Biological roles of angiotensin II via its type 2 receptor during rat follicle atresia

ERI KOTANI,1 MASATAKA SUGIMOTO,2 HACHIROH KAMATA,2 NOBUHARU FUJII,2 MASAHIRO SAITOH,3 SATOSHI USUKI,1 TAKESHI KUBO,3 KEIFU SONG,3 MIZUO MIYAZAKI,3 KAZUO MURAKAMI,2 AND HITOSHI MIYAZAKI2

1Department of Obstetrics and Gynecology, Institute of Clinical Medicine, University of Tsukuba, Ibaraki 305–8572; and 3Department of Pharmacology, Osaka Medical College, Osaka 569, Japan

Kotani, Eri, Masataka Sugimoto, Hachiroh Kamata, Nobuharu Fujii, Masahiro Saitoh, Satoshi Usuki, Takeshi Kubo, Keifu Song, Mizuo Miyazaki, Kazuo Murakami, and Hitoshi Miyazaki. Biological roles of angiotensin II via its type 2 receptor during rat follicle atresia. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E25–E33, 1999.—Type 1 angiotensin II (ANG II) receptors play crucial roles in the regulation of blood pressure and fluid osmolarity, whereas the physiological roles of type 2 ANG II receptors (AT2) remain unclear. Because AT2 is expressed in atretic follicles where granulosa cells undergo apoptosis, we examined the space and time relationship between AT2 expression and follicle atresia in vivo and the effect of AT2 on follicle-stimulating hormone (FSH) actions in vitro. Binding studies, autoradiography, and RT-PCR of AT2 revealed that the AT2 content in granulosa cells was time dependently increased at both protein and mRNA levels in equine choric gonadotropin-treated immature female rats. This increase paralleled the progression of atresia. ANG II suppressed FSH-induced DNA fragmentation, increases in luteinizing hormone receptor content, and estrogen production through AT2 in cultured granulosa cells. Moreover, FSH-induced stimulation of extracellular signal-regulated kinase activity, critical for cell survival, was inhibited by AT2 stimulation. These results suggest that AT2 mediates the progression of follicle atresia through granulosa cell apoptosis by inhibiting FSH actions.

The granulosa layer and theca interna in ovarian follicles contain ANG II receptors that are predominantly AT2 (12, 18, 27, 29). In addition, human follicular fluid contains angiotensinogen, prorenin, ANG I, and ANG II, the concentration of which is more than 11 times greater than that found in plasma (3, 7). The concentration of rat ovarian ANG II is also 8- to 75-fold higher than that found in plasma and is not reduced in bilaterally nephrectomized rats (12). These data indicate that ANG II is locally produced in the ovary, but the physiological roles of ANG II via AT2 in the ovary have not yet been defined. Several reports suggest that ANG II is involved in the regulation of follicle atresia where granulosa cells undergo apoptosis. Daud et al. (5) first showed that AT2 is exclusively expressed in atretic follicles of adult rats during all stages of the estrous cycle, whereas AT1 is associated with other ovarian structures including blood vessels. Pucell et al. (26) demonstrated that AT2 expression is inhibited in rat cultured granulosa cells by follicle-stimulating hormone (FSH), a crucial factor for survival and differentiation of granulosa cells, whereas the expression of most receptors for hormones and growth factors related to follicle maturation, such as epidermal growth factor, luteinizing hormone (LH), and prolactin, is induced by FSH (9). We recently demonstrated that the AT2 content in cultured granulosa cells increases under conditions in which apoptosis of the cells is observed (30). Kitzman and Hutz (15) indicated that ANG II treatment of hamster follicles cultured in vitro reduces the estradiol-to-androgen ratio and induces morphological changes in the theca interna that are observed in atresia. Mukhopadhyay et al. (20) showed that levels of the renin precursor prorenin in follicular fluid are associated with follicle atresia in the bovine ovary. Regardless of these findings, no concrete evidence has shown that ANG II regulates the onset and/or progression of follicle atresia via AT2. This is because the localization of AT2 and the change in its expression level with the onset and progression of follicle atresia are not clear, and the effect of ANG II via AT2 on the roles of FSH also remains obscure.

Recent reports indicated that FSH activates extracellular signal-regulated kinases (ERK1 and ERK2) in rat and porcine cultured granulosa cells (2, 4). ERKs belong to the mitogen-activated protein kinases (MAPKs), which are activated in response to a variety of stimuli involved in cell proliferation, survival, and differentiation (17). For example, withdrawal of nerve growth factor (NGF), a survival and differentiation
factor for neural cells, from rat PC12 pheochromocytoma cells led to inhibition of ERKs, resulting in apoptosis of these cells (31). Also, experiments with PC12 cells suggested that the difference in the duration of ERK activation may be critical for decisions about proliferation vs. differentiation (17). Therefore, the effect of ANG II via AT$_2$ on FSH-induced ERK activation must be very important for determining the fate of granulosa cells.

Moderate doses of equine chorionic gonadotropin (eCG) stimulate the growth and development of ovarian follicles for 2–3 days, after which the follicles undergo atresia because of waning levels of trophic support caused by metabolism of the gonadotropin (24). This eCG-induced model shows follicle atresia morphologically identical to that occurring in untreated adult animals (23). In the present study, we examined AT$_2$ expression at both the mRNA and protein levels during the onset and progression of atresia in vivo with this rat model. Furthermore, we studied the effect of ANG II on the abilities of FSH to prevent apoptosis and to differentiate ovarian granulosa cells and to activate ERKs in vitro prepared from immature female rats treated with diethylstilbestrol (DES) or eCG.

**MATERIALS AND METHODS**

Reagents. Ovine FSH was kindly provided by the National Institutes of Health (Bethesda, MD). This compound (NIH-FSH) is also known as NIDDK-oFSH-15 or AFP-5529C. The potency of NIH-FSH is 20 times greater than that of NIH-FSH-S1. By weight, the LH potency of NIH-FSH is 0.04 times that of NIH-LH-S1, and that of prolactin is less than 0.001 times that of NIH-PRL. eCG, DES, Hanks’ balanced salt solution (HBSS), and myelin basic protein were purchased from Sigma (St. Louis, MO). Modified McCoy’s 5A medium (pH 7.4) was prepared with McCoy’s 5A (GIBCO Life Technologies) supplemented with 25 mM HEPES, 26 mM NaHCO$_3$, 2 mM L-glutamine (Sigma), 100 µg/ml streptomycin, and 100 IU/ml penicillin (GIBCO Life Technologies). ANG II, leupeptin, and antipain were purchased from the Peptide Institute (Osaka, J andan). The nonpeptide antagonists DuP-753 and PD-123319 were provided by Dr. R. D. Smith (DuPONT Merck Pharmaceutical, Wilmington DE) and Dr. J. A. Keiser (Warner-Lambert, Ann Arbor, MI), respectively. The selective AT$_2$ ligand CGP-42112B was provided by Dr. Marc de Gasparo (Ciba-Geigy, Basel, Switzerland). The radioligands [$^3$H]cAMP, [$^3$H]ATP, and 125I-[Sar$^1$,Ile$^8$]ANG II were obtained from Amersham (Bucks, UK) and 125I-human choriionic gonadotropin (125I-hCG) was obtained from New England Nuclear (Boston, MA).

Hormone administration. Immature female Wistar-Imaiichi rats obtained from the Imamichi Institute for Animal Reproduction (Ibaraki, J apan) were housed in air-conditioned quarters with a 12:12-h light-dark cycle. For in vivo experiments, 23-day-old rats were subcutaneously injected with 20 IU of eCG for 3–6 days. The rats were killed by decapitation under mild ether anesthesia on each of days 3-6 after injection, and the ovaries were removed. For autoradiographic studies, ovaries were snap-frozen in a dry ice-isopentane bath and stored at −80°C. For in situ 3'-end labeling analysis, ovaries were immersion-fixed in 10% buffered formalin for 7 days at room temperature. Granulosa cells were prepared as described by Knecht et al. (16) to extract total RNA and for binding studies. For in vitro experiments, one set of rats was implanted subcutaneously with Silastic 10-mm capsules containing DES at 21 days of age to stimulate granulosa cell proliferation. The other set of animals (23 days old) was subcutaneously injected with 20IU of eCG to induce preovulatory follicles. Five days after DES implantation and 48 h after eCG injection, the animals were anesthetized with ether and decapitated, and then the ovaries were removed.

Preparation of granulosa cells. Granulosa cells were prepared according to the method of Knecht et al. (16). Briefly, after the intercellular gap junctions were disrupted with 6.8 mM EGTA and 0.5 M sucrose, granulosa cells were released by puncturing the ovaries with a 27-gauge needle and pressing the remaining cells through a 40-mesh stainless steel grid. Thereafter, cells were washed and pelleted by gentle centrifugation (200 g, 5 min, room temperature) and then snap-frozen in liquid nitrogen at −80°C until DNA and total RNA extraction. The remaining cells were resuspended in McCoy’s 5A medium in 12 × 75-mm polystyrene tubes and were cultured in an incubator at 37°C with a humidified gas mixture of 5% CO$_2$-95% room air for 48 h with or without various additives.

In subsequent experiments of MAPK activity, the granulosa cell cultures were plated for 48 h in 10% fetal calf serum and cultured for an additional day in serum-free media.

In situ 3’-end labeling. Ovaries were paraffin-embedded, sectioned at 8-µm thickness from the center, and mounted on microscope slides. The sections were deparaffinized by heating for 30 min at 60°C followed by two 5-min washes in xylene and were rehydrated through a graded ethanol series and double-distilled water. Protein was removed from tissues by an incubation with 20 µg/ml proteinase K (Sigma) for 15 min at room temperature; then endogenous peroxidase was inactivated by immersion in 3% H$_2$O$_2$ in ethanol for 5 min. DNA was labeled at 3’-ends with biotinylated 2’-deoxyuridine 5’-triphosphate biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany) by an incubation with reaction buffer consisting of 300 mM Tris·HCl (pH 7.4), 140 mM (CH$_3$)$_2$AsO$_3$Na, and 1 mM CoCl$_2$ containing terminal deoxynucleotidyl transferase for 30 min at 37°C. The sections were incubated for 1 h at 37°C in Vectastain avidin-biotin complex-peroxidase standard solution (Vector Laboratories, Burlingame, CA). Diaminobenzidine (Dojin, Kumamoto, J apan), a substrate for peroxidase, was applied to the slides for the color reaction, and then sections were stained with hematoxylin and eosin. Negative controls were sections incubated with reaction mixtures in the absence of terminal deoxynucleotidyl transferase.

ANG II receptor autoradiography. Ovarian sections (20 µm) were cut in a cryostat, attached to gelatinized slides (Wako Pure Chemical, Osaka, J apan), air-dried at 4°C, and stored at −80°C. Sections were incubated for 15 min at room temperature in incubation buffer containing 0.2% BSA and 0.5 mg/ml bacitracin (Nakarai Tesque, Kyoto, J apan) and were placed for 1 h at room temperature in incubation buffer containing 0.5 nM 125I-[Sar$^1$,Ile$^8$]ANG II, 0.2% BSA, and 0.5 mg/ml bacitracin with or without several agonists and antagonists. The sections were then serially rinsed in ice-cold wash buffer (10 mM Na$_2$HPO$_4$/150 mM NaCl/5 mM EDTA/0.02% NaN$_3$) four times and dried under a stream of cold air for 1 h. Slides were exposed to X-ray film (Eastman Kodak, Rochester, NY) for 5 to 6 days and then stained with hematoxylin and eosin.

DNA isolation and analysis. Low-molecular-weight DNA was extracted from granulosa cells isolated from the ovaries of rats killed on each of days 3-6 after eCG injection or from cultured granulosa cells. Cells were gently homogenized in a 300 mM Tris·HCl (pH 7.4) containing 100 mM NaCl, 10 mM EDTA, and 200 mM sucrose, and then incubated in 0.5% SDS at
65°C for 30 min to facilitate membrane and protein disruption. The samples were cooled on ice for 60 min in 1 M potassium acetate to precipitate protein and were clarified by centrifugation at 5,000 g for 10 min at 4°C. The supernatant was then extracted with phenol-chloroform, precipitated in ethanol, and resuspended in water. Thereafter, DNA samples (4 µg) were resolved through 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

Quantitative RT-PCR analysis of AT₂ mRNA. Total RNA (1.2 µg) isolated from granulosa cells was reverse transcribed with random primers. The resultant cDNA mixtures were amplified by PCR to selectively detect AT₂ mRNA in the presence of a known amount of deletion-mutated AT₂ cDNA with a trace amount of [α-32P]dCTP with the following primers (6): 5'-TATGCTCATGGTCTGGCT-3' (sense primer, nucleotides 610–627 of a cDNA for rat AT2) and 5'-CCACTAA-CAGATTTAAGACAC-3' (antisense primer, nucleotides 1,084–1,104) (14, 21). Denaturation, annealing, and the polymerase reaction proceeded at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 1.5 min, respectively. After 20 cycles of amplification, the incubation was continued at 72°C for another 8.5 min to complete polymerization. Native AT₂ cDNA produces a 495-bp fragment, whereas deletion-mutated AT₂ cDNA generates a fragment of 425 bp. The PCR products were size-fractionated on 5% acrylamide gels. The gels were dried and examined with a BAS-2000 imaging analyzer (Fuji Film, Tokyo, J apan). The radioactivity of the PCR product obtained from the target cDNA was divided by that from the deletion-mutated cDNA. The resultant values were normalized to the amount of β-actin mRNA, which was also determined in the same method as AT₂ mRNA at the same time, for correcting variations in RNA loading and RT-PCR efficiency.

Binding of 125I-[Sar¹,Ile⁸]ANG II. Cells (1 × 10⁶ cells/tube) were immersed in acid buffer (50 mM glycine-150 mM NaCl, pH 3.0) on ice for 10 min, washed twice with HBBS, incubated with 4 mM DTT at 22°C for 15 min, and then reacted with 125I-[Sar¹,Ile⁸]ANG II at a final concentration of 50 pM in 200 µl of binding buffer consisting of HBBS containing 0.2% crystallized BSA, 1 mM phenylmethylsulfonyl fluoride, 50 mM leupeptin, and 25 µg/ml antipain at 4°C for 2 h. Nonspecific binding was determined by incubating with 1 µM unlabeled [Sar¹,Ile⁸]ANG II. Free and bound radioligands were separated by centrifugation, and then radioactivity was assayed by γ-scintillation counting.

Binding of 125I-hCG. After culture, cells (1 × 10⁶ cells/tube) were immersed in acid buffer, washed twice with HBBS, and then reacted with 125I-hCG at a final concentration of 10 ng/ml in 200 µl of binding buffer at 22°C for 17 h. Nonspecific binding was determined by incubating the cells with 25 µg/ml unlabeled hCG. Free and bound radioligands were separated by centrifugation; then radioactivity was assayed by γ-scintillation counting.

Measurement of estradiol-17β. Estradiol-17β was measured with an enzyme immunoassay (EIA) kit (Cayman Chemical, MI). The cross-reactivity of the antiserum used in the estradiol-17β assay was 100% for estradiol-17β, 7.5% for estrone, 0.3% for estriol, 0.1% for testosterone and dihydrotestosterone-5α, and <0.01% for C₂₁ and other C₁₉ and C₁₈ steroids. The minimum detectable amount of the steroid assay was 9.2 pg/ml. Intra- and interassay coefficients of variation were <10%.

MAPK activity. Control and various additive-treated granulosa cell cultures were rinsed with three changes of ice-cold phosphate-buffered saline (pH 7.4) and immediately scraped on ice in lysis buffer [20 mM HEPES, pH 7.2, 25 mM NaCl, 2 mM EGTA, 1 mM EDTA, 0.2 mM DTT, 0.1% Triton X-100, 50 mM NaF, 1 mM Na₃VO₄, 25 mM β-glycerophosphate, and protease inhibitor mixture tablet (Boehringer Mannheim)]. Cell lysates were centrifuged at 12,000 g for 10 min at 4°C. Supernatants were removed, and protein concentrations were assayed with the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). MAPK activity within the supernatant was analyzed with the in-gel kinase method. Cell extracts (25 µg total protein/ lane) were separated by 10% SDS-polyacrylamide gel electrophoresis with 0.5 µg/ml of myelin basic protein copolymerized in the running gel. After electrophoresis, the gel was washed twice for 30 min with 50 mM Tris (pH 8.0) and 20% isopropanol alcohol followed by two changes of a buffer containing 50 mM Tris (pH 8.0) and 5 mM β-mercaptoethanol (buffer 1). The gel was then treated with 6 M guanidine HCl in buffer 1 for 1 h to denature the proteins. The kinases in the gel were renatured with buffer 1 containing 0.04% Tween 40 for 16 h at 4°C, and then incubated with 40 mM HEPES buffer (pH 7.5) containing 2 mM DTT, 0.1 mM EGTA, and 20 mM MgCl₂ for 1 h. The kinase assay was performed by layering 10 µl of the same buffer containing 25 µCi of [γ-32P]ATP onto the renatured gel followed by incubation at 25°C for 1 h. Thereafter, the gel was washed with 5% trichloroacetic acid and 1% sodium pyrophosphate to remove unreacted [γ-32P]ATP. Thereafter, the gel was dried and examined with a BAS-2000 imaging analyzer.

Data analysis. Data are presented as means ± SE of several independent experiments. The effects of various treatments on different culture groups were compared by one-way ANOVA, followed by the post hoc Student-Newman-Keuls test. A difference of P < 0.05 was considered significant.

RESULTS

Effects of eCG on ovarian morphology and DNA fragmentation. The apoptotic death of granulosa cells is the molecular mechanism underlying follicle atresia (10). We initially examined the time course of the effect of eCG injection on ovarian DNA degradation, a characterist of apoptosis, by treating immature female rats with 20 IU eCG at 23 days of age. The electrophoretic profile of low-molecular-weight DNA fragments obtained from granulosa cell lysates on each of days 3–6 after eCG injection is shown in Fig. 1. Granulosa cells obtained 3 days postinjection lacked signals indicating low-molecular-weight DNA. However, light fragments were discernible on day 4. On days 5 and 6, oligonucleosomal length DNA fragments formed an obvious ladder.

To study the specific ovarian cell types exhibiting apoptotic DNA degradation after eCG injection, DNA on histological sections of ovaries was 3'-end labeled with biotin-16-dUTP as shown in Fig. 2. Incorporation of biotin-16-dUTP after the terminal transferase reaction was detected with avidin conjugated to peroxidase. In ovaries obtained 4 but not 3 days after eCG injection, low levels of DNA were 3'-end labeled in granulosa cells in a small population of atretic follicles. In contrast, in ovaries obtained on days 5 and 6, biotin-16-dUTP was incorporated into the granulosa cell layer of many atretic follicles. Although oocytes were also labeled in some follicles, theca cells were not labeled in the same follicles where granulosa cells were heavily labeled. In follicles with antral cavities, antral granulosa cells

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were frequently labeled more heavily than mural granulosa cells. In some of these follicles, the antral cavity was also positive, probably because of the presence of cell-free DNA fragments.

Effects of eCG on AT₂ content at the protein and mRNA levels. To investigate the levels of AT₂ during the onset and progression of atresia, granulosa cells were obtained from rats on each of days 3-6 after eCG injection. To specifically detect AT₂, cells were exposed to DTT, which considerably decreases the ligand binding activity of AT₁ and enhances that of AT₂ without affecting antagonist selectivity (18). Figure 3 shows that the binding of 125I-[Sar₁,Ile₈]ANG II, a subtype-nonselective antagonist, was noticeably and time dependently increased 3–6 days after eCG injection.

Because follicles containing AT₂ in the adult rat ovary are mainly atretic throughout the estrous cycle (5), we studied the cellular localization of AT₂ during atresia by autoradiography of 125I-[Sar₂,Ile₈]ANG II binding. To investigate the possibility of heterogeneous ANG II receptor types localizing in the ovaries of rats injected with eCG, we used the selective nonpeptide ANG II receptor antagonists DuP-753 for AT₁ and PD-123319 for AT₂. Figure 4, top, shows that ANG II receptors in granulosa cells were AT₂, whereas those on other ovarian structures were AT₁. As indicated in Fig. 4, bottom, the radioligand binding localizing in the granulosa cell layer was markedly and time dependently increased from days 3 to 6.

To determine the levels of AT₂ mRNA during follicle atresia, total RNA was extracted from ovaries on each
of days 3-6 after eCG injection and analyzed by reverse transcription (RT)-PCR. Figure 5 shows that the AT2 mRNA level increased from days 3 to 6 in a time-dependent fashion. Therefore, the time course of the increased AT2 content at both protein and mRNA levels agrees well with that of the progression of follicle atresia, suggesting a close relationship between AT2 and atresia.

Effects of ANG II on FSH actions in cultured granulosa cells. To determine the biological roles of ANG II via AT2 in follicle atresia, we studied the effects of ANG II on the actions of FSH in cultured granulosa cells, because FSH is a crucial differentiation and survival factor for these cells. We focused on the abilities of FSH to prevent apoptosis (10), to induce the LH receptor expression (9), and to enhance steroidogenesis (9).

As shown in Fig. 6, DNA prepared from rats 2 days after eCG injection was cleaved in serum-free media during a 48-h culture. ANG II did not affect DNA fragmentation. Although FSH decreased apoptotic DNA fragmentation compared with untreated controls, this decrease was prevented by ANG II.

Figure 7 shows the effect of ANG II on 125I-hCG binding to the LH receptor of granulosa cells prepared from DES-treated rats. After a 48-h incubation, FSH caused an 8.5-fold increase in 125I-hCG binding compared with untreated controls (P < 0.05). In contrast, ANG II inhibited the FSH-stimulated binding of 125I-

Fig. 3. 125I-[Sar1,Ile8]ANG II binding to AT2 in granulosa cells obtained on days 3-6 after injection with 20 IU eCG. Binding of 125I-[Sar1,Ile8]ANG II was assayed in granulosa cells isolated from ovaries without culture as described in MATERIALS AND METHODS. Data are means ± SE (vertical bars) of 3 independent experiments, each performed in duplicate.

Fig. 4. Binding sites for 125I-[Sar1,Ile8]ANG II in ovaries obtained on days 3-6 after injection with 20 IU eCG. Ovarian sections (20 µm) were prepared as described in MATERIALS AND METHODS. A–D: autoradiograms of 4 adjacent ovarian sections 6 days after injection of eCG. A, total 125I-[Sar1,Ile8]ANG II binding; B, 125I-[Sar1,Ile8]ANG II binding in presence of PD-123319 (5 µM) to selectively block AT1; C, 125I-[Sar1,Ile8]ANG II binding in presence of DuP-753 (5 µM) to selectively block AT2; D, nonspecific 125I-[Sar1,Ile8]ANG II binding in presence of ANG II. Nonspecific radioligand binding was negligible. E–H: autoradiograms of ovaries obtained 3–6 days after injection with 20 IU eCG. E, F, G, and H represent postinjection days 3, 4, 5, and 6, respectively.
hCG by 48% (P < 0.05). ANG II-induced suppression of this FSH effect was reversed by the AT₂-selective antagonist PD-123319 (P < 0.05) but not by the AT₁-selective antagonist DuP-753.

Similarly, Fig. 8 indicates the effect of ANG II on estrogen production in cultured granulosa cells. After a 48-h incubation, FSH induced a 2.4-fold increase in estrogen production compared with untreated controls (P < 0.05). ANG II suppressed FSH-stimulated estrogen production by 36% (P < 0.05). This ANG II effect was reversed by PD-123319 (P < 0.05) but not by DuP-753.

Fig. 5. Content of AT₂ mRNA in granulosa cells obtained on days 3-6 after injection with 20 IU eCG. AT₂ mRNA in uncultured granulosa cells isolated from ovaries was examined by RT-PCR as described in MATERIALS AND METHODS. A: representative autoradiogram showing native and deletion-mutated cDNA encoding AT₂ (top) and β-actin (bottom). B: quantification of AT₂ mRNA. One arbitrary unit indicates value of AT₂ mRNA level 3 days after eCG injection. Data are means ± SE (vertical bars) of 3 independent experiments, each determined in triplicate.

Fig. 6. Effect of ANG II on DNA fragmentation in cultured granulosa cells from ovaries of immature rats 2 days after eCG injection. Granulosa cells were cultured in presence or absence of follicle-stimulating hormone (FSH) (200 ng/ml) with or without ANG II (1 µM) for 48 h. Low-molecular-weight DNA extracted from cells was loaded on to a 2% agarose gel, resolved by electrophoresis and visualized by ultraviolet. Data represent 3 separate experiments. Similar results were obtained in 2 other experiments. Lane 1, control; lane 2, ANG II; lane 3, FSH; lane 4, FSH plus ANG II.

Fig. 7. Effect of ANG II on luteinizing hormone receptor content in cultured granulosa cells isolated from ovaries of immature rats treated with diethylstilbestrol (DES). Granulosa cells were cultured in presence (+) or absence (−) of FSH (50 ng/ml) or ANG II (100 nM) with or without DuP-753 (10 µM) and PD-123319 (10 µM). Binding of ¹²⁵I-labeled human chronic gonadotropin (¹²⁵I-hCG) was assayed as described in MATERIALS AND METHODS. Data are means ± SE (vertical bars) of 3 independent experiments, each performed in triplicate. *P < 0.05.

Fig. 8. Effect of ANG II on estradiol-17β production in cultured granulosa cells from ovaries of immature rats treated with DES. Experimental conditions were identical to those in Fig. 7. Enzyme immunoassay was performed as described in MATERIALS AND METHODS. Data are means ± SE (vertical bars) of 3 independent experiments, each performed in triplicate. *P < 0.05.
ERKs (ERK1 and ERK2) are key enzymes in the regulation of a variety of cellular events, including cell differentiation, survival, and apoptosis. To know the mechanism of the inhibitory effects of ANG II on FSH actions described above, we measured FSH-induced ERK activation in the presence or absence of ANG II by in-gel kinase assay. The activities of both ERK1 and ERK2 were rapidly increased after FSH treatment, with a peak at 2 min, and declined to basal levels within 30 min (data not shown). ANG II inhibited FSH-stimulated ERK activities (data not shown). To selectively investigate the role of AT2, we examined the effect of the AT2-specific agonist CGP-42112B on FSH-stimulated ERK activities. CGP-42112B is known to act as an agonist for AT2 but as an antagonist for AT1 at a concentration in this study (1 µM). As shown in Fig. 9, after a 2-min incubation, FSH induced a 1.8-fold and a 1.6-fold increase in ERK1 and ERK2 activities, respectively (P < 0.05), whereas CGP-42112B inhibited these FSH-caused increases (P < 0.05). This CGP-42112B effect was reversed by PD-123319 (P < 0.05).

DISCUSSION

In addition to gonadotropins, several growth factors, cytokines, and vasoactive peptides are implicated in the regulation of follicle development and atresia. In contrast to the regulatory mechanisms underlying follicle development, little is known about the regulation of atresia, which is the ultimate fate of most follicles. The vasoactive peptide ANG II is one of such ovarian factors. Ovarian ANG II receptors are predominantly AT1, but their physiological roles remain to be resolved. Therefore, the ovary is a useful model with which to investigate novel roles of the renin-angiotensin system (RAS) through AT2, functions that are unrelated to the regulation of blood pressure and fluid osmolarity. However, although AT1 is predominantly expressed in atretic follicles, AT2 has not been conclusively implicated in the regulation of atresia. In this study, we examined immature model rats injected with eCG, in which ovarian follicles undergo growth and development with subsequent atresia, in addition to investigating granulosa cells in primary culture. Our data suggest that AT2 stimulation causes the progression of follicle atresia through granulosa cell apoptosis by inhibiting FSH actions, mainly because of the following findings. First, the AT2 content in vivo was increased at both protein and mRNA levels in parallel with the progression of follicle atresia. This increase was not observed before the onset of atresia. If AT2 is involved in the fate of follicles, considerable levels of AT2 should be detected in some healthy follicles. Thus the implication of AT2 in the progression of atresia is more likely than in the onset of atresia. Second, AT2 stimulation inhibited the in vitro actions of FSH, which plays crucial roles for survival and differentiation of granulosa cells. That is, FSH-induced DNA fragmentation and FSH-caused increases in the LH receptor content and estrogen production were all inhibited. Also, AT2 stimulation inhibited FSH-induced ERK activation, enzymes that are critical for cell survival and differentiation. Enhancement of estrogen biosynthesis and the increase in the LH receptor content are the parameters for FSH-stimulated differentiation of cultured granulosa cells (9).

In contrast to our findings, several reports (25, 33, 34) indicate that ANG II may be involved in the induction of follicle development, steroidogenesis, oocytes maturation, and ovulation, rather than with atresia. For example, ANG II stimulated estrogen production, oocytes maturation, and ovulation in in vitro-perfused rabbit ovaries, and these ANG II effects were mediated by AT2. No convincing rational can explain this discrepancy, but a species difference might have some relevance. An autoradiographic study has demonstrated a high degree of localization of AT2 in the granulosa cell layers and the stroma of rabbit preovulatory follicles (33, 34), whereas our results and those of another (5) revealed intense signals for AT2 on atretic follicles in the rat ovary. Also, the rabbit estrous cycle differs from cycles of rats and humans. That is, copulatory stimulation induces ovulation in the rabbit. Pucell and co-workers (25, 27) have also demonstrated that ANG II stimulated estrogen secretion in quartered ovaries from eCG-treated immature rats and that ANG II did not affect aromatase activity in cultured granulosa cells prepared from DES-treated rats. Our data are not in

Fig. 9. Effect of CGP-42112B on FSH-stimulated extracellular signal-regulated kinase (ERK) 1 and ERK2 activities in cultured granulosa cells from ovaries of immature rats treated with DES. Serum-starved granulosa cells were incubated at 37°C in presence (+) or absence (−) of FSH (100 ng/ml) or CGP-42112B (1 µM) with (+) or without (−) PD-123319 (100 µM) for 2 min. Cell lysates were subjected to in-gel analysis of ERK1 and ERK2 activities as detailed in MATERIALS AND METHODS. A: representative autoradiogram showing ERK1 and ERK2 activities in each treatment situation. Nos. at right indicate molecular mass in kDa. B: quantification of ERK1 and ERK2 activities. Data are means ± SE (vertical bars) of 5 independent experiments. *P < 0.05.
agreement with these findings. This can be explained by experimental conditions. Pucell et al. (27) showed that ANG II (1-100 nM) does not affect FSH (100 ng/ml)-induced estrogen production. We examined the effect of ANG II on FSH-stimulated estrogen production in the presence of 50, 100, and 200 ng/ml FSH with or without 1, 10, or 100 nM ANG II (data not shown). FSH significantly stimulated estrogen production in a concentration-dependent manner. ANG II did not affect the estrogen production induced by 100 or 200 ng/ml FSH, whereas 100 nM ANG II suppressed the increase in estrogen production induced by 50 ng/ml FSH. Nevertheless, our findings that the increase in AT2 expression exhibits close space and time correlation with follicle atresia in vivo and that AT2 mediates inhibition of FSH actions in vitro are consistent with the fact that AT2 is predominantly expressed in atretic follicles of adult rats at all stages of the estrous cycle. It has been proposed that ANG II receptors would become undetectable in healthy follicles because of downregulation or by blockade of the binding of exogenous radiolabeled ANG II in receptor binding studies, because preovulatory follicles contain high levels of endogenous ANG II. However, we found that AT2 mRNA levels increased in parallel with the progression of atresia, suggesting that this notion is unlikely.

The AT2-mediated induction of apoptosis in granulosa cells is consistent with the results of several investigations. For example, Kakuchi et al. (13) found by in situ hybridization that sites of AT2 expression overlap closely with those of a specific group of cells undergoing apoptosis during nephrogenesis in fetal mice. Yamada et al. (32) reported that ANG II induces the apoptosis of PC12W cells cultured in the presence of low concentrations of NGF via AT2. Xia et al. (31) also found that withdrawal of NGF from PC12 cells led to inhibition of ERK activity, resulting in apoptosis of these cells.

Androgen is a potential atretogenic factor (10). Estrogen is thought to prevent the apoptosis of granulosa cells, in part by suppressing the atretogenic effect of androgen (11). We demonstrated that ANG II inhibits FSH-induced estrogen production via AT2 in granulosa cells. This finding further supports the thought that AT2 is involved in follicle atresia involving granulosa cell apoptosis. On the other hand, FSH induces follicle maturation with an increase in the LH receptor content. Preovulatory follicles expressing increased levels of LH receptors ovulate in response to an LH surge. The present study showed that this FSH-induced increase in the LH receptor content was considerably inhibited by AT2. Therefore, AT2 stimulation should decrease the response of follicles to LH, which would lead to a decrease in the rate of ovulation and progression of atresia.

Some reports suggest that the ovarian RAS is associated in humans with polycystic ovary syndrome (PCOS), which can be identified by various symptoms such as chronic anovulation, inappropriate gonadotropin secretion, and hyperandrogenism, depending on the patient (33). Basal plasma levels of prorenin in PCOS patients are higher than those in follicular-phase controls and significantly correlate with peripheral androgen concentrations. Serum concentrations of total renin are also particularly enhanced in women with PCOS. Moreover, both theca and granulosa cells in large cystic follicles of PCOS intensely immunostain for renin and ANG II, as do granulosa cells of atretic follicles of normal ovaries. These findings raised the possibility that increased levels of ANG II in the ovary may contribute to the cause of PCOS through AT2. These findings suggested that dysregulation of the RAS is involved in the development of PCOS, because AT2 may be able to induce follicle atresia with a low ratio of estrogen to androgen and lower levels of LH receptors as described above. The ovarian RAS may be one target to consider when designing a strategy to treat such functional ovarian disorders.

Recent studies indicated that AT2 activates protein tyrosine phosphatase, serine-threonine phosphatase, and the delayed rectifier K+ current and inhibits I-type Ca2+ channels (1, 19). However, the signaling pathways of AT2 are still far from being completely understood. Our data revealed that stimulation of AT2 inhibits FSH-induced ERK activation in granulosa cells. Further studies are necessary to gain insight into how AT2 mediates apoptosis and how AT2 mediates inhibition of ERK activities and to understand the relation between apoptosis and ERK activities in these cells. Ovarian granulosa cells provide a useful tool for studying not only unknown signaling pathways of AT2 but also novel function of the RAS.

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