Role of the angiotensin II system in regulation of ovulation and blood flow in the rat ovary

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The aim of the present study was to examine the roles of the angiotensin II receptor subtypes, AT₁ and AT₂, in ovulation, and to evaluate the contribution of angiotensin II-mediated pathways in regulation of ovarian blood flow. The AT₁-specific antagonist, losartan, was administered alone or in combination with the AT₂-specific antagonist, PD123319, to preovulatory rat ovaries perfused *in vitro*. Losartan (100 µmol l⁻¹) did not affect the number of ovulations, whereas the combination of losartan (100 µmol l⁻¹) and PD123319 (10 µmol l⁻¹) inhibited ovulation. The angiotensin II antagonists did not affect the ovarian production of oestradiol, progesterone, prostaglandin E₂ (PGE₂), PGF_{2α} or plasminogen activator activity. Ovarian nitric oxide production was inhibited by losartan. Ovarian blood flow was measured

by laser Doppler flowmetry *in vivo* in preovulatory rat ovaries. Intrabursal injection of angiotensin II reduced ovarian blood flow of gonadotrophin-stimulated rats. Losartan had no effect on basal ovarian blood flow but completely blocked the angiotensin II-induced reduction. In contrast, treatment with PD123319 increased basal ovarian blood flow and failed to reverse the effect of exogenously administered angiotensin II, indicating that under physiological conditions, ovarian blood flow of the rat is negatively regulated by angiotensin II mainly through the action of AT₂. Taken together, these results indicate that two different types of angiotensin II receptor facilitate ovulation by cooperative mechanisms and that they regulate ovarian blood flow in a different manner.

Introduction

The renin–angiotensin system has been recognized as an important regulator in mammalian homeostasis, mainly concerning the maintenance of blood pressure and electrolyte balance. Recent studies have shown that the renin–angiotensin system is localized in many extra-renal organs, such as the brain and the heart, with a broad spectrum of paracrine and autocrine functions (Kramar *et al.*, 1997; Chin *et al.*, 1998). All main components of the renin–angiotensin system are present in the ovary (Sealey *et al.*, 1985; Lightman *et al.*, 1987; Speth and Husain, 1988) and there is evidence that the ovarian renin–angiotensin system is involved in mammalian reproduction. The renin–angiotensin system in the ovary is upregulated by gonadotrophins (Fernandez *et al.*, 1985; Sealey *et al.*, 1985; Howard *et al.*, 1988). The concentration of angiotensin II in human follicular fluid increases after treatment with hCG or LH (Lightman *et al.*, 1987) and this result was confirmed by an

in vitro study with rabbit ovarian perfusion (Yoshimura *et al.*, 1994). It has been reported that angiotensin II induces rabbit ovulation (Yoshimura *et al.*, 1996) and that the ovulation rate is decreased by the treatment with angiotensin II antagonists as demonstrated both *in vivo* (Pellicer *et al.*, 1988) and *in vitro* (Peterson *et al.*, 1993).

Angiotensin II exerts its actions through the binding to a group of receptors, which are subclassified into the angiotensin II receptor type 1 (AT₁) and angiotensin II receptor type 2 (AT₂). Most of the well-characterized functions of angiotensin II, such as vasoconstriction and the maintenance of electrolyte balance, are ascribed to the AT₁ receptor, whereas physiological roles of the AT₂ receptor are still uncertain. Both of these angiotensin II receptors are expressed in the ovary, and the presence and distribution patterns of the two receptors differ significantly among species and developmental stages of the follicle (Pucell *et al.*, 1991; Obermüller *et al.*, 1998). It was previously reported that the blockade of the AT₂ receptor in the rat ovary by a specific AT₂ antagonist PD123319 did not inhibit ovulation, whereas a non-selective angiotensin II receptor antagonist saralasin reduced ovulation rate in the *in vitro* perfused ovary (Mikuni *et al.*, 1998). These results indicate the

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importance in ovulation of the pathways mediated exclusively through AT₁ or a combination of AT₁ and AT₂ in the rat ovary.

Angiotensin II is a potent vasoactive substance and part of its functions in the ovary may be related to the modulation of ovarian blood flow. During the growth of the ovarian follicle, the capillaries surrounding the antrum proliferate (Murakami *et al.*, 1988), and ovarian blood flow further increases shortly after the preovulatory LH surge (Janson, 1975; Brännström *et al.*, 1998) with a marked dilatation of the vessels around the ovulating follicles (Kranzfelder *et al.*, 1992). Since sufficient blood supply to the ovary is necessary throughout the ovulatory process and ovarian steroidogenesis (Zackrisson *et al.*, 2000), it is possible that angiotensin II affects ovarian vasculature and thereby ovarian function. However, the roles of angiotensin II and angiotensin II receptors on the regulation of ovarian blood flow have not been reported.

The aim of the present study was to explore further the functions of the angiotensin II receptors in the rat ovary during ovulation. The effects of an AT₁-selective antagonist, losartan, alone or in combination with PD123319, on the ovulation rate and the ovarian production of ovulation-associated mediators were studied in the rat ovaries perfused *in vitro*. The effects of angiotensin II and angiotensin II antagonists on ovarian blood flow were studied *in vivo* by laser Doppler flowmetry allowing real-time measurement of tissue blood perfusion.

Materials and Methods

Animals

Immature female Sprague–Dawley rats (B&K Universal, Sollentuna) were kept under a 14 h light:10 h dark cycle and had free access to pelleted food and water. All experiments were carried out according to the principles and procedures outlined in the NIH *Guide for the Care and Use of Laboratory Animals*, and were approved by the Animal Ethics Committee of Göteborg University.

Hormones and chemicals

Ovine luteinizing hormone (NIDDK-oLH-26) was kindly provided by the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (NIADDK) and National Hormone and Pituitary Program (Rockville, MD). Human CG was purchased from Serono (Rome). Equine chorionic gonadotrophin (eCG), 3-isobutyl-1-methylxanthine (IBMX) and angiotensin II were purchased from Sigma Chemical Company (St Louis, MO); ketamine was from Parke Davis (Barcelona); xylazine was from Bayer (Leverkusen); medium 199 was from GIBCO BRL (Rockville, MD); gentamicin sulphate was from Biological Industries (Kibbutz Beit Haemek); bovine serum albumin (fraction V) was from Boehringer

Mannheim (Mannheim); insulin was from Novo Nordisk (Bagsvaerd) and heparin was purchased from Lövens (Ballerup). The angiotensin II receptor subtype-selective nonpeptide antagonists losartan (selective for AT₁) and PD123319 (selective for AT₂) were kindly donated by DuPont (Wilmington, DE) and Parke-Davis (Ann Arbor, MI), respectively.

For the perfusion experiments, LH, losartan and PD123319 were dissolved in the perfusion media and stored at -70°C until used. IBMX was dissolved just before use in perfusion media. For the laser Doppler experiments, stock solutions of hCG, angiotensin II, losartan and PD123319 were prepared with 0.9% NaCl and kept at -70°C .

Ovarian perfusion

At 28 days of age, the rats were given 20 iu eCG s.c. to promote growth and maturation of a first generation of follicles to reach a large antral stage 48 h later. On the morning of day 30 of age, the rats were anaesthetized with 40 mg ketamine kg^{-1} and 6.5 mg xylazine kg^{-1} , and 300 iu heparin sulphate was injected i.v. through the femoral vein. Laparotomy was performed and the right ovary was surgically removed with its feeding and draining vessels as described in detail by Brännström *et al.* (1987). The bursa was gently opened and the ovary was placed in a perfusion chamber. The perfusion was performed in a recirculating system with 30 ml of medium (Medium 199 with Earl's salts supplemented with 0.026 mol sodium bicarbonate l^{-1} , 0.2 iu insulin ml^{-1} , 50 μg gentamycin sulphate ml^{-1} and 4% (w/v) BSA). The perfusion pressure was maintained at 80 mmHg and the medium was continuously gassed with 5% CO₂ and 95% O₂. The ovaries were perfused for 30–60 min before addition of any compound.

Losartan (100 $\mu\text{mol l}^{-1}$, $n=11$) alone or losartan (10 $\mu\text{mol l}^{-1}$, $n=6$ and 100 $\mu\text{mol l}^{-1}$, $n=5$) in combination with 10 $\mu\text{mol PD123319 l}^{-1}$ was added to the perfusion medium 30 min before the administration of LH and IBMX. IBMX is a non-selective phosphodiesterase inhibitor, which was added to potentiate the LH effects to stimulate optimally the ovulation process *in vitro* (Peterson *et al.*, 1993). Control ovaries were perfused at the same time as the treated ovaries but angiotensin II receptor antagonists were not present ($n=7$ controls for losartan alone and $n=13$ controls for PD123319 + losartan). The perfusion was continued for 20 h after administration of LH + IBMX. Samples of medium (1 ml) were taken at 0, 1, 3, 5, 7, 10 and 20 h and stored at -70°C for later analysis. At the end of the perfusion, the number of ovulations were determined by counting the ovulated oocytes present in the perfusion chamber under a stereomicroscope.

Another set of experiments with ovarian perfusion was conducted to measure the intra-ovarian content of the proposed ovulatory mediators, prostaglandin E₂

(PGE₂), PGF_{2α} and plasminogen activator (PA) activity. The ovaries were perfused as described above for 10 h after LH + IBMX administration (100 μmol losartan l⁻¹, *n* = 5; 100 μmol losartan l⁻¹ + 10 μmol PD123319 l⁻¹, *n* = 6; control, *n* = 5). At the end of the perfusion, the ovaries were removed from the perfusion chamber, snap frozen in liquid nitrogen and stored at -70°C for later analysis (see below).

Assays

Oestradiol and progesterone concentrations in the perfusion media were analysed by radioimmunoassays. Nitric oxide (NO) produced by the ovary was analysed as total amount of nitrite (NO₂⁻) and nitrate (NO₃⁻) in the perfusion media. Ten microlitres each of nitrate reductase and cofactors (Alexis Corporation, Leufelfingen) was added to 100 μl of sample to convert nitrate to nitrite. Greiss reagent (consists of 100 μl 5.8 mmol sulphanylic acid l⁻¹ in 5% (v/v) H₃PO₄ and 10 μl 3.85 mmol *N*-1-naphthylethylenediamine dihydrochloride l⁻¹ in water) was added to each sample. After 60 min incubation at room temperature, the absorbance was measured at 550 nm. Intra-ovarian PGE₂ and PGF_{2α} concentrations were analysed by enzyme immunoassay assay kits (RPN 222 and TRK 900, respectively; Amersham Pharmacia Biotech, Little Chalfont). PA assay was performed according to the method by Espey *et al.* (1985) with minor modifications. Briefly, ovarian tissues were homogenized in ice-cold 0.05 mol Tris-HAc buffer l⁻¹, sonicated and centrifuged (10 000 *g*, 20 min at 4°C). Supernatant or standard solution, 20 μl, was mixed with 100 μl of substrate S-2251 and 20 μl of plasminogen (Chromogenix AB, Mölndal), and after 2 h incubation the absorbance was measured by UV-max® (Molecular Devices, Menlo Park, CA) at 405 nm. Protein concentrations in the supernatant were measured by the BCA assay kit (Pierce, Rockford, IL). Inter- and intra-assay coefficients of variation were <10% for all the assays in this study.

Measurement of ovarian blood flow

Laser Doppler flowmetry. At 26 days of age, all rats were treated with 15 iu eCG s.c. to promote the growth and maturation of a first generation of preovulatory follicles. Some animals were given hCG (15 iu, i.p.) 48 h later to induce the ovulatory cascade with predicted ovulation 12–15 h later.

Longitudinal measurement of ovarian blood flow was performed by laser Doppler flowmetry (Zackrisson *et al.*, 2000). Ovarian blood flow was measured 46–48 h after eCG administration, when the ovary had reached a preovulatory stage, or 6–8 h after hCG, which is a time approximately half-way through the ovulatory stage. This stage corresponds to a time when ovarian blood flow is

maximal after hCG stimulation (Abisogun *et al.*, 1988; Makinoda *et al.*, 1988).

The rats were anaesthetized with s.c. injection of 50 mg ketamine kg⁻¹ and 10 mg xylazine kg⁻¹ and placed on a heating pad to maintain body temperature at 37°C. Tracheal intubation was performed to maintain patent airways and the iliac artery and the femoral vein on the left side were cannulated with PE-20 polyethylene catheters. Arterial blood pressure was measured from the iliac artery using a Grass polygraph (Grass Instruments, Quincy, MA). The animals were continuously infused with 0.5 mg ketamine kg⁻¹ min⁻¹ and 0.1 mg xylazine kg⁻¹ min⁻¹ in 0.9% (w/v) NaCl through the arterial cannula during the experiment.

One ovary was exposed by a flank incision and stabilized by a ligature tied to the periovarian adipose tissue. The laser Doppler probe (Probe 407, Perimed AB, Stockholm) with an adhesive miniholder was placed on the ovarian surface, avoiding larger blood vessels, for the measurement of relative changes in ovarian blood flow. The probe and the incision wound were covered by an aluminum foil shield to minimize the effect of external light. The signal was analysed by a laser Doppler flowmeter (PeriFlux System 5000 with PF5010 laser Doppler perfusion monitor units, Perimed AB) and was continuously recorded by PeriSoft software for Windows (Perimed AB). The ovarian blood flow was quantified as an arbitrary perfusion unit, which is proportional to the number and velocity of moving blood cells in an approximate tissue volume of 1 mm³ (Lissbrant *et al.*, 1997). As the ovarian blood flow measured in different preparations could not be compared directly, the average flow between -5 and 0 min in relation to the initial injection time point was used as a basal level and the relative ovarian blood flow values were used for analysis. All experimental procedures were completed within 90 min after the laparotomy.

Experimental protocols. Ovarian blood flow was measured in rats either in preovulatory or ovulatory stage. The systemic effect was minimized by administering angiotensin II and angiotensin II antagonists locally into the ovarian bursa (intrabursally; i.b.). A polytetrafluoroethylene (PTFE) tube (diameter 0.4 mm, Cole-Parmer International, Vernon Hills, IL), with the tip cut sharp, was attached to a micro-syringe filled with one of the following: 100 ng angiotensin II, 40 μg losartan, 40 μg PD123319 (each in 5 μl 0.9% NaCl) or the same volume of 0.9% NaCl, and was threaded through the periovarian adipose tissue into the ovarian bursa. Two of these preparations, one for the first injection and the other for the second injection, were made in each ovary according to the protocol below (Fig. 1).

After a stable blood flow signal had been recorded for at least 10 min, one of the following: angiotensin II, losartan, PD123319 or 0.9% NaCl as control was

Fig. 1. The experimental protocols used in the rat ovarian blood flow measurement study. PO: preovulatory stage; OV: ovulatory stage; eCG: equine chorionic gonadotrophin; ANG: angiotensin.

injected into the ovarian bursa (first injection). Ten to fifteen minutes after the first injection, angiotensin II (100 ng in 5 µl 0.9% NaCl) was administered intrabursally to all ovaries studied (second injection). The average blood flow between -5 and 0 min in relation to the first injection was used as a basal value and the relative value after each treatment was calculated. All the experimental procedures were completed within 90 min after the laparotomy. Between 30 and 50 µl of solution or air was injected through the tube at the end of each experiment to ensure that the expected bulging of the capsule wall took place, to confirm that the tube had actually been inserted into the bursal cavity.

Statistical analysis

Non-parametric tests were used in data analysis of the perfusion study, since in some of the experiments the data were not normally distributed. The results of intra-ovarian contents of PGE₂, PGF_{2α} and PA activity were analysed by Mann–Whitney U test. Number of ovulations and concentrations of steroids and nitrite or nitrate in the medium at each sample point were evaluated by Kruskal–Wallis rank test followed by Mann–Whitney U test. The results of the laser Doppler experiment were analysed using repeated measures ANOVA followed by Scheffe's test for the comparison within a group. Differences among multiple groups in response to the treatment were evaluated by one-factor ANOVA followed by Scheffe's test. $P < 0.05$ was considered to be statistically significant.

Results

Effects on ovulation and ovarian mediators

The numbers of ovulations observed in the different treatment groups are summarized (Fig. 2). In the con-

trol group ($n=7$), there were 12.0 (median, 25–75% range = 9.0–16.0) ovulations per ovary. No significant difference was seen in the number of ovulations between the control and the group treated with 100 µmol losartan l⁻¹ (median = 8.0, 25–75% range = 5.25–26.75, $n=11$). The administration of losartan in combination with 10 µmol PD123319 l⁻¹ did not affect ovulation rate when 10 µmol losartan l⁻¹ was administered (median = 12.5, 25–75% range = 5.0–20.0, $n=6$ versus median = 12.0, 25–75% range = 9.0–16.0 for control, $n=11$), whereas when 100 µmol losartan l⁻¹ was administered it reduced the number of ovulations (median = 3.0, 25–75% range = 1.75–4.5, $n=5$).

In all groups, there were marked increases in secreted progesterone and oestradiol concentrations in the perfusion media after the administration of LH + IBMX. None of the angiotensin II inhibitors, given alone or in combination, significantly altered the gonadotrophin-induced steroid concentrations (data not shown). The values of ovarian NO production, measured as total concentrations of nitrite and nitrate in the perfusate at 0, 10 and 20 h sampling points (Fig. 3) show that in all treated groups, NO concentrations in the media increased with time after the gonadotrophin stimulation. At the 20 h time point, nitrite or nitrate concentrations were significantly lower in the group with 100 µmol losartan l⁻¹ compared with the control and the group with 100 µmol losartan l⁻¹ + 10 µmol PD123319 l⁻¹.

No significant difference was observed among the tissue concentrations of PGE₂ and PGF_{2α} in ovaries perfused for 10 h of the treated groups (Fig. 4a and b, respectively), although the median values were the lowest for both prostaglandins in the group with 100 µmol losartan l⁻¹ + 10 µmol PD123319 l⁻¹. The administration of angiotensin II inhibitors did not affect PA activity in the ovarian tissue 10 h after the LH + IBMX stimulation (Fig. 5).

Fig. 2. Number of ovulations by rat ovaries after 20 h perfusions with angiotensin II receptor antagonists. The treatment with 100 $\mu\text{mol l}^{-1}$ losartan (Lo) alone ($n=11$) did not affect the number of ovulations compared with that of the control group ($n=7$). The combined treatment with 100 $\mu\text{mol l}^{-1}$ losartan + 10 $\mu\text{mol l}^{-1}$ PD123319 ($n=5$) significantly (** $P < 0.01$) reduced the number of ovulations, whereas 10 $\mu\text{mol l}^{-1}$ losartan + 10 $\mu\text{mol l}^{-1}$ PD123319 ($n=6$) did not affect the number of ovulations compared with the controls ($n=13$). Individual values are shown and medians are indicated by horizontal bars. IBMX: 3-isobutyl-1-methylxanthine.

Fig. 3. Concentrations of nitrite + nitrate in the perfusion media used in the present study of rat ovaries. Administration of losartan (Lo) ($n=11$) resulted in significantly lower concentrations of nitrite + nitrate at 20 h (* $P < 0.05$) compared with the controls (LH + IBMX; $n=13$). Treatment with PD123319 together with losartan ($n=5$) abolished this decline in nitrite or nitrate production by the rat ovary. Bars indicate 10–90% range; boxes indicate 25–75% range and horizontal bars indicate medians. IBMX: 3-isobutyl-1-methylxanthine.

Effects on ovarian blood flow

Preovulatory stage rats. In our preliminary experiment, intravenous administration of 100 ng angiotensin II to preovulatory stage rats caused a rapid and significant increase in mean arterial pressure and a reduction of ovarian blood flow that lasted for 5–10 min (data not shown). It is well established that ovarian blood flow

is affected by changes in the systemic blood pressure (Wiltbank *et al.*, 1990), and we could not exclude the possibility that systemic actions of angiotensin II might modulate ovarian circulation. Therefore, we used intrabursal administration of angiotensin II and angiotensin II antagonists for further experiments to

Fig. 4. (a) Prostaglandin E₂ (PGE₂) contents of rat ovaries perfused for 10 h. Ovarian contents of PGE₂ in ovaries perfused with 100 μmol losartan (Lo) l⁻¹ alone ($n=5$) or 100 μmol losartan l⁻¹ + 10 μmol PD123319 l⁻¹ ($n=6$) were not significantly different from those of controls ($n=5$). Bars indicate 10–90% range; boxes indicate 25–75% range and horizontal bars indicate medians. (b) Prostaglandin F_{2α} (PGF_{2α}) contents in rat ovaries perfused for 10 h. Contents of PGF_{2α} in ovaries perfused with 100 μmol losartan l⁻¹ alone ($n=5$) or 100 μmol losartan l⁻¹ + 10 μmol PD123319 l⁻¹ ($n=6$) were not significantly different from those of controls ($n=5$). Bars indicate 10–90% range; boxes indicate 25–75% range and horizontal bars indicate medians. IBMX: 3-isobutyl-1-methylxanthine.

minimize the systemic effects of these compounds. Intrabursal administration of 100 ng angiotensin II in 5 μl 0.9% NaCl reduced ovarian blood flow by 30.4%, with no significant increase in systemic blood pressure (Fig. 6 and Table 1). The amount of ovarian blood flow returned to the pretreatment value within 5 min. After a 10–15 min interval from the initial injection, the same dose of angiotensin II was given. The effect of this second injection was significantly smaller than that of the initial injection (Fig. 6). Intrabursal injection of 0.9%

NaCl did not alter blood pressure or ovarian blood flow.

Either of the two angiotensin II antagonists, losartan or PD123319, was administered before angiotensin II. Losartan by itself did not alter ovarian blood flow, but pretreatment with losartan completely blocked angiotensin II-induced ovarian blood flow reduction. In contrast to the effect of losartan, intrabursal injection of PD123319 produced a 52.7% increase in ovarian blood flow above the baseline value, and the subsequent

Fig. 5. Plasminogen activator contents in rat ovaries perfused for 10 h. Ovarian plasminogen activator activities in ovaries perfused with 100 μmol losartan (Lo) l^{-1} alone ($n=5$) or 100 μmol losartan l^{-1} + 10 μmol PD123319 l^{-1} ($n=6$) were not significantly different from those of controls ($n=5$). Bars indicate 10–90% range; boxes indicate 25–75% range and horizontal bars indicate medians. IBMX: 3-isobutyl-1-methylxanthine.

Fig. 6. A representative result of ovarian blood flow measurement by laser Doppler flowmetry. The rats were pretreated sequentially with equine chorionic gonadotrophin and hCG (ovulatory-stage rats). The marks at the top of the figure indicate the sequential injections of angiotensin II (100 ng, intrabursally). The ovarian blood flow values are presented in an arbitrary perfusion units (PU).

Table 1. Changes of ovarian blood flow and mean systemic arterial pressure in response to intrabursal injection of angiotensin II or angiotensin II-antagonists (first injection) followed by angiotensin II (second injection) in rats pretreated with equine chorionic gonadotrophin 46–48 h before the experiment (preovulatory stage rats)

Treatment (first injection)	n	Ovarian blood flow (percentage of basal flow)		Mean arterial pressure (mmHg)		
		First injection	Second injection	Before	First injection	Second injection
Control (0.9% NaCl)	6	100.2 ± 0.8	68.5 ± 5.0 ^{††}	76.7 ± 1.7	76.7 ± 1.7	86.7 ± 6.1
Angiotensin II (100 ng)	7	69.6 ± 4.1 ^{**††}	85.1 ± 4.7 ^{*†}	79.1 ± 1.5	85.7 ± 5.3	87.9 ± 5.0
Losartan (40 µg)	6	115.7 ± 8.5	113.7 ± 8.6 ^{**}	76.7 ± 1.7	78.3 ± 1.7	80.0 ± 1.3
PD123319 (40 µg)	6	152.7 ± 11.7 ^{**†}	96.7 ± 10.6 [*]	83.3 ± 2.1	82.5 ± 2.1	87.5 ± 4.8

Values relative to the pretreatment basal values (–5 to 0 min) are mean ± SEM; n = number of animals.

*P < 0.05 and **P < 0.01; value significantly different from control at each time point.

[†]P < 0.05 and ^{††}P < 0.01; value significantly different from the corresponding basal level of the same animal.

Table 2. Changes of ovarian blood flow and mean systemic arterial pressure in response to intrabursal injection of angiotensin II or angiotensin II-antagonists (first injection) followed by angiotensin II (second injection) in rats pretreated sequentially with equine chorionic gonadotrophin and hCG (ovulatory-stage rats)

Treatment (first injection)	n	Ovarian blood flow (percentage of basal flow)		Mean arterial pressure (mmHg)		
		First injection	Second injection	Before	First injection	Second injection
Control (0.9% NaCl)	5	100.2 ± 1.0	63.0 ± 2.5 ^{††}	83.0 ± 4.6	86.0 ± 4.6	87.0 ± 4.4
Angiotensin II (100 ng)	5	63.0 ± 2.5 ^{**††}	91.0 ± 7.8 [*]	83.0 ± 4.6	83.0 ± 4.6	90.0 ± 5.5
Losartan (40 µg)	5	106.8 ± 2.7	104.6 ± 1.9 ^{**}	83.8 ± 5.9	83.8 ± 5.9	91.3 ± 5.2
PD123319 (40 µg)	6	121.2 ± 5.8 ^{*†}	97.7 ± 5.4 ^{**}	86.7 ± 1.1	85.8 ± 1.5	93.3 ± 3.8

The experiments were conducted 6–8 h after hCG. Values relative to the pretreatment basal values (–5 to 0 min) are mean ± SEM; n = number of animals.

*P < 0.05 and **P < 0.01; value significantly different from control at each time point.

[†]P < 0.05 and ^{††}P < 0.01; value significantly different from the corresponding basal level of the same animal.

injection of angiotensin II resulted in a decline of the blood flow similar to the initial value. No significant change in frequency in short-term variations of microvascular flow was observed by the treatment with angiotensin II or angiotensin II antagonists. Arterial blood pressure was not significantly altered by any of these intrabursal treatments.

Ovulatory stage rats. The results of the ovarian blood flow measurement in rats treated sequentially with eCG and hCG are presented (Table 2). After the first injection of angiotensin II, ovarian blood flow declined to 63.0% of the pretreatment value. A second administration of angiotensin II exhibited a lower response than that at the first administration. Losartan did not affect the basal ovarian blood flow and abolished the effect of the following angiotensin II injection. Ovarian blood flow increased by 21.2% after the injection of PD123319 but returned to the initial value after the following angiotensin II treatment. Neither the systemic blood pressure nor the frequency in variation of microvascular flow was altered by the intrabursal treatment with angiotensin II or angiotensin II antagonists.

Discussion

The present study was designed to investigate the contribution of AT₁ to the ovulatory process by using an AT₁ selective antagonist, as our previous study showed that selective inhibition of AT₂ by PD123319 did not decrease the number of ovulations in the *in vitro* perfused rat ovaries (Mikuni *et al.*, 1998). The addition of losartan, a nonpeptide antagonist of angiotensin II with a specific affinity toward AT₁, did not influence the number of ovulations in the LH-stimulated perfused rat ovary, whereas the simultaneous blockade of AT₁ and AT₂ receptors by the combination of losartan and PD123319 reduced ovulation rate almost to the same extent as with saralasin treatment in an identical *in vitro* model (Peterson *et al.*, 1993; Mikuni *et al.*, 1998). These results imply that in the ovulatory process of the rat, the AT₁- and AT₂-mediated pathways function in a cooperative or compensatory way and that the inhibition of either pathway is not sufficient to affect ovulation.

The findings of the present study in the rat differ from those in the isolated rabbit ovarian perfusion model, in which both saralasin and PD123319 inhibited ovulation

whereas the AT₁-specific antagonist was without effect (Kuji *et al.*, 1996; Yoshimura *et al.*, 1996). These results indicate that the action of angiotensin II in the rabbit ovulatory process is mediated solely by the AT₂ receptor. The discrepancy between the previous results and the results in the present study may be related to inter-species differences, especially in the ovarian localization of angiotensin II receptor subtypes. In the rabbit ovary, AT₂ is expressed mainly in the granulosa cells of preovulatory follicles and AT₁ is localized to theca cells and ovarian stroma (Yoshimura *et al.*, 1996). In contrast, in the rat ovary, AT₂ is detected exclusively in granulosa cells of large antral follicles with the signs of atresia, whereas follicles of healthy appearance do not express AT₂ receptor. Angiotensin II receptors expressed in all other structures of the rat ovary examined are of the AT₁ type (Pucell *et al.*, 1991; Obermüller *et al.*, 1998). The absence of AT₂ expression in any viable part of the ovary, including the preovulatory and ovulatory follicles, and the predominant distribution of AT₁ receptor may indicate a relative importance of the AT₁-mediated pathways in the ovulatory process of the rat.

Intra-ovarian contents of PGE₂, PGF_{2α} and PA activity 10 h after LH stimulation were not affected by either of the angiotensin II antagonists administered alone or in combination. Prostaglandins are recognized as important mediators in the ovulatory process (Richards *et al.*, 1998) and it has been reported that in the gonadotrophin-stimulated ovary, the treatment with angiotensin II antagonists resulted in reduced prostaglandin production and also decreased ovulation rate in rabbits (Kuji *et al.*, 1996) and in rats (Mikuni *et al.*, 1998). In the latter studies, prostaglandin concentrations were measured in the media of perfused ovaries, whereas the present study examined the tissue concentrations of these ovulation-associated mediators. Thus, the cause for the discrepancy may be attributable to the difference in the components for the prostaglandin analysis. The ovarian tissue contents at 10 h, which is 2–5 h before anticipated ovulation, would presumably be a more exact indicator of the biological significance of changed synthesis of any ovarian mediators. It has been suggested that angiotensin II upregulates tissue-type PA and PA inhibitor type I, both of which are thought to be involved in the ovulatory cascade through the control of proteolysis and extracellular matrix breakdown (van Leeuwen *et al.*, 1994). The results of the present study indicate that the blockade of AT₁ and AT₂ receptors inhibits ovulation without altering ovarian PA activity.

In the present study, continuous measurements of ovarian blood flow with laser Doppler flowmetry revealed that intrabursal administration of angiotensin II rapidly reduced ovarian blood flow. No significant difference in the reduction was observed between preovulatory stage ovary (30.1% of the pretreatment value) and ovulatory stage ovary (37.0%). This instant

reduction in ovarian blood flow is in line with reports on the effects of angiotensin II on local blood flow in other organs, such as the kidney (Chin *et al.*, 1998) and brain (Kramar *et al.*, 1997). The nadir of the flow lasted for 1–2 min and ovarian blood flow returned to the pretreatment values within 5 min in all observations, as a result of presumably the relatively short half-life (16 s) of angiotensin II in rats (Al-Merani *et al.*, 1978). However, considering the less pronounced effect of the second injection of angiotensin II compared with the first injection, it is possible that angiotensin II activates some type of local compensatory mechanism to maintain ovarian blood flow. Nitric oxide may be one of the mediators involved in this vaso-relaxing property of angiotensin II. In various organs, such as the kidney, NO has a blood sparing function in the presence of angiotensin II (Chin *et al.*, 1998; Walker *et al.*, 1999), and NO synthase (NOS) is expressed also in the ovary, especially in the vascular-rich theca cell layer (Zackrisson *et al.*, 1996). The results of the present study with ovarian perfusion showed reduction in the ovarian production of NO by a selective AT₁ antagonist losartan. This finding implies that NO plays a role in maintaining the ovarian blood flow in the presence of angiotensin II. However, the treatment with a combination of losartan and PD123319 completely abolished this reduction in NO production.

In the next set of experiments, the contributions of the AT₁ and AT₂ receptors in the angiotensin II-mediated reduction of ovarian blood flow were examined. In regulation of systemic blood pressure, the AT₁ receptor is known to mediate the pressor effects of angiotensin II, whereas the AT₂ receptor has been demonstrated to play antagonistic roles to AT₁ (Oliverio *et al.*, 1998; Siragy *et al.*, 1999). Overexpression of the AT₂ receptor in aortic vascular smooth muscle cells in transgenic mice completely abolished the AT₁-mediated pressor effect of angiotensin II (Tsutsumi *et al.*, 1999). However, there appear to be variations in the angiotensin II receptor-mediated regulation of local blood perfusion among different organs. Experiments on the uterine artery of rats indicated the mediation of contractile activity of vascular smooth muscle through AT₁ and vasorelaxation through AT₂ (Zwart *et al.*, 1998). On the contrary in the kidney, AT₂-mediated vasoconstriction and reduced renal blood flow were observed *in vivo* in mice and *in vitro* in an isolated perfusion model of the rat (Müller *et al.*, 1998; Ruan *et al.*, 1999).

In the present study, the blockade of AT₁ receptor with losartan did not affect the basal ovarian blood flow but intrabursal administration of PD123319 significantly increased the blood flow in the ovary. This result could be interpreted as indicating that in the preovulatory rat ovary, blood vessels are mainly constricted through AT₂ and not through AT₁. Considering the tissue-specific localization of angiotensin II receptors in the ovary, this finding may indicate that AT₂, which is

exclusively expressed in the atretic follicles of the rat (Obermüller *et al.*, 1998), selectively constricts blood vessels in this region and reduces blood supply to the atretic follicles. However, pretreatment with losartan completely eliminated the action of angiotensin II to reduce the ovarian blood flow but PD123319 was without effect, indicating that the effect of exogenously added angiotensin II was exerted mainly through the AT₁ receptor.

These findings may be explained by two models. The first model is based on a compartmentalization of local concentrations of angiotensin II within the ovary. As the AT₂ receptor is localized exclusively to the atretic follicles of the rat, if the concentration of angiotensin II in this compartment is higher than in the other parts of the ovary, blood flow in this region may be selectively restricted. If AT₂ activation is almost saturating, any additional angiotensin II will have no further effect. It is of note that higher concentrations of prorenin and ACE are detected in the AT₂-expressing atretic follicles (Speth and Husain, 1988; Schultze *et al.*, 1989). The second model is that there are compensatory mechanisms against AT₁-mediated vasoconstriction in the ovary. These mechanisms may be able to maintain the ovarian blood flow under physiological conditions but cannot counteract the action of the excessive amount of angiotensin II that was administered in the experiment. Nitric oxide may be involved in this compensatory mechanism since NOS coexists with AT₁ in the theca cell layer and ovarian stroma, and the results of the present perfusion study *in vitro* indicate that angiotensin II stimulates ovarian production of NO through AT₁. These models may be in line with the previous observations that vasoconstriction in parts of the ovary redirects blood flow from atretic follicles to healthy preovulatory follicles (Scanes *et al.*, 1982). This redistribution and increase of blood flow in and around the ovulatory follicles is considered to be essential for the ovulatory process to proceed until follicular rupture (Janson, 1975).

In summary, the present findings demonstrate that simultaneous blockade of AT₁ and AT₂ receptors inhibits gonadotrophin-induced ovulation in the rat, whereas only the AT₁ selective antagonism is without effect. The mechanisms underlying this inhibition of ovulation is not likely through the modulation of PG synthesis or PA activity. Ovarian blood flow during the preovulatory period is negatively regulated by angiotensin II and under physiological conditions, AT₂ receptor seems to be the receptor subtype involved. Exogenously administered angiotensin II exerts the vasoconstrictive effect through AT₁ receptor, and the presence of compensatory mechanisms to maintain ovarian blood flow is indicated.

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