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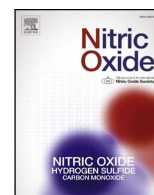

The production of nitric oxide in the coeliac ganglion modulates the effect of cholinergic neurotransmission on the rat ovary during the preovulatory period

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ABSTRACT

The aim of the present work was to investigate whether the nitric oxide produced by the nitric oxide/nitric oxide synthase (NO/NOS) system present in the coeliac ganglion modulates the effects of cholinergic innervation on oxidative status, steroidogenesis and apoptotic mechanisms that take place in the rat ovary during the first proestrous. An *ex vivo* Coeliac Ganglion- Superior Ovarian Nerve- Ovary (CG-SON-O) system was used. Cholinergic stimulation of the CG was achieved by 10^{-6} M Acetylcholine (Ach). Furthermore, 400 μM Aminoguanidine (AG) – an inhibitor of inducible-NOS was added in the CG compartment in absence and presence of Ach. It was found that Ach in the CG compartment promotes apoptosis in ovarian tissue, probably due to the oxidative stress generated. AG in the CG compartment decreases the release of NO and progesterone, and increases the release of estradiol from the ovary. The CG co-treatment with Ach and AG counteracts the effects of the ganglionic cholinergic agonist on ovarian oxidative stress, increases hormone production and decreases Fas mRNA expression. These results suggest that NO is an endogenous modulator of cholinergic neurotransmission in CG, with implication in ovarian steroidogenesis and the apoptotic mechanisms that take place in the ovary during the preovulatory period in rats.

1. Introduction

Through anatomical descriptions mostly derived from rodent studies, it was demonstrated that the extrinsic innervation of the ovary is primarily provided by sympathetic and sensory nerves, as well as a small contingency of parasympathetic nerves. These nerves reach the ovary via two main routes: (i) the ovarian plexus nerve, which travels along the ovarian artery; and (ii) the superior ovarian nerve (SON), which is associated with the suspensory ligament of the ovary [1,2].

The SON is constituted mainly of catecholaminergic fibers and the neurochemical nature of this projection is mainly noradrenergic. This route provides communication between the coeliac ganglion (CG) and the ovary, with implication in steroidogenesis, follicular maturation, ovulation and luteolysis [2,3].

The CG is a component of the prevertebral ganglionic sympathetic pathway with function of receiving and integrating information from the central nervous system and organizes responses that influence ovarian physiology [4]. This structure has a variety of neurotransmitters (acetylcholine, catecholamines, neuropeptides, and nitric oxide) also specific receptors [5,6].

Acetylcholine (Ach) is the principal preganglionic neurotransmitter of the sympathetic ganglionic pathway [7]. The CG has specific structures to respond to cholinergic stimuli, such as nicotinic and muscarinic receptors in the principal cells as well as muscarinic receptors in other neuronal populations [8,9]. It has been shown that the stimulation of such receptors modifies the release of ovarian steroids in prepubertal [10], cyclic [11], pregnant [12] and peripubertal-pubertal rats [3,13,14].

Respect of nitric oxide (NO), this gasotransmitter is produced by three isoforms of NO synthase (NOS): nNOS (neural), iNOS (inducible), and eNOS (endothelial). The presence of the NO/NOS system in rat prevertebral ganglia has been confirmed [15]. In addition, the three isoforms have been shown to influence autonomic neural function in some manner [16–18]. Unlike conventional neurotransmitters, NO is not stored in synaptic vesicles, its action is not limited to the synaptic regions of the neurons and does not interact with receptor proteins. This

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gaseous molecule accordingly regulates many functions, such as vascular tone, immune response, synaptic plasticity, neurotransmission, and oxidation-sensitive mechanisms [19,20], suggesting its participation in the physiological and pathological processes.

In a previous report, Casais and her collaborators [10], using the *ex vivo* CG-SON-O system of prepubertal rat and stimulating both CG and ovary with isoform-selective inhibitors of NOS enzymes, corroborated the existence of the NO/NOS system in CG when obtaining relevant results with aminoguanidine (AG, a selective inhibitor of the iNOS). In addition, they highlighted the existence of a contribution of NO from the CG to the ovary through the SON [10]. Afterwards, using the *ex vivo* CG-SON-O system of rats in the first proestrous (PE), Delsouc et al. [13] demonstrated that the addition of 10^{-6} M Ach into the CG compartment increases NO concentration in the incubation media of ovaries and causes oxidative stress in gonadal tissue. Assuming that highest levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) lead to irreversible cell damage, it was also considered interesting to stimulate the ovary with 400 μM AG. Finally, this study demonstrated that the addition of AG in the ovarian compartment counteracts the effects generated by the ganglionic cholinergic agonist, showing a protective action against induced damage in gonadal tissue [13]. Despite the interesting findings, the influence of the ganglion NO/NOS system on the ovary is not known in depth. It is presumed that both NOS and choline acetyltransferase play a vital role in prevertebral ganglia, complementing each other's functions and synergistically modulating the activity of preganglionic neurons [6,21] and, as such, likely to contribute in ovarian physiology.

Given the important role of NO in physiological and pathophysiological processes, several studies have analyzed the expressions and localization patterns of NOS isoforms in reproductive systems in mouse, rat, sheep, and pig [22,23]. Although neuronal pathways are an important factor in the regulation of ovarian function, our understanding of the regulatory factors governing NOS function and the influences of NO on the CG remains limited. The NO generated in the nerve synapses can easily diffuse through short distances and affect one or more cells, influencing the presynaptic and postsynaptic events of both excitatory and inhibitory synapses [17]. Clearly its mechanism of action is complicated and for this reason additional studies are necessary.

Based on the above and without attempting to oversimplify the complex problem of ganglionic functioning and regulation, the aim of this work was to investigate whether the NO/NOS system present in CG modulates the effects of cholinergic innervation on oxidative status, steroidogenesis and apoptotic mechanisms that take place in rat ovary during the first PE.

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 light on from 07:00 to 19:00 h and at a temperature of 22 ± 2 °C. Animals had free access to food (Cargill SAIC, Saladillo, Buenos Aires, Argentina) and tap water. Groups of six animals were used for the experimental procedure.

The experiments were performed per duplicate according to the procedures approved in the UFAW Handbook on the Care and Management of Laboratory Animals [24]. 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, B-200/15).

2.2. Reagents

L-acetylcholine hydrochloride (Ach), aminoguanidine (AG), 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). The Estradiol (E_2) DIASource ImmnoAssays kit was purchased from DiagnosMed SRL (Buenos Aires, Argentina). Other reagents and chemicals were of analytical grade.

2.3. Experimental procedure

The animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). The CG-SON-O system was removed by dissecting, as previously described by Delgado et al. [3]. 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 (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. Incubations were conducted in a Dubnoff metabolic shaking-water bath.

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. Likewise, the different agents used for each experimental group were added at the same time. To stimulate the CG, Ach was dissolved in 1 ml of Krebs-Ringer solution at a 10^{-6} M final concentration in the CG compartment (Ach group). In addition, to test the role of NO on extrinsic ovarian innervation, AG (a selective inhibitor of the iNOS) was dissolved in 1 ml of Krebs-Ringer solution at a 400 μM final concentration in the CG compartment, with or without 10^{-6} M Ach [10]. The control groups consisted of untreated CG-SON-O systems.

The incubation was performed during 180 min. Periodical extractions of 250 μl were made from the ovary compartment at 30, 120 and 180 min. Liquid samples from the ovary compartment were maintained at −20 °C for further analysis. At the end of the incubation period, the ovaries were collected and frozen at −80 °C for further analysis.

2.4. Nitrite assay

Levels of nitrite, a water-soluble metabolite of NO, were measured spectrophotometrically by Griess reaction [25]. Briefly, 50 μl of each aliquot of incubation medium from the ovaries was mixed with Griess reagent (1% sulfanilamide with 0.1% *N-l*-naphthyl-ethylenediamine/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. Preparation of tissue homogenate

Six ovaries per experimental group were homogenized separately in 150 μl RIPA buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Tissue homogenates were centrifuged at 14,000 × *g* for 15 min at 4 °C to remove nuclei and cell debris. The pellets were discarded whereas the supernatants were collected and used to determine the expression of iNOS, BAX and BCL-2 proteins, the total antioxidant capacity (TAC) and the activity of antioxidant enzymes (catalase and glutathione peroxidase). The concentration of protein carbonyls and thiobarbituric acid reactive

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