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Androstenedione acts on the coeliac ganglion and modulates luteal function via the superior ovarian nerve in the postpartum rat

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ABSTRACT

Androstenedione can affect luteal function via a neural pathway in the late pregnant rat. Here, we investigate whether androstenedione is capable of opposing to regression of pregnancy corpus luteum that occurs after parturition, indirectly, from the coeliac ganglion. Thus, androstenedione was added into the ganglionar compartment of an *ex vivo* coeliac ganglion–superior ovarian nerve–ovary system isolated from non-lactating rats on day 4 postpartum. At the end of incubation, we measured the abundance of progesterone, androstenedione and oestradiol released into the ovarian compartment. Luteal mRNA expression and activity of progesterone synthesis and degradation enzymes, 3β-hydroxysteroid-dehydrogenase (3β-HSD) and 20α-hydroxysteroid-dehydrogenase (20α-HSD), respectively, as well as the aromatase, Bcl-2, Bax, Fas and FasL transcript levels, were also determined. Additionally, we measured the ovarian release of norepinephrine, nitric oxide and luteal inducible nitric oxide synthase (iNOS) mRNA expression. The presence of androstenedione in the ganglion compartment significantly increased the release of ovarian progesterone, androstenedione and oestradiol without modifying 3β-HSD and 20α-HSD activities or mRNA expression. The ovarian release of oestradiol in response to the presence of androstenedione in the ganglion compartment declined with time of incubation in accord with a reduction in the aromatase mRNA expression. Androstenedione added to the ganglion compartment decreased FasL mRNA expression, without affecting luteal Bcl-2, Bax and Fas transcript levels; also increased the release of norepinephrine, decreased the release of nitric oxide and increased iNOS mRNA. In summary, on day 4 after parturition, androstenedione can mediate a luteotropic effect acting at the coeliac ganglion and transmitting to the ovary a signaling via a neural pathway in association with increased release of norepinephrine, decreased nitric oxide release, and decreased expression of FasL.

1. Introduction

The mammalian ovary is innervated by two vias: the ovarian plexus and the superior ovarian nerve (SON), which is the most relevant for the ovarian steroidogenesis. The SON is mainly constituted by adrenergic fibres, most of which originate in the coeliac ganglion (CG) [1]. In the ovary, these fibres form a net of varicosities surrounding the theca and secondary interstitial cells, responsible for the androgens synthesis, without entering the follicle and with little visible relation with the corpus luteum [2] yet express β_1 and β_2 adrenergic receptors that transduce positive inputs to luteal steroidogenesis [3,4]. The CG has an ample microvasculature and contains many neurotransmitters, receptors for neurotransmitters [5], and as shown recently by our laboratory, androgen receptors [6]. Among the neurotransmitters released into the ovarian interstitial tissue are norepinephrine [4,7] and nitric oxide [8]. Several studies support a role of nitric oxide in the regulation of luteal function [9,10] and it is interesting that nitric oxide synthesis enzymes in their different isoforms have been characterized and localized to the ovary [11] and to the peripheral neural system [12].

During pregnancy in the rat, the corpus luteum is the dominant ovarian entity and its main steroid hormone produced is progesterone, fundamental to maintain uterine support for the implanting embryo. This steroid is synthesized from pregnenolone by the action of the enzyme 3β-HSD and is catabolized by the enzyme 20α-HSD into an inactive progestin. Another steroid hormone is oestradiol synthesized from androstenedione upon the action of

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aromatase. Intraluteal oestradiol synthesized during pregnancy is important for progesterone synthesis and to promote the differentiation and growth of the luteal cells [13]. In contrast, little is known about the effect of androstenedione, oestradiol and the regulation of aromatase expression in the regressing corpus luteum after parturition.

Our laboratory has demonstrated that in addition to the mainstream hormonal regulators such as oestradiol and prolactin, the physiology of the rat corpus luteum of pregnancy is modulated by the peripheral nervous system [6,14–17].

After parturition takes place, and as a consequence of the ovulation that occurs in this species within the following 24–36 h, the corpora lutea of previous pregnancy and the newly formed corpora lutea after postpartum ovulation coexist and will regress if lactation is not established [18–21]. These corpora lutea found in the ovary after parturition in the absence of lactation can be rescued, at least in part, by systemic administration of androstenedione or progesterone [18,19], suggesting that the corpora lutea even while regressing maintain certain responsiveness to appropriated hormonal stimuli. Thus, the objective of this work was to investigate whether androstenedione from the CG, via the SON, is capable of leading to the refunctionalization of the corpora lutea of pregnancy in non-lactating rats on day 4 postpartum.

2. Materials and methods

2.1. Reagents

The chemicals and other reagents of analytical grade used for this study were purchased from 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).

2.2. Animals

Virgin Holtzman strain female rats weighing 250 ± 50 g were used in all the experiments. Animals had free access to food (Cargill SACI, Saladillo, Buenos Aires, Argentina) and water. They were kept in a light (lights on from 07:00 to 19:00 h) and temperature-controlled room (24 ± 2 °C).

In our laboratory, rats usually give birth on day 22. The pups were removed immediately after delivery to avoid the establishment of lactation. Groups of six animals on day 4 postpartum were used for experimental group. Each experiment included an experimental and a control group.

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

2.3. Extraction of CG–SON–ovary system of rats on day 4 postpartum

The surgical procedure to remove the CG–SON–ovary system and the incubation conditions were carried out according to Casais et al. [17]. The system was placed in a cuvette with two compartments, one for the CG 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 of 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, buffer was changed in both compartments and 0.1 mg of ascorbic acid/ml was added to the ganglion compartment as antioxidant agent. The CG–SON–ovary system was exposed to 10^{-6} M androstenedione, principal circulating androgen in rat, or vehicle in the ganglion compartment. Periodic extractions of the ovary incubation liquid (250 μl) were carried out at 30, 60, 120 and 180 min and were kept at −20 °C until progesterone, androstenedione, oestradiol and nitrites were assayed. The corresponding corrections were made in all cases, taking into consideration the volume extracted in each tested period.

At the end of the incubation period only the corpora lutea of pregnancy, recognized as larger and less vascularized than the newly-formed corpora lutea, were isolated in ice under stereoscopic lens and frozen at −80 °C until determination of the mRNA levels and enzyme activities of 3β-HSD and 20α-HSD and mRNA expression of aromatase, iNOS, Bax, Bcl-2, Fas and FasL.

2.4. Progesterone, androstenedione and oestradiol assay

Steroids were measured in duplicate by RIA in the ovary incubation liquid. Progesterone and androstenedione concentration was expressed as nanogram per milligram ovary (ng/mg ovary). Oestradiol was expressed as picograms per milligram of ovarian tissue (pg/mg ovary), all against incubation time. The assay sensitivity was <5 ng progesterone/ml, <10 pg androstenedione/ml, and <2.2 pg oestradiol/ml. The inter- and intra-assay coefficients of variation in all the assays were <10.0%.

2.5. Enzyme activities

The activities of the enzymes 3β-HSD and 20α-HSD were measured as reported [22]. 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 $105{,}000 \times g$ for 60 min. The supernatant fluids were used for the assay of 20α-HSD activity. The precipitates were rehomogenized with 0.7 ml of 0.25 M sucrose and centrifuged at $800 \times g$ 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. Sequences primers (5′–3′) are shown below:

3β-HSD: GTCTTCAGACCAGAAACCAAG; CCTTAAGGCACAAGTATGCAG;
20α-HSD: TTCGAGCAGAACTCATGGCTA; CAACCAGGTAGAATGCCATCT;
Aromatase: TGCACAGGCTCGAGTATTTCC; ATTTCCACAATGGGGCTGTCC;
iNOS: GCATGGACCAGTATAAGGCAAGCA; GCTTCTGGTCGATGTCATGAGCAA;
Bax: GATTGCTGACGTGGACACGGACT; TCAGCCCATCTTCTTCCA;
Bcl-2: AGAGGGGCTACGAGTGGGAT; CTCAGTCATCCACAGGGCGA;
Fas: GTGATGAAGGGCATGGTT; TTGACACGCACCAGTCTT;

FasL: CCAGATCTACTGGGTAGA; ATGGTCAGCAACGGTAAG;
S16: CGTTCACCTTGATGAGCCCATT; TCCAAGGGTCCGCTGCAGTC;
β-actin: CGGAACCGCTCATTGCC; ACCCACACTGTGCCCATCTA.

The amplification of the cDNA was performed using a thermalcycler (My Cycler, BioRad); for 3β-HSD, 20α-HSD, aromatase, Bcl-2, β-actin and S16 the reactions were carried out at 95 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min by 35 cycles. For Bax, Fas, FasL and iNOS the parameters were 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min by 40 cycles. All the reactions were terminated with a 5 min extension at 72 °C.

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/). Relative levels of mRNA were expressed as the ratio of signal intensity for the target genes relative to that for the housekeeping genes S16 and β-actin.

2.7. Real time PCR

Relative quantification of aromatase mRNA levels was performed by real-time PCR using the ABI Prism® 7500 thermocycler (Applied Biosystems, USA). Before the real-time PCR was performed, cDNA obtained by RT-PCR was diluted to 20 ng/μl with nuclease-free water. The diluted cDNA (5 μl) was amplified in a 25 μl final volume reaction mix containing 1 × SYBR Green I fluorescent dye (Applied Biosystems, USA) and 500 nM aromatase-specific primers. The reactions were subjected to one step of 95 °C for 5 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Relative expression of the real-time PCR products was determined by the ΔΔCt method. This method calculates relative expression using the equation: *fold induction* = $2^{-[\Delta\Delta Ct]}$, where Ct is the threshold cycle, i.e. the cycle number at which the sample's relative fluorescence rises above the background fluorescence and $\Delta\Delta Ct$ = [*Ct gene of interest* (*unknown sample*) − *Ct housekeeping gene* (*unknown sample*)] − [*Ct gene of interest* (*calibrator sample*) − *Ct housekeeping gene* (*calibrator sample*)]. In this case, β-actin was chosen as housekeeping gene and one of the control samples as calibrator. Each sample was run in triplicate, and the mean Ct was used in the ΔΔCt equation. Data for the normalized transcript level of aromatase is shown as mean ± S.E.M.

2.8. Nitrite assay

Levels of nitrite, a water-soluble metabolite of nitric oxide, were measured in the ovary incubation liquid spectrophotometrically. Samples were immediately mixed with Griess reagent (sulphanilamide with N-1-naphthyl-ethylendiamine/HCl). After a 10 min incubation period at room temperature, the absorbance of 540 nm was measured. 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).

2.9. Catecholamine assay (HPLC)

Norepinephrine was partially purified by batch alumina extraction from 20 μl aliquots of liquid from the ovarian cuvette, separated by reverse-phase high-pressure liquid chromatography using a 4.6 mm × 250 mm Zorbax RxC18 column (Du Pont, USA) and quantified by current produced upon exposure of the column effluent to oxidizing and then reducing potentials in series using a triple-electrode system (Coulochem II, ESA, Bedford, MA). Recovery through the alumina extraction step averaged 70–80% for catecholamines. Catecholamine concentration in each sample was corrected for recovery of an internal standard dihydroxybenzylamine. The results were expressed as picograms of norepinephrine/milligram of ovarian tissue/ml of incubation medium (NE pg/mg ovary/180 min incubation).

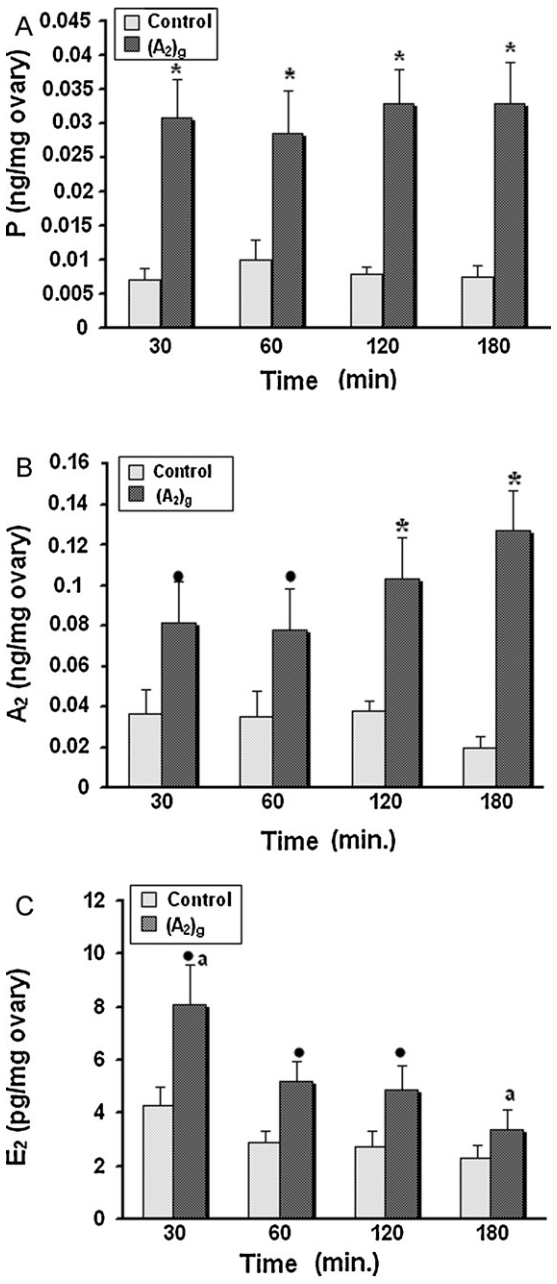

Fig. 1. Coeliac ganglion–superior ovarian nerve–ovary system of day 4 post-partum non-lactating rats. Ganglionic effect of androstenedione on ovarian steroids release. The presence of A_2 in the ganglion compartment increased the release of P (A), A_2, (B) and E_2 (C) into the ovarian compartment. Results are expressed as mean ± S.E.M. of six animals per group. One-way analysis of variance followed by Tukey's test was used. •$p < 0.05$; *$p < 0.025$. [(A_2)g]: androstenedione added into the ganglion compartment; P: progesterone; A_2: androstenedione; E_2: oestradiol.

2.10. 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

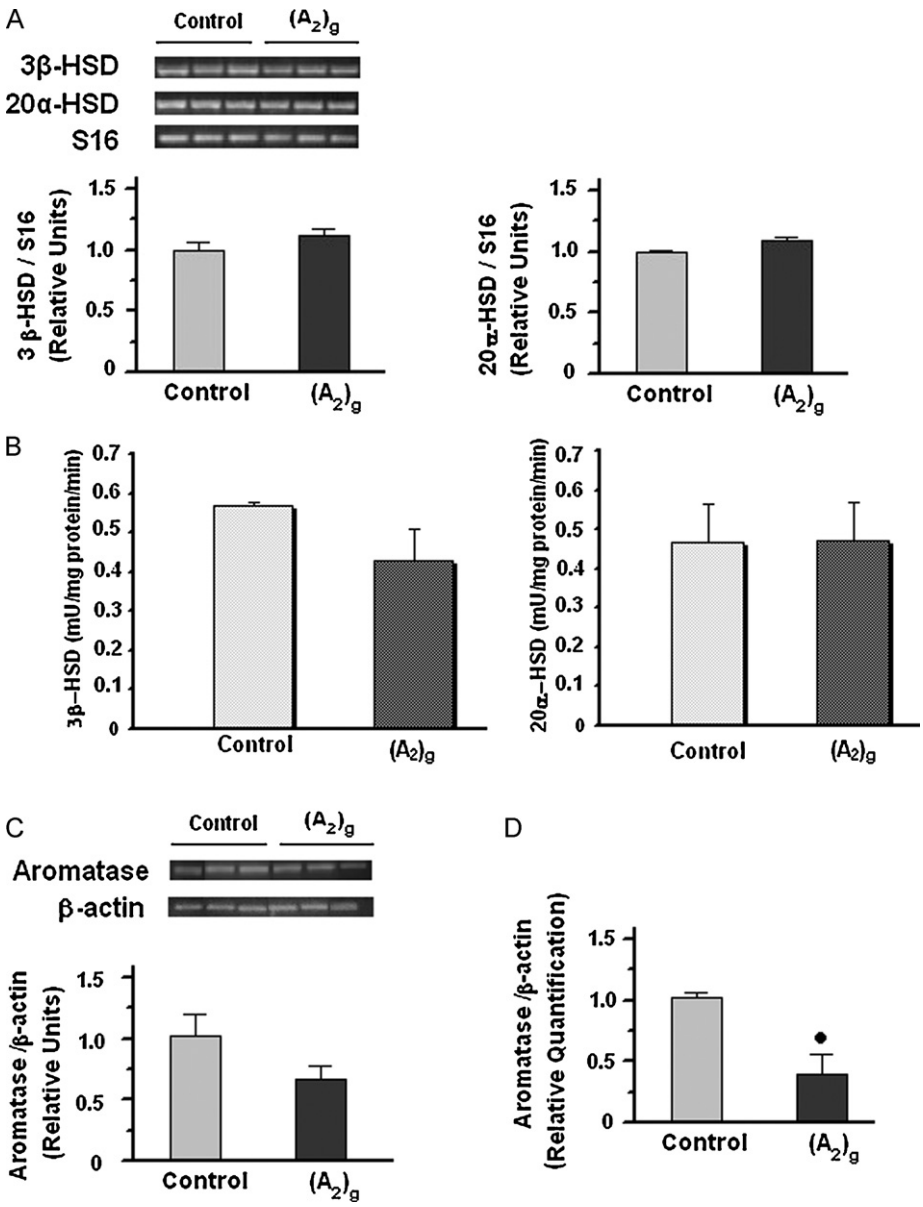

Fig. 2. Coeliac ganglion–superior ovarian nerve–ovary system of day 4 post-partum non-lactating rats. Ganglionic effect of androstenedione on luteal expression and activities of steroidogenic enzymes. (A) Measurement by RT-PCR of expression of 3β-HSD, 20α-HSD and S16 as housekeeping gene. (B) Luteal 3β-HSD and 20α-HSD activities. The mRNA levels and activities of 3β-HSD and 20α-HSD did not modify by the presence of A_2 in the ganglion compartment. (C) Measurement by RT-PCR of expression of aromatase and β-actin as housekeeping gene. (D) Relative quantification of aromatase mRNA levels by real-time PCR. The presence of A_2 in the ganglion compartment decreased the aromatase luteal mRNA expression. Results are expressed as mean ± S.E.M. ($n = 3$). Student's t-test was used. $^{\bullet}p < 0.05$. [(A_2)g]: androstenedione added into the ganglion compartment. 3β-HSD: 3β-hydroxysteroid-dehydrogenase; 20α-HSD: 20α-hydroxysteroid-dehydrogenase.

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 $p < 0.05$.

3. Results

3.1. Ganglionic effect of androstenedione on progesterone, androstenedione and oestradiol release from ovaries of day 4 postpartum, non-lactating rats

The presence of androstenedione in the ganglion compartment increased the release of ovarian progesterone at all the studied times with similar potency ($^{*}p < 0.025$) (Fig. 1A), while also increased ovarian androstenedione release at 30 and 60 min ($^{\bullet}p < 0.05$) and at 120 and 180 min ($^{*}p < 0.025$) respect to the control group (Fig. 1B). The addition of androstenedione into the ganglion compartment also increased significantly the release of ovarian oestradiol at 30, 60 and 120 min ($^{\bullet}p < 0.05$), yet such increase in oestradiol release declined significantly with time of incubation (Fig. 1C).

3.2. Ganglionic effect of androstenedione on mRNA expression of 3β-HSD, 20α-HSD and aromatase and on the activities of 3βHSD and 20αHSD in corpora lutea isolated from ovaries of day 4 postpartum, non-lactating rats

The mRNA levels and activities of 3β-HSD and 20α-HSD did not significantly change in the corpora lutea of pregnancy isolated from ovaries incubated for 180 min upon isolation from non-lactating animals on day 4 after parturition in response to the presence of androstenedione in the ganglion compartment

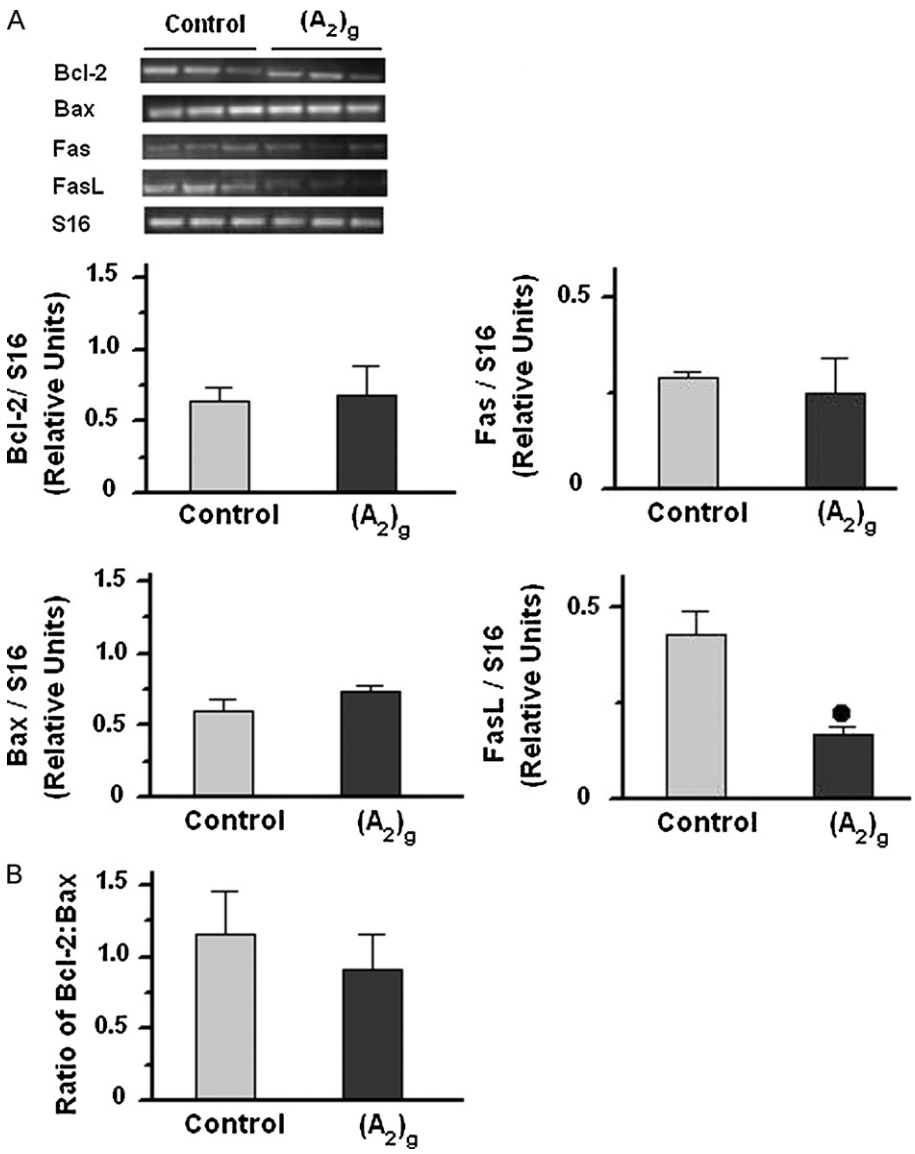

Fig. 3. Coeliac ganglion–superior ovarian nerve–ovary system of day 4 post-partum non-lactating rats. Ganglionic effect of androstenedione on luteal expression of regulators of apoptosis. (A) Measurement by RT-PCR of expression of Bcl-2, Bax, Fas, Fas L and S16 as housekeeping gene. (B) Ratio of Bcl-2 to Bax. The presence of A_2 in the ganglion compartment decreased FasL luteal mRNA expression, without affecting luteal Bcl-2, Bax and Fas expression. PCR products were visualized on agarose gels and stained with ethidium bromide. The gel photographs were quantified using ImageJ and expressed as arbitrary units. Results are expressed as mean ± S.E.M. ($n = 3$). Student's t-test was used. $^{\bullet}p < 0.05$. [(A_2)g]: androstenedione added into the ganglion compartment.

(Fig. 2 A and B). While, the aromatase mRNA expression, analysed by semiquantitative RT-PCR, had a tendency to decline (Fig. 2C) and taking into account the decrease in oestradiol release at the end of incubation we considered interesting to analyse the aromatase mRNA expression by real time RT-PCR. Thus, the results showed that, in fact, such decrease was significant ($^{\bullet}p < 0.05$) (Fig. 2D).

3.3. Ganglionic effect of androstenedione on parameters of luteal regression in corpora lutea isolated from ovaries of day 4 postpartum, non-lactating rats

The presence of androstenedione in the ganglion compartment did not modify Bcl-2 and Bax mRNA expression in the regressing corpora lutea of pregnancy as shown in Fig. 3A; consequently, the ratio Bcl-2/Bax did not change either (Fig. 3B). Moreover, whereas luteal Fas expression was not modified by the action of androstenedione in the CG, FasL expression declined significantly ($^{\bullet}p < 0.05$) (Fig. 3A).

3.4. Ganglionic effect of androstenedione on norepinephrine and nitrites released into the ovarian compartment of day 4 postpartum, non-lactating rats

The presence of androstenedione in the ganglion compartment significantly increased the release of ovarian norepinephrine measured at the end of the incubation period (180 min) ($^{\bullet}p < 0.05$) (Fig. 4A). Whereas control preparations had an increased accumulation of nitrites after 60 min incubation that however declined thereafter, the presence of androstenedione in the ganglion compartment abrogated such increases ($^{\bullet}p < 0.05$) (Fig. 4B). Finally, the expression of luteal iNOS significantly increased in the presence of androstenedione in the ganglion compartment ($^{\bullet}p < 0.05$) (Fig. 4C).

4. Discussion

We have provided evidence that systemic administration of androstenedione or its addition to the ganglion compartment of

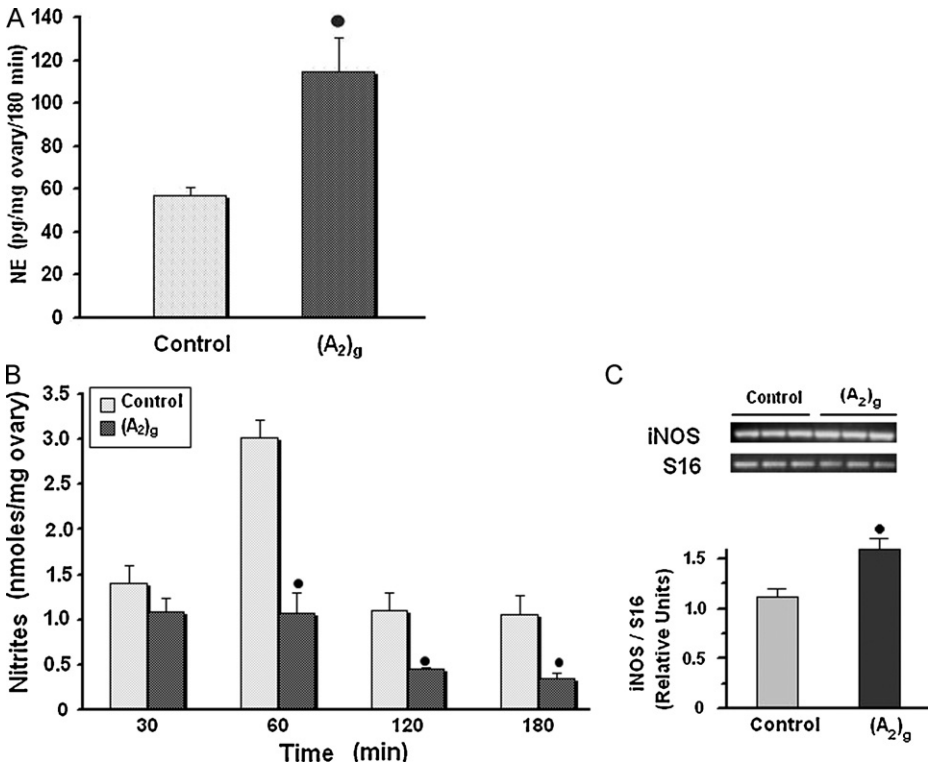

Fig. 4. Coeliac ganglion–superior ovarian nerve–ovary system of day 4 post-partum non-lactating rats. Ganglionic effect of androstenedione on ovarian neurotransmitters. The presence of A_2 in the ganglion compartment increased the norepinephrine (A) and decreased nitrites (B) release into the ovarian compartment. Results are expressed as mean ± S.E.M. of six animals per group. Differences between two groups were analysed with Student's *t*-test. One-way analysis of variance followed by Tukey's test was used for multiple comparisons. $^{\bullet}p < 0.05$. (C) Measurement by RT-PCR of luteal iNOS and S16 as housekeeping gene. The presence of androstenedione in the ganglion compartment increased the expression of luteal iNOS. PCR products were visualized on agarose gels stained with ethidium bromide. The gel photographs were quantified using ImageJ and expressed as arbitrary units. Results are expressed as mean ± S.E.M. ($n = 3$). Student's *t*-test was used. $^{\bullet}p < 0.05$. [(A_2)g]: androstenedione added into the ganglion compartment, NE: norepinephrine; iNOS: inducible nitric oxide synthase.

the CG–SON–ovary system, opposes to the functional regression of the corpora lutea at the end of pregnancy in rats [14–16]. We further demonstrated that androgen receptor immunoreactive proteins are present in the neurons of the CG before parturition, suggesting that the indirect luteotropic activity of androstenedione may be mediated by these receptors and that they can eventually modulate the synthesis of neurotransmitters by the SON [6]. In the present study we utilized the integrated *ex vivo* CG–SON–ovary system to assess whether androstenedione at the ganglion is capable of transmitting a luteotropic signal via the SON to the ovary containing corpora lutea of pregnancy undergoing regression after parturition. To answer the question we selected day 4 postpartum where the corpora lutea of pregnancy are at advanced regression with much reduced capacity to synthesize progesterone and high rate of apoptotic cell death [23]. We also selected rats postpartum but not undergoing lactation to avoid the ovarian influence of prolactin that increases in response to the suckling stimulus of the pups [24] and has antiapoptotic activity in the luteal tissue [20].

The accumulation of progesterone in the ovarian compartment of day 4 postpartum, non-lactating animals that were stimulated with androstenedione in the ganglion compartment reached values that could be observed in untreated ovaries obtained from day 21 of pregnancy [6,15], and without modifying 3β-HSD and 20α-HSD enzyme activities or mRNA expression. This suggests that the regressing corpora lutea of pregnancy present in postpartum ovaries are still capable of functioning if timely stimulated, in this case through a neural stimulus.

In our experimental design, androstenedione in the ganglion compartment led to only a transient major increase in oestradiol in the ovarian compartment after 30 min of incubation whereas the concentration of the steroid declined thereafter. In contrast, androstenedione and progesterone continuously accumulated through the 180 min incubation period. The decline in oestradiol production with time of incubation associated with reduced luteal aromatase mRNA expression is possibly a consequence of the increased progesterone levels in the media. This is because it has been shown that progesterone is capable of inhibiting oestradiol synthesis by blocking aromatase activity in rat ovarian cells [25].

In this work we also report that the presence of androstenedione in the ganglion compartment caused a slight modification in the morphology of the luteal cells, decreasing the number of cells with apoptotic characteristics and increasing the corpora lutea vascularization (staining with H&E, data not shown). Considering that such effect is obtained by indirect action of androstenedione by neural way and at relatively short incubation times, this result led us to explore the expression by RT-PCR of genes that take part in apoptosis. This proposal came from studies performed by Guo et al. [26], who argue that there are many apoptotic-associated genes linked to luteal regression that are up-regulated before the morphological changes are clearly shown. In this respect, the presence of androstenedione in the ganglion compartment does not affect the expression levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 suggesting that the intrinsic (mitochondrial) apoptotic pathway is not affected. However, we observed a significant decline in the expression levels of FasL, which is a known trigger for the extrinsic apoptotic pathway in the regressing rat corpus luteum [24,27–29]. It is highly possible that ovarian progesterone release that increases in response to the presence of androstenedione in the ganglion compartment is ultimately responsible for the decline observed in FasL mRNA expression. Progesterone has been shown to oppose prolactin-induced luteal regression and Fas expression in the rat

corpus luteum of the oestrual cycle [27]. Together these data support the capacity of androstenedione and progesterone to regulate the Fas/FasL system in the rat corpus luteum and suggest that, at least in part, the anti-apoptotic activity of the steroid hormones may be mediated by neural inputs traveling to the ovary via the SON.

The fact that in our experimental design the CG is physically and functionally connected to the ovary by the SON demonstrates that when androstenedione is placed in the ganglion compartment the impact on the physiology of the ovary must be modulated by either an increase or a decrease in neurotransmitters released by the SON. We have shown that under our experimental *ex vivo* conditions the SON releases catecholamines and nitric oxide to the ovarian compartment [6,14,16]. It has been shown that the CG expresses the enzyme tyrosine hydroxylase that controls the synthesis of norepinephrine [30]. Indeed we have shown that the main neuronal bodies forming the CG express androgen receptor immunoreactive proteins [6] and when we mapped 1510 bp upstream of the transcription starting site of the tyrosine hydroxylase gene using the MatInspector software [31], we identified one putative androgen response element and three putative oestrogen response elements (results not shown). It has been reported that the transcriptional activity of the tyrosine hydroxylase gene is transactivated by androgen receptors in a ligand-dependent manner [32]. Yet the fact that oestrogen response elements are also present and considering aromatase expression in ganglionic neurons [33] allows hypothesizing that androstenedione may be converted to oestradiol in the ganglion compartment before regulating the tyrosine hydroxylase gene. We however could not find differences among the levels of oestradiol in the incubation media of the ganglion compartment before and after stimulation with androstenedione (results not shown) suggesting that it is likely an effect of androstenedione *per se* and not mediated by its previous aromatization to oestradiol.

We have shown that androstenedione impacts the CG and modulates steroidogenesis in the ovary increasing norepinephrine and decreasing nitric oxide. Precisely, the nitric oxide has negative impact on steroidogenesis [34], thus its decline in the ovarian compartment caused by the presence of A_2 in the ganglion compartment may explain the increased abundance of steroid hormones in the ovarian incubation media found in the present study. These results are in agreement with results obtained in cows suggesting that adrenergic and nitrergic agents play opposing roles in the regulation of luteal function [35], with norepinephrine stimulating and nitric oxide inhibiting luteal function. The pro-apoptotic role of nitric oxide in the luteal tissue appears mediated by the elevated iNOS activity [36]. We found that the presence of androstenedione in the ganglion compartment ganglion increased iNOS expression in the luteal tissue. This result may be the consequence of a negative feedback mechanism. It is know that nitric oxide has a biphasic positive/negative effect on its own production by regulating the expression of iNOS [37]. Thus, it is possible that the accumulation of progesterone and oestradiol in the ovarian compartment leads to a decline in nitric oxide that cannot perform its negative feedback on iNOS expression.

Thus, in the present work we show that androstenedione can mediate a luteotropic effect acting at the CG and transmitting to the ovary a signaling via the SON that leads to increased progesterone, androstenedione and oestradiol release, all steroids known to be tropic for the rat corpus luteum, while at the same time induces a decline in the expression of proapoptotic FasL. These luteotropic effects caused by the presence of A_2 in the ganglion compartment are associated with the increased levels of norepinephrine and decreased levels of nitric oxide in the ovarian compartment.

In conclusion, these findings indicate that androstenedione, the main circulating androgen in rat, is able to act in the CG and lead, at least in part, to the refunctionalization of the regressing corpus luteum of pregnancy through the SON on day 4 postpartum. This may help understand why certain reproductive disorders may be associated with both hormonal and neural causes.

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