Modulation of the noradrenergic activity index by neural stimulus, and its participation in ovarian androstenedione release during the luteal phase

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Objective: To investigate the participation of catecholamines in the association between peripheral innervation and luteal steroidogenesis.
Design: Animal study.
Setting: University animal laboratory.
Animal(s): Six to eight virgin adult Holtzman-strain female rats in control and experimental groups on diestrus days 1 and 2.
Intervention(s): Removal of the coeliac ganglion-superior ovarian nerve-ovary system, with catecholaminergic agonist or antagonist added in the ganglion compartment (experimental group only). The control group received no treatment.
Main Outcome Measure(s): Ovarian neurotransmitters and their catabolites measured by reverse-phase high-pressure liquid chromatography, and A_2 measured by radioimmunoassay.
Result(s): On day 1, dopamine and catabolite increased whereas norepinephrine decreased, and the noradrenergic neuronal activity index was higher. On day 2, dopamine levels decreased, norepinephrine increased, and dopaminergic neuronal activity was higher. The release of A_2 was decreased by addition of norepinephrine to the ganglions on day 1, but was increased by the norepinephrine antagonist on day 2. Hence, norepinephrine increased A_2 release, and propranolol diminished it.
Conclusion(s): Ganglionic activity is modified by noradrenergic stimulus, leading to different ovarian A_2 release profiles. The peripheral nervous system is a modulator in these homeostatic mechanisms. (Fertil Steril® 2011;95: 1211–6. ©2011 by American Society for Reproductive Medicine.)

Key Words: Androstenedione, neurotransmitters, ovary

The superior ovarian nerve (SON), which belongs to the sympathetic peripheral nervous system, innervates the ovary and is mainly constituted by the axons of the coeliac ganglion (CG) (1, 2). The SON fibers innervate especially the secondary interstitial and theca-interstitial cells, which are responsible for androgen synthesis (3).The fact that the fibers constituting the SON are mostly catecholaminergic suggests that in the ovary they might act through α or β adrenergic receptors (4, 5). It is well known that the in vitro release of ovarian progesterone and androgen is induced by the union of β_2 receptors and adrenergic agonists (5–9). In this neural pathway, the intermediate structure most closely related to the ovary is the CG (10–12), composed of noradrenergic neurons called principal neurons, small intensely fluorescent cells, and peptidergic interneurons (13). The CG has an abundant capillary plexus (14) and a great variety of neurotransmitters with their respective receptors (15, 16).

The importance of studying A_2, the principal androgen in rats, lies in the fact that it is not only the precursor of estrogen synthesis but also exerts a direct trophic action on the corpora lutea (17, 18). In addition, the ovarian level of this hormone determines the tendency to dominance or to atresia of the developing follicles (3, 19), and its level undergoes modification by the presence of catecholamines (20).

The adrenergic system is a modulatory system that includes norepinephrine and epinephrine. Evidence suggests that dopamine may also activate adrenergic signaling and may regulate physiologic processes in the ovary (21, 22). This supports the view that the catecholaminergic system may play a role in intraovarian regulatory mechanisms, although its exact function in ovarian hormone secretion is unclear. The presence in ovary of neurotransmitters released from the SON other than norepinephrine (12, 23) has not been demonstrated.

We explored the presence of catecholaminergic neurotransmitters and their catabolites in the ovary compartment after stimulation by norepinephrine in the ganglion compartment in an ex vivo coeliac ganglion-superior ovarian nerve-ovary (CG-SON-O) system. We then assessed A_2 release in the ovary compartment and evaluated the possible relationship between the catecholamines present and ovarian A_2 release.

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MATERIALS AND METHODS

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 room that was light controlled (lights on from 07:00 to 19:00 hours) and temperature controlled (24° ± 2°C).Vaginal smears were taken daily, and only rats with a 4-day estrus cycle were used. Groups of six to eight animals on diestrus day 1 (D1) and day 2 (D2) were used as the experimental group. Each experiment included an experimental and a control group. Animals were handled according to the procedures recommended in the *Handbook on the Care and Management of Laboratory Animals* (24). The experimental protocol was approved by the University of San Luis Animal Care and Use Committee (number 107 protocol: B17/04, ordinance CD 006/02).

Reagents

The chemicals and other reagents of analytical grade used for this study were purchased from Sigma Chemical (St. Louis, MO). We obtained 1,2,6,7- [^{3}H] Androst 4-ene-3,17 dione (115.0 Ci/mmol) from New England Nuclear Products (Boston, MA).

Surgical and Experimental Procedure

The CG-SON-O system removal, histologic control, characterization, and standardization of incubation conditions were performed as previously described elsewhere (11, 12). The surgical procedure was performed between 15:00 and 16:00 hours. The CG-SON-O system was removed and placed in a cuvette with two separate compartments, one for the ovary and the other for the ganglion, connected by the SON and maintaining the nerve wet with the working solution. The system was stabilized by incubation in a metabolic bath at 37°C for 30 minutes in an atmosphere of 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, and ascorbic acid (0.1 mg/mL in Krebs-Ringer) was added as an antioxidant agent to the ganglion compartment (11, 12). Catecholamines and A_2 released in the ovary compartment under these conditions were considered as the control group.

For the experimental groups, norepinephrine as an agonist and phentolamine as an α and propranolol as a β antagonist were added to the ganglion compartment at incubation time 0, because the CG have α and β adrenergic receptors (16, 25). The different substances were dissolved to a final concentration of 10^{-6} M (11) in a volume (2 mL) of Krebs-Ringer solution. Periodical extractions of 250 μL were made from the ovary compartment at 30, 60, 120, and 180 minutes. Liquid samples from the ovary compartment were maintained at –20°C until determination of A_2 by radioimmunoassay (RIA) and at –80°C until determination of neurotransmitters by reverse-phase high-pressure liquid chromatography only at 180 minutes' incubation and were expressed as picograms of catechol/mg ovary/mL.

Catecholamine Assays

The catecholamines measured were dopamine, norepinephrine, and their catabolites, as previously described elsewhere (26). Aliquots of 20 μL of liquid from the ovarian cuvette (180 minutes) were partially purified by batch alumina extraction, separated by reverse-phase high-pressure liquid chromatography using a 4.6 × 250 mm XBridge C18 column (Waters, Milford, MA). The quantification was made by current produced upon exposure of the column effluent to oxidizing and then reducing the potentials in series using a triple-electrode system (Coulochem II; ESA, Bedford, MA).

Recovery through the alumina extraction step averaged 70% to 80% for catecholamines. Catechol concentrations in each sample were corrected for recovery of an internal standard dihydroxybenzylamine. The detection limit of the assay was about 15 pg per volume assayed for each catechol. The electrochemical response was linear (r = 0.99) for amounts of norepinephrine from 50 to 2000 pg. The interassay variation coefficients were 14% and 15%, and intra-assay variation coefficient was 10% for norepinephrine. Measurements were performed at all times of study, but only the values at 180 minutes are reported because no statistically significant differences were found at the other times.

Steroid Assay

The A_2 contents were measured in duplicate by RIA and were expressed as picograms of A_2 per milligram of ovarian tissue per mL (pg/mg ovary/mL) against incubation time. Appropriate corrections were made in all cases, taking into consideration the volume extracted in each period. The assay sensitivity was <10 pg A_2/mL. The interassay and intra-assay variation coefficients in all the assays were <10%. These assays have been previously validated (18), and the range was of 1–70 pg/mg tissue/mL.

Ovary Incubation

To confirm the participation of the peripheral nervous system in the ovarian response observed in the integrated system, a comparison was performed with a traditional ovary incubation scheme (6) on D1 and D2. The incubation conditions were similar to those described for the CG-SON-O system (11). This group was considered as the control.

Statistical Analysis

All data are presented as mean ± standard error of the mean in each group. Analysis of variance (ANOVA I) followed by Duncan's multiple range test was used for several comparisons. $P<.05$ was considered statistically significant (27).

RESULTS

Effect of Norepinephrine Addition to CG on Ovarian Release of Norepinephrine, Dopamine, DHPG, and DHPAC

It has been demonstrated in rat brain that short-term accumulation of the dopamine and norepinephrine metabolites provides a good indication of the activity of dopaminergic and noradrenergic neurons (28–30). Even more efficient is the determination of the uptake of dopamine (3,4-dihydroxyphenylacetic acid [DHPAC]/dopamine concentration ratio) and norepinephrine (3,4-dihydroxyphenylglycol [DHPG]/norepinephrine concentration ratio) to estimate rapid changes in neuronal activity.

On D1, in the experimental group, dopamine reached the highest levels in the ovary compartment in relation to the respective control group (26.00 ± 5.9 vs. 57.80 ± 4.0; $P<.001$) (Fig. 1A, I). Its catabolite increased only in the experimental group (96.16 ± 12; $P<.001$) (see Fig. 1A, II), indicating an elevated dopaminergic index of neuronal activity (see Fig. 1A, III).

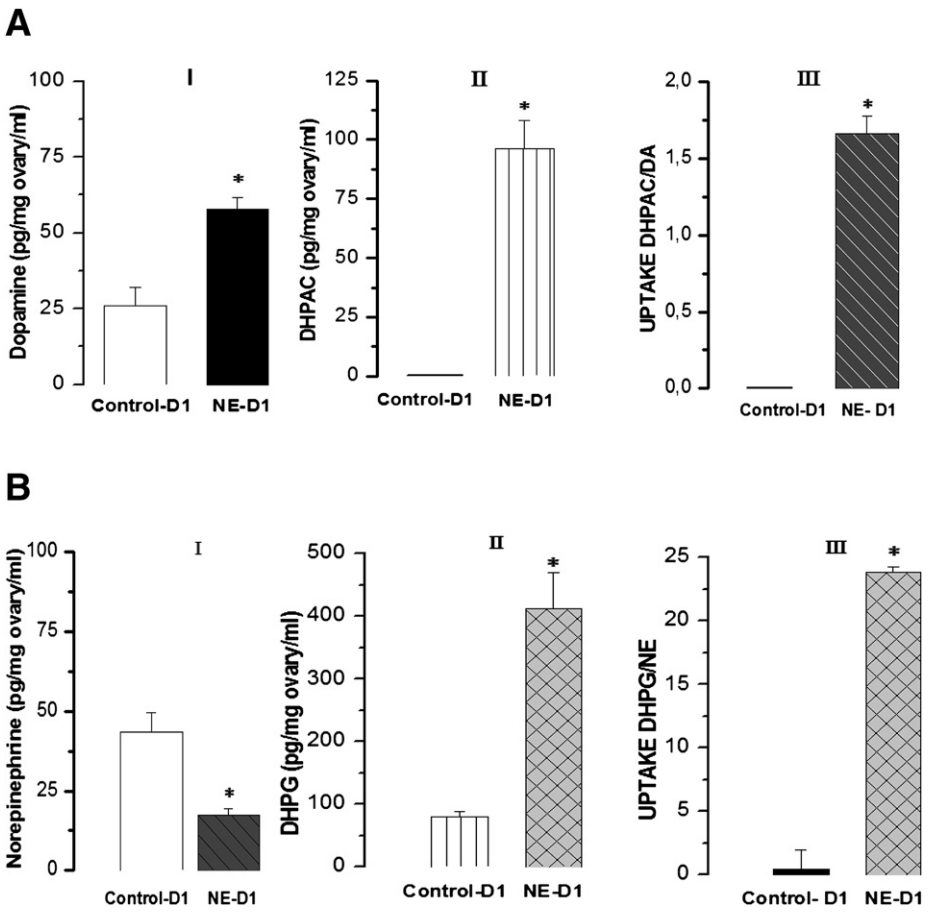
The addition of norepinephrine to the CG diminished norepinephrine release in relation to the control (43.5 ± 6 vs. 17.3 ± 2.1; $P<.001$) (see Fig. 1B, I), while DHPG increased with respect to the control (80.23 ± 9 vs. 411.69 ± 57.89). It is to be noted that this value is the highest found in the study ($P<.001$) (see Fig. 1B, II), which shows that the noradrenergic index of neuronal activity is markedly increased (see Fig. 1B, III).

On D2, the addition of norepinephrine to the CG decreased dopamine release in relation to the control (54.30 ± 0.9 vs. 4.20 ± 1.0; $P<.001$) (Fig. 2A, I), while no changes were observed in its catabolite (see Fig. 2A, II). However, we found that the levels of the index of neuronal activity were increased (see Fig. 2A, III). In the control group, dopamine content in the ovary compartment was lower on D1 as compared with D2 (see Fig. 1A, I vs. Fig. 2A, I).

The presence of norepinephrine in the CG increased ovarian norepinephrine release in relation to its control ($P<.001$) (see Fig. 2B, I), but no statistically significant changes in the level of DHPG was observed (see Fig. 2B, II). It must be noted that under control conditions the level of norepinephrine in the ovary compartment was statistically significantly higher on D1 than on D2 (see Fig. 1B, I vs. Fig. 2B, I). In addition, the noradrenergic activity index was low and decreased with norepinephrine at the ganglionic level (see Fig. 2B, III).

FIGURE 1

Ganglionic effect of adrenergic agents in the coeliac ganglion-superior ovarian nerve-ovary (CG-SON-O) system on (**A**) ovarian dopamine, DHPAC release, and dopamine uptake index; (**B**) ovarian norepinephrine and DHPG release and norepinephrine uptake index on diestrus day 1 (D1). The results are the mean ± standard error of the mean from six animals per experimental group, compared with the control group. DA: dopamine, and DHPAC: 3,4-dihydroxyphenylacetic acid, dopamine catabolite; NE: norepinephrine, and DHPG: 3,4-dihydroxyphenylglycol, norepinephrine catabolite. *$P<.001$ compared with the control group (Student's *t* test and analysis of variance–Duncan multiple range test).



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Effect of the Catecholamines in the Coeliac Ganglion on the Androstenedione Release on Diestrus Days 1 and 2

On D1, norepinephrine in the CG decreased the A_2 release at all the studied times ($P<.001$), while the addition of phentolamine or propranolol increased A_2 in relation to the control ($P<.001$). On D2, norepinephrine in the CG increased the A_2 release in comparison with its control at all the studied times ($P<.001$) (Table 1). On the other hand, A_2 values were not modified by the addition of phentolamine, but they were decreased by addition of propranolol at 60, 120, and 180 minutes ($P<.001$) (see Table 1).

Androstenedione Release in Ovary-Only Incubation and in the CG-NOS-Ovary System

The A_2 levels on D1 and D2 were lower in incubations of the CG-SON-O system under control conditions ($P<.001$) than in the ovary-only incubations at all the studied times (Table 2).

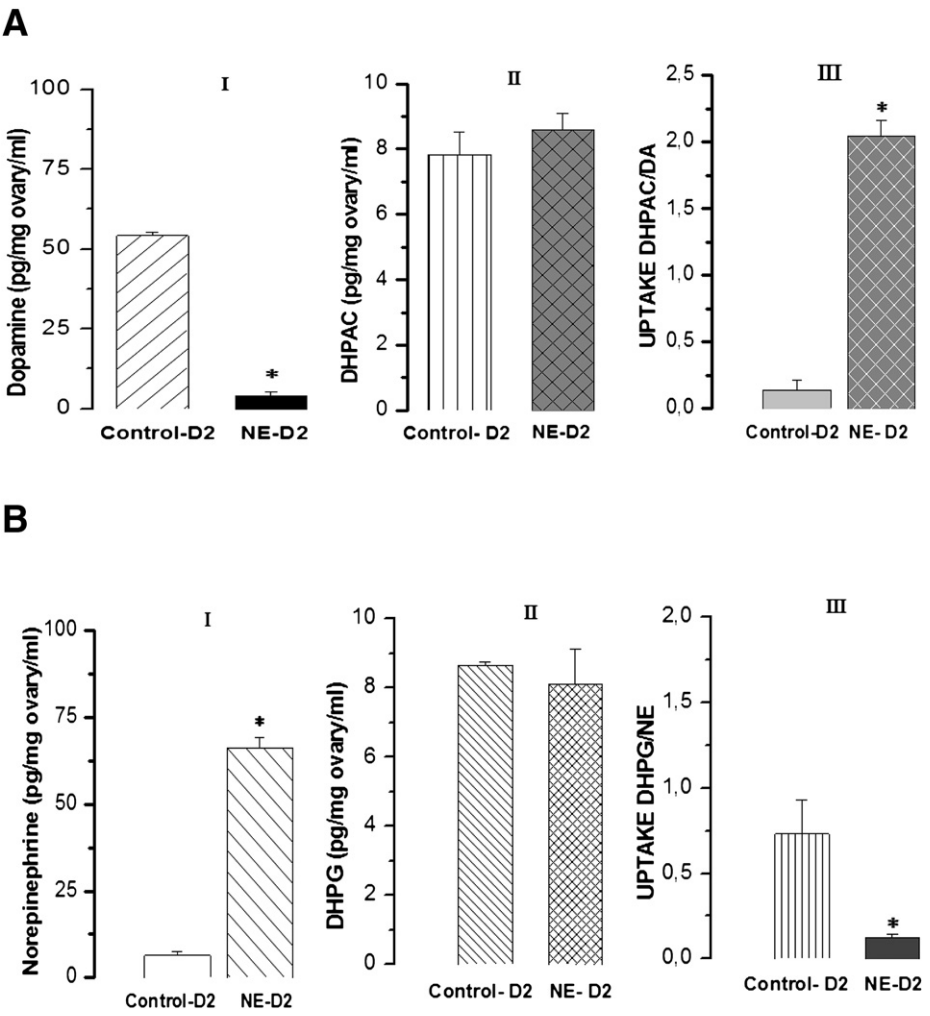
DISCUSSION

Our present study investigated the participation of the catecholamines in the association between peripheral innervation and luteal steroidogenesis. We found that on D1 the addition of norepinephrine to the CG inhibited norepinephrine release, increased dopamine, and also increased both catabolites in the ovary compartment.

The increase of dopamine might be assumed to be the result of the direct action of norepinephrine on the ganglionic receptors, which, through the SON, release neurotransmitters that have an impact on the intraovarian cells, which are capable of synthesizing dopamine (31). Further reasons for dopamine increase might be its liberation through the nervous terminals of the SON or a combination of both phenomena. Thus, our results are in agreement with Mayerhoffer et al. (32), who described the presence of the dopamine-I receptor in the ovary and suggested that dopamine diffuses its action through the follicular space in events associated with the follicular development and/or the regulation of the corpora lutea.

FIGURE 2

Ganglionic effect of norepinephrine in the coeliac ganglion-superior ovarian nerve-ovary (CG-SON-O) system on the (**A**) ovarian dopamine, DHPAC release, and dopamine uptake index; (**B**) ovarian norepinephrine and DHPG release and norepinephrine uptake index on diestrus day 2. The results are the mean ± standard error of the mean from six animals per experimental group, compared with the control group. DA: dopamine, and DHPAC: 3,4-dihydroxyphenylacetic acid, dopamine catabolite; NE: norepinephrine, and DHPG: 3,4-dihydroxyphenylglycol, norepinephrine catabolite. *$P<.001$ compared with the control group (Student's *t* test and analysis of variance–Duncan multiple range test).



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On the other hand, on D2, norepinephrine increased but dopamine decreased, with no variations in their catabolites. This effect on D2 might be related to the transformation of dopamine into norepinephrine at this stage. This is in agreement with Kotwica et al. (33), who postulated that dopamine is the precursor in the biosynthetic pathway of norepinephrine. Thus, concentrations of dopamine within the corpus luteum highly correlate with those of norepinephrine during the estrous cycle, and are higher in the newly formed corpus luteum of D1 than in the developed corpus luteum of D2 or the regressed corpus luteum.

It must be noted that addition of norepinephrine to CG decreased the levels of norepinephrine on D1 but increased them on D2, with very low control levels on D2. This is in agreement with the results reported by investigators using other experimental schemes (23). These results deserve particular analysis. The decrease of norepinephrine in the ovary compartment on D1 might be attributed to an increase in the reuptake and/or metabolization of norepinephrine, as observed by Lara et al. (34).

Taken together, our results obtained indicate that, besides leading to an increase of dopamine in the ovary compartment, the ganglionic noradrenergic stimulus on D1 produces a higher norepinephrine uptake and metabolization index. This suggests that the neural action at the level of the ovary may be mostly exerted by dopamine. Following the same line of analysis for D2, we observed that, in this phase, the neurotransmitter released in the highest proportion via ganglionic noradrenergic stimulation was norepinephrine, and that the dopamine turnover and metabolization index was higher. In other words, the index of neuronal activity on D2 is reversed as compared

TABLE 1

The effect of the catecholamines in the coeliac ganglion on the androstenedione release in rat on dioestrus days 1 and 2.

Group	Incubation time (min)			
	30	60	120	180
Dioestrus 1				
Control CG-SON-O	2.57 ± 0.16	3.39 ± 0.09	2.68 ± 0.13	4.39 ± 0.32
Norepinephrine	0.50 ± 0.09[a]	0.49 ± 0.06[a]	0.38 ± 0.06[a]	0.37 ± 0.03[a]
Phentolamine	13.7 ± 1.67[a]	15.92 ± 1.47[a]	22.94 ± 1.4[a]	13.18 ± 1.29[a]
Propranolol	16.62 ± 0.49[a]	18.64 ± 1.29[a]	22.27 ± 1.96[a]	15.58 ± 2.00[a]
Dioestrus 2				
Control GC-SON-O	3.3 ± 0.3	5.28 ± 0.51	6.84 ± 0.49	5.42 ± 0.31
Norepinephrine	12.48 ± 0.83[a]	19.3 ± 3.08[a]	29.53 ± 3.70[a]	44.77 ± 1.88[a]
Phentolamine	4.08 ± 0.27	5.37 ± 0.78	7.87 ± 0.53	5.26 ± 0.46
Propranolol	3.69 ± 0.42	2.43 ± 0.20[a]	3.54 ± 0.36[a]	2.54 ± 0.50[a]

Note: The results are the mean ± standard error of the mean from six animals per experimental group, compared with the control group. GC-SON-O = coeliac ganglion-superior ovarian nerve-ovary.

[a] $P < .001$, statistically significant, norepinephrine, phentolamine, and propranolol groups compared with GC-SON-O control group on days 1 and 2, respectively (Student's *t* test and analysis of variance–Duncan multiple range test).

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with D1, and, as a consequence, the effect might be exerted by a mainly noradrenergic action, as suggested by Kotwica et al. (35).

Considering the marked difference in the levels of dopamine and norepinephrine on D1 and D2, we explored the relation between the neural stimulus and ovarian steroidogenesis by measuring A_2 liberation. The addition of norepinephrine to the CG produced a marked inhibition of A_2 release on D1, which is in agreement with the dopamine inhibitory action already described by other investigators. On the other hand, A_2 increased on D2, as has been observed in previous experiments (12). The results obtained with our experimental scheme coincide with those of Morimoto et al. (36).

On the basis of these results, the next step was to evaluate the effects of the addition of catecholaminergic antagonists to the ganglion compartment on the release of A_2 on D1 and D2. Phentolamine and propranolol were added to the ganglion compartment without the agonist, based on previously obtained results (11, 12). Both phentolamine and propranolol led to an increase in A_2 release on D1; on D2, phentolamine did not produce changes, and propranolol markedly diminished the A_2 release. These results suggest that the effect of norepinephrine and their antagonists in the CG depends on the estrus cycle stage, which supports the functionality of the system previously described elsewhere (11, 12, 37).

Finally, for confirming the neural influence on A_2 ovarian release, the control levels of A_2 obtained from the ovary compartment of the CG-SON-O were compared with the control levels of A_2 from the ovary incubations under identical experimental conditions. This experiment clearly showed the differences between the incubations of ovaries alone versus ovaries with the nerve and the ganglion. The results showed that the presence of the CG and the nerve led to a decrease in the liberation of A_2 at all the studied times. It has been observed in previous studies that SON dissection leads to modifications in the release of neurotransmitters, which has been interpreted as an indication that different neurotransmitters are released through this nerve to the ovary (38). The inhibitory effect observed on the liberation of A_2 indicates that neurotransmitters with inhibiting capacity are being released from the nervous terminals (11, 20, 37).

There is ample evidence suggesting that the sympathetic nervous system is involved in pathologies associated with the reproductive system. Polycystic ovary syndrome, a common cause

TABLE 2

Androstenedione release in ovary incubation only and in the coeliac ganglion-superior ovarian nerve-ovary (CG-SON-O) system in dioestrus days 1 and 2 under the control conditions.

Ovary control	Incubation time (min)			
	30	60	120	180
Dioestrus 1				
Ovary only	8.75 ± 0.73	9.3 ± 0.48	14.85 ± 1.33	15.88 ± 2.1
Control CG-SON-O	2.57 ± 0.16[a]	3.39 ± 0.09[a]	2.68 ± 0.13[a]	4.39 ± 0.32[a]
Dioestrus 2				
Ovary only	6.2 ± 0.52	8.33 ± 0.34	9.66 ± 0.68	12.43 ± 3.69
Control GC-SON-O	3.3 ± 0.3[a]	5.28 ± 0.51[a]	6.84 ± 0.49[a]	5.42 ± 0.31[a]

Note: The results are the mean ± standard error of the mean from six animals per experimental group, compared with the control group.

[a] $P < .001$, statistically significant, GC-SON-O control group compared with ovary-only incubation on days 1 and 2, respectively (Student's *t* test and analysis of variance–Duncan multiple range test).

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of infertility in women during their reproductive years, is associated with an increased capacity to produce androgens (39). The results of our present study demonstrate that adrenergic activation of the CG has an impact on ovarian androgen production in rats and on the accumulation of catecholamines at the ovary level, thus providing evidence for the participation of peripheral sympathetic nerves in ovarian function under normal conditions and possibly under pathologic conditions such as polycystic ovary syndrome. In this system where the ovarian innervation are intact as well as paracrine and autocrine regulation within the gland, steroidogenesis might be controlled by a balance between stimulatory and inhibitory effects, as the peripheral nervous system modulator in these homeostatic mechanisms, steroidogenesis might be controlled by a balance between stimulatory and inhibitory effects, as the peripheral nervous system modulator in these homeostatic mechanisms.

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