Localized accumulation of angiotensin II and production of angiotensin-(1–7) in rat luteal cells and effects on steroidogenesis

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Submitted 9 May 2005; accepted in final form 26 January 2006

The formation of angiotensin II (ANG II) occurs as the result of a cascade reaction that is initiated by activation of prorenin to renin. The substrate angiotensinogen is hydrolyzed by renin, releasing the decapeptide ANG I, which in turn is substrate for angiotensin-converting enzyme (ACE). ACE hydrolysis of ANG I results in the formation of the octapeptide ANG II, considered the major biologically active peptide of the cascade, and its proteolysis produces ANG III [ANG-(2–8)]. Historically, ANG II was considered an adrenal hormone restricted to renal function, but evidence that ANG II is produced by other tissues, including the ovaries, began to emerge in the 1980s. The demonstration of ovarian angiotensinogen, ANG II receptors, and renin and ACE activity has been reviewed (52, 82), and this ovarian renin angiotensin system (OVRAS) appears to be under gonadotropin control. Prorenin/renin and ANG II/ANG III immunoreactivity is present in follicular fluid of women at significantly higher concentrations than in serum, indicating local ovarian synthesis (21, 39), and these concentrations increase after gonadotropin treatment or during the midcycle gonadotropin surge (39, 40, 63). Similarly, in the rat, gonadotropin regulation of the OVRAS is implicated, because granulosa cells of immature developing follicles show no immunostaining for renin or ANG II, but at the time of the gonadotropin surge, granulosa cells of the preovulatory follicle stain intensely for both these antigens (38). However, the role of the OVRAS in ovarian function remains to be clarified.

For example, ANG II mediates ovulation in the rat (50, 53), but ANG II receptors in preovulatory follicles of this species have not been demonstrated (65). ANG II receptors are classified as type 1 (AT1) or type 2 (AT2) receptor antagonists, were used in progesterone production studies. ZPP significantly reduced LH-stimulated progesterone, and PCMS blocked LH-sensitive cAMP accumulation. Losartan inhibited progesterone production in permeabilized but not intact luteal cells and was reversed by ANG II. PD-123319 had no significant effect on luteal steroidogenesis. Confocal microscopy showed ANG II distributed within the cytoplasm and nuclei of luteal cells. HPLC analysis showed that ANG II was synthesized by bovine luteal cells, identify other bioactive angiotensin peptides, and investigate a role for angiotensin peptides in luteal steroidogenesis. ANG II inhibits cholesterol side chain cleavage activity and reduces pregnosterone production (66). Inhibitory effects of ANG II on luteal steroidogenesis may be modulated by angiotensin peptides that act in part through intracellular AT1 receptors.

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hydrolysis of ANG I results in the formation of ANG II that is further proteolytically degraded by aminopeptidase A to ANG III [ANG-(2–8)] or by aminopeptidase D to ANG IV [ANG-(3–8)] and other fragments (57). An additional pathway for lysis of ANG I is via prolyl endopeptidase (PEP; EC 3.4.21.26) or neutral endopeptidase (NEP; EC 3.4.24.11), resulting in the formation of ANG-(1–7). However, ANG-(1–7) can also be directly formed from ANG II by PEP or prolyl carboxypeptidase activity. Thus, in addition to ANG II, other bioactive angiotensin peptides, including ANG III, ANG-(1–7), and ANG IV, can be produced by the renin-angiotensins cascade (20, 27). Further biological complexity of the renin-angiotensin system is bestowed by specific angiotensin peptide receptors. The AT1 and AT2 receptors are recognized to mediate different actions (11, 78), whereas receptors for ANG IV and ANG-(1–7) are also implicated because they often have opposing effects to those of ANG II (11, 16, 20, 43), and recently, the orphan receptor Mas was proposed to be the ANG-(1–7) receptor (62). Clearly, a combination of angiotensin peptides and their receptors in any given tissue can confer a highly complex regulatory system, and such complexity is indicated in the ovary (13). It is the hypothesis of this study that, because the corpus luteum is a source of ANG II (32, 48), other angiotensin peptides also are produced and that different angiotensin peptides may differentially regulate luteal steroidogenesis. Therefore, we intend to determine whether ANG-(1–7) is formed by the corpus luteum and to investigate the effects of angiotensin peptides on progesterone production.

METHODS

Animals

Immature (26–27 days old) female rats (CD strain; Charles River Laboratories, Wilmington, MA) were injected subcutaneously with 50 IU pregnant mare serum gonadotropin (Gestyl; Organon Pharmaceuticals, West Orange, NJ) to induce superovulation. Fifty-four hours later, 25 IU human chorionic gonadotropin (hCG; Wyeth-Ayerst Laboratories, Rouses Point, NY) were injected to induce ovulation and pseudopregnancy. All procedures were in accordance with institutional animal care and use committee guidelines.

Luteal Cell Isolation

Rat luteal cells were isolated using methods previously described (51). Briefly, 5 days after injection of hCG, the ovaries were removed and finely minced with a razor blade. The ovarian fragments were suspended in 5 ml of Ca2+-free minimal essential medium (medium 1, no. 1380; Gibco, Grand Island, NY) containing 0.1% bovine serum albumin (BSA; Gibco), 2,000 IU collagenase ( Worthington Biochemical, Freehold, NJ), and 3,000 IU deoxyribonuclease (DNase; Worthington) per gram of tissue for 1 h at 37°C under 95% air-5% CO2. The isolated luteal cells were enriched by density gradient sedimentation on a discontinuous density gradient (Percoll; Pharmacia Fine Chemicals, Uppsala, Sweden) (51). The cells were washed with 10 ml of medium 1, collected by centrifugation, and resuspended in Dulbecco’s modified Eagle’s/Ham’s F-12 medium at 0.5 million–1.0 million cells/ml containing 0.1% FCS, penicillin (100 U/ml), streptomycin (0.1 μg/ml), and amphotericin (0.25 μg/ml; all Gibco), hereafter referred to as DMEM. Cell number was determined by means of a hemocytometer, and cell viability was >90%, as assessed by exclusion of Trypan blue dye.

Immunochemistry

Luteal cell suspensions in DMEM containing BSA (0.1%) were cultured in Lab-Tek culture slides for 24 h, after which they were fixed with 4% paraformaldehyde in Dulbecco’s phosphate-buffered saline (DPBS; pH 7.4) for 1 h. Inhibition of endogenous luteal peroxidase was achieved with 5% hydrogen peroxide (1 h). The cells were washed with DPBS, incubated with 0.1% Triton X in DPBS for 30 min at room temperature, and then washed twice (5 min each) with DPBS. To recover antigenicity, we placed cells in a microwave oven for 1 min at maximum power in citrate buffer (50 mM, pH 6.0) (54) and then washed cells once with DPBS for 5 min. Nonspecific binding sites were blocked with Power Block (BioGenex, San Ramon, CA) incubated at 37°C for 20 min, followed by washing twice with DPBS for 5 min. The cells were incubated overnight at 4°C with ANG II antibody (diluted 1:300 in DPBS containing 3% BSA) in a humidified chamber. Before secondary labeling with streptavidin/diaminobenzidine was performed, the cells were washed in DPBS (1 × 5 min, 1 × 30 min, 1 × 10 min). The cells were then incubated with MultiLink (BioGenex) for 10 min, washed three times (5 min each) in DPBS, and incubated with horseradish peroxidase-conjugated streptavidin (BioGenex) for 15 min. Subsequently, cells were incubated with 3'-3'-diaminobenzidine plus hydrogen peroxide (BioGenex) for 5 min. The cells were counterstained with hematoxylin for 1 min and NH4OH for 3 min, followed by a wash with deionized H2O before being mounted.

In the case of confocal and fluorescence microscopy studies, paraformaldehyde-fixed cells were incubated with Triton X-100 (0.1%) in DPBS for 30 min and washed three times with DPBS (10 min each). Cells were incubated for 1 h at 37°C with DPBS containing 5% normal rabbit serum and 3% BSA (blocking solution), followed by three washes with DPBS (10 min each). Immunolabeling was achieved with rabbit anti-ANG-II antibody (1:400) in DPBS containing 3% BSA incubated overnight at 4°C, followed by three washes with DPBS (15 min each) and incubation for 45 min at room temperature with fluorescein-labeled goat anti-rabbit IgG Fab (Cappel, Irvine, CA) (diluted 1:100 in blocking solution). Unbound label was removed by washing the cells three times with blocking solution, followed by two washes with DPBS alone. The slides were mounted with Slowfade (Molecular Probes, Portland, OR) containing propidium iodide.

HPLC Analysis

Angiotensin peptides were resolved using HPLC, based on published methods (9). Isolated luteal cells were aliquoted to tubes, centrifuged (1,200 g, 10 min), and resuspended with intracellular (IC) buffer (110 mM KCl, 10 mM NaCl, 1 mM KH2PO4, 5 mM KHCO3, and 20 mM HEPES, pH 7.1) containing Triton X (0.1%) at 4°C. The cell suspension was sonicated on ice for 10 s at 50% cycle with a miniprobe. Aliquots were added to tubes containing quinapril, Z-(Leu3)-3-125 I-angiotensin II (1:400) in DPBS containing 3% BSA incubated overnight at 4°C, followed by three washes with DPBS (15 min each) and incubation for 45 min at room temperature with fluorescein-labeled goat anti-rabbit IgG Fab (Cappel, Irvine, CA) (diluted 1:100 in blocking solution). Unbound label was removed by washing the cells three times with blocking solution, followed by two washes with DPBS alone. The slides were mounted with Slowfade (Molecular Probes, Portland, OR) containing propidium iodide.
Cell Incubations

Isolated luteal cells were washed with 10 ml of medium 1, collected by centrifugation, and resuspended in DMEM.

Whole cells. Luteal cells were incubated in 12 × 75-cm glass assay tubes at a cell concentration of 1–2 × 10^5 cells/ml DMEM. Experiments adopted a paradigm of a preincubation with protease inhibitors for 30–60 min as specified in legends, followed by an acute 1-h incubation with and without LH (100 ng/ml). Cell incubations for ACE assays were carried out in 24-well plates at an initial cell concentration of 1 × 10^6 cells/ml.

Permeabilized cells. Permeabilization of rat luteal cells was based on published methods that describe the use of saponin to allow exogenous bioactive molecules access to the intracellular milieu with continued cell function (36, 76). Luteal cells were washed with IC buffer (containing in mM: 20 HEPES, 110 KCl, 10 NaCl, 1 KH2PO4, 5 KHCO3, and 0.03 MgCl2, pH 7.1), centrifuged, and resuspended at 1 × 10^7 cells/ml with IC buffer supplemented with MgCl2 (3.3 mM), ATP (4.4 mM), and saponin (0.1 mg/ml). The cells were incubated for 2–5 min at 20°C, when permeabilization was achieved with 100% cells taking up Trypan blue. The permeabilized cells were centrifuged and resuspended with IC buffer containing MgCl2 (3.3 mM), ATP (4.4 mM), creatine phosphokinase (10 U/ml), and creatine phosphate (20 mM) and aliquoted at 2 × 10^5 cells/ml to tubes in the presence of LH (100 ng/ml) and in the absence and presence of ANG-(1–7), ANG II, losartan, or PD-123319. After incubation for 1 h at 37°C, the tubes were immersed in an 80°C water bath for 10 min before storage at −20°C awaiting progesterone radioimmunoassay (RIA).

ACE Activity

ACE activity was measured by means of o-aminobenzoyl-Gly-p-nitro-Phe-Pro (ABGP), which forms a fluorescent product upon cleavage by ACE (7). Luteal cells were incubated with DPBS (500 µl) containing glucose (1 g/l) and ABGP (1 mM) dissolved in dimethyl sulfoxide (final concentration 0.5%). Cells were separated from the medium at the end of the incubation period by centrifugation (800 g, 5 min). Aliquots (200 µl) of supernatant were added to 2 ml of Tris (0.2 M, pH 8.2) containing EDTA (0.1 M) at 4°C, and the fluorescence in the medium was measured with a fluorimeter (Deltascan PTI; Photon Technology International, Brunswick, NJ) (excitation, 360 nm; emission, 460 nm). Fluorescence of substrate blanks that contained all reagents except for cells was subtracted from that of experimental reactions. Preliminary experiments with luteal cells and ABGP (1 mM) determined a linear time-dependent increase in fluorescence of product over 12 h and indicated that substrate concentration was not limiting for product formation in the assay.

Statistical Analysis

Statistical differences between multiple treatments were determined using one-way analysis of variance (ANOVA) or two-way ANOVA, and specific differences among groups were identified using post hoc Student-Newman-Keuls or a paired t-test. A significant difference was established at the level of P < 0.05. Each experiment included at least three replications per treatment, and data are presented as means ± SE of n experiments as expressed in legends.

Hormones, Drugs, and Reagents

Ovine luteinizing hormone (oLH no. 26) was supplied by the National Institute of Diabetes and Digestive and Kidney Diseases. ZPP was generously donated by Dr. K. B. Brosnihan (Wake Forest, NC). Rabbit anti-ANG II antibody was kindly donated by Dr. T. Inagami (Vanderbilt University, Nashville, TN). Quinapril and PD-123319 were obtained from Parke-Davis (Ann Arbor, MI), and losartan was obtained from Merck, Sharpe & Dohme (Rahway, NJ). ANG II, porcine renin substrate tetradecapeptide (angiotensinogen), and all other chemicals were purchased from Sigma Chemical (St. Louis MO).

RESULTS

Figure 1 shows images of luteal cells obtained under differential interference contrast microscopy. The presence of ANG II immunoreactivity was confirmed in luteal cells by the presence of intense dark immunostaining, but when the primary antibody was omitted in negative control incubations, no
immunostaining was apparent. The distribution of immunolabeled ANG II in cultured luteal cells was further studied with confocal fluorescence microscopy, and Fig. 2 is a composite image formed from six stacked images obtained at 1-μm sections. Not only is ANG II distributed throughout the cytoplasm of the luteal cells, but also intense patches of ANG II are present within the nuclei. (No labeling of ANG II in rat luteal cells was observed in the absence of anti-ANG II primary antibody).

To further characterize the presence of angiotensin peptides in luteal cells, we used HPLC analysis to identify and estimate the relative amounts of the bioactive angiotensin peptides ANG-(1–7), ANG II, and ANG III. Figure 3A shows an HPLC elution profile of synthetic angiotensin standards that were extracted from IC buffer, and the elution of ANG-(1–7), ANG II, and ANG III occurred at 16.4 ± 0.2, 25.1 ± 0.2, and 26.1 ± 0.2 min, respectively. Integration of the area under the peaks of absorbance of known concentrations of peptide standards and linear regression analysis showed a highly significant correlation ($P < 0.0001$) between peptide concentration and integrated area ($r^2 = 0.960, 0.989, 0.977$ for ANG-(1–7), ANG II, and ANG III, respectively). The efficiency of extraction of peptides was calculated by comparing the integrated areas obtained from extracted standards with those areas obtained from nonextracted standards. Recovery was 92.9 ± 5.0, 85.5 ± 8.8, and 83.3 ± 5.3% ($n = 4$) for ANG-(1–7), ANG II, and ANG III, respectively. Extracts from luteal cells displayed peaks that correlated with ANG-(1–7), ANG II, and ANG III, but because we wanted to investigate angiotensin processing pathways and effects of protease inhibitors, we incubated luteal cells with angiotensinogen, as described in METHODS, to prevent substrate depletion effects that would compromise interpretation of the data. Figure 3B shows a luteal cell chromatogram with absorbance peaks that correlated with ANG-(1–7), ANG II, and ANG III, and the relative amounts of these peptides were determined to be 586.5 ± 63.1, 622.1 ± 87.2, and 244.1 ± 55.9 pmol/mg ($n = 8$) for ANG-(1–7), ANG II, and ANG III, respectively.

Protease inhibitors that may modulate angiotensin peptide include quinapril, a specific nonpeptide, nonsulphhydryl competitive inhibitor of ACE (12); PCMS, a sulphydryl protease inhibitor; ZPP, an inhibitor of PEP (61); and PMSF, a serine protease inhibitor. Luteal cells were incubated for 60 min in the presence of each inhibitor (100 μM) in DMEM containing 0.1% BSA and were then centrifuged and resuspended with IC buffer before a secondary incubation with protease inhibitor and angiotensinogen, as described in METHODS. Peptides were extracted and subjected to HPLC. Table 1 shows the effects of protease inhibitors on accumulation of angiotensin peptides by luteal cells. Quinapril significantly reduced ANG III levels ($P < 0.05$), but a trend toward reduced ANG II did not reach statistical significance. ANG-(1–7) also was unaffected by quinapril. ZPP significantly increased accumulation of ANG II ($P < 0.05$) but was without statistically significant effect on ANG-(1–7) or ANG II levels. When cells were incubated with ZPP and quinapril combined, the accumulation of ANG-(1–7) was significantly reduced ($P < 0.05$), but ANG II and ANG III were not significantly different from control levels. The sulphydryl protease inhibitor PCMS significantly reduced accumulation of ANG-(1–7), ANG II, and ANG III, but no effect of PMSF on peptide accumulation was detected.

Figure 4 summarizes data of studies that investigated the effects of protease inhibitors on basal and LH-stimulated steroidogenesis. Under basal conditions, quinapril and PMSF had no statistically significant effects; however, PCMS at higher concentrations (100 μM) significantly reduced progesterone production ($P < 0.05$). ZPP, however, enhanced progesterone output compared with the effects of the other protease inhibitors, and at 100 μM, the level of progesterone production was elevated compared with that of its control group ($P < 0.05$). In the case of LH-stimulated cells, the protease inhibitors tended to reduce progesterone production. PCMS and PMSF induced a dose-dependent inhibition that reached ~70 and 50% inhibition, respectively, at the maximally tested concentration (100 μM). The effects of quinapril and ZPP were more subtle than those of PCMS and PMSF: ZPP (100 μM) inhibited LH-driven progesterone production ~15% compared with the control group ($P < 0.05$); however, progesterone production in the presence of quinapril (100 μM) was not statistically different from control levels, although an approximate 10% reduction in its output was detected compared with cells incubated with 10 μM quinapril ($P < 0.05$). Other protease inhibitors were tested at concentrations up to 100 μM and found to have no effect on...
basal or LH-stimulated progesterone production including benzamidine, pepstatin, and aprotinin (data not shown).

The effects of the protease inhibitors on LH action may be mediated through the second messenger cAMP; therefore, we investigated their effects on cAMP accumulation, and the results from these experiments are summarized in Fig. 5. Under basal conditions, no statistically significant dose-response effects were detected within the protease inhibitor treatments. However, among the treatment groups, cells treated with PCMS accumulated less cAMP than did cells treated with quinapril, ZPP, or PMSF ($P < 0.05$). LH-stimulated cAMP accumulation also was unaffected by quinapril, ZPP, and PMSF, but in the presence of PCMS, a dose-dependent inhibition occurred ($P < 0.05$). We further investigated the regulatory effect of PCMS on LH-stimulated luteal cells and tested whether degradation of cAMP was enhanced in this treatment group. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) prevents cAMP hydrolysis, and the data of

| Table 1. Effects of protease inhibitors on accumulation of angiotensin peptides by luteal cells |
|-----------------|-----------------|-----------------|
|                | ANG-(1–7), % control | ANG II, % control | ANG III, % control |
| PMSF 4         | 102.4±14.8       | 94.9±8.9        | 106.7±13.9        |
| PCMS 8         | 59.9±12.6*       | 67.2±6.5*       | 30.1±6.0*         |
| Quinapril 5    | 94.5±14.4        | 80.4±9.3        | 49.2±10.0*        |
| ZPP 5          | 91.3±5.5         | 135.9±14.8*     | 90.7±14.5         |
| Quinapril + ZPP| 56.8±3.6*        | 89.3±7.4        | 83.3±9.2         |

Levels of ANG-(1–7), ANG II, and ANG III in control incubations that contained no inhibitors were set at 100%, and the effects of the inhibitors quinapril, Z-proprolin (ZPP), parachloromercurylsulfonic acid (PCMS), and phenylmethylsulfonyl fluoride (PMSF) are expressed as percentages of the controls. Data are means ± SE. *$P < 0.05$, statistically significant differences from controls.

Fig. 3. HPLC elution profiles. A: an elution profile for standards of ANG-(1–7), ANG II, and ANG III (each 200 pmol). B: a chromatogram showing the elution profile of luteal cell homogenate incubated with angiotensinogen (25 μM).

Fig. 4. Dose-response effects of quinapril, Z-proprolin (ZPP), parachloromercurylsulfonic acid (PCMS), and phenylmethylsulfonyl fluoride (PMSF) on progesterone production. Luteal cells were preincubated with protease inhibitors (PMSF and PCMS for 30 min, quinapril and ZPP for 60 min) and subsequently further incubated in the absence (top) or presence (bottom) of luteinizing hormone (LH; 100 ng/ml) for 60 min. Statistical analysis with 2-way ANOVA was conducted on arcsine transformed data; however, the nontransformed data are depicted, expressed as percentages of control incubations in the absence of inhibitor (100%). Values are means ± SE of a minimum of 3 repeated experiments for each inhibitor. *$P < 0.05$, significant differences from control, as revealed with 2-way ANOVA and Student-Newman-Keuls post hoc test. **$P < 0.05$, significantly different means.
Fig. 6 show that LH-stimulated cAMP accumulation was enhanced ($P < 0.05$) in the presence of IBMX (200 μM). However, IBMX did not block the inhibitory effect of PCMS on either cAMP accumulation or progesterone production in LH-stimulated cells. In addition, the nonmetabolized cAMP analog dibutyryl cAMP (DBcAMP) was tested, and PCMS also was inhibitory on DBcAMP-stimulated progesterone.

Transport of cholesterol to the mitochondria may be a target for protease inhibitors; therefore, we used 22-OH-cholesterol that can enter mitochondria independently of LH action and transporters. We found that, under these conditions, the inhibitory effect of PMSF on progesterone production was reversed (Fig. 7); however, addition of 22-OH-cholesterol only partially reversed the inhibitory effect of PCMS.

The protease inhibitors quinapril and ZPP target the proteases ACE and PEP, which are enzymatic pathways for production of ANG II and ANG-(1–7), respectively. We investigated the effects of quinapril and ZPP on progesterone production and found that they had an additive inhibitory effect on LH-regulated progesterone production (Fig. 8). However, addition of ANG II or ANG-(1–7) to cells incubated with quinapril and ZPP did not reverse their inhibitory effect. In contrast, 22-OH-cholesterol did reverse the inhibition produced by quinapril and ZPP, and the levels of progesterone produced were not significantly different from that of control cells (Table 2).

The effects of exogenous ANG II and ANG-(1–7) on luteal steroidogenesis also were studied. In acute 1-h incubations, no
significant effect of ANG II or ANG-(1–7) on LH-stimulated progesterone was found (Table 3). Similarly, no effect of exogenous ANG I (3 μM) on progesterone production was detected: LH, 9.9 ± 0.5 ng/10^5 cells; LH + ANG I, 9.4 ± 0.3 ng/10^5 cells (n = 4).

Expression of ACE activity in rat granulosa cells is linked to the LH surge (38), and we wanted to investigate whether LH-regulated ACE activity occurs in luteal cells. Luteal cells were incubated for 24 h with and without LH (100 ng/ml), washed, and further incubated for 3 h in the presence of ABGP (1 mM). Some incubations included quinapril (100 μM) to verify that the hydrolysis product was dependent on ACE activity. Luteal cells consistently hydrolyzed ABGP to a fluorescent product, and quinapril inhibited this activity. Furthermore, LH treatment of luteal cells resulted in an increase in fluorescence of ABGP product that was sensitive to quinapril (Fig. 9). LH consistently elevated ACE activity in three experiments tested, but the degree of stimulation showed some variability, ranging from 25 to 500%.

Addition of extracellular ANG II had no significant effect on progesterone production, but protease inhibitors that can affect angiotensin processing were inhibitory to steroidogenesis.

**Fig. 7.** Reversal of inhibitory effects of PCMS and PMSF on progesterone production. Rat luteal cells were preincubated for 30 min in the absence or presence of PCMS (100 μM; top) or PMSF (100 μM; bottom). Subsequently, cells were further incubated for 60 min in the presence of LH (100 ng/ml) and in the absence or presence of 22-OH-cholesterol (20 μg/ml). Data from representative experiments are shown. Values are means ± SE (n = 4).

**Fig. 8.** Top: combined effects of ZPP and quinapril on LH-stimulated progesterone production. Luteal cells were incubated in the presence of increasing concentrations of ZPP with or without quinapril (100 μM) for 60 min and then incubated for 60 min with LH (100 ng/ml). Statistical analysis with 2-way ANOVA was performed using arcsine transformed data and showed significant differences among treatments (*P < 0.05). *P < 0.005, paired t-test comparison of ZPP vs. ZPP + quinapril. Bottom: cells were incubated with quinapril and ZPP (each 100 μM) as described at top. ANG II (1 μM) or ANG-(1–7) (1 μM) was added at the same time as LH, and cells were incubated for 60 min. Data are expressed as percentages of the progesterone produced by cells incubated with LH alone (100%). Values are means ± SE of 3–5 experiments. *P < 0.05 vs. control (ANOVA).
These data and the observed accumulation of cytoplasmic and nuclear ANG II suggest a potential intracellular regulatory site of action that may affect cell function. Therefore, we set out to modulate intracellular actions of angiotensin peptides by permeabilization of the luteal cell membrane with saponin and treatment with angiotensin peptides and receptor antagonists. Figure 10 summarizes the data of experiments with permeabilized luteal cells and ANG II or ANG-(1–7). Treatment with ANG II or ANG-(1–7) did not significantly affect progesterone production compared with control levels, although cells incubated with ANG II produced significantly (P < 0.05) more progesterone than did cells incubated with ANG-(1–7). When permeabilized cells were incubated with losartan (1 μM), progesterone production was significantly reduced (P < 0.05), and this effect was reversed when ANG II (1 μM) was included with the antagonist. The AT2 receptor antagonist PD-123319 had no significant effect on progesterone production. In whole cells (nonpermeabilized), losartan had no significant effect on progesterone production, which was 97.7 ± 3.3% compared with control incubations (100%; means ± SE from 4 replicated experiments, P > 0.05).

DISCUSSION

ANG II can no longer be considered the only biologically active end point of the renin-angiotensinogen cascade, because other angiotensin peptides have biological activity, including ANG III, ANG-(1–7) (3, 16), and ANG-(3–8) (27). ANG III, ANG-(1–7), and ANG-(3–8) have some overlapping actions with ANG II because of partial occupancy of ANG II receptors, but importantly, ANG-(1–7) and ANG-(3–8) have their own receptors that mediate opposing actions to ANG II (27, 47). In the present study, HPLC analysis of luteal cell incubations showed peaks that eluted with the same retention times as ANG II, ANG III, ANG-(1–7), and ANG-(3–8) have some overlapping actions with ANG II because of partial occupancy of ANG II receptors, but importantly, ANG-(1–7) and ANG-(3–8) have their own receptors that mediate opposing actions to ANG II (27, 47). In the present study, HPLC analysis of luteal cell incubations showed peaks that eluted with the same retention times as ANG II, ANG III, ANG-(1–7), and ANG-(3–8), and their relative quantities were ANG II ≡ ANG-(1–7) > ANG III. These observations suggest that ANG II and ANG-(1–7) are significant products of angiotensinogen processing in the rat corpus luteum.

Table 3. Effect of ANG II on LH-stimulated progesterone production

<table>
<thead>
<tr>
<th>Concentration</th>
<th>ANG II</th>
<th>ANG-(1–7)</th>
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<tbody>
<tr>
<td>1 nM</td>
<td>N/A</td>
<td>94.1 ± 2.3 (5)</td>
</tr>
<tr>
<td>10 nM</td>
<td>89.6 ± 8.4 (5)</td>
<td>91.5 ± 2.1 (5)</td>
</tr>
<tr>
<td>100 nM</td>
<td>94.5 ± 7.4 (5)</td>
<td>89.8 ± 2.7 (5)</td>
</tr>
<tr>
<td>1 μM</td>
<td>89.0 ± 7.5 (6)</td>
<td>89.7 ± 2.9 (5)</td>
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Luteal cells were incubated with LH (100 ng/ml) for 1 h in the absence or presence of increasing concentrations of ANG II or ANG-(1–7). Data are means ± SE and are expressed as percentages of progesterone produced by control incubations that contained LH alone (100%). N/A, not assayed. Numbers in parentheses indicate the number of repeated experiments.

Fig. 10. Effect of modulation of intracellular angiotensin peptides on progesterone production. Luteal cells were permeabilized as described in METHODS and incubated for 60 min with LH (100 ng/ml) and ANG II (1 μM) or ANG-(1–7) (1 μM) (top) or losartan (1 μM), PD-123319 (1 μM), or losartan + ANG II (each 1 μM) (bottom). Data are expressed as percentages of control incubation with LH alone (100%). Values are means ± SE (n = 3 experiments). *P < 0.05 vs. control (ANOVA). a,bP < 0.05, significantly different means.
formation and regulation of ANG II and ANG-(1–7) in the ovary may have consequences for health and disease. ANG II and ANG-(1–7) are frequently counterregulatory: ANG II is a potent vasoconstrictor, stimulates mitogenesis, and has angio-
genic effects, whereas ANG-(1–7) is a vasodilator, reduces mitogenic activity, and opposes angiogenesis (5, 19, 33, 41, 69). Thus the balance of production of these two peptides may play a role in formation of the corpus luteum and also may play a role in ovarian pathologies such as ovarian cancer, thereby underlining the importance to understand their biochemical pathways.

PCMS, the sulphydryl protease inhibitor, significantly inhib-
ited accumulation of ANG-(1–7), ANG II, and ANG III. The broad inhibitory effects of PCMS on angiotensins are likely due to the sulphydryl groups of ACE that are susceptible to PCMS (14, 23), thereby inhibiting hydrolysis of ANG I to ANG II that can be a precursor for both ANG-(1–7) and ANG III. In addition, a sulphydryl-containing peptideidase identified in a neuroblasta-glioma cell line (8) also may occur in luteal cells and contribute to PCMS-sensitive inhibited ANG-(1–7) accumulation. The serum protease inhibitor PMSF was without effect on accumulation of angiotensin peptides. In some tissues PMSF inhibits activation of prorenin to renin and also reduces ANG II accumulation (60, 70), but the present data do not identify a role for serum protease activity in angiotensinogen processing in luteal cells. The broad specificity of PCMS and PMSF directed studies with quinapril and ZPP that represent more specific inhibitors of proteases of angiotensin processing. Quinapril specifically inhibits ACE-mediated hydrolysis of ANG I to ANG II (12), and ZPP inhibits the PEP pathway that hydrolyzes ANG I or ANG II to ANG-(1–7) (8, 61). Treatment of luteal cells with quinapril significantly reduced accumula-
tion of ANG III, but a trend of reduced accumulation of ANG II did not reach statistical significance. These findings suggest that the proteolytic cascade of ANG I to ANG II to ANG III via ACE activity is not a major pathway in luteal cells. Alternative angiotensinogen processing that can give rise to ANG II production includes direct proteolysis of angiotensinogen to ANG II independently of renin and ACE activity (20); in addition, chymase/cathepsin G appears to be the major en-
zyme that converts ANG I to ANG II in heart tissue (29). It is not clear whether a similar pathway may occur in luteal cells, because chymase/cathepsin G is a serine protease, and in our studies we found no significant effect of the serine protease inhibitor PMSF on accumulation of ANG II. Further studies are necessary to identify whether pathways in addition to ACE generate ANG II from ANG I in the rat corpus luteum. However, alternative ovarian pathways of ANG II production could explain the lack of effect of competitive ACE inhib-
itors on ovulation observed in studies with rats and rabbits (15, 59), species known to be dependent on ANG II for ovulation (50, 84).

Under basal conditions, incubation of luteal cells with ZPP that inhibits PEP statistically enhanced ANG II accumulation but did not affect accumulation of ANG-(1–7) or ANG III. PEP can utilize ANG II as substrate to form ANG-(1–7) (61, 81), thus ZPP-enhanced accumulation of ANG II may be explained by reduced metabolism of ANG II to ANG-(1–7). Therefore, enhanced levels of ANG II in ZPP-treated luteal cells suggest the presence of a hydrolytic pathway that utilizes PEP hydro-
lysis of ANG II to produce ANG-(1–7) in this tissue. Further-
more, inclusion of quinapril with ZPP reduced ANG-(1–7) and abrogated the increased accumulation of ANG II, indicating that ACE hydrolysis of ANG I to ANG II and its PEP-mediated hydrolysis to ANG-(1–7) may occur in luteal cells. Although the net accumulation of ANG-(1–7) was unaffected by ZPP by itself, we cannot rule out the possibility that reduced levels of production coupled with a low turnover maintain ANG-(1–7) levels in luteal cells. In addition, ANG-(1–7) production di-
rectly from ANG I via NEP that is independent of ANG II and ACE activity can occur in some cell types (61), and a similar route in luteal tissues may contribute to accumulated ANG-
(1–7) levels. The effect of alternative pathways of angio-
tensinogen processing includes the result that shutting down one pathway can drive enhanced accumulation of other angio-
tensin peptides. It is conceivable that in vivo, different path-
ways may operate in specific conditions of health or disease. Indeed, levels of circulating ANG-(1–7) and ANG II increase during pregnancy in women, and a change in the ratio of their concentration is implicated in the onset of preeclampsia (45). However, it remains to be determined whether the imbalance in their ratios arises from differential angiotensin peptide production by the corpus luteum of pregnancy.

The effects of the protease inhibitors on basal steroidogen-
esis correlated with their effects on ANG II accumulation. Quinapril and PMSF had no significant effects on progesterone production or ANG II accumulation, and PCMS at higher concentrations inhibited progesterone and ANG II levels, whereas ZPP enhanced basal progesterone production and ANG II accumulation. These effects of protease inhibitors on basal progesterone production may be unrelated to angiotensin processing and attributable to effects on the steroidogenic machinery. However, other studies have investigated ACE inhibition and ovarian steroidogenesis and have reported various effects. Inhibition of ACE activity in the frog ovary enhanced estradiol and progesterone production (4); in a hy-
perstimulated rabbit model, progesterone was unaffected and estradiol production was decreased (59); and in PMSG-treated rats, ACE inhibition by captopril infusion reduced serum levels of progesterone and enhanced estradiol output (2).

The varying physiological responses to ACE inhibition may be due to alternative angiotensinogen processing pathways in different species, but a further complicating factor is likely to be gonadotropin treatment. Previous studies identified an inter-
action between LH and the OVARAS. LH stimulates bovine thecal cell production and secretion of renin/prorenin through a cAMP-dependent mechanism (6), and in vivo, gonadotropin stimulation leads to enhanced ovarian ANG II levels in bovines and women (1, 39). Whether this effect is a direct response to gonadotropin or a secondary event is unclear. Bovine luteal ACE has been located to the endothelial cell population, and its activity is upregulated by a combination of estradiol and vascular endothelial growth factor (VEGF) but not estradiol alone (25, 33). However, the testicular ACE isoform (ACE2) is regulated by gonadotropin via a cAMP response element on the ACE gene (30, 75). We found an LH-dependent increase in ACE activity in cultured rat luteal cells that may be mediated by the LH second messenger cAMP. It is possible that en-
hanced ACE activity occurred in a population of endothelial cells as a secondary event to LH-stimulated endocrine cells. However, the luteal cells are purified by density gradient sedimentation and are principally steroidogenic cells (73),
suggesting a direct effect of LH on luteal ACE. The hypothesis of LH-regulated luteal ACE activity also is supported by a report that inhibition of ACE in women during normal menstrual cycles is without effect on serum steroid levels, but in gonadotropin-stimulated women during the luteal phase of in vitro fertilization (IVF) cycles, ACE inhibition reduces serum levels of progesterone and elevates estradiol (46). In our studies, progesterone output was unaffected by quinapril under basal conditions, but in LH-stimulated cells, an inhibitory effect was indicated when quinapril was included with ZPP. Taking these data together, inhibition of ACE activity may principally have steroidogenic effects in gonadotropin-stimulated cells, but it remains to be determined whether enhanced ACE activity and corresponding ANG II production play a role in LH-stimulated steroidogenesis.

LH-stimulated progesterone production also was inhibited by ZPP, PCMS, and PMSF. PCMS and PMSF had the most profound inhibitory effects on progesterone production, but only PCMS affected accumulation of ANG II and ANG-(1–7). We did not test the effect of exogenous ANG II or ANG-(1–7) with PCMS, but these peptides had no significant effect on progesterone production when added by themselves. The actions of PCMS and PMSF may be related to cholesterol transport (see below), and further studies are required to identify whether their antisteroidogenic sites of action include angiotensin processing.

ANG-(1–7) stimulates progesterone output in perfused rat ovaries from gonadotropin-primed animals (13), but we found no effect of exogenous ANG II or ANG-(1–7) on progesterone production in luteal cells. Furthermore, ZPP that inhibits PEP, a mediator of ANG-(1–7) production, had differential effects on progesterone that related to gonadotropin-stimulated accumulation. ANG-(1–7) is a mediator of ANG-(1–7) production, but PEP alone had no effect on progesterone accumulation. This effect was not due to enhanced degradation of ANG II by phosphodiesterase activity, because the phosphodiesterase inhibitor IBMX did not reverse PMSF-induced effects. We cannot rule out the possibility that a protease-dependent requirement for LH binding to its receptor (22) is a factor or that PCMS may have a direct effect on adenylyl cyclase activity (42). However, PCMS also exerted effects on steroidogenesis at a site postadenylate cyclase, because DB-cAMP-stimulated progesterone was blocked by PCMS. Basal progesterone was largely unaffected by PMSF and PCMS, except for a high concentration of PCMS, but a more pronounced inhibition of progesterone production occurred in LH-stimulated cells that could be related to cholesterol transport. Investigations of the postadenylate cyclase site of action utilized 22-OH-cholesterol that permeates mitochondria independently of transport mechanisms. PMSF-dependent inhibition of steroidogenesis was fully reversed, whereas PCMS-dependent inhibition was mostly restored by 22-OH-cholesterol. Therefore, PMSF and PCMS may affect cholesterol transport to the mitochondria and/or cytochrome P-450scc at sites including sterol carrier protein 2 (SCP2, which regulates cholesterol transport from the lipid droplet to the mitochondria), steroidogenesis activator polypeptide, and steroid acute regulatory peptide (Star). However, SCP2 is slowly turned over (58) and therefore seems unlikely to be affected by the acute treatment in our studies. On the other hand, Star is rapidly synthesized in response to LH and cAMP and is a key protein for transport of cholesterol from the outer to the inner mitochondrial membrane, where the p450scc resides (10, 56). During its import to the inner membrane, Star is proteolytically degraded from a 37-kDa precursor to a 30-kDa protein (55, 67) and may be sensitive to PCMS and PMSF. Indeed, in adrenal and Leydig cells, PMSF blocks stimulated steroidogenesis, at least in part, by inhibition of turnover of the 37-kDa Star precursor (44). It is unknown whether quinapril or ZPP affects the 37-kDa Star precursor, but their combined inhibitory effect was reversed by 20-OH-cholesterol, suggesting an inhibitory site of action before P-450scc.

Better understanding of the complexity of the OVRAS may allow new diagnostic and treatment strategies. In beginning to examine such complexities, we conclude from these studies that angiotensin processing in luteal cells includes the formation of ANG II, ANG-(1–7), and ANG III. Exogenous ANG II or ANG-(1–7) does not affect the acute production of progesterone in either basal or LH-stimulated paradigms; however, inhibitors of ACE and PEP cause inhibition of LH-stimulated steroidogenesis. This effect is not mediated by adenylyl cyclase activity and includes an inhibitory site of action before

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ACKNOWLEDGMENTS

We gratefully acknowledge DuPont Merck for donating losartan and Parke-Davis for providing quinapril and PD-123319.

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