Direct effect of angiotensin II on in-vitro perfused rabbit ovary

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Summary. The effects of angiotensin II (AII) and its receptor blocker, saralasin (SAR), on ovulation and oocyte maturation were investigated in an isolated, in-vitro perfused rabbit ovary. Ovulation and oocyte maturation were induced by AII in the absence of human chorionic gonadotrophin (hCG). SAR inhibited ovulation induced by AII or hCG, but not oocyte maturation. AII appears to play a critical role in follicle rupture, but not in resumption of oocyte meiosis.

Keywords: angiotensin II; ovulation; ovary; oocyte maturation; saralasin; rabbit

Introduction

Human preovulatory follicular fluid concentrations of prorenin, renin and angiotensin II (AII) are higher than those in peripheral blood in gonadotrophin-stimulated and natural cycles (Fernandez *et al.*, 1985; Culler *et al.*, 1986; Glorioso *et al.*, 1986; Lightman *et al.*, 1987). The intracellular presence of renin and AII has been demonstrated in luteal, thecal, stromal and luteinized granulosa cells, but not in other granulosa cells (Lightman *et al.*, 1988; Palumbo *et al.*, 1989). Administration of saralasin (SAR), a specific AII receptor blocker, to gonadotrophin-stimulated rats has been reported to inhibit ovulation and oocyte maturation (Palumbo *et al.*, 1988; Pellicer *et al.*, 1988). AII has been demonstrated to stimulate ovarian steroidogenesis in rats (Pucell *et al.*, 1987). Ovarian AII receptors have been localized autoradiographically on rat granulosa, theca, and luteal cells (Speth *et al.*, 1986; Husain *et al.*, 1987; Daud *et al.*, 1988; Lightman *et al.*, 1988). These receptors, predominantly on the granulosa cells, display cyclical variation over the rat oestrous cycle (Daud *et al.*, 1988; Lightman *et al.*, 1988). Although these findings raise the possibility of a role for AII in ovulation and oocyte maturation, controversy exists because AII receptors were not detected on rat granulosa cells containing luteinizing hormone (LH) receptors (Daud *et al.*, 1989).

This study investigated whether AII has a direct effect on isolated rabbit ovary in the absence of gonadotrophin and has a physiological role in the ovary. The major objective was to examine the effects of AII and its receptor blocker, SAR, on ovulation and oocyte maturation, using an in-vitro perfused ovary system. In addition, alterations in the follicular microvasculature following stimulation with AII or gonadotrophin were compared by scanning electron microscopy (SEM).

Materials and Methods

Animals. Twenty-nine sexually mature New Zealand White female rabbits weighing 3.5–4.5 kg were caged individually for at least 3 weeks before use. Animals were given water and a diet of rabbit chow *ad libitum*. Rabbits were

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anaesthetized with intravenous sodium pentobarbitone (32 mg/kg), treated with heparin sulphate (120 U/kg) for anticoagulation and then subjected to laparotomy. All studies were performed in accordance with the National Institutes of Health (1985) *Guide for the Care and Use of Laboratory Animals*.

In-vitro perfusion. The cannulation procedure and perfusion apparatus have been described (Lambertsen *et al.*, 1976; Kobayashi *et al.*, 1981). Briefly, each ovarian artery was isolated and cannulated *in situ* after ligation of major anastomotic connections. Each ovary with its vein, cannulated artery and supportive connective tissue was removed and placed in the perfusion chamber. The perfusion system consisted of an ovarian perfusion chamber, a reservoir, an oxygenator and a pulsatile roller pump that maintained flow at 1.5 ml/min, the approximate blood flow to the rabbit ovary (Ahren *et al.*, 1972). Each ovary was perfused with 150 ml of tissue culture medium 199 (TCM199, M. A. Bioproducts, Walkersville, MD, USA) supplemented with 200 U heparin sulphate, 20 U insulin, 50 mg streptomycin and 75 mg penicillin G/l) at 37°C for 10.5 h. Ovaries were examined at 15-min intervals for evidence of follicular growth and rupture. In several experiments, arterial pressure was measured with a manometer at 3-h intervals over the course of perfusion. Ovulatory efficiency, the percentage of mature follicles (≥ 1.5 mm in diameter) that proceed to rupture during perfusion, was calculated for each ovary. Ovulated ova and follicular oocytes from unruptured follicles were assessed for stage of maturity as described by Kobayashi *et al.* (1981).

Direct effects of AII on ovulation and oocyte maturation in the absence of gonadotrophins. AII (Sigma Chemical Co., St Louis, MO, USA) was added to the perfusate of each experimental ovary at 15 ng, 1.5 μ g or 150 μ g for a final concentration of 0.1, 10 or 1000 ng/ml, respectively, at the onset of perfusion. The control ovaries received only the saline vehicle. AII administration was repeated 2 h later. Six ovaries were perfused for each dose. A total of 12 rabbits was used for this experiment.

Effects of SAR on AII-induced ovulation and oocyte maturation. One ovary of each of 4 rabbits was perfused with TCM199 containing 1.0 μ g of SAR/ml ([Sar¹, Val⁵, Ala⁸]-AII, Sigma Chemical Co.); the contralateral control ovary was perfused with medium alone; 30 min after the onset of perfusion, 15 ng AII was added to the perfusate and perfusion was continued for a further 10 h.

Effects of SAR on hCG-induced ovulation and oocyte maturation. SAR (1.0 μ g/ml, 10 μ g/ml) was incorporated in the perfusate of experimental ovaries; medium alone was used for control ovaries. Thirty minutes later, 50 iu hCG (Pregnyl, Organon, West Orange, NJ, USA) was added to the perfusate of experimental and control ovaries. To investigate the effect of SAR alone, 7 ovaries were perfused with 10 μ g SAR/ml and exposed to saline vehicle in place of hCG. Thirteen rabbits were used for this experiment.

Morphology of preovulatory follicles treated with AII or hCG. One ovary of each of 4 rabbits was perfused with 0.1 ng AII/ml, while the contralateral ovary was perfused with 50 iu hCG. The agent was added to the perfusate 30 min after the onset of ovarian perfusion. Perfusion was terminated 4–6 h after drug addition to obtain preovulatory follicles for scanning electron microscopy (SEM) (Kitai *et al.*, 1985b).

Statistical analysis. Data were analysed using the χ^2 test with Yates' correction (ovulatory efficiency and ovum stage) and ANOVA (time of ovulation).

Results

Direct effects of AII on ovulation and oocyte maturation in the absence of gonadotrophins

AII induced ovulation at all concentrations (0.1, 10, and 1000 ng/ml), in the absence of hCG. The greatest ovulatory efficiency (75%) was observed at the highest dose tested (Table 1). Increasing AII concentration reduced ovulation time, but the difference was not significant (Table 1). As the concentration of AII increased, the ovulated ova and follicular oocytes with germinal vesicle breakdown (GVBD) increased to 35.1% and then declined to 17.5% (Table 1). AII did not affect arterial pressure during perfusion at the lower doses, 0.1 and 10 ng/ml, while the peak pressure observed with 1000 ng/ml was no higher than the peak observed with the control (data not shown).

Effects of SAR on AII-induced ovulation and oocyte maturation

Ovulatory efficiency was significantly ($P < 0.05$) lower when ovaries were perfused with AII plus SAR than with AII alone (Table 2). The interval from addition of agent to ovulation

Table 1. Angiotensin II-induced ovulation in rabbits

	0	Angiotensin II (ng/ml)		1000
		0.1	10	
No. of ovaries perfused	6	6	6	6
Time to ovulation (h)	7.54 ± 1.71	5.72 ± 0.24	5.95 ± 0.31	4.74 ± 0.22
No. of mature follicles	37	43	37	40
No. of ovulated ova	2	20	20	30
No. showing GVBD†	0	7	8	6
No. of follicular oocytes	35	23	17	10
No. showing GVBD	2	8	5	1
Total GVBD (%)	5.4	34.9*	35.1*	17.5
Ovulatory efficiency (%)‡	5.4	46.5**	54.1**	75.0**

* $P < 0.01$ compared with control; ** $P < 0.001$ compared with control.

†GVBD is germinal vesicle breakdown.

‡Ovulatory efficiency is the percentage of mature follicles that rupture during perfusion.

Table 2. Saralasin inhibition of ovulation induced by 0.1 ng angiotensin II/ml in rabbits

	Saralasin (µg/ml)	
	0	1
No. of ovaries perfused	4	4
Time to ovulation (h)	5.08 ± 0.35	7.50 ± 0.52*
No. of mature follicles	24	29
No. of ovulated ova	10	3
No. showing GVBD†	3	0
No. of follicular oocytes	14	26
No. showing GVBD	4	4
Total GVBD (%)	29.2	13.8
Ovulatory efficiency (%)‡	41.7	10.3**

* $P < 0.005$.

** $P < 0.05$.

†GVBD is germinal vesicle breakdown.

‡Ovulatory efficiency is the percentage of mature follicles that rupture during perfusion.

Table 3. Effect of saralasin on ovulation in rabbits in the presence and absence of human chorionic gonadotrophin (hCG)

	hCG (iu)			
	50		0	
	Saralasin (µg/ml)			
	0	1	10	10
No. of ovaries perfused	6	6	6	7
Time to ovulation (h)	5.84 ± 0.27	5.90 ± 0.29	7.02 ± 0.38*	8.25
No. of mature follicles	37	32	49	47
No. of ovulated ova	33	26	9	1
No. showing GVBD†	26	19	9	0
No. of follicular oocytes	4	6	26	44
No. showing GVBD	3	4	21	1
Total GVBD (%)	78.4	71.9	85.8	2.2
Ovulatory efficiency (%)‡	89.2	81.3	18.4*§	2.1

* $P < 0.05$, ** $P < 0.001$ compared with 0 or 1 µg/ml.

§ $P < 0.01$ compared with saralasin (10 µg/ml) alone.

†GVBD is germinal vesicle breakdown.

‡Ovulatory efficiency is the percentage of mature follicles that rupture during perfusion.

Fig. 1. Scanning electron microscopy of rabbit follicular microvascular casts ($\times 140$). (a) Control ovary 6 h after ovulation induction with human chorionic gonadotrophin. Note the dilation of vessels and the filling defect at the apex of the follicle (arrow).

(b) Ovary induced to ovulate with 0.1 ng angiotensin II/ml, 4 h after drug addition. Vessels appear to be dilated, and a filling defect is apparent (arrow) as in control animals treated with hCG.

lengthened from 5.08 ± 0.35 h to 7.50 ± 0.52 h during perfusion with SAR (Table 2). SAR did not significantly affect GVBD of ovulated ova or follicular oocytes (Table 2).

Effects of SAR on hCG-induced ovulation and oocyte maturation

Ovulatory efficiency was reduced to 18.4% ($P < 0.001$) and the mean time to ovulation increased from 5.84 ± 0.27 h to 7.02 ± 0.38 h ($P < 0.02$) when ovaries receiving hCG were perfused with $10 \mu\text{g}$ SAR/ml (Table 3). hCG-induced GVBD in ovulated or follicular oocytes was not

affected by SAR. SAR when given alone at 10 µg/ml had no effect on induction of ovulation or GVBD (Table 3).

Morphology of preovulatory follicles treated with AII or hCG

SEM revealed that the microvasculature of ovarian follicles in both groups displayed filling defects at the site of stigma formation and dilation of surrounding vessels (Fig. 1a, b). There was no apparent difference between the 2 agents and no evidence for pharmacological vasoconstriction in response to AII administration.

Discussion

The results of the present study, demonstrating that AII induces ovulation in the absence of gonadotrophin and that ovulation induced by hCG or AII can be inhibited by SAR, raise the possibility that specific AII receptors may be involved in ovulation in rabbits. The microvasculature in AII- and hCG-stimulated follicles was similar by SEM (Fig. 1), suggesting that AII administration does not cause pharmacological constriction of the follicular microvasculature. This was further supported by the perfusion pressure, which was not affected by AII administration. The perfusion system maintains a constant flow; therefore, changes in microvasculature, i.e. resistance, would have resulted in a change in pressure. The similarity in alteration of microvasculature and the 80% inhibition of hCG-induced ovulation in the presence of 10 µg SAR/ml suggest that AII plays a critical role in the ovulatory cascade initiated by hCG. Our results confirm the findings of Pellicer *et al.* (1988) that SAR blocks hCG-induced ovulation *in vivo*.

AII induced meiotic resumption in ovulated ova and follicular oocytes. Although the effectiveness of AII was less than half that observed by stimulation with hCG, other nongonadotrophic agents (e.g. PGF-2α, plasminogen activator and bradykinin) that induce ovulation in this in-vitro system do not promote oocyte maturation (Kitai *et al.*, 1985a; Yoshimura *et al.*, 1987; Yoshimura *et al.*, 1988). Higher concentrations of AII were not as effective as lower concentrations in promoting GVBD, suggesting the possibility of down-regulation of AII receptors. This effect differs from the effects of AII on follicle rupture, in which ovulatory efficiency varied directly with dose of AII throughout the range used. In our system, meiotic resumption induced by AII or hCG was not affected by SAR. In contrast, Palumbo *et al.* (1988) demonstrated that SAR inhibits meiotic resumption in rats. In rabbit ovary, the AII receptors responsible for inducing meiotic resumption may fail to bind SAR; AII may be inducing resumption of meiosis indirectly or via a process that is not receptor mediated.

In summary, AII has a direct effect on the ovary, stimulating both ovulation and oocyte maturation in the absence of gonadotrophin. AII may play a critical role in the ovulatory cascade initiated by hCG. The effect of AII on meiotic resumption appears to be mediated by a process distinct from that mediating follicle rupture.

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