Nitric oxide in prepubertal rat ovary contribution of the ganglionic nitric oxide synthase system via superior ovarian nerve

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Submitted: November 22, 2006 *Accepted: December 28, 2006*

Key words: **nitric oxide; nitric oxide synthase; ovary; coeliac ganglion; acetylcholine**

Neuroendocrinol Lett 2007; **28**(1):39–44 **PMID:** 17277728 NEL280107A07

Abstract

Both peripheral innervation and nitric oxide (NO) participate in ovarian steroidogenesis. Considering the existence of the nitric oxide/ nitric oxide synthase system in the peripheral neural system and in the ovary, the aim of this work was to analyze if the liberation of NO in the ovarian compartment of prepubertal rats is of ovarian and/or ganglionic origin. The analysis is carried out from a physiological point of view using the experimental coeliac ganglion – Superior Ovarian Nerve – ovary model with and without ganglionic cholinergic stimulus Acetylcholine (Ach) 10^{-6} M. Non selective and selective inhibitors of the synthase nitric oxide enzyme were added to the ovarian and ganglionic compartment, and the liberation of nitrites (soluble metabolite of the nitric oxide) in the ovarian incubation liquid was measured. We found that the non-selective inhibitor L-nitro-arginina methyl ester (L-NAME) in the ovarian compartment decreased the liberation of nitrites, and that Aminoguanidine (AG) in two concentrations in a non-dose dependent form provoked the same effect. The addition of Ach in ganglion magnified the effect of the inhibitors of the NOS enzyme. The most relevant results after the addition of inhibitors in ganglion were obtained with AG 400 and 800 µM. The inhibition was made evident with and without the joint action of Ach in ganglion. These data suggest that the greatest production of NO in the ovarian compartment comes from the ovary, mainly the iNOS isoform, though the coeliac ganglion also contributes through the superior ovarian nerve but with less quantity.

ORIGINAL ARTICLE

Introduction

The formation of nitric oxide (NO) from L-arginine is catalyzed by a diverse family of nitric oxide synthase (NOS) isoenzymes [1]. The isoforms were divided into two functional classes on the basis of their dependence on calcium for their enzymatic activity. The inducible or non-constitutive isoform (iNOS) is calcium independent whereas the neuronal and the endothelial isoforms are calcium dependent, both of which are constitutive (nNOS and eNOS respectively) [2]. The NOS isoforms differ with respect to function and the quantities of NO that they produce. NO liberated by nNOS and eNOS at low concentrations undertakes a bewildering array of regulatory tasks, whereas NO generated by iNOS in higher amounts is involved mainly in immunological processes [3, 4]. Van Voorhis et al. [5] and Jablonka-Shariff and Olson [6] suggest that of the three NOS isoenzymes, ovaries express eNOS and iNOS but not nNOS. Additionally, Srivastava et al. [7] reported that both iNOS and nNOS are expressed in the ovary of immature rats, and the expression of ovarian iNOS mRNA and protein showed developmental changes both before and after the first ovulation, thus providing evidence in support of the hypothesis that the NO/NOS system plays a role in ovarian maturation and function [8].

Although expressed in different cell types, all the isoforms of NOS are also present in the nervous system. Keilhoff et al. [9] demonstrated that the success of fiber regeneration in the peripheral nervous system depends on a concerted regular expression of the three NOS isoform with a dominant role of nNOS.

Earlier studies demonstrated that the extrinsic innervation of the ovary consists of sympathetic, parasympathetic and sensory fibers derived from neurons of the peripheral nervous system. Such fibers enter the gland mainly through the Superior Ovarian Nerve considered as the main neural pathway related to the ovarian steroidogenesis [10,11,12]

Delgado et al. [13], working with the integrated *ex vivo* coeliac ganglion-superior ovarian nerve-ovary system in prepubertal rat, showed that the cholinergic ganglionic stimulation increases the liberation of NO in the ovarian compartment while the release of the steroids is inhibited, which might indicate that the cholinergic action is probably mediated by NO.

Considering the existence of the NO/NOS system in the peripheral neural system and in the ovary, the aim of this work was to analyze if the liberation of NO in the ovarian compartment of prepubertal rats is of ovarian and/or ganglionic origin. The analysis is carried out from a physiological point of view using the experimental coeliac ganglion-superior ovarian nerve-ovary model with and without ganglionic cholinergic stimulus. Inhibitors (non-selective and selective) of the isoenzymes of the NOS were added in the ovarian and ganglionic compartment.

Materials and methods

Animals

Virgin Holtzman strain female prepubertal rats of 30 days of age (60±10 g body weight) were used in all the experiments. The rats were kept under controlled conditions with lights on from 0700 to 1900 h and at a temperature of 24±2 °C. Animals had free access to food (Cargill SACI, Saladillo, Buenos Aires, Argentina), and tap water was available *ad libitum*. Groups of six animals were used for the experimental procedure.

The experiments were performed in accordance with the UFAW Handbook on file Care and Management of Laboratory Animals, volume 1-Terrestrial vertebrates–seventh edition, edited by Trevor Poole (1999) and the Guide for Animal Use and Handling of the National University of San Luis, Argentina.

Reagents

The following drugs: L-Acetylcholine Hydrochloride, dextrose, ascorbic acid, bovine serum albumin fraction V (BSA), Sulfanilamide, N-1-naphthyl-ethylenediamine, L-nitro-arginina methyl ester (L-NAME), Aminoguanidine (AG) were provided by the Sigma Chemical Co (St. Louis, Mo, USA). Other reagents were of analytical grade.

Experimental procedure

The surgical procedure used for removing the system and its characterization were performed as described previously [13]. Briefly, a piece of tissue containing the left ovary, the fibres constituting the superior ovarian nerve and the coeliac ganglion were removed. The strip of tissue was carefully dissected avoiding contact between the surgical instruments and the nerve fibres or the ganglion in order to prevent spontaneous depolarisation of file nerves. The total surgical procedure was completed in 1–2 min. The coeliac ganglion-superior ovarian nerve-ovary system was washed with the incubation medium and immediately placed in the cuvette consisting of two compartments.

Each compartment contained 1 ml of Krebs-Ringer bicarbonate buffer, pH 7.4 solution in the presence of dextrose (0.1 mg/ml) and BSA (0.1 mg/ml). The ganglion was placed in a compartment and the ovary, in the other one, both joined by the superior ovarian nerve that had to remain humid with the working solution. The system was stabilized by preincubation in metabolic bath at 37 °C for 15 min in an atmosphere composed by 95% O_2 and 5% CO_2. The end of the preincubation period was considered as incubation time 0. At this time, the buffer was changed in both compartments; ascorbic acid (0.1 mg/ml in Krebs-Ringer) was added as an antioxidant agent to the ganglion compartment (Control group).

Likewise, the different agents used for each experimental group were added at the same time. The cholinergic

agent added in the ganglion compartment was L-Acetylcholine Hydrochloride (Ach 10^{-6} M) dissolved in 1 ml Krebs-Ringer plus ascorbic solution (Ach group).

Non-selective L-NAME in concentration 100 μM and selective inhibitors of the iNOS isoform AG in concentration 400 and 800 μM were used. The inhibitors were added in the ovarian compartment dissolved in 1 ml of Krebs-Ringer solution, or in the ganglion compartment dissolved in Krebs-Ringer plus ascorbic acid, according to the following distribution:

With addition of the inhibitors into ovarian compartment:

Groups:
L-NAME
Ach+L-NAME
AG 400
Ach+AG 400
AG 800
Ach+AG 800

With addition of the inhibitors in the ganglion compartment:

Groups:
L-NAME
Ach+L-NAME
AG 400
Ach+AG 400
AG 800
Ach+AG 800

The samples of liquid from the ovarian compartment (250 μl) were collected at 15, 30, 60 and 120 min from the beginning of the incubation. The nitrites were measured by Griess method. The respective corrections were made in all cases considering the volume in each period tested.

Nitrite assay

Levels of nitrites were measured spectrophotometrically [14]. Samples were immediately mixed with Griess reagent (sulfanilamide with N-l-naphthyl-ethylenediamine/HCl). After a 10-minute incubation period at room temperature, they were read for absorbance of 540 nm. The assay sensitivity was less than 2.5 nmol/ml. The intraassay coefficients of variation for all the assays were less than 10.0 %. The results were expressed as nanomol of nitrite per milligram of ovarian tissue (nmol/mg ovary).

Statistical Analysis

Results are presented as mean±SEM in each group. Student's *t*-test was used to assay significant differences between means of two groups. Analysis of the variances (ANOVA) followed by Tau's multiple range test was used for several comparisons. A value of $p<0.05$ was accepted as statistically significant [15].

Results

The levels of nitrites in the ovarian compartment in the control and Ach groups obtained in the present work are similar to those obtained in Delgado et al. [13].

Presence of nitric oxide in the ovarian compartment:

1– Effect of the addition of inhibitor agents of the NOS in the ovarian compartment on the release of nitrites

The non-selective inhibitor L-NAME in the ovarian compartment significantly decreased the nitrites liberation when compared with the control group [▪] at all the studied times ($p<0.001$).

The joint effect of Ach in CG and L-NAME in ovary caused a significant decrease in the liberation of nitrites compared with the Ach groups [*] at all the studied times ($p<0.001$). The Ach+L-NAME group did not show a significant difference in relation to the L-NAME group (Figure lA).

The selective inhibitor AG 400 in the ovarian compartment decreased the liberation of nitrites compared with the control group [▪] ($p<0.01$).

The joint effect of Ach in CG and AG 400 in ovary decreased the liberation of nitrites in the ovarian compartment in relation to the Ach group [*] at all the studied times ($p<0.001$). The Ach+AG 400 group decreased the liberation of nitrites in relation to the AG 400 group [#] (15 and 30 min $p<0.01$, 60 and 120 minutes $p<0.05$) (Figure lB).

The selective inhibitor AG 800 in the ovarian compartment decreased the liberation of nitrites at 15 and 30 minutes ($p<0.05$) compared with the control group [▪].

The joint effect of Ach in CG and AG 800 in ovary inhibited the liberation of nitrites in the ovarian compartment compared with the Ach group [*] at all the studied times ($p<0.001$). The Ach+AG 800 group decreased the liberation of nitrites in relation to the AG 800 group [#] (15 and 30 min $p<0.01$; 60 min $p<0.05$) (Figure 1C).

2– Effect of the addition of inhibitor agents of the NOS in the ganglion compartment on release of nitrites

The non-selective inhibitor L-NAME added to the ganglion compartment did not show any significant changes compared with the control group.

The joint effect of Ach+L-NAME in CG provoked a significant decrease of the liberation of nitrites compared with the Ach group [*] only at 30 min of incubation ($p<0.0l$). The Ach+L-NAME group in CG increased the liberation of nitrites in relation to the L-NAME group [#] at all the studied times ($p<0.001$) (Figure 2A).

The selective inhibitor AG 400 in the ganglion compartment decreased the liberation of nitrites compared with the control [▪] at all the studied times ($p<0.05$).

The joint effect of Ach+AG 400 in CG did not show significant differences in relation to the Ach group. The

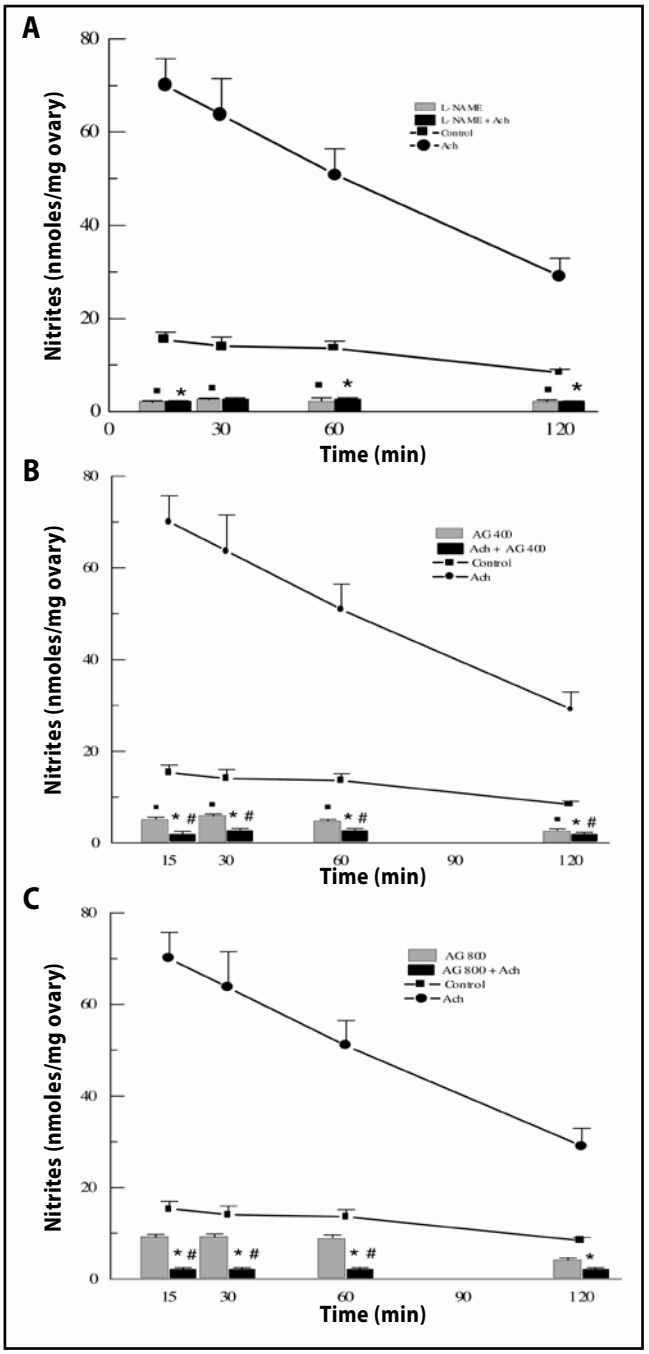

Figure 1. Effect of non selective and selective inhibitors of the inducible isoform of Nitric Oxide Synthase in ovarian compartment on the release of ovarian nitrites in the coeliac ganglion-superior ovarian nerve-ovary system removed from prepubertal rats. The system was incubated in Krebs Ringer solution, at 37 °C in an atmosphere of 95% O_2-5% CO_2 for 120 min without (Control group) and with Acetylcholine 10^{-6} M added to the ganglion compartment (Ach group). Values are the mean ± SEM of six animals per experimental group. Student's t-test was used to assay significant differences between means of two groups. Analysis of the variances (ANOVA) followed by Tau´s multiple range test was used for several comparisons.
1A – L-NAME vs Control: (■) p<0.001; Ach+L-NAME vs Ach (*) p<0.001.
1B – AG 400 vs Control: (■) p<0.01; Ach+AG 400 vs Ach: (*) at all the studied times p<0.001; Ach+AG 400 vs AG 400: (#) 15 and 30 min p<0.01, 60 and 120 min p<0.05.
1C – AG 800 vs Control: (■) 15 and 30 min p<0.05; Ach+AG 800 vs Ach: (*) p<0.001; Ach+AG 800 vs AG 800: (#) 15 and 30 min p<0.01; 60 min p<0.05.

liberation of nitrites in the Ach+AG 400 group was higher in relation to the AG 400 group [#] at all the studied times (p<0.00l) (Figure 2B).

The selective inhibitor AG 800 in the ganglion compartment decreased the liberation of nitrites after 30 min (p<0.05) compared with the control group [▪].

The joint effect of Ach+AG 800 in CG decreased the liberation of nitrites in relation to Ach group [*] at all the studied times (p<0.001). The liberation of nitrites in the Ach+AG 800 group was higher in relation to the AG 800 group [#] at all the studied times (15, 30 and 60 min p<0.01 and 120 min p<0.05) (Figure 2C).

Discussion

The results of the present study show that the addition of L-NAME (non selective inhibitor of the NOS) and AG (selective inhibitor of the iNOS) in the ovarian compartment decreased the liberation of NO, though not completely, in relation to the control and the Ach in CG groups. When those inhibitors were added to the ganglion compartment, the NO decreased in ovary in a proportion lower than previously mentioned. This suggests that the origin of NO is mainly ovarian and that there might exist a ganglionic contribution through the superior ovarian nerve.

The *ex vivo* CG-superior ovarian nerve-O system permits to explore the functionality of the isoenzymes of the NOS in the ovary as well as in the CG. For this analysis, non selective and selective inhibitors of the iNOS were added in both compartments in control conditions and with ganglionic cholinergic stimulus, and the liberation of NO in the ovarian compartment was determined.

The presence in the ovary of the non-selective L-NAME inhibitor and of the selective inhibitor of the iNOS AG in both concentrations (400 and 800 μM) decreased the liberation of NO in both conditions (control and Ach in CG). The response to the AG action was not dose-dependent. Considering the results obtained in the control situation, we corroborated from the physiological point of view that the NO/NOS system is present in ovary as has been suggested by Matsumi et al. [16] who postulated that the NO synthesis enzymes in their different isoforms have been characterized and located in the ovary of the prepubertal rat.

In relation to the joint action of Ach in CG and the inhibitors of the NOS in ovary and considering that the only addition of Ach in CG increases the liberation of NO in ovary [13], we postulate that the ganglionic cholinergic action might sensitize the NO/NOS ovarian system through the superior ovarian nerve.

The NO/NOS system is present in the granulosa cells of immature follicles [16] as well as in neurons of the intrinsic ovarian neural system [17]. Yet, it is not possible with the present study to determine the origin of the NO released in the ovarian compartment.

Considering the analysis of the effect of both inhibitors in the ovarian compartment, we conclude that this

effect is mainly caused by the inducible isoform of the NOS, which might indicate that the higher proportion of NO comes from this isoform. This was in accordance with specified by [7]. The remnant of NO after the inhibition in all the experiences might be the result of an incomplete inhibition since Garvey et al. [18] proved that N-(3-(Aminomethylbenzyl) acetomidine (1 400 W) is a more potent and selective inhibitor of the iNOS. Another possibility might be the contribution of NO from the CG via the superior ovarian nerve. In relation to this, Quinson et al. [19] have stated that the presence of NO in the prevertebral ganglia is now well established and they have demonstrated that the endogenous NO (as well as other neurotransmitters such as Ach, noradrenaline, serotonine and enkephaline) also plays a role in the modulation of the ganglionic nicotinic transmission by exerting excitatory and inhibitory effects (these NO-mediated modulations of the synaptic activation strengthen the integrative properties of the prevertebral ganglia).

The NOS or NADPH diaphorase positive nerve fibers have been described in the prevertebral ganglia, which may have a central or a peripheral origin [20]. Moreover, the possible presence of the NOS in the postganglionic neurons of the CG in rats should be considered as demonstrated by Sheng et al. [21] in the bovine superior cervical ganglion or by Hohler et al. [22] in guinea pig sympathetic ganglia.

Considering these antecedents and having standardized the *ex vivo* integrated system that permits to study the neuroendocrine interactions without humoral influence, we decided to investigate the effect of the inhibitors of the NOS in ganglion in basal situation and also with the addition of Ach in ganglion, and to determine the liberation of NO in ovary.

According to the results obtained with the inhibitors in CG in basal situation, we deduce that the AG selective inhibitor shows a significant non-dose dependent inhibition of NO. It is important to emphasize that these results might indicate the existence of iNOS activity in CG.

The most interesting aspect of the joint action of Ach and the inhibitors in CG occurred when working with AG at an 800 μM dose. In this case the contribution of ganglionic NO mainly by the action of iNOS could be observed. We might also suggest that Ach sensitizes the ganglionic NO/NOS system.

Our results add a functional significance to the presence of NOS in the coeliac ganglion and in the ovary of prepubertal rat.

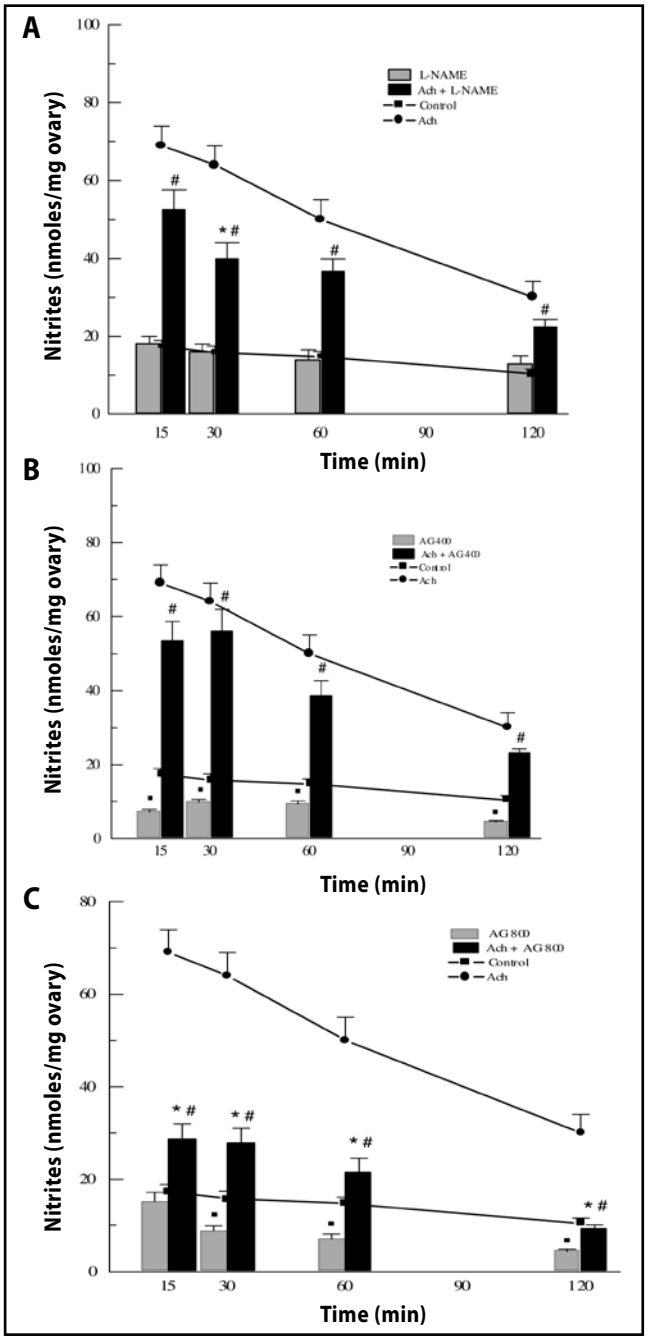

Figure 2. Effect of non selective and selective inhibitors of the inducible isoform of Nitric Oxide Synthase in ganglion compartment on the release of ovarian nitrites in the coeliac ganglion-superior ovarian nerve-ovary system removed from prepubertal rats. The system was incubated in Krebs Ringer solution, at 37 °C in an atmosphere of 95% O_2-5% CO_2 for 120 min without (Control group) and with Acetylcholine 10^{-6} M added to the ganglion compartment (Ach group). Control and Ach groups (Delgado et al., 2004). Values are the mean ± SEM of six animals per experimental group. Student's t-test was used to assay significant differences between means of two groups. Analysis of the variances (ANOVA) followed by Tau´s multiple range test was used for several comparisons.
2A – Ach + L-NAME vs Ach: (*) 30 min $p<0.01$; Ach+L-NAME vs L-NAME (#) $p<0.001$.
2B – AG400 vs Control: (■) $p<0.05$; Ach+AG400 vs AG400: (#) $p<0.001$.
2C – AG 800 vs Control: (■) 30, 60 and 120 min $p<0.05$; Ach+AG 800 vs Ach: (*) $p<0.001$; Ach+AG 800 vs AG 800: (#) 15, 30 and 60 min $p<0.01$, 120 min $p<0.05$.

Acknowledgements

This research was supported by Grant 9302 from Universidad Nacional de San Luis, Argentina. This work is part of the doctoral thesis of Silvia Marcela Delgado.

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