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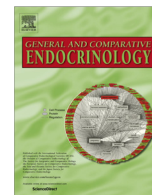

Research paper

Participation of the extrinsic cholinergic innervation in the action of nitric oxide on the ovarian steroidogenesis in the first proestrous in rats

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A B S T R A C T

An *ex-vivo* Coeliac Ganglion-Superior Ovarian Nerve-Ovary (CG-SON-O) system and an ovary without peripheral neural influence from virgin rats in the first proestrous were used to test whether ovarian extrinsic innervation and nitric oxide (NO) affects steroidogenesis in the ovary. The CG and the ovary were placed in separate buffered-compartments, connected by the SON. Stimulation of the CG was achieved by 10^{-6} M acetylcholine (Ach). The ovary without peripheral neural influence was placed alone in a buffered-compartment. To test a possible role of NO in the ovarian response to peripheral neural influence, 100 μM sodium nitroprusside (SNP, an NO donor) and 100 μM N^G-nitro-L-arginine methyl ester (L-NAME, an inhibitor of NO synthase) were added to the ovarian compartment separately. In the CG-SON-O system, SNP into the ovarian compartment increased the concentration of NO, reduced the release of progesterone and increased the release of estradiol (E_2), increasing the mRNAs related to their synthesis enzyme. The addition of L-NAME to the ovarian compartment caused an opposite effect. In the ovary alone, NO manifested an antisteroidogenic effect on both hormones. These results show that the ovarian extrinsic innervation maintains a direct relationship between NO and E_2, both needed at high levels during the follicular phase, allowing the continuity of the estrous cycle.

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1. Introduction

Numerous studies emphasize the participation of the peripheral nervous system (PNS) in the ovarian physiology (Delgado et al., 2010, 2004; Madekurozwa, 2008). The ovarian sympathetic innervation includes two pathways that enter the gland through the hilum: the ovarian nervous plexus and the superior ovarian nerve (SON). The central nervous system (CNS) exerts a regulating influence on the ovary through these sympathetic nerves (De Bortoli et al., 1998; Gerendai et al., 2002), and through intermediate structures such as the coeliac ganglion (CG) (Casais et al., 2001; Delgado et al., 2010, 2004; Sosa et al., 2000). The CG is the biggest ganglion of the PNS and has a variety of specific receptors for neurotransmitters, acetylcholine (Ach) among them (Matthews, 1989). Ach is considered the classic preganglionic neurotransmitter of the sympathetic ganglionic pathway (Berthoud and Powly, 1996; Eccles and Libet, 1961). Indeed, the CG has specific structures to respond to cholinergic stimuli, such as nicotinic and muscarinic receptors (Quinson et al., 2000; Sosa et al., 2004). The former are mainly located in the principal neurons, and the latter are found in both principal neurons and interneurons (Prud'Homme et al., 1999; Reid and Perry, 1995). The muscarinic receptors provide synaptic responses, through systems of second messengers, which are slower but more constant than the responses provided by the nicotinic receptors (Dutar and Nicoll, 1988).

In order to know more about the influence of the PNS on the gonadal function, an *ex vivo* system constituted by the CG, the SON and the ovary (CG-SON-O) was isolated and characterized, allowing the emulation of *in vivo* conditions, but without the influence of humoral factors which may interfere with the neural action (Delgado et al., 2004; Sosa et al., 2000). In previous works, using this system, we reported that the *input* from the CG via the SON is a fine modulator of the steroidogenic activity and NO release, in the rat ovary during different reproductive stages (Casais

Abbreviations: Ach, acetylcholine; CG, coeliac ganglion; E_2, estradiol; eNOS, endothelial nitric oxide syntase; iNOS, inducible nitric oxide syntase; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; P, progesterone; PE, proestrous; PNS, peripheral nervous system; P450arom, P450 aromatase; SNP, sodium nitroprusside; SON, superior ovarian nerve.

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et al., 2006, 2001; Delgado et al., 2010, 2004; Sosa et al., 2004; Vallcaneras et al., 2009). Thus, using the CG-SON-O system isolated from rats in different stages of the first estral cycle, Delgado et al. (2010), reported that the ganglionic stimulation with Ach modifies the ovarian release of NO, increasing significantly its concentration in the proestrous stage (PE).

NO is a gaseous neurotransmitter able to flow freely into the cellular cytoplasm, due to its low molecular weight (30 Da), its lipophilic nature and its high diffusibility, thus leading to rapid and precise responses (Flora Filho and Zilberstein, 2000). To our knowledge, three isoforms of the NO synthase (NOS) have been identified: the neuronal or type I (nNOS), the endothelial or type II (eNOS) and the inducible or type III (iNOS). Even though they catalyze the NO synthesis in the same way, NOS isoforms are codified by different genes and present different localization, kinetics and regulation (Bogdan, 2001; Stuehr et al., 2004). For example, in the rat ovary, the nNOS has been localized in granulosa cells, ovarian stroma, oocytes and in the pregnancy corpus luteum (Yang et al., 2003; Zik et al., 2012) while the eNOS has been localized in granulosa cells, theca cells, ovarian stroma and on the oocytes surface inside the developing follicles (Jablonka-Shariff et al., 1999; Jablonka-Shariff and Olson, 1997; Yamagata et al., 2002; Zackrisson et al., 1996; Zik et al., 2012). In addition, the iNOS has been localized in somatic cells of ovarian follicles and in luteal cells (Jablonka-Shariff and Olson, 1997; Yamagata et al., 2002). Given the NO influence on the regulation of several physiological functions, including ovulation and steroidogenesis, in the ovary, this gaseous neurotransmitter has been found to be a potential regulator of the follicular development (Matsumi et al., 2000; Tamanini et al., 2003). However, the mechanism underlying NO-mediated regulation is not completely clear yet.

Considering the above antecedents, we investigated the participation of the PNS in the NO action on the ovarian steroidogenesis, using an NO donor and an NOS inhibitor administered into ovary incubations, with and without peripheral neural influence, in the first PE in rats.

2. Materials and methods

2.1. Animals

Female 37-day-old virgin Holtzman rats in their first PE and weighing 100 ± 10 g were used in all the experiments. The rats were kept under controlled conditions with lights on from 07:00 to 19:00 h and temperature of 24 ± 2 °C. Animals had free access to food (Cargill SAIC, Saladillo, Buenos Aires, Argentina) and tap water. Groups of six animals each were used for the experimental procedures.

The experiments were performed in duplicate according to the procedures approved in the UFAW Handbook on the Care and Management of Laboratory Animals (Poole, 1999). The experimental procedures used in this study were approved by the Institutional Animal Care and Use Committees of the National University of San Luis (Protocol#B-96/12).

2.2. Reagents

L-Acetylcholine hydrochloride (Ach), sodium nitroprusside (SNP), N^G-nitro-L-arginine methyl ester (L-NAME), dextrose, ascorbic acid, bovine serum albumin-fraction V (BSA), sulfanilamide, N-1-naphthylethylenediamine were 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). Estradiol DIASource ImmnoAssays kit was purchased from DiagnosMed SRL (Buenos Aires, Argentina). Other reagents and chemicals were of analytical grade.

2.3. Experimental procedure

For our purpose, we conducted two experimental schemes. In the first experimental scheme, the CG-SON-O system was removed by dissecting, as previously described by Delgado et al. (2010, 2004), after anesthetizing the animal with i.p. injections of 80 mg/kg ketamine and 10 mg/kg xylazine. In order to prevent spontaneous depolarization of the nerves, the strip of tissues was carefully dissected avoiding contact between the surgical instruments and the nerve fibers or the ganglion, and the total surgical procedure was completed within 1–2 min. The CG-SON-O system was rinsed with incubation medium and immediately 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 was 1 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, with 0.1 mg/ml dextrose and 0.1 mg/ml BSA at 37 °C in a saturated atmosphere of 95% O_2 and 5% CO_2.

The CG-SON-O system was pre-incubated for 15 min, and the end of this pre-incubation period was considered incubation time 0. After this pre-incubation time, Krebs-Ringer solution (1 ml) was changed in both compartments, and 0.1 mg/ml ascorbic acid was added as an antioxidant agent to the CG compartment.

To stimulate the CG, Ach was dissolved in 1 ml of Krebs-Ringer solution plus ascorbic acid at a 10^{-6} M final concentration in the CG compartment. In addition, to test the role of NO in ovarian steroidogenesis, SNP (an NO donor) and L-NAME (an inhibitor of the NOS) were separately dissolved in 1 ml of Krebs-Ringer solution at a 100 μM final concentration in the ovarian compartment, with and without 10^{-6} M Ach in the CG compartment.

In the second experimental scheme, whole ovaries were removed from the rats and incubated under the same conditions of the *ex vivo* CG-SON-O system (ovary incubations without peripheral neural influence).

In both experimental schemes, the incubation was performed during 180 min. At 120 and 180 min, aliquots of incubation medium (250 μl) in the ovarian compartment were removed and frozen at −20 °C until the biochemical determinations: nitrites by the Griess method, and progesterone (P) and estradiol (E_2) by radioimmunoassay (RIA). Appropriate corrections were made in all cases according to the volume of liquid extracted in each tested period from the ovarian compartment. At the end of the 180 min-incubation, the ovary was collected and frozen at −80 °C, until the analysis of iNOS, eNOS and P450 aromatase (P450arom, E_2 synthesis enzyme) expression by RT-PCR.

2.4. Nitrite assay

The nitrite concentration in the incubation medium from the ovarian compartment was measured spectrophotometrically (Egami and Taniguchi, 1974). Briefly, 50 μl of sample was mixed with Griess reagent (1% sulfanilamide with 0.1% *N*-L-naphthylethylenediamine/HCl in 1% phosphoric acid). After 10 min incubation at room temperature, the absorbance was read at 540 nm. A solution of nitrite of known concentration was used to prepare a standard curve. The assay sensitivity was <2.5 nmol/ml. The intra-assay coefficients of variation for all the assays were less than 10%. The results were expressed as nmol of nitrite per milligram of ovarian tissue (nmol/mg ovary).

2.5. Progesterone and estradiol assay

Steroids were measured in the ovarian incubation liquid per duplicate by radioimmunoassay (RIA). The P antiserum, provided by Dr. R. Deis (IMBECU, Mendoza, Argentina), was produced in rabbits against progesterone conjugated to bovine serum albumin at the 11 position. The antiserum was highly specific for P with low cross-reactivities, <2.0% for 20α-dihidro-progesterone and

deoxy-corticosterone and 1.0% for other steroids. The sensitivity was less than 5 ng/ml and the inter- and intra-assay coefficients of variation were less than 10%. This assay has been previously validated (Bussmann and Deis, 1979). P concentration was expressed as nanogram per milligram of ovarian tissue (ng/mg ovary).

The E_2 concentration was determined using a double antibody RIA Diasource kit (DiagnosMed SRL) following the manufacturer's instructions. The percentages of cross-reactions were 1.8% for estrone, 1.2% for estriol, 0.0011% for androstenedione and 0.0002% for P. The assay sensitivity was <2.2 pg E_2/ml. The inter- and intra-assay coefficients of variation in all the assays were <10.0%. E_2 was expressed as picograms per milligram of ovarian tissue (pg/mg ovary).

2.6. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from three pools of two ovaries each per experimental group. All RNA isolations were performed using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) as suggested by the manufacturers. Purified total RNAs were then quantified and assessed for purity by measurement of the 260/280 and 260/230 ratio using a UV spectrophotometer Beckman DU-640 B (CA, USA). Only samples with 260/280 ratio of 1.8–2.0 and 260/230 ratio of 2.0–2.2 were used. The integrity of the total RNAs were checked on a denaturing agarose gel. After GelRed™ (Biotium) staining, RNA bands were visualized with a UV transilluminator, and 28S and 18S rRNA band patterns were analyzed. Two micrograms of total RNA were reverse-transcribed at 37 °C with 200 units of M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) using random hexamers in a 26 µl reaction mixture to produce cDNA according to the manufacturer's instructions. For amplification of the cDNAs, 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. The PCR primers were designed using Primer Express 3.0 software (Applied Biosystems, USA) on the basis of the respective published rat DNA sequences. The primers information is shown in Table 1.

PCRs were performed using a thermocycler (My Cycler; BioRad) programmed at 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min by 35 cycles, for iNOS and eNOS. Instead, 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 P450arom and β-actin. All the reactions were terminated with a 5 min extension at 72 °C. The reaction products were electrophoresed on 2% agarose gels, visualized with GelRed (0.05 µl/ml) and examined by ultraviolet transillumination. Band intensities of RT-PCR amplicons 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 between the signal intensity of target genes and that for the housekeeping gene β-actin.

2.7. Statistical analysis

All data are presented as means ± SEM for each group of six rats. The differences between the two groups were analyzed with the Student's *t*-test. For multiple comparisons made along the incubation times, a repeated measures analysis of variance followed by Tukey's test was used. For multiple comparisons not involving repeated measures, one-way analysis of variance followed by Tukey's test was used. A difference was considered to be statistically significant when $p < 0.05$.

3. Results

3.1. Effect of 100 µM SNP in ovary incubations with and without peripheral neural influence on the nitrite concentration

In the CG-SON-O system, the addition of 100 µM SNP into the ovarian compartment increased nitrite concentration at both 120

Table 1
Primers used for PCR amplification.

Gen	Sequences (sense above, antisense below; 5′-3′)	GenBank accession #	Amplicon length (bp)	Sequence references
iNOS	GCATGGACCAGTATAAGGCAAGCA GCTTCTGGTCGATGTCATGAGCAA	S71597	219	Markewitz et al. (1993)
eNOS	CGAGATATCTTCAGTCCCAAGC GTGGATTTGCTGCTCTGTAGG	NM_021838	164	Pfeil et al. (2014)
P450arom	TGCACAGGCTCGAGTATTTCC ATTTCCACAATGGGGCTGTCC	M33986	266	Hickey et al. (1990)
β-Actin	ACCCACACTGTGCCCATCTA CGGAACCGCTCATTGCC	NM_031144	289	Parvin et al. (2002)

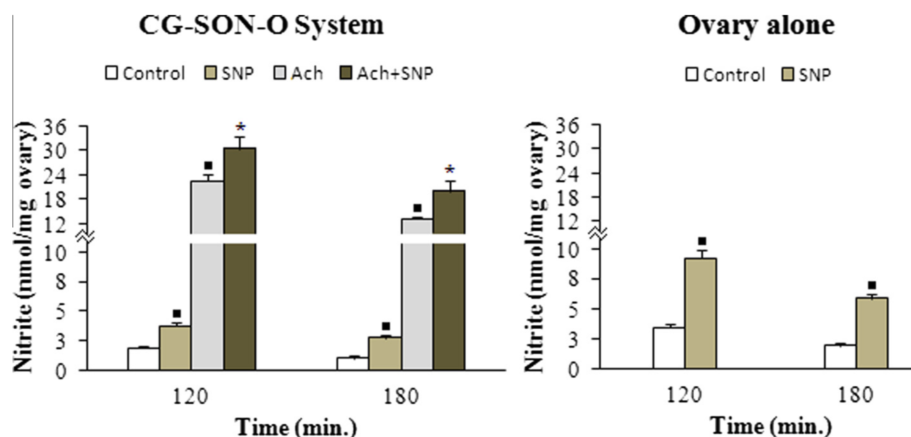

Fig. 1. Effect of 100 µM SNP in ovary incubations with and without peripheral neural influence in the first PE in rats, on the nitrite concentration expressed in nmol/mg ovary. The results are presented as mean ± SEM of six animals per experimental group. Repeated measures analysis of variance followed by Tukey's test was used for several comparisons: (*) $p < 0.05$, (■) $p < 0.001$.

and 180 min (■p < 0.001), as compared to the control group (Fig. 1).

In order to analyze whether the extrinsic cholinergic innervation affects the ovary ability to convert SNP into NO metabolites in the GC-SON-O system, we stimulated the CG with Ach. The joint effect of 100 μM SNP into the ovarian compartment and 10^{-6} M Ach into the CG compartment increased nitrite concentration in the ovary incubation medium at 120 and 180 min (*p < 0.05), as compared to the Ach group (Fig. 1).

In ovary without peripheral neural influence (alone), the addition of 100 μM SNP also increased nitrite concentration at 120 and 180 min (■p < 0.001), as compared to the control group (Fig. 1).

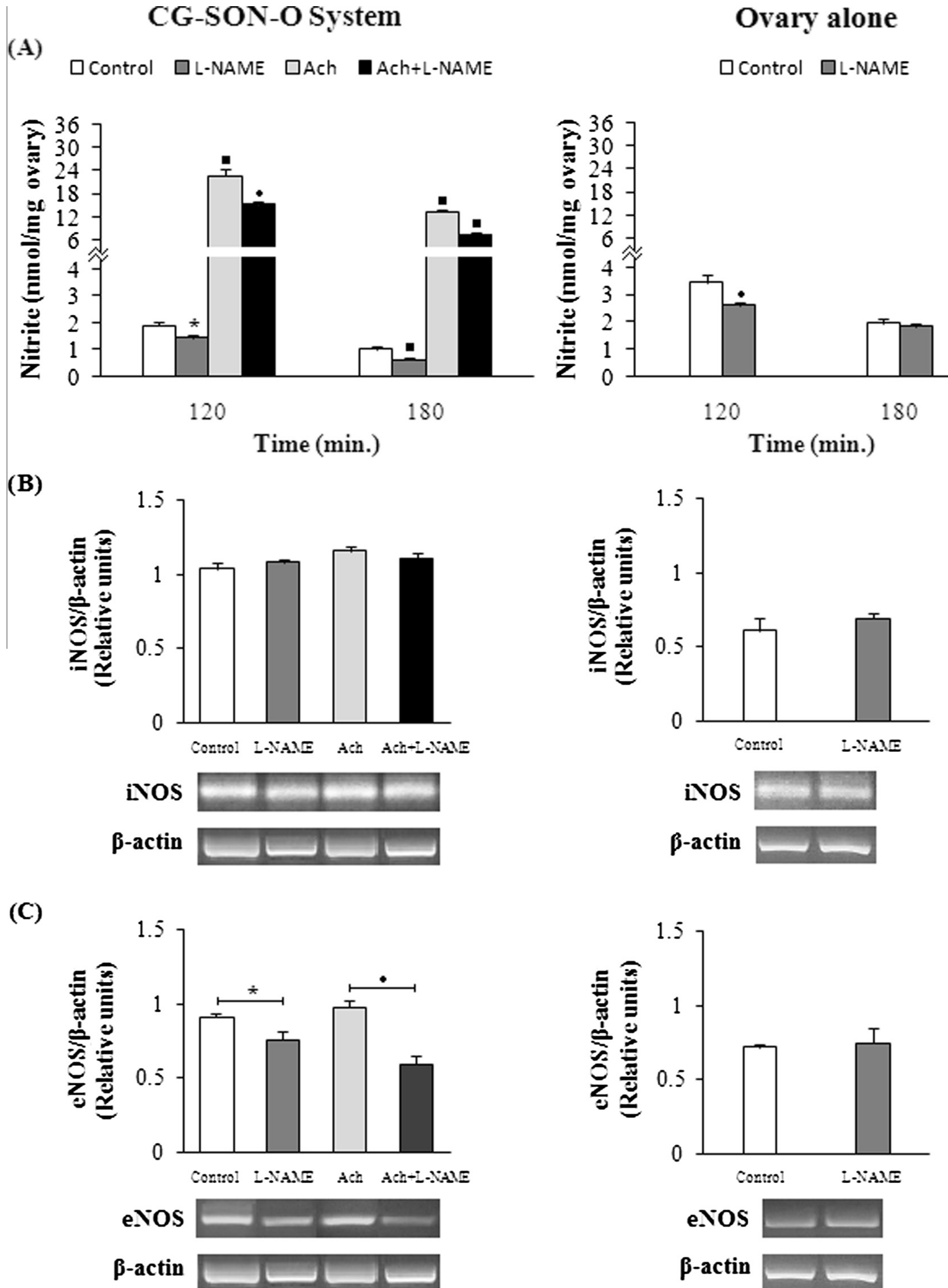

Fig. 2. Effect of 100 μM L-NAME in ovary incubations with and without peripheral neural influence in the first PE in rats, on the nitrite concentration expressed in nmol/mg ovary (A; upper panel) and the mRNA expression of iNOS (B; middle panel) and eNOS (C; lower panel). The results are presented as mean ± SEM of six animals per experimental group for nitrite determination. Repeated measures analysis of variance followed by Tukey's test was used for several comparisons: (*) p < 0.05, (●) p < 0.01, (■) p < 0.001. PCR products: iNOS (219 bp), eNOS (164 bp) and β-actin (289 bp) as endogenous control, were visualized on agarose gels stained with GelRed. The gel photographs were quantified using ImageJ and expressed as arbitrary units. Results are expressed as mean ± SEM (n = 3) for the expression analysis.

3.2. Effect of 100 μM L-NAME in ovary incubations with and without peripheral neural influence on the nitrite concentration and iNOS and eNOS mRNA expression

In the CG-SON-O system, the addition of 100 μM L-NAME into the ovarian compartment decreased the nitrite concentration at 120 ($^{*}p < 0.05$) and 180 min (▪$p < 0.001$) (Fig. 2A) as well as the eNOS mRNA expression in the ovarian tissue at 180 min ($^{*}p < 0.05$), as compared to the control group (Fig. 2B and C). The addition of 10^{-6} M Ach into the CG compartment did not cause significant changes in iNOS and eNOS mRNA expression in the ovarian tissue at 180 min, as compared to the control group (Fig. 2B and C).

The addition of 100 μM L-NAME into the ovarian compartment and 10^{-6} M Ach into the CG compartment decreased the nitrite concentration in the ovary incubation medium at 120 (•$p < 0.01$) and 180 min (▪$p < 0.001$) as well as the eNOS mRNA expression in the ovarian tissue at 180 min (•$p < 0.01$), as compared to the Ach group (Fig. 2A and C).

In the ovary alone, the addition of 100 μM L-NAME decreased the nitrite concentration only at 120 min (•$p < 0.01$) (Fig. 2A), without showing any changes in iNOS and eNOS mRNA expression in the ovarian tissue at 180 min, as compared to the control group (Fig. 2B and C).

3.3. Effect of 100 μM SNP in ovary incubations with and without peripheral neural influence on the release of ovarian P

In the CG-SON-O system, the addition of 100 μM SNP into the ovarian compartment decreased the release of ovarian P at 120 and 180 min (•$p < 0.01$), as compared to the control group (Fig. 3). On the contrary, the joint effect of 100 μM SNP into the ovarian compartment and 10^{-6} M Ach into the CG compartment did not show significant changes in the release of ovarian P, as compared to the Ach group (Fig. 3).

In the ovary alone, the addition of 100 μM SNP decreased the release of ovarian P at 120 ($^{*}p < 0.05$) and 180 min (▪$p < 0.001$), as compared to the control group (Fig. 3).

3.4. Effect of 100 μM L-NAME in ovary incubations with and without peripheral neural influence on the release of ovarian P

In the CG-SON-O system, the addition of 100 μM L-NAME into the ovarian compartment increased the release of ovarian P at 120 ($^{*}p < 0.05$) and 180 min (•$p < 0.01$), as compared to the control group (Fig. 4).

The addition of 100 μM L-NAME into the ovarian compartment and 10^{-6} M Ach into the CG compartment decreased the release of ovarian P at 120 and 180 min (▪$p < 0.001$), as compared to the Ach group (Fig. 4).

In the ovary alone, no significant effect was observed in P release after the ovarian stimulation with 100 μM L-NAME, as compared to the control group (Fig. 4).

3.5. Effect of 100 μM SNP in ovary incubations with and without peripheral neural influence on the release of ovarian E_2 and P450arom mRNA expression

In the CG-SON-O system, the addition of 100 μM SNP into the ovarian compartment increased the release of ovarian E_2 at 120 and 180 min (•$p < 0.01$) and the P450arom mRNA expression in the ovarian tissue at 180 min ($^{*}p < 0.05$), as compared to the control group (Fig. 5A and B). On the contrary, the joint effect of 100 μM SNP into the ovarian compartment and 10^{-6} M Ach into the CG compartment decreased the release of ovarian E_2 and the P450arom mRNA expression in the ovarian tissue at 180 min ($^{*}p < 0.05$), as compared to the Ach group (Fig. 5A and B).

In the ovary alone, the addition of 100 μM SNP decreased the release of ovarian E_2 at 120 min ($^{*}p < 0.05$) without showing any changes in the P450arom mRNA expression at 180 min, as compared to the control group (Fig. 5A and B).

3.6. Effect of 100 μM L-NAME in ovary incubations with and without peripheral neural influence on the release of ovarian E_2 and P450arom mRNA expression

In the CG-SON-O system, the addition of 100 μM L-NAME into the ovarian compartment decreased the release of ovarian E_2 at 180 min ($^{*}p < 0.05$) without showing significant changes in the P450arom mRNA expression, as compared to the control group (Fig. 6A and B).

No significant modification in E_2 release was observed when 100 μM L-NAME was added into the ovarian compartment and 10^{-6} M Ach was added into the CG compartment, as compared to the Ach group, but the P450arom mRNA expression decreased in the ovarian tissue at 180 min ($^{*}p < 0.05$) (Fig. 6A and B).

In the ovary alone, the addition of 100 μM L-NAME increased the release of ovarian E_2 at 120 and 180 min (•$p < 0.01$) without showing changes in the P450arom mRNA expression at 180 min, as compared to the control group (Fig. 6A and B).

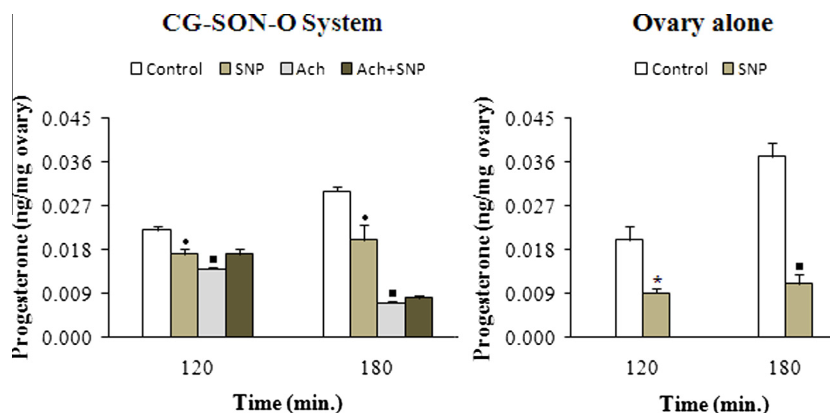

Fig. 3. Effect of 100 μM SNP in ovary incubations with and without peripheral neural influence in the first PE in rats, on the release of ovarian P expressed in ng/mg ovary. The results are presented as mean ± SEM of six animals per experimental group. Repeated measures analysis of variance followed by Tukey's test was used for several comparisons: (∗) $p < 0.05$, (●) $p < 0.01$, (▪) $p < 0.001$.

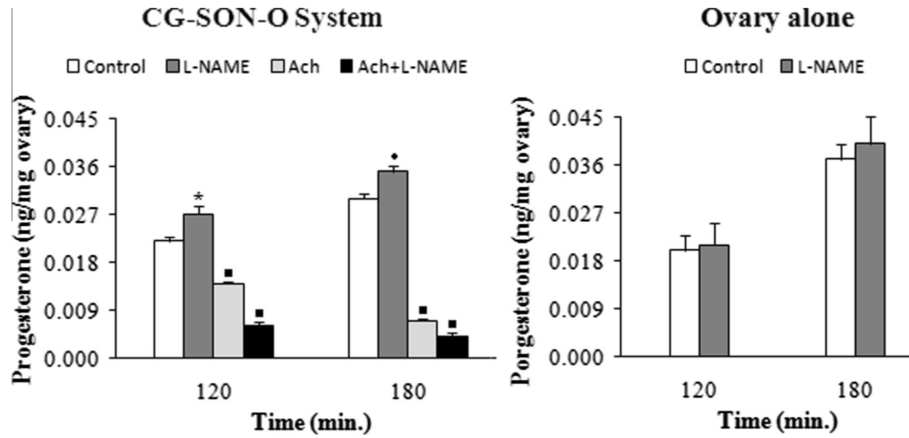

Fig. 4. Effect of 100 μM L-NAME in ovary incubations with and without peripheral neural influence in the first PE in rats, on the release of ovarian P expressed in ng/mg ovary. The results are presented as mean ± SEM of six animals per experimental group. Repeated measures analysis of variance followed by Tukey's test was used for several comparisons: (⁎) $p < 0.05$, (●) $p < 0.01$, (■) $p < 0.001$.

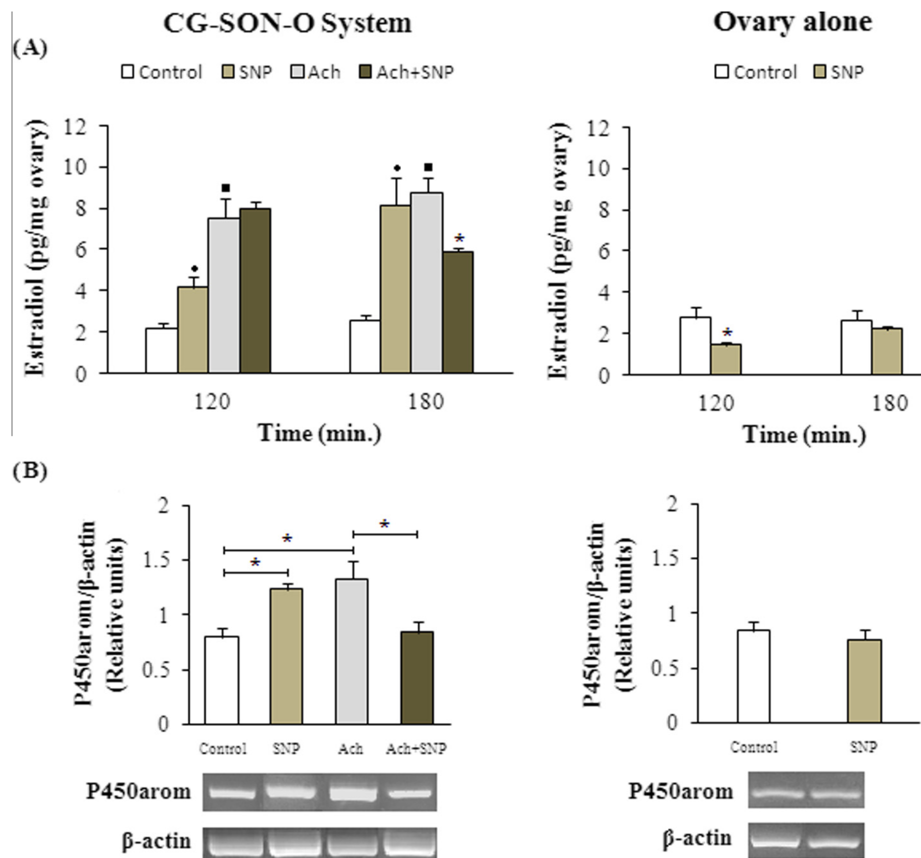

Fig. 5. Effect of 100 μM SNP in ovary incubations with and without peripheral neural influence in the first PE in rats, on the release of ovarian E_2 expressed in pg/mg ovary (A; upper panel) and P450arom mRNA expression (B; lower panel). The results are presented as mean ± SEM of six animals per experimental group for E_2 determination. Repeated measures analysis of variance followed by Tukey's test was used for several comparisons: (⁎) $p < 0.05$, (●) $p < 0.01$, (■) $p < 0.001$. PCR products: P450arom (266 bp) and β-actin (289 bp) as endogenous control, were visualized on agarose gels stained with GelRed. The gel photographs were quantified using ImageJ and expressed as arbitrary units. Results are expressed as mean ± SEM (n = 3) for the expression analysis.

4. Discussion

The participation of the sympathetic innervation and NO in the gonadal function has been extensively demonstrated (Basini and Grasselli, 2015; Delgado et al., 2010, 2004; Madekurozwa, 2008; Tamanini et al., 2003; Vallcaneras et al., 2013).

We have recently reported that the stimulation of the CG with Ach caused oxidative stress in the ovary, associated with an increase in the release of NO, which modified the steroidogenesis in the first PE in rats. We also observed that aminoguanidine (a selective inhibitor of iNOS) in a concentration of 400 μM exerts protective effects against the oxidative stress and the altered

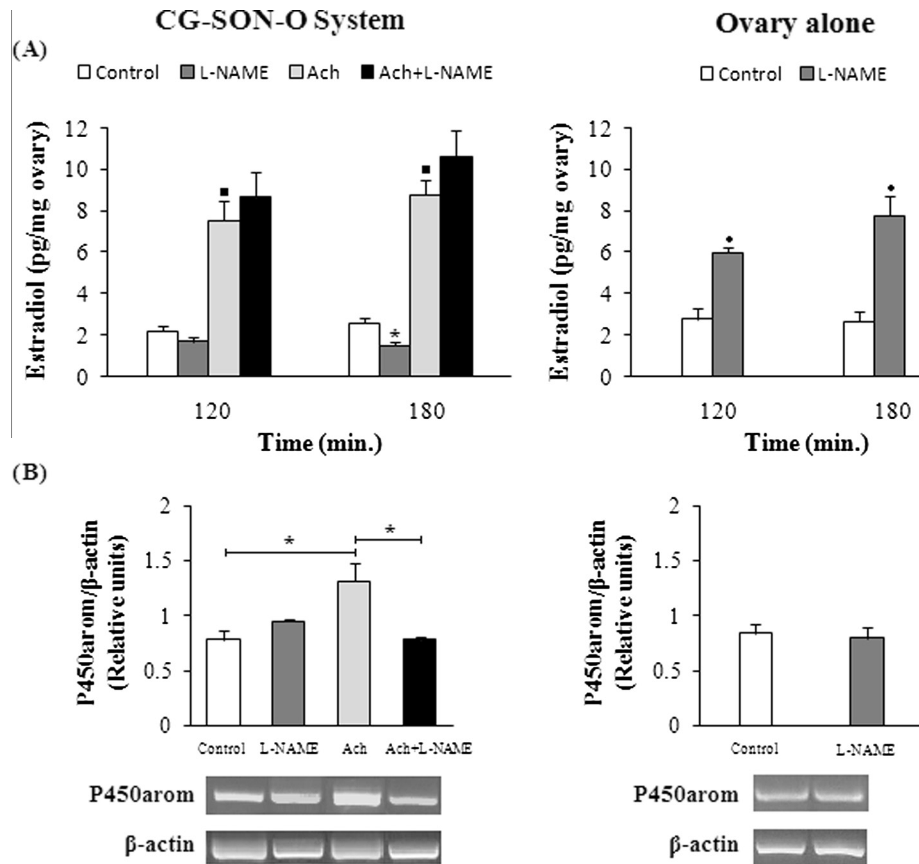

Fig. 6. Effect of 100 μM L-NAME in ovary incubations with and without peripheral neural influence in the first PE in rats, on the release of ovarian E_2 expressed in pg/mg ovary (A; upper panel) and P450arom mRNA expression (B; lower panel). The results are presented as mean ± SEM of six animals per experimental group for E_2 determination. Repeated measures analysis of variance followed by Tukey's test was used for several comparisons: (•) $p < 0.01$, (▪) $p < 0.001$. PCR products: P450arom (266 bp) and β-actin (289 bp) as endogenous control, were visualized on agarose gels stained with GelRed. The gel photographs were quantified using ImageJ and expressed as arbitrary units. Results are expressed as mean ± SEM (n = 3) for the expression analysis. (∗) $p < 0.05$ (Student's *t*-test).

metabolism of ovarian steroids, both of which are a product of the stimulation of the extrinsic cholinergic innervation (Delsouc et al., 2016).

Considering those findings and taking into account that the environment in which the ovarian follicles develop is not completely known, we thought it is interesting to further explore the effects of the extrinsic cholinergic innervation and NO in the preovulatory process. We focused on the first PE since the ovarian extrinsic innervation reaches biochemical and functional maturity during this stage (Ricu et al., 2008), thus favoring the development of the follicles which will give place to the first ovulation. Thus, the aim of the present work was to investigate the participation of the PNS in the action of NO on the ovarian steroidogenesis, using an NO donor (SNP) and an inhibitor of NOS (L-NAME), administered in incubations of ovary with and without peripheral neural influence, in the first PE in rats.

Two experimental schemes were used, one is the CG-SON-O system (Delsouc et al., 2016; Delgado et al., 2010; Sosa et al., 2000) and the other is the ovary without peripheral neural influence (Bronzi et al., 2015, 2013). Given the well-proven influence of Ach neurotransmitter on the NO/NOS system and the secretion of steroid hormones in ovary through the SON, the CG was stimulated with 10^{-6} M Ach in the CG-SON-O system. Using the CG-SON-O system of rats during different stages of the first estral cycle, Delgado et al. (2010) reported that the addition of this cholinergic agonist in the ganglion compartment increases the release of NO as well as the synthesis of E_2 and it decreases the release of P in the ovary incubation medium, in the first PE. In the present work, ganglionic stimulation with 10^{-6} M Ach was performed for 180 min of incubation, simultaneously to the other experimental groups, to analyze possible changes in the synthesis and release of NO and steroid hormones in the ovary, thus strengthening the results previously mentioned.

It is interesting to note that in the first PE in rats, the ganglionic stimulation with 10^{-6} M Ach increased NO concentration in the ovary compartment without altering mRNA expression of iNOS and eNOS at 180 min of incubation. Casais et al. (2007) demonstrated that there is a contribution of NO from the ganglionic NO/NOS system to the ovary through the SON. However, it is worth highlighting that Ach increases the iNOS protein expression in the ovary (unpublished data). A possible mechanism through which Ach increases the iNOS protein expression and the release of NO might be an increase in the activity of the reactive oxygen species (ROS). We have recently reported that the stimulation of the extrinsic-cholinergic innervation of the ovary causes oxidative stress (Delsouc et al., 2016), and it is known that an increase in ROS activity upregulates iNOS expression through the activation of the nuclear factor kappa B (NFkappaB) (Aktan, 2004; Zhen et al., 2008).

In addition, SNP increased the concentration of nitrite in both experimental schemes, as would be expected, because it is a drug that liberates NO spontaneously through an independent reaction of the enzymatic pathway that uses L-arginine as substrate (Dubey et al., 2011; Feelisch and Noack, 1991).

The addition of L-NAME, which is considered to be more effective at inhibiting eNOS than the iNOS (Leal et al., 2007; Pfeiffer

et al., 1996), decreased the release of ovarian NO in both experimental schemes. This effect was more significant in the CG-SON-O system at 180 min of incubation, in relation to the ovary alone, both compared with their respective control. The inhibitory effect of L-NAME was even higher in the CG-SON-O system under ganglionic cholinergic stimulation in agreement with a decrease in the expression of eNOS in ovary. Casais et al. (2007) reported similar results when working with the CG-SON-O system of prepubertal rats, which showed a sensitization of the ovarian NO/NOS system caused by a cholinergic stimulation.

In relation to steroidogenesis, the exogenous donor of NO decreased the release of ovarian P in both experimental schemes, thus providing new evidence of an antisteroidogenic effect of NO on P, previously observed during the preovulatory period in rat (Dave et al., 1997; Delgado et al., 2010). These observations are supported by the opposite result obtained with the competitive inhibitor of NOS in the absence of Ach in CG. However, L-NAME in the presence of cholinergic ganglionic stimulation decreased ovarian release of P independently of its inhibitory effect on the NO synthesis. In recent works, it has been demonstrated that the cholinergic stimulation of the CG increases the release of GnRH and neurotransmitters, such as catecholamines and the vasoactive intestinal peptide (VIP), which are all intraovarian regulators with a well-known influence on P (Bronzi et al., 2015; Daneri et al., 2013; Rosas et al., 2015). For this reason, and given the complex interaction between those factors and their effects on P release, the most sensitive steroid to neural action (Aguado et al., 1982; Casais et al., 2006; Delgado et al., 2010), further studies are needed.

In relation to E_2, our results in the CG-SON-O system demonstrate that its release is directly related with the production of NO in accordance with that reported by Delgado et al. (2010) and recently by Delsouc et al. (2016). This is in agreement with the fact that NO and E_2 favor the local vasodilation, increasing the blood flow and benefitting the follicular development and the subsequent ovulation (Masuda et al., 2001), both being needed in higher concentrations during the PE of the estral cycle. However, NO in excessive levels decreases the release of ovarian E_2 and the expression of P450arom, confirming its role in the follicular development and the *in vitro* maturation of oocytes in a concentration-dependent way (Dubey et al., 2011), an effect that was observed as a result of co-treatment of Ach and SNP in the CG-SON-O system. In addition, previous studies have demonstrated that NO, besides being an intraovarian steroidogenic regulator, may also modulate the sympathetic tone (Jordan et al., 2001; Prast and Philippu, 2001; Schultz, 2009). This might explain our results since the physicochemical characteristics of NO allow this little gaseous molecule to diffuse in an autocrine and paracrine way in order to have an effect on the pre-synaptic and post-synaptic events (Schultz, 2009).

In ovary incubations without peripheral neural influence, the results in relation to E_2 were opposite to those obtained in the CG-SON-O system. The release of this steroid hormone was inhibited by SNP and it was stimulated by L-NAME. Dubey et al. (2011) reported that SNP in concentrations higher than 10 μM inhibits *in vitro* the survival, growth and steroidogenesis of the preantral follicles, inducing the apoptosis of the oocytes in buffalo ovaries. Considering this antecedent and the results obtained from the ovary stimulation with 100 μM SNP, it is interesting to emphasize that even though NO is necessary during the follicular development, it alters the ovulatory process in isolated ovary since it decreases the release of E_2. This effect was modulated in the CG-SON-O system by the peripheral neural influence.

In short, our study has made evident the importance of the ovarian extrinsic innervation in maintaining a direct relationship between NO and E_2, both needed in high concentrations during the follicular phase (Chun et al., 1995; Masuda et al., 2001) in order to favor the continuity of the estral cycle. This result is in agreement with the work of Bronzi et al. (2013) who, working with the CG-SON-O system during diestrus stage in adult rat, demonstrated that the PNS exerts a protective effect on the ovary against the apoptotic mechanisms. However, the physiological balance is delicate since previous works have demonstrated that the sympathetic hyperactivation for a long period of time increases the concentration of catecholamines and E_2 in ovary (Bronzi et al., 2015, 2011; Paredes et al., 2011), favoring the development of the polycystic ovary syndrome (Bernuci et al., 2008; Dorfman et al., 2003; Greiner et al., 2005). In relation to this, after the experimental induction of the polycystic ovary syndrome in rodents, Barria et al. (1993) reported that the ablation of the SON allows reestablishing the estral cyclicity and the ovulatory capacity. This is in agreement with our results obtained in incubations of isolated ovary, in which the release of E_2 decreased due to an increase in the concentration of NO (by the SNP action) and a possible reduction in the levels of catecholamines in ovary because of the absence of the *input* coming from the CG (Barria et al., 1993; Bronzi et al., 2015).

Our findings contribute with new evidence on the modulating effect of NO on the ovarian steroidogenesis and the importance of the neuroendocrine integration for the correct reproductive function.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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