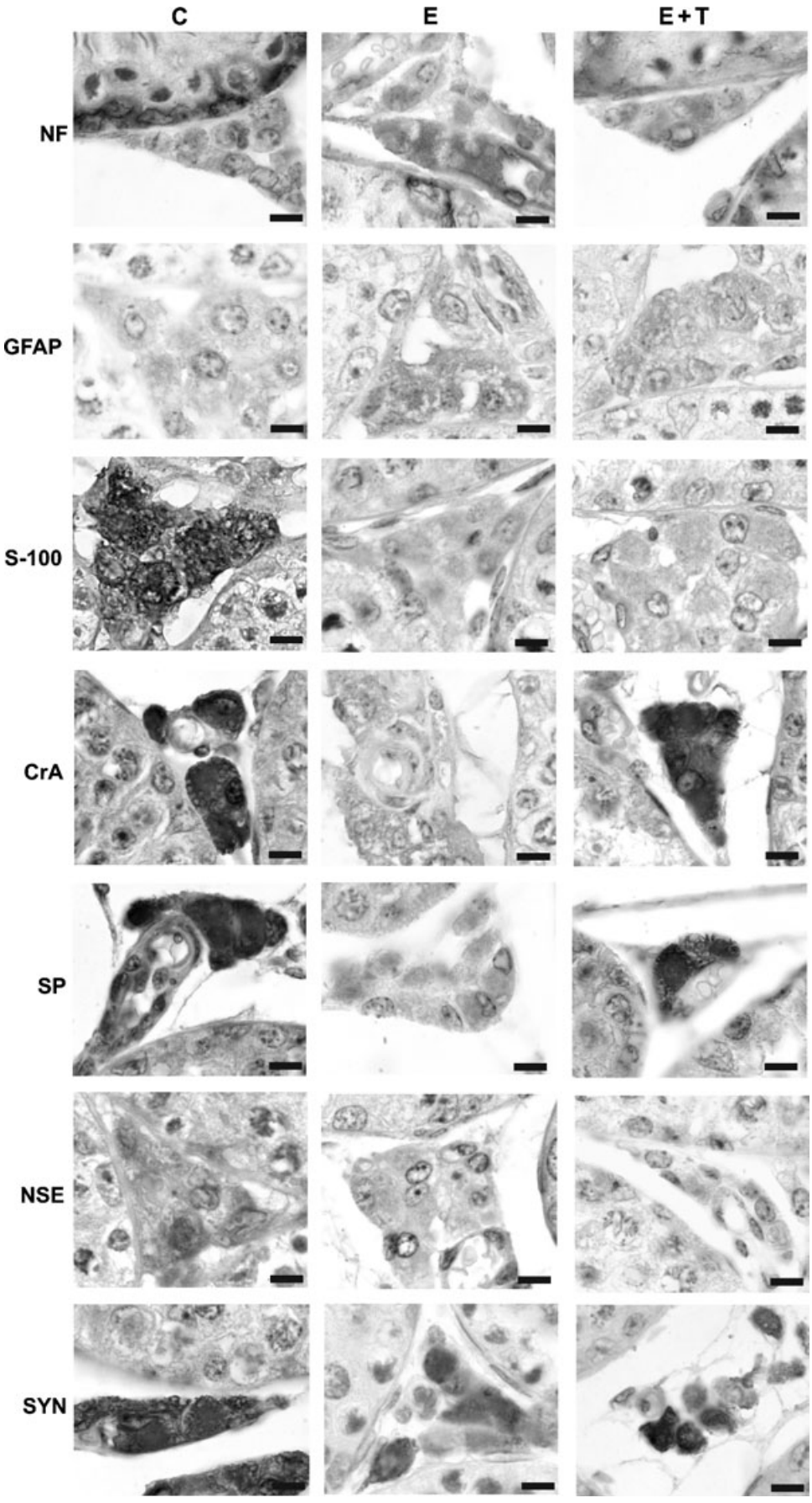

Fig. 2. Immunoreactivity for different neuroendocrine markers within the Leydig cell in control (C), oestradiol-treated (E) and testosterone-supplemented, oestradiol-treated (E + T) rats. Bar = 10 μm

receptor α (ERα) has been found in Leydig cells, which suggest that this hormone might play a role in the regulation of Leydig cell function. On the other hand, as Leydig cells, which produce testosterone, also contain ARs, it might be suggested that, in this cell type, androgens exert an autocrine or paracrine activity (Pelletier et al. 2000).

It is well established that adult Leydig cell testosterone production depends upon LH, which has effects on testosterone production and on Leydig cell structure. In its action, LH binds to specific receptors on, initiating a cascade of events that transform cholesterol to testosterone (Zirkin et al. 1997). Although the available information is few, it is very probable that at least some of the neuroendocrine-related substances detected in Leydig cells play a role in the autocrine regulation of steroidogenesis in these cells. Gerendai et al. (1984) have shown that endogenous pro-opiomelanocortin-derived peptides can modulate testosterone secretion in rat Leydig cells. A modulation of the steroid hormone production of golden hamster gonads by SP was also established in *in vitro* experiments with cultured testicular fragments (Angelova et al. 1991). Also, effects of oestrogens on SP expression in rat pituitary (Brown et al. 1991) and on the regulation of neuropeptide expression (Simerly et al. 1989) have been described. In agreement with these results, if we consider that the supplementation with testosterone revert the reduction in SP and CrA expression, we could suppose its participation in the regulation of steroideogenesis *in vivo*.

On the other hand, some of these neuroendocrine markers might have functional importance during testicular development. Others, such as the catecholamines, calcium-binding proteins (S-100) and hormones of Leydig cells may participate in autocrine and/or paracrine regulatory mechanism (Bardin et al. 1987; Skinner 1991; Davidoff et al. 1993). In this sense, similar differences in staining intensity were described in human patients treated with oestrogen or anti-androgen therapy for long periods (Middendorff et al. 1993).

Intermediate filaments (NF and GFAP) perhaps are stable structural (cytoskeletal) components of Leydig cells and they are not altered by disturbances in gonadotrophins stimulation. Nevertheless these substances seem to belong to a basic equipment of the Leydig cells and their expression may be not affected by hormonal influences.

Although the nature and functions of many of these neuroendocrine tissue associate substances in Leydig cells still remain unknown, these results are consistent with the hypothesis that the expression of some of the neuroendocrine markers is hormonally controlled. Also, in addition to possible gonadotrophin control, we show that treatment with testosterone abolishes the differences in response to oestrogens of some of these markers, suggesting that some of these substances play a role in the paracrine control of testicular function.

In summary, the present findings add to the evidences that the expression of neuroendocrine markers in Leydig cells has not only structural function but rather they also participate in the regulation of the cellular metabolism. It would seem that the substances expressed by the Leydig cells can contribute to the local regulation of the gonadal function.

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