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Angiotensin II directly induces follicle rupture and oocyte maturation in the rabbit

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To investigate the possible direct involvement of angiotensin II (Ang II) in ovulation and oocyte maturation, Ang II at 100 or 10 μg was administered at 2-h intervals in the in-vitro perfused rabbit ovaries. The addition of Ang II in the perfusate induced ovulation in vitro in the absence of gonadotropin, while ovulation did not occur in any contralateral control ovaries. However, the ovulatory efficiency in the Ang II-treated ovaries was significantly lower than in hCG-treated ovaries. Ang II significantly stimulated the meiotic maturation of ovulated ova and follicular oocytes. Concomitant addition of the specific receptor antagonist of Ang II, saralasin, 30 min before the onset of Ang II administration blocked Ang II-induced ovulation in a complete manner. Although saralasin did not inhibit completely hCG-induced ovulation and oocyte maturation, these results suggest that Ang II produced in the ovary may act locally in the process of ovulation.

Angiotensin II; Angiotensin-receptor antagonist; Follicle rupture; Oocyte maturation; Rabbit

1. INTRODUCTION

A functional local renin-angiotensin system (RAS) is known to exist in the ovary [1,2]. Recently, Pellicier et al. [1] demonstrated that intraperitoneal administration of Ang II receptor antagonist, saralasin, blocked ovulation in immature rats treated with pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). This suggests that Ang II has an obligate role in ovulation as an intermediary of hCG action. In contrast, an autoradiographic study using the same procedure and animals [3] revealed the lack of Ang II receptors in preovulatory follicles containing the LH receptor. These results, in conjunction with data showing that Ang II receptor blockade by intraperitoneal injection of saralasin has no effect on ovulation [3], do not support the involvement of intraovarian Ang II in ovulation. The discrepancy of the information about the effects of Ang II on gonadotropin-induced ovulation in rats impeded a better understanding of the physiological role of Ang II in ovulation. No clear evidence of a direct role for Ang II in the process of ovulation has been obtained. Therefore, we investigated the direct effect of Ang II in the process of follicle rupture and oocyte maturation in the in vitro perfused rabbit ovaries. This model is useful for exploring the direct effects of various substances on ovulation independent of any systemic influences [4,5].

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2. MATERIALS AND METHODS

2.1. *Materials*

Forty-eight sexually mature female Japanese white rabbits weighing 3.5–4.5 kg, which were cared according to Kyorin University School of Medicine guideline, were isolated for a minimum of 3 weeks. Animals were caged individually and fed water and a diet of Purina rabbit chow ad libitum under controlled light and temperature. Rabbits were anesthetized with intravenous sodium pentobarbital (32 mg/kg), given heparin sulfate (120 U/kg) for anticoagulation, and then subjected to laparotomy. Ovaries were excluded from further study if they appeared immature. They also were excluded if 50% or more of the surface follicles were hemorrhagic.

2.2. *Ovarian perfusion*

Each ovarian artery was cannulated in situ after ligation of the major anastomotic connections. The ovary with its artery, vein, and supporting adipose tissue was removed and immediately placed in a perfusion chamber. The perfusion fluid consisted of 150 ml of Medium 199 (Gibco, Grand Island, NY) containing 1% bovine serum albumin (BSA; Fraction V powder, Sigma Chemical Co., St. Louis, MO, USA), which was supplemented with heparin sulfate, insulin, streptomycin and penicillin G and adjusted to a pH of 7.4. The cannulation procedure and the perfusion technique have been described in detail [6]. The perfusate was introduced through the ovarian artery cannula and was collected from the unligated ovarian vein and recycled. In the selected experiments, the ovarian vein was also cannulated using Teflon tubing beveled at 45° as previously described [5,7]. In a modification of this technique, 1% BSA was added to the basic perfusion fluid to increase the oncotic pressure and decrease edema formation [7]. Ovaries were observed every 15 min for evidence of follicle growth and rupture throughout the 12 h of perfusion.

At the time of follicle rupture, the ovulated ovum surrounded by its cumulus mass was recovered carefully from the ovarian surface using a Pasteur pipette. The time interval from hCG or Ang II administration to follicle rupture was recorded. The ovulatory efficiency (percent of follicles > 1.5 mm that ovulated) was calculated for each group. At the end of perfusion, follicular oocytes were recovered by aspiration from mature follicles > 1.5 mm in diameter. Both ovulated ova and

follicular oocytes were assessed for stage of maturity and signs of degeneration, as previously described [5,8]. Oocytes were placed on slide, fixed in 2.5% glutaraldehyde and then stained with 0.25% lacmoid in 45% acetic acid for microscopic evaluation. The degree of ovum maturity was expressed as the percentage of ova achieving germinal vesicle breakdown (GVBD). Ova were also assessed for degenerative changes including vacuolation, cytolysis, necrosis, fragmentation and loss of spherical shape.

2.3. *Experimental design*

In the preliminary experiment using 6 ovaries, a single injection of angiotensin II (Ang II; Sigma Chemical Co., St. Louis, MO) at 100 μg in 0.1 ml phosphate-buffered saline (PBS) was administered to the perfused rabbit ovary and the perfusate samples were withdrawn at 15, 30, 45, 60, 75, 90, 105, 120, and 240 min after the onset of perfusion. In the absence of a rabbit ovary, the concentration of Ang II in the perfusate was also measured at 15, 60, 90, 120, and 240 min after the administration of 100 μg Ang II. In the first experiment, Ang II at 100 or 10 μg was added to the perfusate every 2 h for 10 h of perfusion. The perfusate of the contralateral ovary was treated with 0.1 ml PBS alone or with 50 IU of hCG (CH-446, biological activity 3830 IU/mg; Organon, Oss, The Netherlands). In 6 hCG-treated ovaries and 6 ovaries perfused with medium alone, the secretion rates of Ang II by perfused rabbit ovaries were calculated by determining the difference in concentrations between the venous and arterial samples [5]. In a related experiment, saralasin [(Sar^1,Val^5,Ala^8)-Ang II, Sigma Chemical Co.] was added to the perfusate of one ovary while the contralateral ovary which served as a control was perfused simultaneously with medium alone. Thirty minutes after the onset of perfusion, 100 μg Ang II was administered every 2 h to the perfusate for 10 h. Following exposure to Ang II the ovaries were perfused for a total of 12 h. Six ovaries from 6 rabbits were used in each treatment group. In the second experiment, one ovary was perfused with medium alone and served as a control. The contralateral ovary of each rabbit was perfused simultaneously with saralasin at 2×10^{-8}, 2×10^{-7}, or 2×10^{-6} M in a separate chamber. Thirty minutes after the onset of perfusion, 50 IU of hCG was administered to the both ovaries. Six rabbits were used to assess the effect of saralasin at each dose. Ovaries were perfused for a total of 12 h after hCG exposure.

2.4. *Radioimmunoassay (RIA) for Ang II*

The extraction of Ang II was performed as described by Beadwell [9] with minor modification. After the addition of [^{125}I]Ang II to estimate the recovery rate, the perfusate samples (0.1~0.6 ml) were suspended in 30 mg florisil to absorb Ang II in the perfusate. The mixtures were centrifuged at 400 × *g* for 10 min, and washed twice with 1 ml distilled water. The precipitates were eluted twice with 0.5 N hydrochloric-aceton solution and centrifuged at 400 × *g* for 5 min. One ml of petroleum ether was added to the supernatant to separate unsubstituted fatty acids. The aqueous extracts were evaporated under a stream of nitrogen gas. The extracts containing Ang II were reconstituted in 500 μl of 0.05 M borate buffer (pH 8.5) containing 0.2% bovine serum albumin, 0.5% NaCl, and 0.1% EDTA-2Na. Aliquots of these extracts were used for RIA of Ang II and determination of recovery rates. The average recovery rate for Ang II was 89.5%. Ang II immunoreactivity in the perfusate was examined by RIA using [^{125}I]Ang II purchased from New England Nuclear Co. (Boston, MA) and a rabbit antiserum against an Ang II-BSA conjugate [10]. The antiserum crossreact 0.3% with angiotensin I, 10^{-4}% with [Sar^1-Ala^8]-Ang II, 30.8% with angiotensin III, 46.0% with des-[Arg^1,Asp^2]-Ang II, and 100% with [Val^5]-Ang II. The standard curve for this assay ranged from 0.75 to 500 pg per tube. The intra-assay and inter-assay coefficients of variation for Ang II were 11.7% and 12.9%, respectively.

2.5. *Statistical analysis*

All data regarding the numbers of ovulation per ovary, ovulatory efficiency, time of ovulation, % GVBD, and the concentrations of Ang II are represented as the mean ± S.E.M. Statistical analysis was performed analysis of variance (ANOVA) and then Scheffé's test was used to establish whether the two categories differed one from another. Differences were considered significantly if P was < 0.05.

3. RESULTS AND DISCUSSION

In order to determine the best way to introduce Ang II in perfused rabbit ovaries, Ang II concentration in the perfusate was measured during the 2 h perfusion period. After a single injection of 100 μg Ang II in the presence of the perfused rabbit ovary, the concentration of Ang II in the perfusate was 656 ± 14 ng/ml ((5.7 ± 0.1) × 10^{-7} M) at 15 min and decreased thereafter. At 4 h after Ang II administration, the concentration of Ang II in the perfusate reduced to 68 ± 10 ng/ml. In the absence of a rabbit ovary, the concentration of Ang II in the perfusate did not change substantially for 2 h. This shows that Ang II is not absorbed by the glassware or tubing of the perfusion system and that the apparent degradation of Ang II could be neglected, in the absence of the perfused rabbit ovary. The fact that the concentration of Ang II in the perfusate decreased as the increasing duration of ovarian perfusion suggests that Ang II could be metabolized in the perfused rabbit ovaries. In the subsequent experiment, therefore, Ang II was added to the perfusate every 2 h.

The addition of 100 μg of Ang II at 2-h intervals to the perfusate induced follicle rupture in all perfused ovaries in the absence of gonadotropin in vitro while

Table I

Ovulation in ovaries perfused with medium alone, Ang II, Ang II plus saralasin, or hCG

	Ang II (per 2 h)			Control (medium alone)	hCG (50 IU)
	100 μg	10 μg	100 μg + saralasin (2×10^{-6} M)		
No. of ovaries perfused	12	6	6	12	6
No. of ovaries ovulating	12	3	0	0	6
No. of ovulations per ovary	2.83 ± 0.22^{a,b}	1.00 ± 0.51^{b,c}	0	0	5.50 ± 0.43^{a,c}
Ovulatory efficiency (%)*	39.4 ± 4.7^{d}	13.3 ± 8.3^{e}	(−)	(−)	83.0 ± 4.1^{d,e}
Time of ovulation (h)	8.31 ± 0.78	8.78 ± 0.68	(−)	(−)	8.57 ± 0.56

*The percentage of mature follicles that proceeded to rupture.

Values with the same superscripts differ significantly: ^{a,d} $P < 0.01$; ^{b} $P < 0.05$; ^{c,e} $P < 0.001$. Values are mean ± S.E.M.

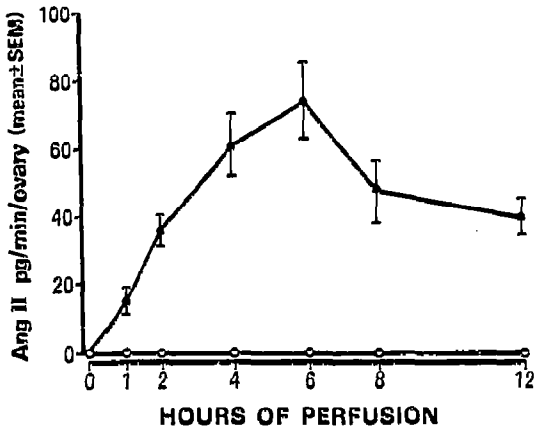

Fig. 1. Changes in angiotensin II (Ang II) production by perfused rabbit ovaries. Rabbit ovaries were perfused with medium alone (○——○) or 50 IU hCG (●——●). Ovarian secretion rates (pg/min/ovary) were calculated by determining the difference in concentrations between venous and arterial samples. Data regarding hCG-treated ovaries represent the mean ± S.E.M. of 6 perfused rabbit ovaries.

ovulation did not occur in any contralateral ovary perfused with medium alone (Table I). Saralasin added to the perfusate 30 min before the onset of Ang II administration completely blocked Ang II-induced ovulation. However, both the ovulatory efficiency, percent of follicles ≥ 1.5 mm which ovulated, and the number of ovulations per ovary in the Ang II-treated ovaries were significantly lower than in hCG-treated ovaries ($P < 0.01$). This implies that other components within the preovulatory follicular environment may also be required for follicles to rupture. Daud et al. [11] recently demonstrate that the follicular Ang II receptor is suppressed in healthy follicles as they are sensitized to circulating gonadotropin. The fact that ovulatory efficiency in Ang II-treated ovaries is low, regardless of the administration of pharmacological dosage of Ang II, as compared to the hCG-treated control, may also be due to the suppression of Ang II receptor during the preovulatory maturational process. In the in vivo studies described by Pellicer et al. [1] and Daud et al. [2], the possibility cannot be completely excluded that the Ang II receptor antagonist has non-specific effects in the process of ovulation with the systemic influence or that the existence of the renal RAS affects a physiological action of Ang II in gonadotropin-induced ovulation. However, the present data showing that Ang II stimulates directly follicle rupture in the absence of any systemic influence suggests the possible involvement of Ang II in the process of ovulation.

In the present study, pharmacological dosages of Ang II were added to the perfusate when compared the concentrations of Ang II secreted by the perfused rabbit ovaries in response to gonadotropin exposure (Fig. 1). However, exogenous Ang II did not increase the degeneration rate of either ovulated ova (Ang II at 100 μg, 9.72 ± 6.2%; 10 μg, 11.1 ± 6.4%) as compared to the hCG-treated control (9.4 ± 3.8%) or follicular oocytes (Ang II at 100 μg, 12.5 ± 5.6%; 10 μg, 12.2 ± 5.3%) as compared to the untreated control (5.7 ± 3.9%).

To assess the role of endogenous Ang II in the preovulatory process, the effect of saralasin on hCG-induced ovulation in vitro was determined (Table II). Saralasin alone neither induced follicle rupture nor stimulated the meiotic maturation of follicular oocytes. All ovaries treated with hCG alone or with hCG plus saralasin at 2×10^{-7} or 2×10^{-8} M ovulated. Three of six ovaries treated with hCG plus saralasin at 2×10^{-6} M failed to ovulate. The mean number of ovulations per ovary was significantly reduced in ovaries perfused with saralasin, as compared to hCG-treated controls. The addition of saralasin to the perfusate blocked hCG-induced ovulation in a dose-dependent manner (Table II). The secretion rate of Ang II by the perfused rabbit ovaries was not detectable in the absence of gonadotropin. The addition of hCG to the perfusate significantly increased Ang II production throughout the entire perfusion periods (Fig. 1). The secretion rate of Ang

Table II

Effects of saralasin on hCG-induced ovulation and oocyte maturation in the in vitro perfused rabbit ovaries

	Saralasin + hCG (50 IU)			hCG (50 IU)	Saralasin (2×10^{-6} M)
	2×10^{-6} M	2×10^{-7} M	2×10^{-8} M		
No. of ovaries perfused	6	6	6	18	6
No. of ovaries ovulating	3	6	6	18	0
No. of ovulations per ovary	1.00 ± 0.51^{a}	2.75 ± 0.42^{b}	2.83 ± 0.75^{c}	$4.89 \pm 0.42^{a,b,c}$	0
Ovulatory efficiency (%)*	$18.9 \pm 8.9^{d,e,g}$	$43.2 \pm 6.3^{e,f}$	53.8 ± 4.3^{g}	$79.1 \pm 1.9^{d,f}$	0
Time of ovulation (h)	8.97 ± 1.20	8.53 ± 0.26	7.66 ± 0.28	8.51 ± 0.23	(–)
% GVBD†					
Ovulated ova	38.9 ± 14.2^{h}	78.1 ± 9.3	65.5 ± 11.5	86.6 ± 6.0^{h}	(–)
Follicular oocytes	77.8 ± 10.2	89.3 ± 5.8	83.3 ± 16.6	94.4 ± 6.7	5.2 ± 3.3

*The percentage of mature follicles that proceeded to rupture.
†The percentage of oocytes that achieved germinal vesicle breakdown (GVBD).
Values with the same superscripts differ significantly: ^{a,f} $P < 0.01$; ^{b,c,e,g,h} $P < 0.05$; ^{d} $P < 0.001$. Values are mean ± S.E.M.

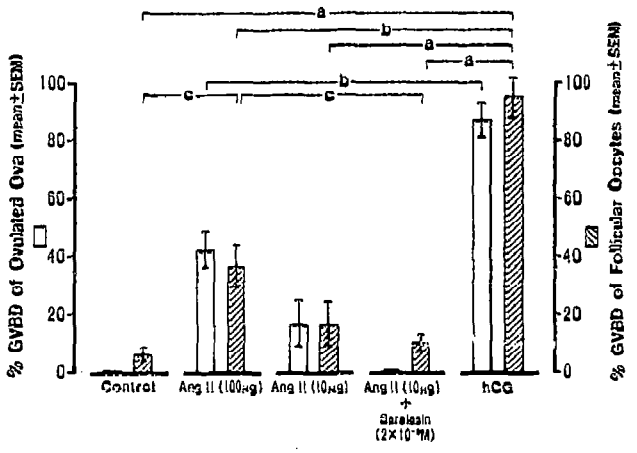

Fig. 2. Oocyte maturation in ovaries perfused with medium alone, Ang II, Ang II plus salarasin, or hCG. The degree of oocyte maturation in ovulated ova and follicular oocytes was expressed as the percentage of oocytes achieving germinal vesicle breakdown (GVBD). a, $P < 0.001$; b, $P < 0.01$; c, $P < 0.05$.

II by the perfused rabbit ovaries reached its maximum 6 h after exposure to hCG and then declined. This implies that a small amount of Ang II can be synthesized by rabbit ovaries in response to gonadotropin. These data, taken together with other data showing that the increase in plasma prorenin concentration occurred almost exactly at the time of ovulation [12,13] and that gonadotropin stimulates renin activity and Ang II immunoreactivity in follicular fluid [14], support the existence in the ovary of an intrinsic RAS, which is responsible as a possible intermediary of gonadotropin-induced ovulation. Husain et al. [15] could not find the expected autoradiographic distribution of Ang II receptors in every developing follicle, suggesting that not all preovulatory follicles respond necessarily to exogenous or endogenous Ang II. Alternatively, the failure of Ang II to induce ovulation with an efficiency comparable to that of hCG implies that the response to Ang II of preovulatory follicles leading to ovulation can be attributed to the functional heterogeneity of follicles within the ovary.

Recent data derived from human in-vitro fertilization program demonstrate that the prorenin concentration in the follicular fluid correlates with follicular development, oocyte-cumulus-complex maturity, and oocyte viability resulting in the successful pregnancy [16,17]. In the present study, Ang II stimulated significantly the meiotic maturation of ovulated ova and follicular oocytes in the absence of gonadotropin stimulus (Fig. 2). Furthermore, concomitant addition of saralasin at 2×10^{-6} M inhibited significantly hCG-induced oocyte maturation (Table II). In oocytes from *Xenopus laevis*, Ang II has been also shown to promote progesterone-induced oocyte maturation by mobilizing Ca^{2+} via a signal transferred through the gap junctions between the follicular cells and the oocyte [18]. These observations, in conjunction with data showing the immunolocalization of angiotensin converting enzyme in the rabbit oolemma [19], suggests that the ovarian RAS may be involved in the mechanism of mammalian oocyte maturation as well. Further experiments are necessary, however, to verify this hypothesis since concomitant addition of saralasin did not inhibit completely hCG-stimulated oocyte maturation (Table II), and because the active renin content within the ovary was recently shown not to correlate with oocyte maturity [11].

In conclusion, these results indicate that Ang II has a direct role in follicle rupture and oocyte maturation and support the hypothesis that Ang II produced in the ovary may act locally in the process of ovulation. However, the physiological role of ovarian RAS may vary greatly as the ovarian follicle develops.

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