Gonadotropin Stimulation Increases the Expression of Angiotensin-(1–7) and Mas Receptor in the Rat Ovary

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We have previously shown the presence of immunoreactive angiotensin-(1–7) [Ang-(1–7)] in rat ovary homogenate and its stimulatory effect on estradiol and progesterone production in vitro. In the current study, we investigated the presence and cellular distribution of Ang-(1–7) and the Mas receptor, the expression of Mas and angiotensin-converting enzyme 2 (ACE2) messenger RNA (mRNA), and the enzymatic activity in the rat ovary following gonadotropin stimulation in vivo. Immature female Wistar rats (25 days old) were injected subcutaneously (SC) with equine chorionic gonadotropin (eCG, 20 IU in 0.2 mL) or vehicle 48 hours before euthanasia. Tissue distributions of Ang-(1–7), Mas receptor, and ACE2 were evaluated by immunohistochemistry, along with angiotensin II (Ang II) localization, while the mRNA expression levels of Mas receptor and ACE2 were evaluated by real-time polymerase chain reaction (PCR). In addition, we determined the activity of neutral endopeptidase (NEP), prolyl endopeptidase (PEP), and ACE by fluorometric assays. After eCG treatment, we found strong immunoreactivity for Ang-(1–7) and Mas primarily in the theca-interstitial cells, while Ang II appeared in the granulosa but not in the thecal layer. Equine chorionic gonadotropin treatment increased Mas and ACE2 mRNA expression compared with control animals (3.3- and 2.1-fold increase, respectively; $P < .05$*). Angiotensin-converting enzyme and NEP activities were lower, while PEP activity was higher in the eCG-treated rats (*$P < .05$*). These data show gonadotropin-induced changes in the ovarian expression of Ang-(1–7), Mas receptor, and ACE2. These findings suggest that the renin-angiotensin system (RAS) branch formed by ACE2/Ang-(1–7)/Mas, fully expressed in the rat ovary and regulated by gonadotropic hormones, could play a role in the ovarian physiology.*

Key words: Angiotensin-(1–7), Mas receptor, ovary, ACE2 mRNA, equine chorionic gonadotropin.

INTRODUCTION

Folliculogenesis is a continuous process that comprises the growth, development, and differentiation of ovarian follicles until they ovulate or undergo atresia. In addition to the well-established control by gonadotropins and gonadal steroids, an intraovarian regulation of folliculogenesis has been described, in which several peptides and growth factors regulate ovarian functions.[1,2]

Many studies point to an involvement of the ovarian renin-angiotensin system (RAS) as a paracrine/autocrine

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Reproductive Sciences Vol. 16 No. 12 December 2009 1165-1174
DOI. 10.1177/1933719109343309

regulator of follicular development, steroidogenesis, oocyte maturation, ovulation, and atresia. Prorenin, angiotensinogen, angiotensin-converting enzyme (ACE), angiotensin II (Ang II), and Ang II receptors have been identified in the ovary, as reviewed by Yoshimura.[3] In spite of these studies, data regarding the role of Ang II in ovarian physiology are very discrepant. Studies related to ovulation showed that saralasin, a nonspecific antagonist for Ang II type 1 (AT_1) and Ang II type 2 (AT_2) receptors, blocked gonadotropin-induced ovulation in rats[4] and rabbits.[5] Kuji et al[6] demonstrated inhibition of ovulation in rabbits by PD123319, a specific AT_2 blocker, an effect not produced by CV11974, a specific AT_1 blocker. In contrast, captopril, an ACE inhibitor, does not affect ovulation in rats[7] or rabbits.[8] Other studies of RAS and steroidogenesis in ovaries revealed a stimulatory effect of Ang II on estradiol,[9,10] androstenedione,[11] and testosterone production.[12] However, captopril does not have any effect on estradiol and progesterone secretion in rats,[13,14] suggesting the possibility of other RAS peptides involvement in these actions.

Angiotensin-(1–7) [Ang-(1–7)] is a biologically active member of the RAS, formed by an ACE-independent pathway, that may have similar or opposite actions to Ang II.[15] It can be generated from Ang I by neutral endopeptidase (NEP) and prolyl endopeptidase (PEP), or from Ang II by PEP.[16] In addition, it can be inactivated by aminopeptidases (AMPs) or by ACE, generating smaller fragments.[17,18] Recently, an Ang-(1-7)-forming enzyme homologous to ACE was discovered and named ACE2. This carboxyaminopeptidase is insensitive to captopril action and is able to generate Ang-(1-7) directly from Ang II or indirectly from Ang I.[19]

We have previously demonstrated the presence of Ang-(1–7) in the rat ovary and its variation during the estrous cycle.[20] This study also showed that Ang-(1–7) induces estradiol and progesterone production in equine chorionic gonadotropin (eCG)–stimulated rat ovaries perfused in vitro, an effect blocked by A-779, a specific Ang-(1–7) antagonist. Taken together, these data demonstrate an important role of Ang-(1–7) in ovarian physiology. However, at that point, the potential targets for Ang-(1–7) within the ovary remained uncertain and a local receptor specific for this peptide was not discerned.

A number of studies were carried out in search of a specific Ang-(1–7) receptor. A protein belonging to the class of G-protein-coupled receptors with 7 transmembrane domains, coded by the *mas* proto-oncogene, was first shown to be involved in some Ang II actions.[21] Subsequently, Mas messenger RNA (mRNA) expression was verified in several tissues, with abundant expression in the rat testis.[22] More recently, it has been demonstrated that Ang-(1-7) is an endogenous ligand for the Mas protein.[23]

Due to the importance of the new RAS branch formed by Ang-(1–7) and its receptor Mas in many physiological systems[15] and the potential effects of this peptide on ovarian function, the aims of the current study were to (1) determine the topographic distribution of Ang-(1–7), Mas receptor, and ACE2, as well as the mRNA expression levels of Mas and ACE2 in the rat ovary; and (2) investigate whether the ovarian ACE2/Ang-(1–7)/Mas axis can be regulated by gonadotropins.

MATERIALS AND METHODS

Animals

Immature (25 days old) female Wistar rats (n = 46), obtained from CEBIO-UFMG (Belo Horizonte, Brazil), were cared for according to the international guidelines for animal care, and the experimental protocol was approved by the Ethics Committee in Animal Experimentation of Federal University of Minas Gerais. Animals (5 per cage) were maintained under controlled light and temperature conditions (lights on from 05:00-19:00 hours, 23°C ± 3°C) and had free access to tap water and standard rat chow (Nuvital Nutrients Ltd, Colombo, Brazil).

Experimental Protocol

All the experiments were performed between 10:00 and 11:00 a.m. Treated animals (n = 23) received a single subcutaneous (SC) injection of eCG 20 IU in 0.2 mL (Sigma-Aldrich Corp, St Louis, Missouri), while control rats (n = 23) received SC 0.2 mL saline solution. All animals were sacrificed 48 hours later.

For peptides, Mas receptor and ACE2 immunolocalization, we followed a previously described procedure.[24] The animals (n = 7 per group) received 500 IU/100 g heparin sulfate intraperitoneally (IP; Liquemine, Roche, Rio de Janeiro, Brazil), and 15 minutes later, they were anesthetized with IP sodium pentobarbital (30 mg/kg). After laparotomy and thoracotomy, the animals were perfused through the left ventricle with 0.05 mol/L phosphate-buffered saline (PBS) containing protease inhibitors (10^{-5} mol/L phenylmethylsulfonyl fluoride, 10^{-5} mol/L pepstatin A, 10^{-5} mol/L EDTA, 10^{-5} mol/L parahydroxybenzoate, and 9×10^{-4} mol/L orthophenanthroline; all of them were purchased from

Sigma-Aldrich Corp) followed by perfusion with 4% paraformaldehyde for about 10 minutes. The ovaries were removed, cleaned of fat, immersed in 4% paraformaldehyde, and kept for 2 hours in a refrigerator (4°C). Subsequently, they were fixed in Bouin solution for 4 hours at room temperature and were transferred into 70% ethanol that was replaced periodically until the picric acid was removed from the tissue. Then, the ovaries were embedded in paraffin and cut into 4 μm sections, which were mounted on gelatinized slides for immunohistochemistry staining.

The remaining animals were killed by decapitation and their ovaries were immediately removed, quickly frozen in liquid nitrogen, and stored at –80°C until RNA extraction (n = 10 animals per group) or enzyme activity assays (n = 6 animals per group).

Immunohistochemistry Staining

The ovarian sections were processed by the avidin-biotin-peroxidase method as previously described,[25,26] using the Vectastain ABC Kit (Vector Laboratories, Burlingame, California). Briefly, the slides were deparaffinized in xylene and rehydrated in graded alcohols followed by distilled water. Subsequently, they were preincubated in methanol/1% hydrogen peroxide solution for 30 minutes followed by normal goat serum for 30 minutes to block endogenous peroxidase and nonspecific binding, respectively. For Ang II staining, antigen recovery was enhanced by microwave heating for 5 minutes in sodium citrate solution before peroxidase block. Sections were then incubated overnight at 4°C with primary antibodies diluted in PBS containing 1% bovine serum albumin (BSA) as follows: rabbit polyclonal anti-Ang-(1-7) 1:30 000, mouse polyclonal against Mas receptor 1:1000, rabbit polyclonal against ACE2 1:1000 (Abcam Inc, Cambridge, Massachusetts), and rabbit polyclonal anti-Ang II 1:1000 (Peninsula Laboratories Inc, Belmont, California). The anti-Ang-(1-7) and anti-Mas antibodies have been used elsewhere and do not cross-react with other RAS members.[27,28] Later, slides were rinsed with PBS and incubated for 30 minutes with biotinylated anti-rabbit and antimouse immunoglobulin G (IgG) 1:200. Slides were washed with PBS and incubated in the avidin–biotin complex 1:200 for 60 minutes. The immunostaining was visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co, St Louis, Missouri) and counterstained with hematoxylin.

Negative controls were obtained by incubation of the sections with normal rabbit or mouse serum instead of the primary antibody. Additional negative controls for Ang-(1–7) and Ang II were performed by preadsorption of the primary antibody with peptides in excess concentration (10^{-3} mol/L).

RNA Extraction and Reverse Transcription

Pairs of ovaries were thawed and homogenized in phenol-guanidine isothiocyanate (Trizol, Gibco BRL, Gaithersburg, Maryland) and total RNA was extracted by addition of chloroform and centrifugation at 12 000*g*/4°C for 15 minutes. The supernatant was separated by adding isopropanol and centrifuged at 12 000*g*/4°C for 10 minutes, thus obtaining an RNA pellet. RNA samples were washed with 70% ethanol, centrifuged at 9600*g*/4°C for 5 minutes, resuspended in 50-μL diethylpyrocarbonate-treated water (DEPC water), and quantified by spectrophotometry at 260 nm. Reverse transcription was carried out in 30 μL final volume. First strand complementary DNA (cDNA) was synthesized from 1 μg total RNA using the SuperScript First-Strand Kit purchased from Invitrogen (Carlsbad, California). Samples were incubated in 6 μL RT (reverse transcription) First Strand Buffer 5× containing 3 μL hexanucleotides, 3 μL deoxynucleotide triphosphate (dNTP), 1 μL RNAsin, 10 mmol/L dithiothreitol (DDT), and DEPC water 30 μL. After preheating at 70°C for 5 minutes, 1 μL of Moloney Murine Leukemia Virus Reverse (M-MLV; RT enzyme—Invitrogen) was added and samples were incubated at 37°C for 1 hour, 90°C for 5 minutes, and kept on ice for 5 minutes.

Real-Time Polymerase Chain Reaction

The determination of the expression of Mas and ACE2 mRNAs in immature rat ovaries was carried out by real-time polymerase chain reaction (PCR) following a protocol described by Pereira et al,[24] using the fluorescent dye SYBR Green Master Mix (Applied Biosystems, Foster City, California). All reactions were run in duplicate on 96-well optical PCR plates (Applied Biosystems) in a final reaction volume of 25 μL. Specific primers manufactured by Invitrogen (Brazil) are listed in Table 1. Two picomoles of each primer were used. Polymerase chain reaction parameters were 1 cycle at 52°C for 2 minutes, 1 cycle at 95°C for 10 minutes, and 40 cycles at 95°C for 30 seconds and 50°C for 1 minute. Quantity of mRNA was calculated using the ΔΔCt method. For each quantitative PCR (Q-PCR), the threshold cycle (Ct) was determined. Expression levels were normalized to the housekeeping gene *S-26* according to the following

Table 1. Primers of Mas Receptor and Angiotensin-Converting Enzyme 2 (ACE2) for Real-Time Polymerase Chain Reaction (PCR)

Genes	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Length bp
MAS	TTGTGGGCAGCAGTAAGAAG	GATACAGTGTTGCCATTGCC	97
ACE2	TTAAGCCACCTTACGAGCCT	TCCCAGTGACGATCAGGATA	73
S-26	CGTGCTTCCCAAGCTCTATGT	CGATTCCTGACAACCTTGCTATG	102

formula: $\Delta Ct = Ct^{Target} - Ct^{S-26}$. Subsequently, the respective mRNA levels were calculated using the ΔΔCt method, that is, ΔΔCt = ΔCt eCG-treated − ΔCt saline (control) and the relative mRNA levels were calculated as $2^{-\Delta\Delta Ct}$ based on the results of control experiments. Negative control samples were prepared without reverse transcriptase and were run simultaneously.

Determination of Enzymatic Activities of ACE, PEP, and NEP

Ovaries were thawed, weighed, and homogenized in pairs, at 4°C in 0.32 mol/L saccharose solution/50 mmol/L sodium borate (pH 7.4; 1 mL/100 g of tissue). Samples were centrifuged (800*g*/4°C for 10 minutes) and the supernatant was stored at –20°C, until the assays for determination of ACE, PEP, and NEP activities. To normalize the results of enzyme activity assays, protein concentration was measured in all samples using the method described by Lowry et al.[29]

In the determination of ACE activity, we used a fluorometric method previously described by Santos et al,[30] which is the measurement of quantity of the dipeptide His-Leu released from incubation of the sample with the substrate 5 mmol/L hippuryl-1-histidyl-1-leucine (Hip-His-Leu). A total of 10 μL of ovarian homogenate was incubated in 500 μL 0.4 mol/L Hip-His-Leu in borate sodium buffer pH 8.3 for 20 minutes at 37°C. Reaction was interrupted by the addition of 1.2 mL 0.34 mol/L NaOH solution. Then, 100 μL of the fluorescent compound orto-phthaldialdehyde (Sigma-Aldrich Corp., St Louis, Missouri, USA) was added and the fluorescence was determined at 365 nm excitation and 495 nm emission wavelengths.

Prolyl endopeptidase activity was determined by a procedure described by Stanziola et al,[31] with modifications. A total of 10 μL of ovarian homogenate was added to 7.2 mL 20 mmol/L Tris-HCl buffer pH 8.3, with EDTA and ditiothreitol (DTT; Amersham Biosciences, Piscataway, New Jersey) and incubated for 10 minutes at 37°C with the substrate 0.25 mmol/L CBZ-Gly-Pro-MCA (Bachem Bioscience Inc, Pennsylvania). The enzymatic reaction was interrupted by the addition of 1 mL 1 mol/L sodium acetate buffer pH 4.2. Fluorescence was read at 380 nm excitation and 455 nm emission wavelengths.

Neutral endopeptidase activity was measured following the protocol described by Medeiros et al.[32] Briefly, 350 μL 50 mmol/L Tris buffer pH 8.0 was dispensed in a quartz cuvette, followed by 1 μL of the substrate 1 μmol/L Abz-DArg-Arg-Gly-Leu-EDDnp. Then, 10 μL of the sample was pipetted in the solution, and after 120 seconds, the samples were read at 420 nm emission and 320 nm excitation wavelengths.

Statistical Analysis

Data were normally distributed and therefore are expressed as means ± SEM. Differences between the 2 experimental groups were analyzed by unpaired Student *t* test, considering $P < .05$ statistically significant.

RESULTS

Immunolocalization of Ang-(1–7), Mas Receptor, ACE2, and Ang II in Immature and Superovulated Ovaries

In immature unstimulated ovaries, Ang-(1–7) immunostaining was detected in the stromal cells (Figure 1A). On eCG treatment, both antral and preovulatory follicles exhibited intense labeling in theca and interstitial cells (Figure 1E, I). In both groups, positive staining for Ang-(1–7) was observed in oocytes. Absence of Ang-(1–7) immunoreactivity was observed in granulosa cells of both groups.

In immature rat ovaries, immunostaining for Mas was absent or very weakly positive (Figure 1B). In contrast, the ovaries from eCG-treated rats showed intense Mas staining in theca and interstitial cells of antral and

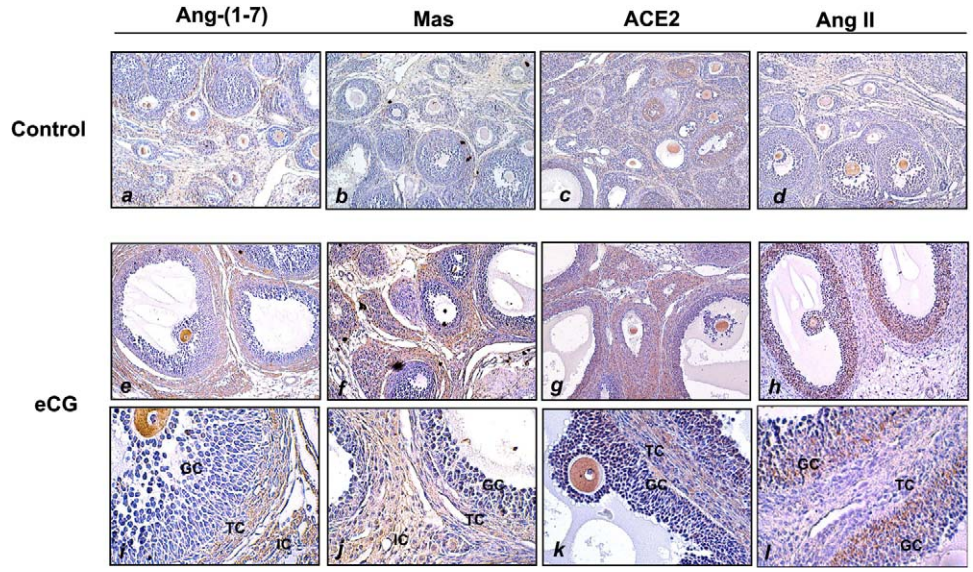

Figure 1. Immunolocalization of angiotensin-(1–7) [Ang-(1–7)], Mas receptor, angiotensin-converting enzyme type 2 (ACE2), and angiotensin II (Ang II) in ovaries of saline (A-D) and equine chorionic gonadotropin (eCG)-treated (E-L) immature rats. Equine chorionic gonadotropin treatment increased immunoreactivity for all of them. Strong immunolabelings for Ang-(1–7) and Mas were present in theca-interstitial cells (IC; E, F, I, J). Ang II immunoreactivity was observed in granulosa cells (GC; H, L). Moderate-to-strong staining for ACE2 can be observed in granulosa (GC) and theca cells (TC; G, K). Note the positive labeling for Ang-(1–7), Ang II, and ACE2 in oocytes and negative for Mas receptor in both immature and eCG-treated ovaries. Original magnification: A-D: ×80; E-L: ×200.

preovulatory follicles. Negative Mas immunoreactivity was observed in granulosa cells (Figure 1F, J) and oocytes.

Angiotensin-converting enzyme 2 immunoreactivity was detected in both, immature and eCG-treated ovaries. In immature ovaries, weak staining was observed in stroma and moderate-to-strong staining was present in granulosa cells from primary and secondary follicles (Figure 1C). In the treated ovaries, we detected strong staining in granulosa, including cumulus cells, inner and outer theca layer, and interstitial cells from antral and preovulatory follicles (Figure 1G, K). In both groups, the oocytes presented positive ACE2 labeling in all slices examined.

Weak immunostaining for Ang II was detected in stroma of immature ovaries (Figure 1D). Ang II immunoreactivity was strongly present in both antral and preovulatory follicles from eCG-treated rats, particularly in mural granulosa cells, contrasting with the lack of staining in the theca cells (Figure 1H, L). Immunoreactivity for Ang II was also observed in oocytes from immature and eCG-treated rats.

Ovarian Expression of mRNA for Mas Receptor and ACE2

All ovarian samples evaluated expressed the mRNAs for both the Mas receptor and the ACE2, whereas no amplification was observed in the PCR runs of samples prepared without reverse transcription (negative controls, not shown).

As shown in Figure 2, expression of mRNA for Mas receptor increased by 3.3-fold, while the expression of mRNA for ACE2 increased by 2.1-fold in the ovaries of eCG-treated rats compared with the control group ($P < .05$).

ACE, PEP, and NEP Activities

Lower activity of ACE was observed in the eCG-treated ovaries (1.62 $\pm$ 0.15 nm/min per mg) compared with control (3.96 $\pm$ 0.65 nm/min per mg, $P < .01$). Equine chorionic gonadotropin treatment also reduced ovarian NEP activity (0.25 $\pm$ 0.02 μmol/L/min per mg) compared with control (0.42 $\pm$ 0.03 μmol/L/min per mg, $P < .01$). However, PEP activity was increased in the eCG-treated rat ovaries (1.59 $\pm$ 0.05 nmol/L/min per mg) compared with controls (1.35 $\pm$ 0.07 nmol/L/min per mg, $P < .05$, Figure 3).

DISCUSSION

The current study shows the topographic distribution of Ang-(1–7) as well as its specific receptor Mas and ACE2

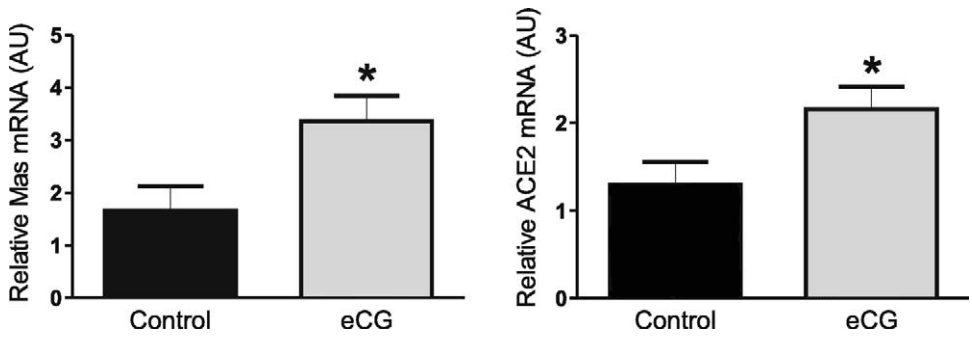

Figure 2. Real-time polymerase chain reaction results of Mas (A) and angiotensin-converting enzyme type 2 (ACE2) (B) messenger RNA (mRNA) expression in ovarian homogenates from saline (control) and equine chorionic gonadotropin (eCG)-treated rats. Data represent the mean ± SEM of 10 animals per group and are expressed in arbitrary units (AU). $*P < .05$ (Student *t* test).

in the rat ovary and the changes induced by eCG stimulation. Mas and ACE2 mRNA expression were quantified and the activities of ACE, PEP, and NEP were also determined. Taken together, these results show that the RAS branch formed by ACE2/Ang-(1–7)/Mas is fully expressed in the rat ovary and is regulated by gonadotropic hormone.

Angiotensin-(1–7) and Mas immunoreactivity are present in the thecal layer of antral follicles and in the theca-interstitial cells of eCG-treated ovaries. Theca-interstitial cells, also known as secondary interstitial cells, are endocrine cells present in abundance in rodent and rabbit ovaries, with some characteristics associated with steroidogenesis, such as lipid droplets in the cytoplasm and high number of mitochondria and endoplasmic reticulum. Carithers and Green[33] demonstrated that the rat theca-interstitial cells are regulated by gonadotropin hormones. The presence of luteinizing hormone (LH) receptors as well as P450scc in these cells and the absence of P450 aromatase reinforce the description of these cells as a source of androgen production in the rat ovary.[34]

The eCG treatment increased the expression of both Ang-(1–7) and Mas in the theca-interstitial cells. The presence of immunoreactivity for ACE2 and mRNA expression in eCG-treated ovaries reinforces the possibility that this enzyme contributed to the local production of Ang-(1–7). The enhancement of ACE2 expression induced by eCG treatment could explain the higher expression of Ang-(1–7) in these ovaries.

Prolyl endopeptidase activity was increased by treatment with eCG, which could also explain, at least in part, the higher expression of Ang-(1–7) in these ovaries. This finding is in accordance with Ohta et al,[35] which demonstrated higher PEP activity in mouse ovaries in the estrous phase compared with diestrus. In this way, we could correlate the high PEP activity in ovaries from the estrous phase and from eCG treatment with an environment rich in estrogen, suggesting a regulation by steroid hormones. In addition, the lower activity of ACE in eCG-treated ovaries would presumably reduce the cleavage of Ang-(1–7) to smaller fragments, thereby contributing to increased Ang-(1-7) levels in gonadotropin-stimulated ovaries. In relation to NEP, another Ang-(1-7)-forming enzyme, we found lower activity in stimulated ovaries, raising the possibility of an absence of action in rat ovary or the participation in the regulation of other ovarian peptides, such as atrial natriuretic peptide. Corroborating our findings, a study demonstrated a decrease in NEP levels in the rat uterus following treatment with estrogen,[36] so we can speculate that the reduced NEP activity in our model could be due to high levels of estrogen characteristic of eCG-treated ovaries.

Another interesting finding of the current study was the positive staining for Ang-(1–7), Ang II, and ACE2 in oocytes, which was negative for the Mas receptor. Other studies have shown immunoreactivity for Ang II and receptors AT_1 and AT_2 in human or porcine oocytes,[37-39] but to our knowledge, it is the first time that the presence of Ang-(1-7) and ACE2 in oocytes is described. The presence of Ang-(1-7) and ACE2 in the oocyte points to a putative role as a product that could act on the surrounding cells as part of the intrafollicular cross talk. Many studies have been demonstrating that oocytes secrete growth factors and proteins, such as growth differentiation factor-9 (GDF-9) and bone morphogenic protein (BMP-15), which participate in the regulation of early follicular development, somatic cell proliferation, and steroidogenesis[40,41], and our findings suggest that Ang-(1–7) may play a similar role. Further evidence required to confirm that the immunoreactivity observed in the oocytes corresponds to locally produced Ang-(1–7) was supplied by the finding of ACE2 in oocytes. However, whether

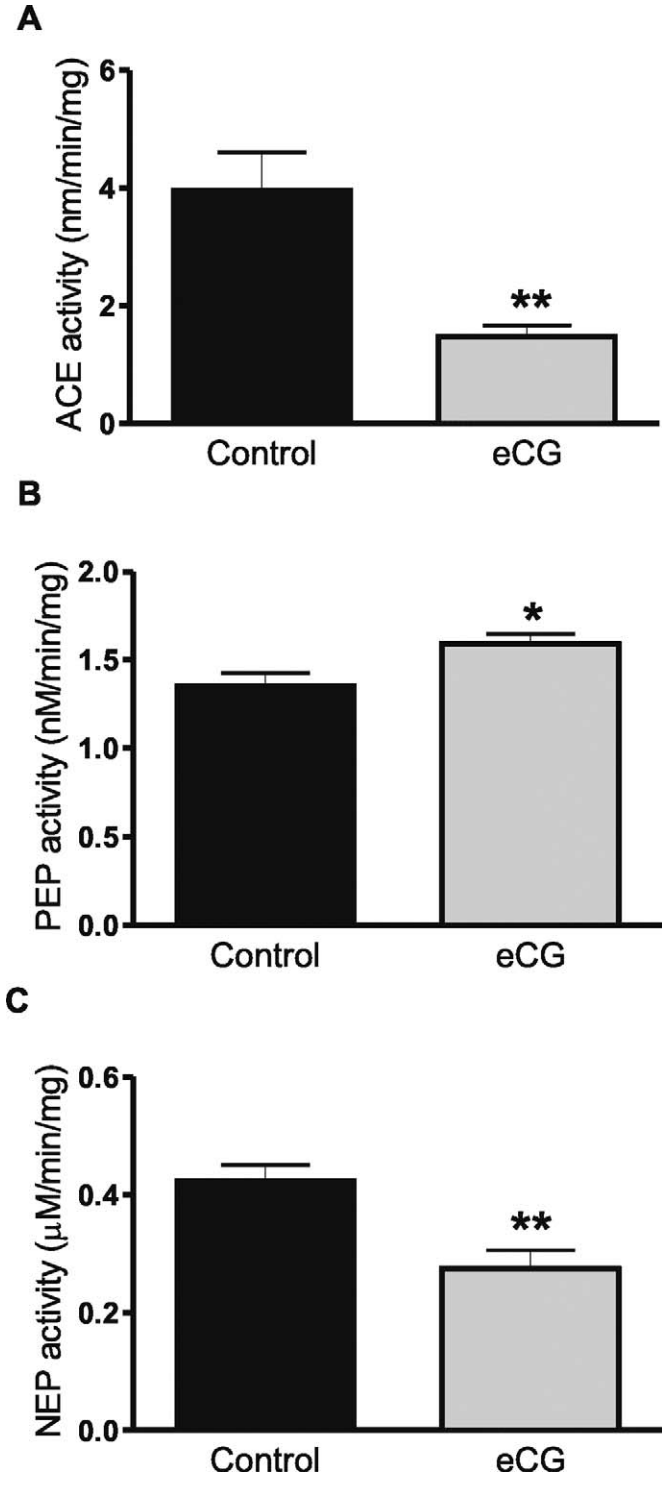

Figure 3. (A) Enzymatic activity of angiotensin-converting enzyme (ACE), (B) prolyl endopeptidase (PEP), and (C) neutral endopeptidase (NEP) in ovarian homogenates from saline (control) and equine chorionic gonadotropin (eCG)-treated rats. Data are reported as the mean ± SEM of 6 animals per group. $\star P < .05$; $\star\star P < .005$ (Student *t* test).

Ang-(1–7) is released and binds to the Mas receptor located in the theca and interstitial cells remains to be determined.

The discrepant findings about the role of Ang II in the ovary[4,7] contribute to our idea that some roles attributed to Ang II could actually be played by Ang-(1–7). In this study, we demonstrated that Ang-(1–7) and its forming enzyme ACE2 are present in the rat ovary. A cautious perfusion of the ovaries was done to exclude the possibility of contamination by peptides from circulation and strengthens the evidence that these peptides are locally produced. It should be noted that granulosa cells of antral and preovulatory follicles were negative for Ang-(1–7). Using a different model, Pepperell et al[42] have shown the presence of Ang-(1-7) in luteal cells, but no effect on progesterone production was observed on exogenous administration of Ang-(1-7). In the current work, we have used immature eCG-treated rats with no corpora lutea. Follicular granulosa cells were rich in Ang II, similarly to that observed by Palumbo et al[43] in granulosa cells of antral and preovulatory follicles of gonadotropin-stimulated human ovaries. However, a recent study showed strong immunoreactivity for Ang II in mid-luteal corpora lutea from unstimulated human ovary, whereas occasional weak staining was observed in follicles of every stage.[37] This suggests an integrated local RAS species-specific system involving different sources, targets, and ovarian status for each active peptide.

Several RAS components have been described in the ovary, including renin,[44] angiotensinogen,[45] ACE,[46] AT_1, and AT_2 receptors.[13] The presence of Ang-(1–7), Mas receptor, and ACE2 completes the panel of RAS members in the ovary and leads to the natural question of how this system works. Figure 4 shows an up-to-date scheme of the possible formation and degradation of the main components of the RAS in the ovary. On the basis of the current findings, showing that Ang-(1–7) and Mas are located in the theca-interstitial cells, while Ang II predominates in the granulosa cells; and considering previous evidence that both peptides are able to stimulate ovarian steroidogenesis,[12,20] it is plausible that the Ang-(1–7)/Mas complex could modulate androgen production in the theca-interstitial compartment, while Ang II promotes the conversion of androgen to estradiol in granulosa cells. We have previously shown that Ang-(1-7) and Ang II had similar effects on estradiol and progesterone production, indicating that some effects attributed to Ang II could be due to its conversion to Ang-(1-7).[20] The Ang-(1–7) antagonist A-779 blocked the progesterone production induced by Ang II but had no effect on the estradiol

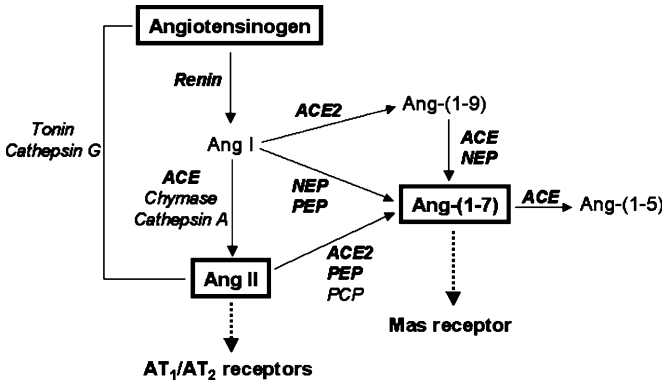

Figure 4. Renin-angiotensin system pathways. Components already described in ovaries are shown in bold and enzymes are in italics. ACE indicates angiotensin-converting enzyme; Ang, angiotensin; AT_1, Ang II type 1 receptor; AT_2, Ang II type 2 receptor; Mas, Ang-(1–7) receptor Mas; NEP, neutral endopeptidase 24.11; PCP, prolyl carboxypeptidase; PEP, prolyl endopeptidase.

production, indicating that Ang II and Ang-(1–7) could play complementary roles on steroidogenesis. Even though we had not analyzed the effect of Ang-(1–7) on the ovulatory process in the current study, we could suppose that the failure of several laboratories to obtain complete control of ovulation, while blocking with saralasin could be accounted for the effect of locally produced Ang-(1–7).

In conclusion, the rat ovary expresses the active peptide Ang-(1–7) and its receptor Mas, which are located mainly in the theca cells of antral follicles and in the ovarian stroma. The comparison between eCG-stimulated and unstimulated immature rats provides evidence for gonadotropin-induced changes in the ovarian expression of Ang-(1–7), Mas, and ACE2 and in the local activity of nonspecific proteases such as ACE, NEP, and PEP. These findings show that the RAS branch formed by ACE2/Ang-(1–7)/Mas is fully expressed in the rat ovary and is regulated by gonadotropin.

ACKNOWLEDGMENTS

This research was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais. VMP was recipient of scholarships from CNPq (PhD) and CAPES-PRODOC (postdoc). We thank Dr Adriana Carmona (INFAR-UNIFESP, São Paulo) for NEP measurements and Solange de Aquino Ferreira for excellent technical assistance.

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