Steroids 74 (2009) 526–534

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Androgen receptors in coeliac ganglion in late pregnant rat

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ARTICLE INFO

Article history:
Received 1 July 2008
Received in revised form
23 December 2008
Accepted 28 January 2009
Available online 21 February 2009

Keywords:
Pregnancy
Coeliac ganglion
Ovary
Androgen

ABSTRACT

The ovarian function is controlled by endocrine factors and neural influence. In late pregnant rat, androstenedione, from the coeliac ganglion, has a luteotrophic effect in the ex vivo coeliac ganglion-superior ovarian nerve–ovary system. In this work we investigate the presence of androgen receptors in the coeliac ganglion of late pregnant rats by immunohistochemistry. We also explore, from a physiological point of view, the potential participation of these receptors in the androstenedione ganglionic action on progesterone release and metabolism, as well as on nitrites release in the ovary compartment. The coeliac ganglion was isolated after being fixed in situ and immunohistochemistry was performed. In the system, three experimental groups were used with the addition of (a) androstenedione, (b) flutamide, and (c) androstenedione plus flutamide in the ganglion compartment. Progesterone and nitrite concentrations were determined in the ovary compartment at different incubation times. Corpora lutea samples isolated at the end of incubation were used to determine the expressions and activities of the progesterone synthesis (3β-hydroxysteroid-dehydrogenase, 3β-HSD) and degradation (20α-hydroxysteroid-dehydrogenase, 20α-HSD) enzymes. Immunohistochemistry revealed cytoplasmatic androgen receptor immunoreactivity in neural somas in the coeliac ganglion. In the coeliac ganglion-superior ovarian nerve–ovary system, androstenedione addition increased 3β-HSD and decreased 20α-HSD, showed a tendency to decrease 20α-HSD expression, and increased nitrites release in relation to control. Androstenedione plus flutamide decreased progesterone and nitrites release in relation to the androstenedione group. This work demonstrates the presence of androgen receptors in neurons of celiac ganglion and provides evidence for the luteotrophic action of androstenedione via a neural pathway that may be mediated by these receptors.
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1. Introduction

In the rat, the corpus luteum is the ovarian structure responsible for the secretion of progesterone, an essential hormone during pregnancy for the establishment and maintenance of gestation. The level of progesterone secreted by the corpus luteum depends on the amount of progesterone synthesized by the enzyme 3β-hydroxysteroid-dehydrogenase (3β-HSD) and on the expression of the enzyme 20α-hydroxysteroid-dehydrogenase (20α-HSD) that catabolizes progesterone into the inactive progestin, 20α-dihydroprogesterone (20α-DHP). It is known that little, if any, 20α-HSD activity is found in the rat corpus luteum throughout pregnancy. However, an elevated activity is observed just before parturition, concomitant with the rapid decrease of progesterone concentration in serum [1].

In mammals the ovary is controlled by endocrine and neural factors [2–7]. It receives innervation by two routes: the ovarian plexus, which innervates the blood vessels musculature, and the superior ovarian nerve (SON), which is the most relevant for the ovarian steroidogenesis [8]. Burden and Lawrence [2] found that denervation on days 10, 14 and 18 of pregnancy caused a decrease in the activity of the 3β-HSD enzyme which synthesizes progesterone through pregnenolone.

The superior ovarian nerve is mainly constituted by adrenergic fibres, most of which originate in the coeliac ganglion [9]. In the ovary, these fibres form a net of varicosities surrounding the theca and secondary interstitial cells that are responsible for the androgens synthesis, without entering the follicle and with little visible relation with the corpus luteum [10].

The coeliac ganglion is included in the sympathetic prevertebral chain, it is constituted by principal neurons and interneurons [11–14], and it has a profuse capillary plexus that constitutes a microcirculation among the different ganglionic structures [15]. Sympathetic ganglia possess a great variety of specific receptors and neurotransmitters, among them catecholamines [16],

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0039-128X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2009.01.009

neuropeptides [17] and nitric oxide (gaseous neurotransmitter) [18].

Nitric oxide has been shown to participate in numerous physiological processes, which are essential for maintaining homeostasis [19,20] and is considered to be a neurotransmitter and biological mediator of the neuroendocrine axis [21,22]. It is interesting that nitric oxide synthesis enzymes in their different isoforms have been characterized and localized to the ovary [23] and to the peripheral neural system [24].

At the end of pregnancy, the corpus luteum suffers a regression process involving two stages. The first, functional regression, is associated with the loss of the luteal progesterone production, and the second, structural regression, involves the involution of the luteal tissue, including steroidogenic and non steroidogenic cells [25].

Besides, androstenedione is the principal circulating androgen in the rat and possesses, in the ovary, a well-known luteotrophic action, which occurs by either its intraluteal conversion to estradiol [26] or by a non genomic mechanism [27–29]. It may also have a primary non genomic effect that might lead to the activation of a complex cascade of signals transduction [30] and a subsequent genomic effect. In addition, Casais et al. [5] demonstrated that in the coeliac ganglion-superior ovarian nerve–ovary system, androstenedione can reverse the functional regression of the corpus luteum when added to the ganglion compartment; this action on the ovary is exerted via the superior ovarian nerve. In relation to this, other authors have demonstrated immunoreactivity and expression of androgen receptors in dorsal [31] and pelvic ganglia [32].

The aims of this work are: (1) to investigate the presence of the androgen receptor in the coeliac ganglion of pregnant rats by immunohistochemistry, and (2) to investigate, from a physiological point of view, the potential participation of these receptors in the androstenedione ganglionic action leading to the release and metabolism of progesterone and to the nitrites release in the ovary compartment at the end of pregnancy.

2. Materials and methods

2.1. Animals

Holtzman strain adult female rats bred in our laboratory aged 2–3 months and weighing 220 ± 50 g were used. The rats were housed under controlled light (lights on from 07:00 to19:00 h) and temperature (24 ± 2 °C) conditions, and allowed ad libitum access to water and food (rat chow—Cargill SACI; Saladillo, Buenos Aires, Argentina).

To induce pregnancy, female rats were caged individually with fertile males on the afternoon of proestrus. Positive mating was verified on the following morning by identifying sperm or copulation plugs in the vagina. This day was designated as day 0 of pregnancy. In our laboratory, rats usually give birth on day 22.

Animals were handled according to the procedures approve in the UFAW Handbook on the Care and Management of Laboratory Animals [33]. The experimental protocol was approved by the University of San Luis Animal Care and Use Committee (number protocol: B17/04, ordinance CD 006/02).

2.2. Reagents

The following drugs: 4-androsteno-3, 17-dione, 2-methyl-N-[4-nitro-3-(trifluormethyl)-phenyl]propanamide (Flutamide), bovine serum albumin fraction V (BSA), ascorbic acid, dextrose, sulphanilamide, N-1-naphtyl-ethylendiamine were purchased by the Sigma Chemical Co.(St Louis, MO, USA). 1,2,6,7-[^{3}H]-Progesterone (107.0 Ci/mmol) was provided by New England Nuclear Products (Boston, MA, USA). Other reagents and chemicals were of analytical grade.

2.3. Surgical and experimental procedures

2.3.1. Extraction of coeliac ganglia fixed in situ for localization of androgen receptors by immunohistochemistry

Rats in the 21st day of pregnancy ($n = 3$) were anaesthetized under ether bell. The coeliac ganglion and the nearby ganglia were fixed in situ with Bouinĭs solution for subsequent application of the immunohistochemical technique. The fetuses were removed and killed in an atmosphere of ether and the mothers were sacrificed by decapitation.

2.3.2. Extraction of coeliac ganglion-superior ovarian nerve–ovary system of late pregnant rats

Groups of six animals on day 21 of pregnancy were used for each experimental procedure. The surgical procedure to remove the coeliac ganglion-superior ovarian nerve–ovary system and the incubation conditions were carried out according to Casais et al. [3]. Animals were anaesthetized under ether bell, the fetuses were removed and killed in an atmosphere of ether, the system was extracted and the mothers were sacrificed by decapitation. The complete system was removed by surgery, avoiding contact between the surgical instruments and the nerve fibres in order to prevent spontaneous depolarisation of the nerves. The piece of tissue removed consisted of the left ovary, the fibres that constitute the SON, that are inserted in the suspensory ligament, and the coeliac ganglion accompanied by some the small ganglia surrounding it. The total surgical procedure was completed in 1–2 min. The system was washed with incubation medium and placed in a cuvette with two compartments, one for the coeliac ganglion and the other for the ovary, both joined by the SON. The incubation medium used was Krebs–Ringer bicarbonate buffer, pH 7.4 with the addition of 0.1 mg glucose/ml and 0.1 mg albumin/ml at 37 °C in an atmosphere composed by 95% of O_2 and 5% of CO_2.

The system was preincubated for 30 min, and the end of this period was considered incubation time 0. After this time, the buffer was changed in both compartments and 0.1 mg of ascorbic acid/ml was added to the ganglion compartment as an antioxidant agent.

The experimental groups proposed were: (a) coeliac ganglion-superior ovarian nerve–ovary system with the addition of androstenedione (A_2) 10^{-6} M in the ganglion compartment [$(A_2)_g$]; (b) coeliac ganglion-superior ovarian nerve–ovary system with the addition of flutamide 10^{-4} M (androgen receptor antagonist [34]) in the ganglion compartment [$(Flu)_g$]; and (c) coeliac ganglion-superior ovarian nerve–ovary system with the addition of flutamide 10^{-4} M and A_2 10^{-6} M 5 min later in the ganglion compartment [$(A_2 + Flu)_g$]. The controls were coeliac ganglion-superior ovarian nerve–ovary system without treatment [Control].

Periodic extractions of the ovary incubation liquid (250 μl) were carried out at 30, 60 and 120 min. and were kept at −20 °C until progesterone was determined by radioimmunoassay (RIA) and nitrites, by the Griess method. The corresponding corrections were made in all cases, taking into consideration the volume extracted in each tested period.

At the end of the total incubation period, the corpora lutea of the control group and of the $(A_2)_g$ group were isolated in ice under stereoscopic lens and frozen at −80 °C until determination of the enzymatic activities and expression levels of 3β-HSD and 20α-HSD.

2.4. Immunohistochemistry

The tissue sections were first deparaffinized with xylene and hydrated through decreasing concentrations of ethanol. After rinsing in phosphate-buffered saline (PBS, 0.01 M, pH 7.4) the sections

were transferred to 0.01 M Na-citrate buffer (pH 6.0) and treated for 3 min in a microwave oven at 900 W, after which they were rinsed with PBS. Endogenous peroxidase activity was blocked by treatment with 3% H_2O_2 in water for 20 min. After this, the sections were rinsed with distilled water and PBS. Nonspecific binding sites for immunoglobulins were blocked by incubation for 15 min with 0.25% casein in PBS and rinsed with distilled water and PBS. Sections were then incubated overnight in a humidified chamber at 4 °C with rabbit polyclonal antibody raised against a peptide mapping at the N-terminus of androgen receptor (AR) of rat origin (AR N-20 sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:200. After they were rinsed with PBS for 10 min, the immunohistochemical visualization was carried out using the Super Sensitive Ready-to-Use Immunostaining Kit (BioGenex, San Ramon, Calif., USA) at 20 °C. The Biotin-Streptavidin amplified system (B-SA) was used as follows: sections were incubated for 30 min with diluted biotinylated anti-rabbit IgG, and after being washed in PBS, they were incubated for 30 min with horseradish peroxidase-conjugated streptavidin, and finally washed in PBS. The reaction site was revealed by 100 μl 3,3′-diaminobenzidine tetrahydrochloride (DAB) chromogen solution in 2.5 ml PBS and 50 μl H_2O_2 substrate solution. The sections were dehydrated and mounted.

In order to confirm the specificity of the immunoreactive procedures, adjacent sections were stained according to the above described protocol, but incubation in the primary antiserum was omitted. In addition, normal rabbit serum was used instead of primary antiserum. No positive structures or cells were found in these sections. Sections of rat prostate were used as positive control of AR.

2.5. Enzyme activities

The activities of the enzymes were measured according to Tellería and Deis [35]. The corpora lutea from each animal were homogenized in 0.7 ml of Tris-HCl, 0.1 mM EDTA (pH 8) with a glass homogenizer. The homogenates were centrifuged at 105.000 *g* for 60 min. The supernatant fluids were used for the assay of 20α-HSD activity. The precipitates were re-homogenized with 0.7 ml of 0.25 M sucrose and centrifuged at 800 *g* for 5 min. The supernatants were used as the enzyme solution for the assay of 3β-HSD activity. The substrates for 3β-HSD and 20α-HSD were pregnenolone (5 μg) and 20α-hydroxypregn-4-en-3-one (12.5 μg), respectively. Both enzyme activities were assayed spectrophotometrically, dependent on the increase in NADH or NADPH in 1 min at 37 °C and the values were expressed as mU/mg protein/min.

2.6. RNA isolation and RT-PCR analysis

Total luteal RNA was isolated using TRIZOL Reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. Two micrograms of total RNA were reverse transcribed at 37 °C using random primers and M-MLV Reverse Transcriptase (Promega Inc.) in a 26 μl reaction mixture. For amplification of the reverse transcription (RT) products, the reaction mixture consisted of 1× Green Go Taq reaction buffer, 0.2 mM deoxynucleoside triphosphates, 0.5 μM specific oligonucleotide primers and 1.25 U Go Taq DNA polymerase (Promega Inc.) in a final volume of 50 μl. Amplification was carried out for 35 cycles using 95 °C for denaturing (1 min), 59 °C for annealing (1 min), and 72 °C for extension (1 min) in My Cycler thermal cycler. Specific primers for 3β-HSD were (5′-GTCTTCAGACCAGAAACCAAG-3′ and 5′-CCTTAAGGCACA AGTATGCAG-3′); and for 20α-HSD (5′-TTCGAGCAGAACTCATGGCTA-3′ and 5′-CAACCAGGTAGAATG CCATCT-3′). Each reaction also included primers (5'-CGTTCACCT-TGATGAGCCCATT-3' and 5'-TCCAAGGGTCCGCTGCAGTC-3') to amplify ribosomal protein S16 as an internal control. The predicted sizes of the PCR-amplified products were 447 pb for 3β-HSD, 440 pb for 20αHSD and 100 pb for S16. Reaction products were electrophoresed on 2% agarose gels, visualized with ethidium bromide, and examined by ultraviolet transillumination. Band intensities of RT-PCR products were quantified using ImageJ (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/) and expressed as arbitrary units.

2.7. Progesterone assay

Progesterone was measured by RIA using antiserum raised against progesterone-11-BSA conjugate in rabbits, provided by Dr R.P.Deis (Laboratorio de Reproducción y Lactancia Mendoza, Argentina). The sensitivity, variability and cross-reaction of this RIA have been reported previously. The sensitivity of the assay is less than 5 ng progesterone/ml serum, and the inter- and intra-assay coefficients of variation were less than 10%. This assay has been previously validated [36].

The results were expressed as nanograms of progesterone/mg of ovarian tissue (ng/mg ovary).

2.8. Nitrite assay

Levels of nitrite, a water-soluble metabolite of nitric oxide were measured spectrophotometrically. Samples were immediately mixed with Griess reagent (sulphanilamide with N-1-naphthyl-ethylendiamine/HCL). After a 10 min incubation period at room temperature, they were read for absorvance of 540 nm. The assay sensitivity was <2.5 nmol/ml. The intra-assay coefficients of variation for all the assays were <10.0%.

The results were expressed as nmol of nitrite per milligram of ovarian tissue (nmol/mg ovary) [37].

2.9. Statistical analysis

All data are presented as means ± S.E.M in each group of six rats. Differences between two groups were analysed with Student's *t*-test. For multiple comparisons made along the time of incubation, repeated measures analysis of variance followed by Tukey's *test* was used. Instead, for multiple comparisons not involving repeated measures, one-way analysis of variance followed by Tukey's *test* was utilized. A difference was considered to be statistically significant at $p < 0.05$ [38].

3. Results

3.1. Localization of androgen receptors in coeliac ganglion of pregnant rats by immunohistochemistry

The histological study of the coeliac ganglion showed a thin capsule of connective tissue and the parenchyma exhibited several neuronal somas with intensely basophilous cytoplasm and nucleus of lax chromatin with one or two evident nucleoli (Fig. 1A). The immunoreactivity was observed in neuronal somas of the celiac ganglion, and its pattern was cytoplasmic and heterogeneous (Fig. 1B–F). No positive structures or cells were observed in the coeliac ganglion sections when the primary antiserum was not added (negative control) (Fig. 1G,H). Sections of rat prostate were used as negative and positive control for androgen receptors immunoreactivity (Fig. 1I,J).

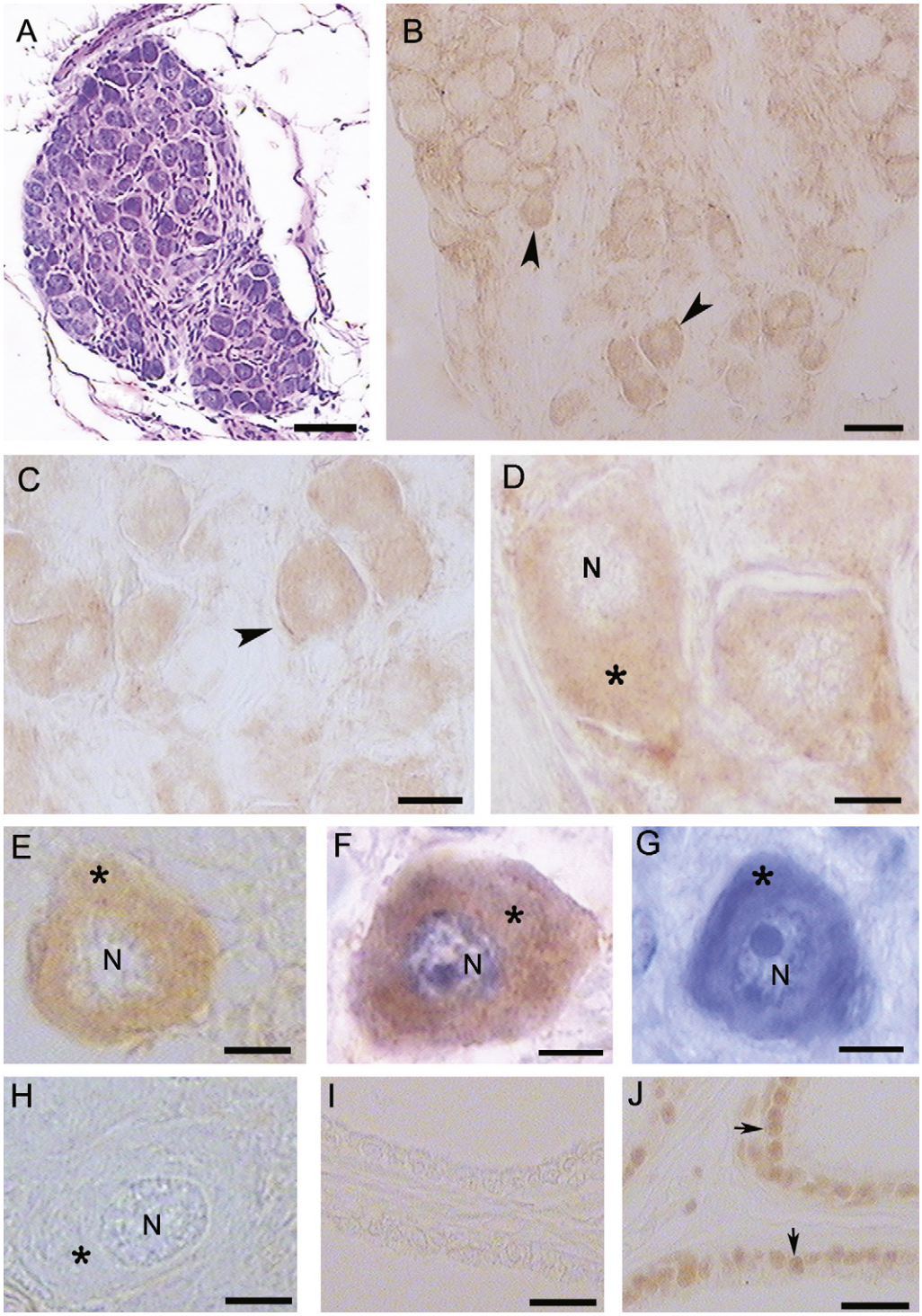

Fig. 1. (A): Photography of the coeliac ganglion showing numerous neuronal somas of pyramidal and oval shape. Hematoxylin-eosin. Scale bar, 100 μm. (B–E): Sections of the immunolabeled coeliac ganglion with the serum anti-AR (N-20). The localization of androgen receptors was observed in the cytoplasm of neuronal soma (arrowheads). N: nucleus. *: immunolabeled cytoplasm. Scale bar: (B), 50 μm; (C), 25 μm; (D–E), 10 μm. (F): immunolabeled neuron with anti-AR and counterstained with hematoxylin. N: nucleus with nucleolus. *: immunolabeled cytoplasm. Scale bar: 10 μm. (G): Negative control of immunohistochemistry counterstained with hematoxylin. N: nucleus with nucleolus. *: cytoplasm. Scale bar: 10 μm. (H): Image of a slide with a negative control of immunohistochemistry without staining of hematoxylin. N: nucleus. *: cytoplasm. Scale bar: 10 μm. (I,J): Image showing prostate of rat, a negative control (I) and a positive control (J) of immunoperoxidase staining with anti-AR (N-20). Arrows: immunolabeled nuclei of epithelial cells. Scale bar: 25 μm.

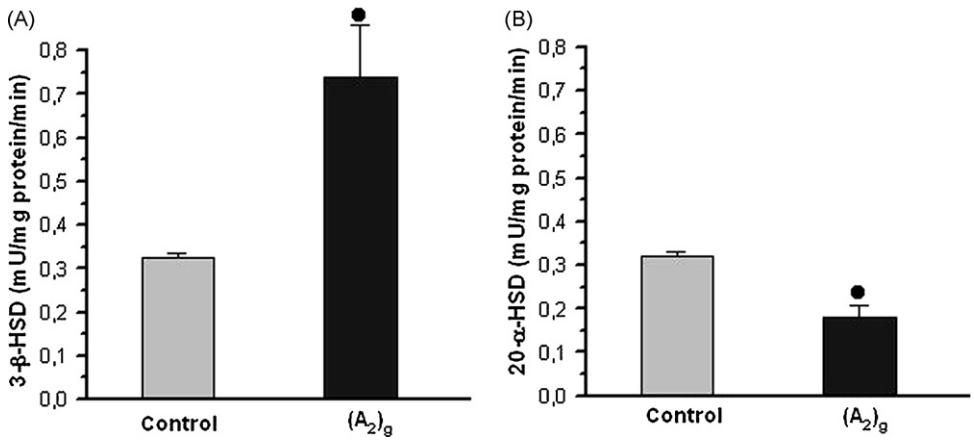

Fig. 2. (A): Ganglionic effect of $(A_2)_g$ on the enzymatic activity of ovarian 3ß-HSD expressed as (mU/mg protein/min.) in the coeliac ganglion-superior ovarian nerve-ovary system in 21-day pregnant rats. (B): Ganglionic effect of $(A_2)_g$ on the enzymatic activity of ovarian 20α-HSD expressed as (mU/mg protein/min.) in the coeliac ganglion-superior ovarian nerve-ovary system in 21-day pregnant rats. Results are expressed as mean ± S.E.M. (●) $p < 0.05$. Student's *t*-test.

3.2. Coeliac ganglion-superior ovarian nerve–ovary system of late pregnant rats

3.2.1. Ganglionic effect of androstenedione on the enzymatic activities of luteal 3β-HSD and 20α-HSD

When analyzing the action of androstenedione on the coeliac ganglion, it was observed that the activity of 3β-HSD increased in relation to the control (•) $p < 0.05$ (Fig. 2A); while the activity of 20α-HSD decreased in relation to the control (•) $p < 0.05$ (Fig. 2B).

3.2.2. Ganglionic effect of androstenedione on the luteal mRNA abundance of 3β-HSD and 20α-HSD

In order to determine whether the action of androstenedione on the coeliac ganglion also affects the expression of enzymes involved in progesterone synthesis and degradation, we measured the luteal mRNA abundance of 3β-HSD and 20α-HSD. The addition of A_2 10^{-6} M in the ganglion compartment did not modify the expression of 3β-HSD, but caused a tendency to reduce 20α-HSD mRNA, in relation to the Control. (Fig. 3)

3.2.3. Ganglionic effect of androstenedione and flutamide on the release of ovarian progesterone

The presence of A_2 10^{-6} M in the ganglion compartment $(A_2)_g$ increased the release of ovarian progesterone in relation to the Control at all the studied times (a,b,c) $p < 0.025$. The action of A_2 10^{-6} M plus flutamide 10^{-4} M in the coeliac ganglion $(A_2 + Flu)_g$ significantly decreased the release of ovarian progesterone at 30 and 120 min in relation to $(A_2)_g$ (*) $p < 0.025$. The only addition of flutamide 10^{-4} M in the coeliac ganglion $(Flu)_g$ increased the release of ovarian progesterone in relation to the control group at all the studied times (d,e,f) $p < 0.05$. In each group, there were no differences in progesterone release at different times. (Fig. 4)

3.2.4. Ganglionic effect of androstenedione and flutamide on the release of nitrites in the ovary compartment

The addition of A_2 10^{-6} M in coeliac ganglion $(A_2)_g$ increased nitrites release in the ovary compartment at all the studied times in relation to the control group (a,b,c) $p < 0.025$. The action of A_2 10^{-6} M plus flutamide 10^{-4} M in the coeliac ganglion $(A_2 + Flu)_g$ significantly decreased nitrites release in the ovary compartment in

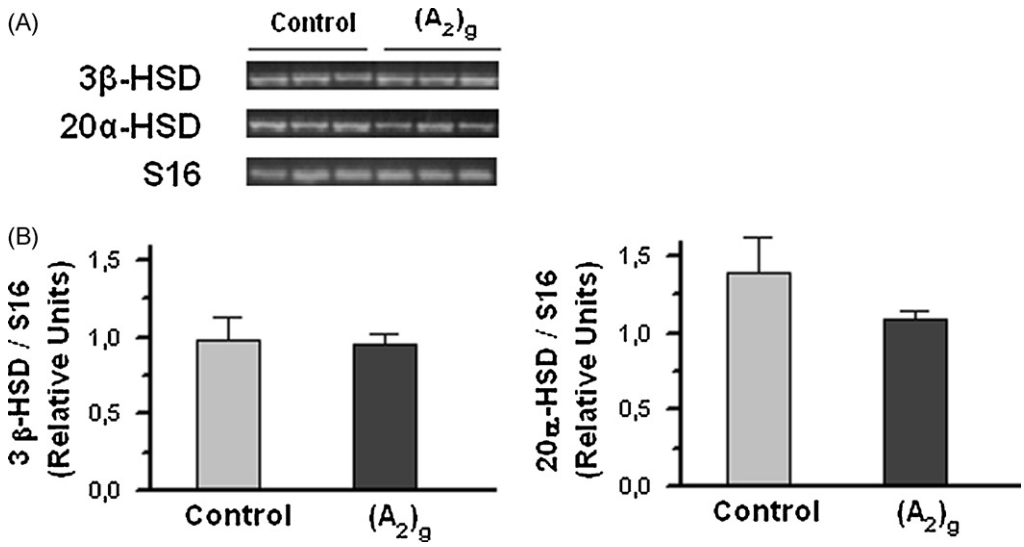

Fig. 3. Ganglionic effect of A_2 on luteal mRNA abundances of enzymes 3β-HSD and 20α-HSD in the coeliac ganglion-superior ovarian nerve-ovary system in 21-day pregnant rats. (A): Measurement by RT-PCR of expression of 3β-HSD, 20-α-HSD and S16. Ethidium bromide fluorescence photograph of the gel electrophoresis of the amplification products (B): Expression of 3β-HSD and 20-α-HSD relative to S16 respectively. The gel photographs were quantified using ImageJ and expressed as arbitrary units. Results are expressed as mean ± S.E.M ($n = 3$).

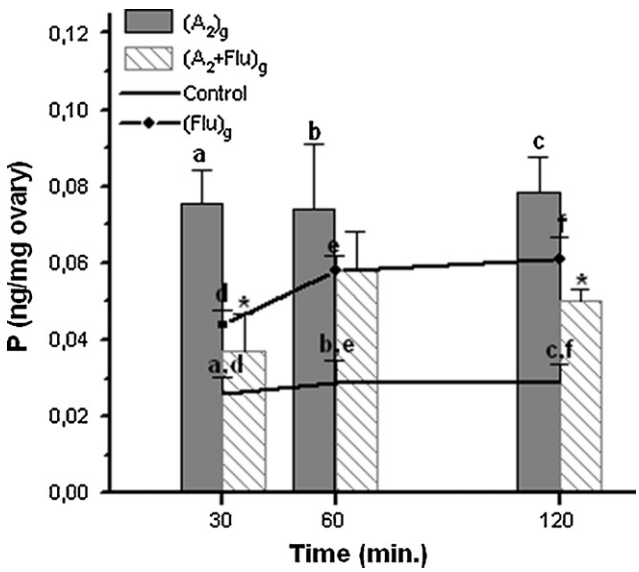

Fig. 4. Ganglionic effect of A_2 and flutamide in the coeliac ganglion-superior ovarian nerve–ovary system on the ovarian progesterone release expressed as ng/mg ovary in 21-day pregnant rats. The systems were incubated in Krebs Ringer buffer, plus 0.1 mg ascorbic acid/ml at 37 °C in an atmosphere of 95% O_2–5% CO_2 for 120 min, without (Control) and with addition of A_2 10^{-6} M $(A_2)_g$, Flutamide 10^{-4} M $(Flu)_g$, or A_2 10^{-6} M + Flutamide 10^{-4} M $(A_2 + Flu)_g$ to the ganglion compartment. Results are expressed as mean ± S.E.M. of six animals per group. One-way analysis of variance followed by Tukey's *test* was used. The same font denote differences of statistical significance; (a,b,c) $p < 0.025$ in $(A_2)_g$ group compared with control group; (d,e,f) $p < 0.05$ in $(Flu)_g$ group compared with control group. (*) $p < 0.025$ in $(A_2 + Flu)_g$ group compared with $(A_2)_g$ group.

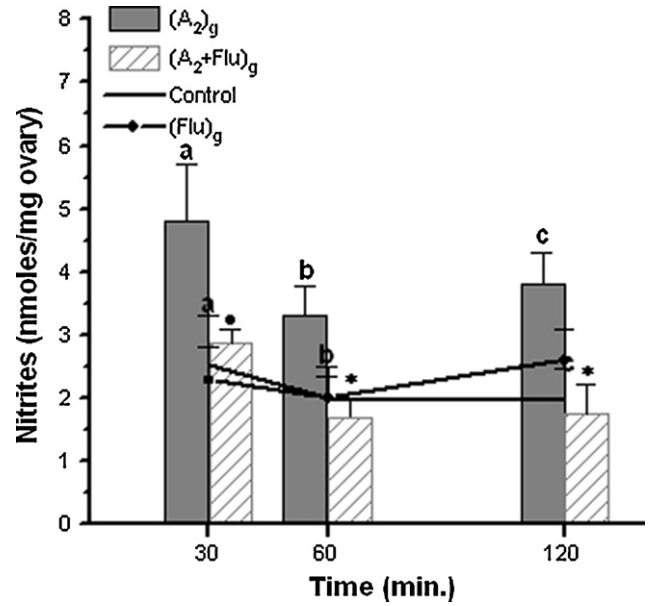

Fig. 5. Ganglionic effect of A_2 and flutamide in the coeliac ganglion-superior ovarian nerve-ovary system on the nitrites release expressed as nmoles/mg ovary in 21-day pregnant rats. The systems were incubated in Krebs Ringer buffer, plus 0.1 mg ascorbic acid/ml at 37 °C in an atmosphere of 95% O_2–5% CO_2 for 120 min, without (Control) and with addition of A_2 10^{-6} M $(A_2)_g$, Flutamide 10^{-4} M $(Flu)_g$, or A_2 10^{-6} M plus Flutamide 10^{-4} M $(A_2 + Flu)_g$ to the ganglion compartment. Results are expressed as mean ± S.E.M. of six animals per group. One-way analysis of variance followed by Tukey's *test* was used. The same font denote differences of statistical significance; (a,b,c) $p < 0.025$ in $(A_2)_g$ group compared with control group; (•) $p < 0.05$ (30 min) (*) $p < 0.025$ (60 and 120 min) in $(A_2 + Flu)_g$ group compared with $(A_2)_g$ group.

relation to $(A_2)_g$ 30′ (•) $p < 0.05$, 60′ and 120′ (*) $p < 0.025$. The only addition of flutamide

10^{-4}M in the coeliac ganglion $(Flu)_g$ did not modify the release of nitrites in the ovary compartment in relation to the control group. In each group, there were no differences in progesterone release at different times (Fig. 5).

4. Discussion

The protective action of androstenedione on the pregnancy corpus luteum has always been the leading interest of our research group [5,27,39]. In Casais et al. [5] we reported that androstenedione can reverse the functional regression of the late pregnancy corpus luteum when administered systemically or when added directly to the ganglion compartment of the coeliac ganglion-superior ovarian nerve–ovary system via the superior ovarian nerve. Based on this result and using the same system standardized in our laboratory, the aims of this work were: (1) to investigate the presence of androgen receptors in coeliac ganglion of pregnant rats by immunohistochemistry, and (2) to investigate, from a physiological point of view, the potential participation of these receptors on the ganglionic action of androstenedione, on the release and metabolism of progesterone and on the release of nitrites in the ovary compartment at the end of pregnancy.

The coeliac ganglion-superior ovarian nerve–ovary system permits in vitro emulation of in vivo conditions, preserving innervation as well as paracrine and autocrine regulation within the gland without humoral influence. A further advantage of this system is that it possesses its own neural tone, as has been demonstrated in previous reports [3,40]. In this work, the morphological study carried out by haematoxylin-eosin staining confirmed the presence of the coeliac ganglion. Previous works by Sosa et al. [40] demonstrated the ganglion presence in the same system for the estral cycle.

Having confirmed the presence of the coeliac ganglion and considering reports by Keast and Gleeson [31], which demonstrated the distribution of androgen receptor immunoreactivity in L_6 and S_1 dorsal root ganglia of male rats, and by Kanjhan et al. [32], who determined the expression of androgen receptors by many neurons of the rat pelvis ganglion, we investigated the existence of androgen receptors in the rat coeliac ganglion on day 21 of pregnancy. It must be noted that the study was performed on ganglia fixed in situ, this is to say, on their natural location. Surprisingly, the immunohistochemical analysis showed positive immunoreactivity for the androgen receptor in neuronal somas of the coeliac ganglion, as can be observed by comparison with the respective negative control and the rat prostate positive control. On the other hand, the receptor cytoplasmatic distribution was found to be in agreement with previous studies reporting that the androgen receptor remains largely in the cytoplasm in the absence of hormone [34,41]. Tyagi et al. [42] have studied the dynamics of the androgen receptor and have shown that the unliganded androgen receptor is primarily localized in the cytoplasm and, upon hormone exposure, rapidly migrate into the nucleus (within 15–60 min). Upon androgen withdrawal, the androgen receptor migrates back to the cytoplasmic compartment and maintains its ability to re-enter the nucleus on subsequent exposure to androgen. These authors conclude that members of the steroid hormone receptor family can be classified into two distinct groups, one that requires ligand binding for nuclear translocation (e.g. glucocorticoid receptor and androgen receptor), and another that is nuclear, even without ligand binding (e.g. estrogen receptor and progesterone receptor). There is also evidence suggesting that the androgen receptor, in addition to its known transcriptional activity, may exhibit some cytoplasmic effect by mimicking the action of growth factors that modulate several signalling pathways [43]. This notion is further supported by immunocytochemical studies that have demonstrated an extranuclear localization for

both the androgen receptor [44] and the estrogen receptor [45,46] in neurons, based on which the possibility of nongenomic effects of aromatizable and nonaromatizable androgens on the brain has been claimed.

Although studies of androgen receptor dynamics have not been carried out, the above results demonstrate the existence of androgen receptors in the coeliac ganglion. Subsequently, experiments were designed to account for the androgen receptor involvement from a physiological point of view using the coeliac ganglion-superior ovarian nerve–ovary system. Thus, we performed incubations of the system by adding in ganglion androstenedione $(A_2)_g$, and androstenedione with previous addition of flutamide, an androgen receptor antagonist, $[(A_2 + Flu)_g]$.

Addition of androstenedione alone produced an increase of the ovarian progesterone release with a pattern similar to that found by Casais et al. [5]. Moreover, it was found that not only did progesterone increase, but also there was an increase on the enzymatic activity of 3β-HSD (progesterone synthesis enzyme) and a decrease on 20α-HSD (progesterone degradation enzyme) in corpora lutea after the system incubation (120 min) in relation to the control. The determination of the enzymatic activities was carried out at the end of incubation since there were no differences in progesterone release at different times. The activities were modified at 120 min by neural stimulus. The results obtained in this work are surprising since the antecedents in relation to the modulation of the enzymatic activities of luteal 3β-HSD and 20α-HSD in late pregnancy were modified at prolonged times after 36 h in luteal cells culture without neural influence and without the paracrine and autocrine relations that are present in our ex vivo system [39,47]. It is important to emphasize that the activities of 3β-HSD and 20α-HSD were determined in the microsomal and the cytoplasmic fraction, respectively. Chapman et al. [48], working with mice, found that on days 15 and 20 of gestation, the activity of 3β-HSD was greater in the microsomal fraction than in the mitochondrial fraction. They also observed that the activity of the mitochondrial 3β-HSD increased after the LH peak. It is interesting to note that our model is an ex vivo system isolated from humoral influence, and it does not receive any influence from the serum LH, therefore the determination of 3β-HSD was carried out in the microsomal fraction. To investigate whether the increase in 3β-HSD and decrease in 20α-HSD activity observed in the celiac ganglion-superior ovarian nerve–ovary system stimulated with $(A_2)_g$ in coeliac ganglion is also reflected in the mRNA expression of these enzymes, we analyzed the abundance of 3β-HSD and 20α-HSD mRNA relative to S16 by PCR. The expression results did not correlate with those obtained for the activity. However, 20α-HSD showed a tendency to decrease in the stimulated group with respect to the control. Although it is known that in the pregnant rat corpus luteum, 3β-HSD is constitutively expressed, several researchers have shown that it is regulated by PRL. It is also known that the expression and activity of 20α-HSD are downregulated during pregnancy by PRL and placental lactogens. Before parturition, mRNA expression of 20α-HSD increases, accompanied by sharp increase in 20α-DHP and a concomitant fall in progesterone [49]. Based on these antecedents, it is possible that in our experiments the expression of 20α-HSD is more sensitive to the neural stimulus than that of 3β-HSD. It is also likely that the chosen expression time (120 min post incubation) for the analyzed enzymes is not appropriate.

Androstenedione with previous addition of flutamide in the ganglion compartment provoked a decrease in ovarian progesterone release at 30 and 120 min of incubation $[(A_2 + Flu)_g$ vs. $(A_2)_g]$, which indicates that the presence of flutamide in ganglion blocks the androstenedione effect. It must be emphasized that the progesterone release values in $(A_2 + Flu)_g$ did not reach those of the control group, but compared to those of the $(Flu)_g$ group. It is possible that flutamide not only has an inhibiting effect on the action of androstenedione but also has a *per se* effect. This has also been suggested by other researchers, such as Mac Lusky et al. [50], who studied the effect of androgens (DHT and DHEA) and of flutamide on the density of dentritic spine synapses in the CA1 subfield of the rat hippocampus, in males and gonadectomized females. These authors concluded that flutamide did not inhibit the effects of those androgens on the density of the synapsis, but it increased them. Yeh et al. [51] have demonstrated that flutamide can act under some circumstances as a partial androgen agonist in androgen-sensitive cancer cells, activating androgen receptor-dependent gene transcription. The same kind of response could contribute to the *per se* effect of flutamide noted here.

Finally, on day 21 of pregnancy the presence of androstenedione in the ganglion probably caused modifications in the release of different neurotransmitters at the ovary level that might be responsible for the observed effects on the release of progesterone and the activity of its synthesis and degradation enzymes. In previous works we demonstrated that different ganglionic stimuli using the same ex vivo system provoked modifications in the release of catecholamines and nitrites at the ovary level [5,52–54]. Evidence has been reported that supports the involvement of nitric oxide in ovary physiology [53–56]. Casais et al. [56], working with the ex vivo celiac ganglion-superior ovarian nerve–ovary system of prepubertal rats, added non selective and selective inhibitors of the synthase nitric oxide enzyme in the ovary and ganglion compartments. Their results demonstrated that the release of nitric oxide in the ovary compartment had ovarian and ganglionic origin, which might indicate the existence of synthase nitric oxide activity in the ovary and the coeliac ganglion. However, there is little information about the role played by the neural factors on nitric oxide release in the ovary during rat pregnancy.

In the present study, nitrites increased in the ovary compartment due to the addition of androstenedione to the coeliac ganglion when compared with the control group. This effect might be attributed to the androgen direct action on the activity of one or more isoforms of the nitric oxide synthesis enzyme present in the ganglion. It is also possible that androstenedione in the ganglion favours the release of some neurotransmitters in ovary that might act on the ovarian nitric oxide synthesis enzyme. These hypotheses will be the subject of future studies. Regarding flutamide, its presence in the ganglion did not produce variations in nitrites content in the ovary compartment and completely blocked the stimulating effect of androstenedione, providing additional evidence of the participation of androgen receptors in the coeliac ganglion.

Both luteolytic and luteotrophic effects have been reported for nitric oxide [22]. In our work, the nitric oxide increase by the androstenedione action in the ganglion is likely to have a trophic effect on the corpus luteum. It is also likely that, under the same circumstances, the increase of progesterone has a protective effect on the corpus luteum that prevails over the luteolitic effect of the nitric oxide. A possible future study will attempt to identify the released neurotransmitters provoking the different responses in which the nitric oxide is evidently involved.

In conclusion, the presence of androgen receptors in neurons of celiac ganglion has been demonstrated by immunohistochemistry as well as physiologically. This finding might indicate that the luteotrophic action of androstenedione, by a neural pathway, is carried out through the androgen receptors in the coeliac ganglion. Since this implies that the coeliac ganglion influences the ovarian physiology at the end of pregnancy, new questions arise in relation to the importance of the hormone receptors participation in the ganglion.

Acknowledgements

This manuscript is dedicated to the memory of Dr. Luis I Aguado (1946-2003) and supported by Grant 9302 from Universidad Nacional de San Luis, Argentina. We thank Dr. R.P. Deis from the Laboratorio de Reproducción y Lactancia (LARLAC-CONICET) who provided the progesterone antiserum, Dr R. Calandra from the Instituto de Biología y Medicina Experimental (IByME-CONICET) who provided the androgen receptor antiserum (AR N-20 sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and Luis Villegas for technical support. We wish to specially thank Dr A.C. Anzulovich for his expert assistance in RT-PCR technique. This work is part of the doctoral thesis of Sandra Vallcaneras.

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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