Involvement of the ganglion cholinergic receptors in gonadotropin-releasing hormone, catecholamines, and progesterone release in the rat ovary

Cristina Daneri,[a] Adriana Vega Orozco, Ph.D.,[a] Daniela Bronzi,[a] Claudia Mohn, Ph.D.,[b] Ana M. Rastrilla, Ph.D.,[a] and Zulema Y. Sosa, Ph.D.[a]

[a] Laboratorio de Biología de la Reproducción (LABIR), Graduate Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, San Luis, and [b] Laboratorio de Neuroendocrinología del Centro de Estudios Farmacológico y Botánicos (CEFyBO-CONICET), Graduate Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

Objective: To investigate whether cholinergic ganglionic stimulus modifies the release of gonadotropin-releasing hormone (GnRH), catecholamines, and progesterone at the ovarian level.
Design: Animal study.
Setting: University animal laboratory.
Animal(s): Six to eight virgin adult Holtzman rats.
Intervention(s): Superior mesenteric ganglion–ovarian nerve plexus–ovary system removed and placed in one cuvette with two compartments, with acetylcholine added to the ganglion in the experimental group.
Main Outcome Measure(s): Measurement of ovarian liquid obtained from catecholamines by high-performance liquid chromatography; measurement of progesterone (P_4), GnRH, and luteinizing hormone (LH) by radioimmunoassay; and measurement of gene expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 20α-hydroxysteroid dehydrogenase (20α-HSD) by reverse-transcriptase polymerase chain reaction (RT-PCR).
Result(s): The study focused on the estrus and diestrus II (DII) stages. On the estrus days, the release of GnRH, NA, and 20α-HSD increased, while P_4 and 3β-HSD decreased. On the DII days, GnRH, P_4, and 3β-HSD increased, while 20α-HSD and NA decreased. The ovarian liquid with GnRH showed biologic activity, namely, an increase in LH release during the DII stage and a decrease during the estrus stage.
Conclusion(s): Neural stimulus from the superior mesenteric ganglion influences the release of NA, adrenaline, and GnRH. We also have demonstrated that these neurotransmitters participate in the atretogenic processes of the ovary, thus providing evidence of the necessity of the sympathetic neural pathway. (Fertil Steril® 2013;99:2062–70. ©2013 by American Society for Reproductive Medicine.)
Key Words: Catecholamines, GnRH, ovary, peripheral nervous system, progesterone

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Reprint requests: Zulema Y. Sosa, Ph.D., Laboratorio de Biología de la Reproducción (LABIR), Facultad de Química, Bioquímica y Farmacia. Universidad Nacional de San Luis, Chacabuco 917, (5700) San Luis, Argentina (E-mail: zsosa@unsl.edu.ar).

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Our research group previously has shown that the periepheral nervous system from the prevertebral sympathetic chain through the superior ovarian nerve and ovarian nervous plexus is a nexus of information between the ovary and the central nervous system (1–6). Most of the sympathetic nerves that innervate the ovary are formed by nerve fibers originating from ganglion neurons,

mostly in the celiac-mesenteric ganglia complex. The superior mesenteric ganglion (SMG), which is included in the sympathetic prevertebral chain, possesses a great variety of specific receptors and neurotransmitters, among then catecholamines (6), neuropeptides (7), and acetylcholine, which are considered to be the classic preganglionic neurotransmitters of the sympathetic ganglionic pathway (8–10). In mammals, two main nerve pathways reach the ovary: the superior ovarian nerve (SON), which in rats is associated with the suspensory ligament, and the ovarian nerve plexus (ONP), which accompanies the ovarian artery at its entrance by the ovarian hilum (8). The ONP is composed of sympathetic, sensory fibers and, in minor proportion, of parasympathetic fibers (8, 11). Acetylcholine, neuropeptide Y, substance P, noradrenaline (NA), and nitric oxide (NO) constitute the main postganglionic neurotransmitters released by this neural pathway (5, 9, 10).

The sympathetic system is considered a modulator system, whose principal endogenous ligands are NA and adrenaline. There is evidence that catecholamines can activate adrenergic signals and regulate numerous physiologic processes in the ovary (12). Our group has studied the importance of catecholamines in the control of various ovarian functions studied through the SON (1, 13) and ONP (6) in different reproductive stages in the rat.

It has also been demonstrated that the ONP can diminish the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD), the enzyme that synthesizes progesterone (P_4) (14). In addition, Vega Orozco et al. (6) have confirmed that stimulus of acetylcholine in the SMG modulates the liberation of ovarian steroids on diestrus days.

Immunohistochemical studies in sympathetic ganglia have demonstrated the release of acetylcholine and GnRH-like peptide from the preganglionic fibers (15, 16). The release of GnRH from postganglionic nerve terminals to the ovary has yet to be detected, but variations have been found in the number of receptors for this peptide in the ovary. Also, GnRH-like proteins in ovary of the rat have been demonstrated, albeit with different characteristics from those found at central nervous system level, that have an inhibitory effect on ovarian function (17–19). The presence of GnRH receptors has been found in various ovarian structures, such as the atretic follicles, granulose cells, preantral follicles, and the corpus luteum (19–21). Marchetti and Cioni (20), have postulated that an increase in the activity of these receptors may trigger mechanisms that lead to neuroendocrine reproductive dysfunction in adult female rats. Bauer-Dantoin and Jameson (22) have argued that the GnRH generated at the ovary level induces follicular atresia, and it has been observed that the location of the GnRH receptor and its mRNA expression change significantly during the estrous cycle in rats. These antecedents would indicate that the nervous peripheral system may take part in the regulation of ovarian physiology through the presence of conventional neurotransmitters as NA and adrenaline, and a peptide such as GnRH.

We investigated whether cholinergic ganglionic stimulus would modify at the ovarian level [1] the release of GnRH, demonstrating its biologic activity during estrus (E) and diestrus II (DII) days, and [2] the release of NA and adrenaline in the ovarian compartment in the ex vivo SMG-ONP-ovary system. We also investigated whether [3] neural stimulus modifies the release of P_4 and the expression of ovarian 3β-HSD and 20α-HSD (enzymes that synthesize and degrade P_4) on estrus and DII days.

MATERIALS AND METHODS

Animals

Virgin Holtzman strain adult female rats weighing 250 ± 50 g were used in all the experiments. The rats were maintained under light from 07:00 to 19:00 hours, at a temperature of 24 ± 2°C. The animals had free access to food (Cargill; SACI), and tap water was available ad libitum. Vaginal smears were obtained daily, and only the rats exhibiting at least two 4-day consecutive estrous cycles were used. Groups of six animals were used for the experimental procedure. The experiments were performed in duplicate and according to the procedures approved in the UFAW guidelines (23). The experimental protocol was approved by the University of San Luis Animal Care and Use Committee (protocol number B17/04, ordinance CD 006/02).

Reagents

The following drugs were purchased from Sigma Chemical: L-acetylcholine hydrochloride (acetylcholine), ascorbic acid, and bovine serum albumin fraction V (BSA). The other reagents and chemicals were of analytic grade. We obtained 1,2,6,7-[^{3}H]progesterone (107.0 Ci/mmol) from New England Nuclear Products.

Surgical and Experimental Procedure

The superior mesenteric ganglion–ovarian nerve plexus–ovary (SMG-ONP-O) system removal and the histologic control, characterization, and standardization of incubation conditions were performed as previously described elsewhere (5, 6). The surgical procedure was performed between 15:00 and 16:00 hours, with previous descriptions of the anatomic trajectory of this neural pathway as a guide (5, 6, 8). Each system consisted of the ovaries, the fibers constituting the ONP, parallel to the ovarian artery, and the SMG surrounded by some small ganglia. The total surgical procedure was completed in 1 to 2 minutes. The systems were immediately placed in a cuvette with two separate compartments, each compartment containing 3 mL of work solution, Krebs-Ringer bicarbonate buffer (pH 7.4) in the presence of dextrose (0.1 mg/mL), and BSA (0.1 mg/mL), as previously described elsewhere (1, 5).

The ganglion was placed in one compartment and the ovary in the other, both joined by the ONP, which remained humid in the work solution. The system was preincubated in a metabolic bath at 37°C in a 95% O_2–5% CO_2 atmosphere for 15 minutes to stabilize the system. After 15 minutes of preincubation (incubation time 0), the Krebs-Ringer solution was changed in both compartments, and ascorbic acid (0.1 mg/mL in Krebs-Ringer) was added as an antioxidant agent (1, 5). The P_4 levels measured under these conditions were

considered the control value (control group). For the experimental group, acetylcholine was dissolved in Krebs-Ringer solution plus ascorbic acid at a 10^{-6} M final concentration in the ganglion compartment (5).

Liquid samples (50 μL) from the ovary compartment were collected at 60 and 120 minutes and maintained at −20°C until the P_4 measurement by radioimmunoassay (RIA); 200 μL was collected for measurement of the catecholamines. We also subjected 1,000 μL of the ovary compartment liquid at each of the incubation times to a 10-minute ebullition to inactivate the proteases that degrade to GnRH, and then stored the result at −20°C until the determination of GnRH by RIA. When the system incubation was finished (120 minutes), the ovaries were maintained at −80°C until the determination of 3β-HSD and 20α-HSD gene expression.

Progesterone, LH, and GnRH Assay

The P_4, GnRH, and luteinizing hormone (LH) contents were measured by RIA. The P_4 antiserum was kindly provided by IMBECU (Instituto de Medicina y Biología Experimental de Cuyo). The values of P_4 and GnRH were expressed in ng/mg ovary/mL. The assay sensitivity by P_4 was less than 5 ng/mL. The inter-assay and intra-assay coefficient variations in all the assays were less than 10.0%. The assay sensitivity for GnRH-RIA was 0.2 pg per tube, and the standard curve maintains linearity to 100 pg/tube of GnRH. The interassay and intra-assay coefficients of variation were 9% and 7%, respectively.

We assayed LH in pituitary incubation liquid with kits supplied by the National Pituitary Program, with results expressed as ng LH/mL of liquid in terms of NIAMDD-Rat-RP-I reference preparation respectively. The respective corrections were made in all cases considering the volume extracted in each tested period.

Catecholamines Assay

The catecholamines measured were NA and adrenaline, as previously reported elsewhere (24). Aliquots of 20 μL of liquid from the ovarian cuvette were partially purified by batch alumina extraction, then separated by reverse-phase high-pressure liquid chromatography using a 4.6 × 250 mm Bridge × C18 column (Waters). Recovery through the alumina extraction step averaged 70% to 80% for catecholamines. Catechol concentrations in each sample were corrected for recovery of the internal standard dihydroxy benzylamine. The detection limit of the assay was about 15 pg/volume assayed for each catechol. The electrochemical response was linear ($r = 0.99$) for amounts of NA from 50 to 2,000 pg. The interassay variation coefficients were 14% and 15%, respectively, and intra-assay variation coefficient was 10% for NA and adrenaline.

GnRH Biologic Activity-Incubations of Pituitary

To measure whether the peptides detected via incubation of the ex vivo system presented biologic activity, we incubated pituitary glands. The pituitary glands were obtained by decapitation of adult male rats. Once the glands had been extracted, they were immediately placed in a cuvette containing 2 mL of Krebs-Ringer bicarbonate buffer (pH 7.4) in the presence of dextrose (0.1 mg/ mL) and BSA (0.1 mg/mL). The cuvettes were immediately preincubated in a metabolic bath at 37°C in 95% O_2–5% CO_2 atmosphere for 15 minutes to stabilize the gland. After 15 minutes of preincubation (incubation time 0), the Krebs-Ringer solution was changed. The pituitaries were divided into three experimental groups stimulated with [1] ovarian incubation liquid from the ex vivo system of the control group (control), [2] ovarian compartment liquid from the ex vivo system with acetylcholine added to the ganglions from the estrus days, and [3] ovarian compartment liquid from the ex vivo system with acetylcholine added to the ganglions from the DII days. Each group was incubated for 60 and 180 minutes, and liquid samples were collected and stored at −20°C until the determination of LH concentrations by RIA.

RNA Isolation and RT-PCR Analysis

After the ovaries were defrosted, the total ribonucleic acid (RNA) was extracted using the TRIZOL-Reagent method (Invitrogen Life Technologies), following the manufacturer's instructions for the RNA extraction (25). Two μg of total RNA were reverse transcribed at 37°C using a Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) and random primers in a 26 μL reaction mixture. For amplification of the RT products, the reaction mixture consisted of 10 μl Green Go Taq reaction buffer, 0.2 mM deoxynucleoside triphosphates, 0.5 μM specific oligonucleotide primers, and 1.25 IU Go Taq DNA polymerase (Promega Inc.) in a final volume of 50 μL. Amplification was performed for 35 cycles using 93°C for denaturing (1 minute), 59°C for annealing (1 minute), and 72°C for extension (15 minutes) in an Eppendorf Cycler thermal cycler. Specific primers for 3β-HSD and 20α-HSD were used, and each reaction also included primers to amplify protein β-actin as an internal control as well as the predicted sizes of the products amplified by polymerase chain reaction (PCR) (4). The 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, http://rsb.info.nih.gov/ij/) and were expressed as arbitrary units.

Statistical Analysis

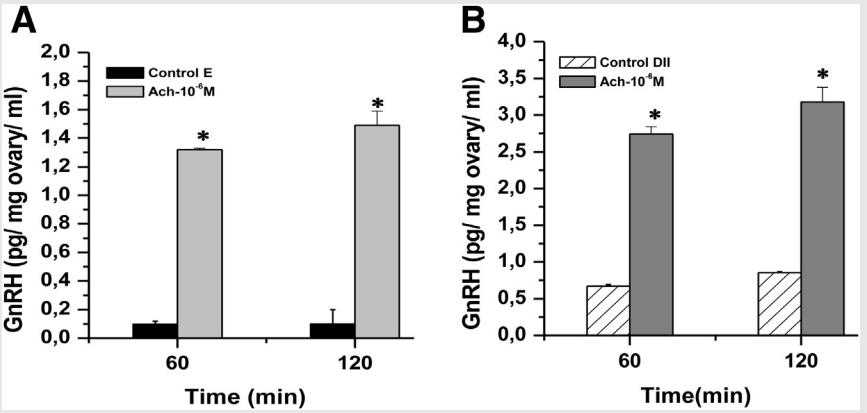
The data are presented as mean ± standard error of the mean (SEM) in each group of six rats. Differences between two groups were analyzed with Student's t test. Analysis of variance (ANOVA I) followed by Duncan's multiple range test was used for several comparisons. $P < .05$ was considered statistically significant (26).

RESULTS

Effect of Acetylcholine on GnRH Release at the Estrus and DII Stages

The addition of acetylcholine to the ganglions statistically significantly increased the GnRH release at 60 and 120

FIGURE 1



Effect of cholinergic agonist in ganglion compartment on ovarian GnRH release in the superior mesenteric ganglion–ovarian nerve plexus–ovary system removed from rats on (A) estrus (E) days and (B) diestrus II (DII) days. Each *bar* represents the mean ± SEM of six animals per experimental group. *$P<.001$ compared with the control group (Student's *t* test; ANOVA, Duncan). Ach = acetylcholine; GnRH = gonadotropin-releasing hormone.

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minutes on estrus and DII days ($P<.001$) in comparison with the control group (Fig. 1A and B), with a greater response found at the DII stage at both times compared with the estrus stage. When comparing the control values with that of the two stages, DII was higher.

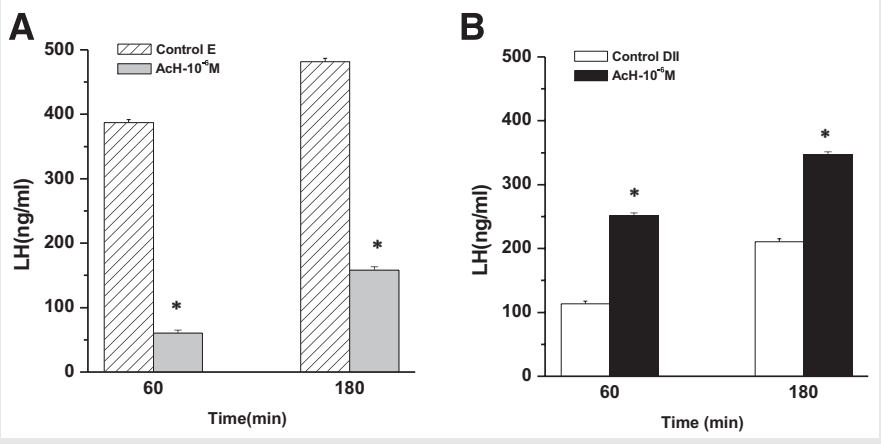
Pituitary Incubation with Liquids Obtained from ex Vivo Systems from the Estrus and DII Stages

To evaluate whether the GnRH-like peptide found in the liquid of the ovarian compartment has biologic activity, we incubated it with the pituitary glands of male rats. The results of the incubations with the ovarian liquid from the estrus days showed a decrease in LH release at 60 and 180 minutes in comparison with the control group ($P<.001$) (Fig. 2A). In contrast, the liquid obtained from the DII stage showed a statistically significant increase in LH release at 60 and 180 minutes in comparison with the control group ($P<.001$) (Fig. 2B).

Effect of Acetylcholine on the Ganglion Release of NA and Adrenaline on Estrus and DII Days

On estrus days, the acetylcholine addition caused a statistically significant increase in the release of NA at 15 minutes, 30 minutes ($P<.05$), and 120 minutes ($P<.001$) (Fig. 3A). The addition of acetylcholine to ganglion did not modify the release of adrenaline in the ovarian compartment at any of the studied times when compared with the controls (see Fig. 3B).

FIGURE 2



The LH release in pituitary incubation with ovarian liquid compartment obtained from the ex vivo superior mesenteric ganglion–ovarian nerve plexus–ovary system on (A) estrus (E) days and (B) diestrus II (DII) days. Each *bar* represents the mean ± SEM of six animals per experimental group. *$P<.001$ compared with the control group (Student's *t* test; ANOVA, Duncan). Ach = acetylcholine; LH = luteotrophic hormone.

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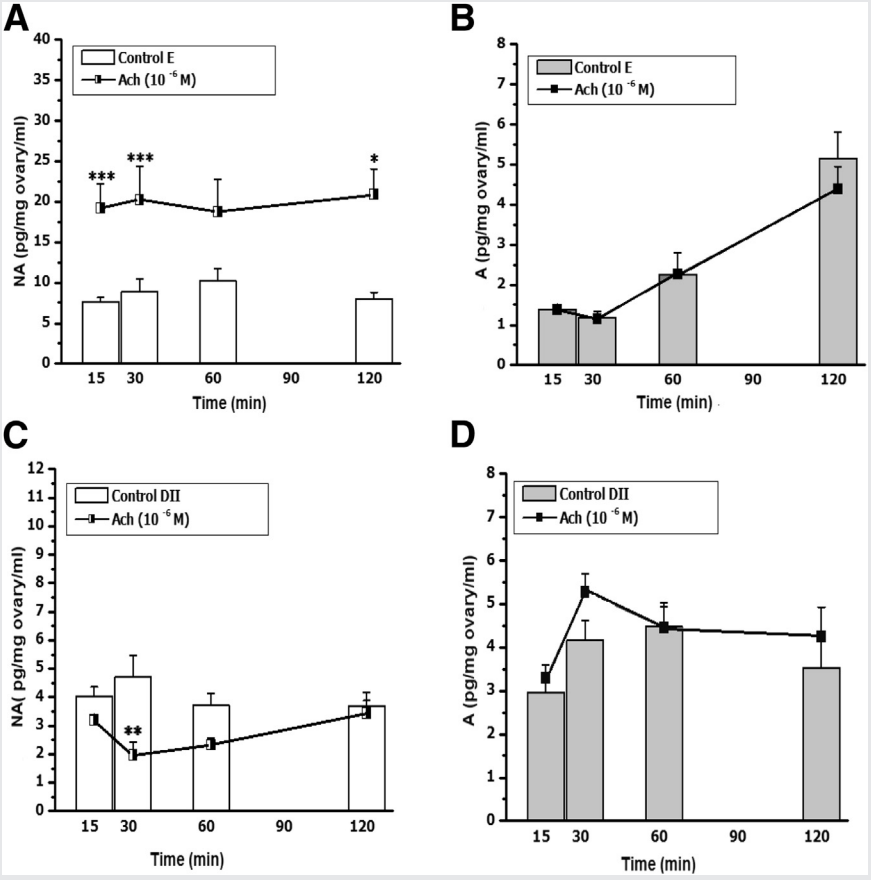
FIGURE 3



Effect of a cholinergic agonist on the superior mesenteric ganglion–ovarian nerve plexus–ovary system at the ovarian level. (A, B) Noradrenaline and adrenaline release on estrus (E) days. (C, D) Noradrenaline and adrenaline release on diestrus II (DII) days. Each *bar* represents the mean ± SEM of six animals per experimental group. *P<.001; **P<.01; ***P<.05 compared with the control group (Student's t test; ANOVA, Duncan). A = adrenaline; Ach = acetylcholine; NA = noradrenaline.

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On DII days, acetylcholine caused a statistically significant decrease in NA release in the ovarian compartment at 30 minutes (P<.01) compared with the control group (see Fig. 3C) but caused no changes in adrenaline release (see Fig. 3D). It is, however, noteworthy that adrenaline was present at the different incubation times in the controls at both stages. In comparison with the control values, NA was statistically significantly higher on estrus days; adrenaline was higher on DII days, except at 120 minutes when the values were lower compared with the estrus days (P<.001) (see Fig. 3A and B).

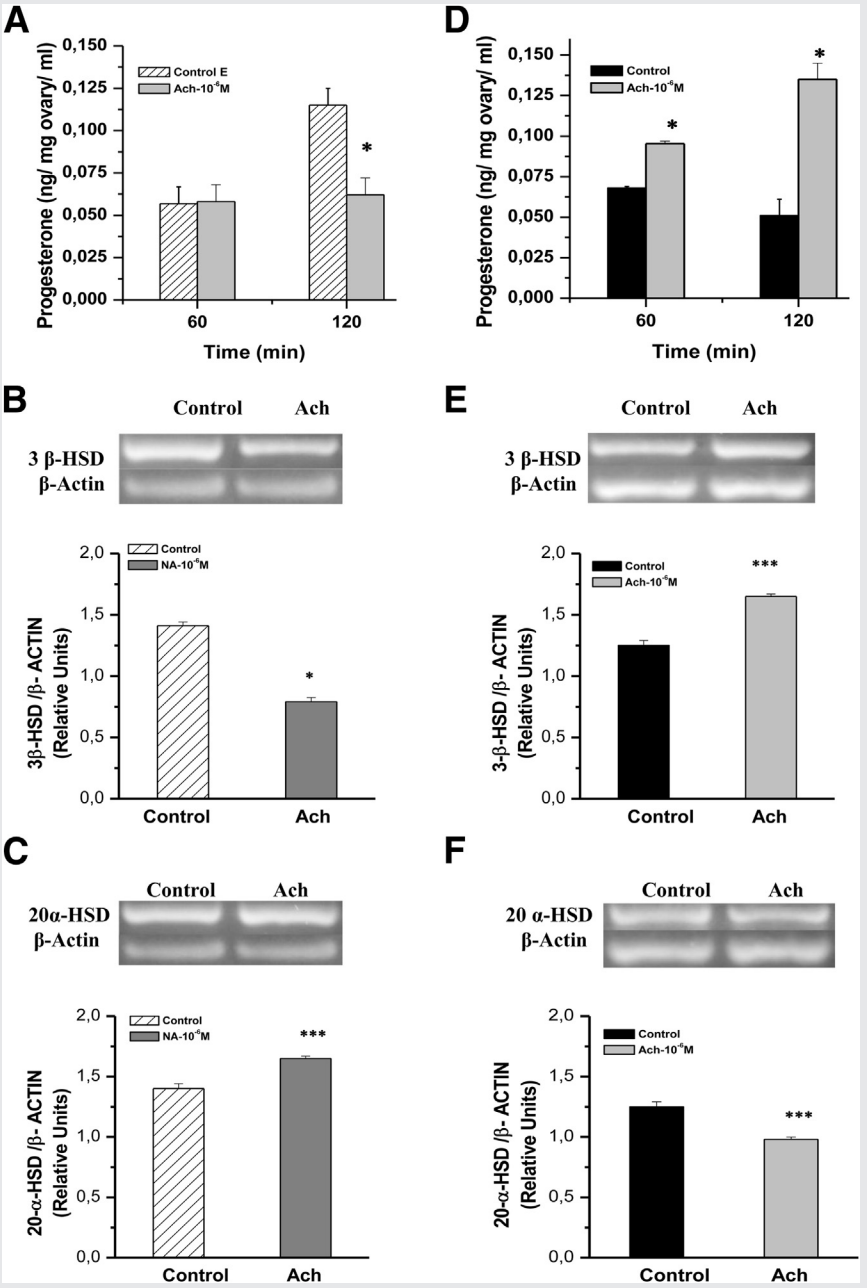
Effect of Acetylcholine on P_4 Release and Ovarian 3β-HSD and 20α-HSD Gene Expression on Estrus and DII Days

Progesterone has proved to be the steroid most sensitive to neural influences in vitro, we determined its level under the influence of acetylcholine. On the estrus days, acetylcholine caused a statistically significant decrease in ovarian P_4 release from ganglion cells at 120 minutes (P<.001) (Fig. 4A) as well as a decrease in ovarian 3β-HSD expression (P<.001) (see Fig. 4B); the ovarian 20α-HSD expression increased (P<.05) (see Fig. 4C). On the DII days, the addition of acetylcholine caused an increase in ovarian P_4 release at both times (P<.001) (see Fig. 4D) and also increased the 3β-HSD gene expression (P<.05) (see Fig. 4E); the expression of the 20α-HSD gene also was inhibited (P<.05) (see Fig. 4F).

DISCUSSION

The SMG is composed of specific structures that respond to cholinergic stimulus, such as the nicotinic and muscarinic receptors in the ganglionic neurons called principal neurons and peptidergic cells (27, 28). The SMG-ONP-ovary system permits in vitro emulation of in vivo conditions, preserving innervation as well as paracrine and autocrine regulation within the gland without humoral influence; in addition, the system possesses its own neural tone (1, 5). Numerous studies have suggested that GnRH is involved in the apoptosis of ovarian granulose cells that occurs during follicular atresia (22, 29), accompanied by an inhibitory effect on ovarian steroidogenesis (22).

FIGURE 4



Effect of cholinergic agonist in the superior mesenteric ganglion-ONP-ovary system on the ovarian. (A–C) Progesterone release, gene expression of 3β-HSD mRNA, and gene expression of 20α-HSD and β-actin, respectively, on estrus (E) days. (D–F) Ovarian progesterone release, gene expression of 3β-HSD mRNA, and gene expression of 20α-HSD mRNA and β-actin on diestrus (DII) days, respectively. Each *bar* of progesterone represents the mean ± SEM of six animals per experimental group. Ach = acetylcholine. *P<.001. The results of the gene expression analyses are expressed as mean ± SEM. ***P<.01. Ethidium bromide fluorescent photograph of the gel electrophoresis of the amplification products. (Student's *t* test; ANOVA, Duncan.)

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The results of our study show that in this system GnRH is actually present in basal conditions and is also expressed via neural stimulus at the ovarian level during estrus and DII days, with significant differences between both stages. These data are in agreement with what has been postulated by Schirman-Hildesheim et al. (30) and Bauer-Dantoin and Jameson (22). The finding that atretic follicles exhibit the greatest degree of GnRH receptor gene expression is consistent with a role for GnRH in the induction of follicular atresia. However, the mechanism that regulates the expression of these GnRH receptors is not known. It remains to be elucidated whether the mechanism that regulates the

expression of these receptors is a direct neural effect or is indirect, as has been postulated by Marchetti and Cioni (20).

In our system, the ganglionic cholinergic stimulation caused an increase in the content of the peptide in comparison with the control in both stages. However, although the liquid obtained during the estrus stage inhibited LH release, the liquid obtained from the DII stage was active on gonadotrophs, thus increasing the release of LH. The GnRH released during the DII stage showed biologic activity, which may indicate that this peptide could be responsible for the fall in the number of receptors at this stage, as posited by Bauer-Dantoin and Jameson (22). On the other hand, it is important to note that one of the neurotransmitters released from the ovarian nerve plexus to the ovary is NA. This is not most important neurotransmitter in this neural pathway, but it has been implicated in ovarian steroidogenesis (1, 2, 6, 31). It has been proposed that under certain physiologic conditions catecholaminergic sympathetic nerves amplify the effect of circulating gonadotropin on the secretion of P_4 in vitro (32). In fact, activation of the sympathetic nerves induced by acute cold stress can induce the morphologic changes in follicles that precede the conditions of polycystic ovaries (33).

In our study, for almost all incubation times we observed that cholinergic ganglionic stimulation during the estrus days increased the presence of NA in the ovarian compartment as compared with the control. Although adrenaline was detected, it did not change with respect to the control; the presence of adrenaline in both the control and experimental groups had not been expected. Fernández-Pardal et al. (34) have demonstrated that the phenylethanolamine-*N*-methyltransferase, an enzyme that catalyses the final reaction of the biosynthesis of adrenaline, is present in the follicular wall, which indicates that catecholamines are involved in the luteinization process. It is interesting that the levels of NA that we found on estrus days are consistent with the increase in the density of catecholaminergic nerve fibers that we also detected at this stage with the histochemical studies (35). Research on this subject has suggested that the effect of catecholamines at the ovarian level is associated with the various cell types to which extrinsic innervation arrives (10, 35). In the DII stage, the addition of acetylcholine to the ganglion cells did not cause any statistically significant changes in the release of ovarian NA (only at 30 minutes) through this neural pathway in comparison with the control group. However, adrenaline was present in the control group, and the adrenaline concentration also was higher at the DII stage. These differential results between the two stages suggest that ovarian sensitivity to catecholamines depends on ovarian physiologic state, the relationship of that state with the hormones that control the cycle, and the neural pathway involved (2).

As regards the release of P_4 after cholinergic ganglionic stimulation, different release patterns in both stages were observed. On estrus days, the release of this steroid decreased in the ovarian compartment in comparison with the control group. These data support the proposal by Bauer-Dantoin and Jameson (22) that follicular atresia in this stage occurs by action of GnRH. Note that although the release of NA is increased at this stage by the cholinergic stimulus, the final effect observed on P_4 release may be due to an increase of ovarian GnRH after ganglionic stimulus. Maruo et al. (36) have demonstrated that GnRH causes a delay of cyclic adenosine 3′:5′ monophosphate (cAMP) in porcine granulosa cells and may be responsible for the inhibition of P_4 production by ovarian cells. The inhibitory actions of GnRH or its agonist on gonad steroidogenesis involve suppression of gonadotropin receptors (37) or intermediary enzymes involved in the steroidogenic pathway such as 3β-HSD (38).

It is necessary to clarify that, besides catecholamines and GnRH, other neurotransmitters such as NO may be liberated from the ovarian nerve plexus by ganglionic stimulus and thus modify P_4 release (6, 39). Importantly, the decrease in the release of ovarian P_4 by neural stimulation coincides with a decrease in ovarian 3β-HSD expression and an increase in 20α-HSD expression at the estrus stage. It is evident that the decreased levels of P_4 on estrus days are largely due to an increase in the expression of ovarian 20α-HSD. In the rat, this enzyme is subject to hormonal regulation (40, 41).

In the DII stage, neural stimulation caused an increase in the release of ovarian P_4 in comparison with the control group. In this case, the increase of ovarian P_4 may be due to the stimulatory effect of GnRH limiting the enzyme activity of ovarian steroidogenesis. Both GnRH and its agonist exert direct inhibitory and stimulatory effects on the ovaries of animals from several species (42–44). Furthermore, it is possible that the observed effect on P_4 levels resulting from neural stimulation may be due to some peptides present in this neural pathway (45, 46). The presence of neuropeptides in this neural pathway is important because it has been demonstrated that sympathetic ganglia have a slow postsynaptic potential, which has the characteristic of being dependent on GnRH but independent of acetylcholine and NA (47). It is likely that this postsynaptic potential with an antiapoptotic effect on the corpus luteum is responsible for the increase in the ovarian P_4 release during the DII stage, because NA decreases at the ovarian level during that period. This effect might also be due to the presence of the gaseous neurotransmitter NO (5, 6). Motta et al. (48) demonstrated in rat that endogenous NO increased the production of P_4 in the corpora lutea in the middle stage of development, thus showing its dual (protective or pro-oxidizing) effect as dependent on the stage of the estrous cycle.

Our results have indicated that the neural stimulus from the SMG influences ovarian physiology, specifically in the presence of GnRH, NA, and adrenaline at the ovarian level. These results demonstrate for the first time the participation of GnRH and NA in the atretogenic processes that occur in the ovary, providing evidence that the sympathetic neural pathway is necessary for these events to occur. This constitutes the first step for future research aimed at elucidating the possible action mechanisms of GnRH and NA.

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