Increase in ovarian kallikrein activity during ovulation in the gonadotrophin-primed immature rat

L. L. Espey, N. Tanaka, V. Winn and H. Okamura

Department of Biology, Trinity University, San Antonio, Texas 78284, USA; and Department of Obstetrics and Gynecology, Kumamoto University Medical School, Kumamoto 860, Japan

Summary. The ovulatory process was initiated in 25-day-old rats by injecting them with hCG (10 i.u., s.c.) 2 days after the animals had been primed with PMSG (10 i.u., s.c.). At 2-h intervals after hCG, the ovaries were extracted and assayed for glandular kallikrein activity by using a chromogenic substrate (H-D-Val-Leu-Arg-*p*-nitroanilide) which exhibits optical density (at 405 nm) upon hydrolysis. In 0-h control ovaries the activity was 12.5×10^{-3} kallikrein units (KU)/mg protein and it increased to a peak of 56.6×10^{-3} KU/mg at 12 h after hCG, when the follicles first began to rupture. The kallikrein activity was distinguishable from ovarian plasminogen activator activity on the basis of pH optima and response to trypsin inhibitor (SBTI). The activity was inhibited by a s.c. dose of indomethacin of 0.3 mg/rat, or higher, and this dosage inhibited ovulation. The results suggest that kallikrein activity contributes to the degradation of Graafian follicles during ovulation in mammals.

Keywords: ovulation; kallikrein; plasminogen activator; indomethacin; rat

Introduction

Mammalian ovaries undergo inflammatory-like changes at the time of ovulation (Espey, 1980). Ovaries become hyperaemic (Tanaka *et al.*, 1989), and there is a local increase in a number of agents that are known to mediate inflammation. Prostaglandins are especially well known for their role in ovulation (Orczyk & Behrman, 1972; Espey *et al.*, 1986b). There is also evidence that bradykinin is released in ovulatory follicles (Smith & Perks, 1983; Espey *et al.*, 1986a; Yoshimura *et al.*, 1988). More recently, lipoxygenase activity and leukotrienes have been implicated in the process (Reich *et al.*, 1985; Espey *et al.*, 1989). In addition to these vasoactive substances, ovulatory follicles contain serine proteases that are characteristic of acute inflammatory reactions. Collagenolytic activity (Espey & Coons, 1976; Curry *et al.*, 1985) and plasminogen activator (PA) activity (Strickland & Beers, 1976; Espey *et al.*, 1985) are the two proteases which have received the most attention.

In view of this growing evidence that the ovulatory process resembles an inflammatory reaction, the purpose of the present study was to examine ovulatory tissues for still another serine protease, namely kallikrein (EC 3.4.21.8), which is commonly involved in inflammation (Solomkin & Simmons, 1983; Marceau *et al.*, 1983).

Materials and Methods

Ovulation induction. Wistar rats were bred in our own animal facility and housed under a 14-h daily light cycle which began at 07:00 h. The young females in the litters were weaned at approximately 22 days of age and were distributed equally by weight (40–50 g) into the different experimental groups. At 08:00 h on the day after weaning, the immature rats were injected s.c. with 10 i.u. PMSG (G-4877, Sigma Chemical Co., St Louis, MO, USA). At 08:00 h 2 days

later, the ovulatory process was initiated by a s.c. injection of 10 i.u. hCG (CG-5, Sigma Chemical Co.). The animals began ovulating 10–12 h later.

Inhibitor administration. Indomethacin (I-7378, Sigma Chemical Co.) was dissolved in distilled water containing 4 mg Na_2CO_3 for every 10 mg indomethacin. The pH of the solution was adjusted to 7.4 by adding 0.1 N-HCl during vigorous stirring. The indicated amounts of indomethacin were administered s.c. at 1 h before the animals were given hCG to induce the ovulatory process. Subsequently, the ovaries were removed at 12 h after hCG administration for assay. Trypsin inhibitor (SBTI; T-9003, Sigma Chemical Co.) was not injected into the animals, but was added to the final assay mixtures for kallikrein and PA at concentrations of 5 and 500 $\mu\text{g}/\text{ml}$.

Ovulation determination. The animals were killed by cranial impaction at the designated intervals, and both ovaries were extirpated. The oviducts were separated from the ovaries and examined under a dissecting microscope with substage illumination. The numbers of ova were counted in each pair of oviducts to calculate the ovulation rate per rat.

Ovary extraction. The ovaries were cut free from the mesovarian ligament and blotted on a paper towel to remove blood from the surface. One ovary from each animal was placed in 1.0 ml ice-cold 0.05 M-Tris buffer at pH 7.4 for kallikrein assay and pH 8.0 for PA assay (except for the different pH values designated in the experiment on optimum pH). The ovaries were homogenized for 30 sec in a glass/glass homogenizer (S62 pestle and S73 tube, Tri-R Instruments, Inc., Rockville Center, NY, USA) connected to a stirrer (S63, Tri-R Instruments, Inc.) set at medium speed (~ 5000 rev./min). The homogenates were centrifuged routinely at 10 000 g for 20 min, at 4°C, and the supernatant fluids were transferred to a polyethylene tube, capped, and stored at -20°C until a complete series of experimental samples could be assayed simultaneously. (The procedure required ~ 10 min from the time the animals were killed to the time the ovarian homogenates were placed in the refrigerated centrifuge.) Aliquants of the samples were assayed for protein content (Lowry reagent kit; P-5656, Sigma Chemical Co.) to express the enzyme activity per mg protein.

Kallikrein assay. The kallikrein substrate was a chromogenic peptide (S-2266; H-D-Val-Leu-Arg-p-nitroanilide dihydrochloride, KABIVITRUM, Stockholm, Sweden, distributed by Helena Laboratories, Beaumont, TX, USA) which has been used to measure glandular kallikrein in pancreas, urine and saliva, but which is not effective for plasma kallikrein (Amundsen *et al.*, 1979). The method for the determination of activity is based on the optical density (at 405 nm) that is generated by the enzymic release of p-nitroanilide (pNA) from the original substrate. The assay solution consisted of: (1) 800 μl 0.05 M-Tris buffer, pH 7.4; (2) 100 μl of a 2.0 mM solution of S-2266 substrate in distilled water; and (3) 100 μl ovarian extract, or kallikrein standard. Standard curves were based on the hydrolysis of substrate by known kallikrein units (KU) of pig pancreatic kallikrein (K-3627, Sigma Chemical Co.). The mixtures were incubated in a shaker bath at 37°C for 90 min. The reaction was stopped by adding 1.0 ml 25% glacial acetic acid to each test tube. The absorbance (at 405 nm) of the final reaction mixture was measured by a photometer (RESPONSE UV-VIS Spectrophotometer, Gilford Instruments Laboratories, Oberlin, OH, USA). In each experiment, the absorbance was also measured in blanks containing ovarian extract only, and these values were subtracted from the absorbance of the main assay solution in order to calculate the actual kallikrein activity in the ovarian samples. Following this procedure, the kallikrein activity was expressed as KU per mg protein in the extracts. The intra-assay coefficient of variation for this assay was 5.6%.

PA assay. The substrate for tissue PA activity was also a chromogenic peptide (S-2251; H-D-Val-Leu-Lys-pNA; KABIVITRUM). The assay procedure was modified from a previous description (Espey *et al.*, 1985) in that the assay solution consisted of: (1) 920 μl 0.05 M-Tris buffer, pH 8.0; (2) 40 μl 3.8 mM solution of S-2251 substrate in distilled water; (3) 20 μl of a plasminogen solution containing 10 casein units/ml distilled water; and (4) 20 μl ovarian extract, or PA standard. Standard solutions were prepared from tissue PA extracted from human melanoma cell cultures (t-PA, KABIVITRUM). The PA activity was expressed in IU per mg protein in the extracts. The intra-assay coefficient of variation for this assay was 2.1%.

Statistical analysis. All data are presented as the mean $\pm$ s.e.m. The significances of the differences among the means during the 24 h periovulatory period and in the indomethacin dose-response study were determined by Duncan's Multiple Range Test. The significances of the differences of the means of the SBTI groups were determined by the *t* test for a difference between two independent means. The level of significance was taken as $P < 0.05$.

Results

Onset of ovulation

In the 8 animals that were used for the kallikrein assays at each test interval during ovulation (Fig. 1), all of the oviducts were examined for the presence of ova at the time the ovaries were extirpated. Ova first appeared at 10 h after hCG, with a total of only 5 ova present in the 9 pairs of oviducts. By 14 h after hCG, the oviducts contained 50.6 ± 5.0 ova/rat, and this increased to 72.3 ± 5.3 at 20 h after hCG.

Fig. 1. Increase in ovarian kallikrein activity during the 24 h periovulatory period. Values are means $\pm$ s.e. for 8 rats/group.

Increase in kallikrein activity during ovulation

Kallikrein activity was assayed simultaneously in ovaries from separate groups of 8 rats at 0, 2, 4, 6, 8, 10, 12, 14, 16, 20 and 24 h after the administration of hCG. The activity in the 0 h control ovaries was $12.5 \pm 1.9 \times 10^{-3}$ KU/mg protein, and increased ($P < 0.01$) to a peak of $56.6 \pm 6.3 \times 10^{-3}$ at 12 h after hCG when the ovarian follicles had just begun to rupture (Fig. 1).

Dose-dependent inhibition of kallikrein activity by indomethacin

Indomethacin was administered s.c. at 1 h before hCG in graded doses from 0.03 mg/rat to 10.0 mg/rat to groups of 8 rats each. Subsequently, the ovaries were removed at 12 h after hCG and assayed for kallikrein activity. At a dose of 0.3 mg/rat, indomethacin significantly reduced ($P < 0.01$) the normal amount of ovarian kallikrein activity at 12 h after hCG (Fig. 2). The inhibitory effect of indomethacin was not the consequence of any direct action on kallikrein activity because the addition of indomethacin to the assay solution in doses as high as 200 μ g/ml did not significantly change the kallikrein activity in ovarian extracts. In 5 assays of pooled extracts of ovaries taken at 12 h after hCG, control samples had 0.138 ± 0.003 KU/ml extract, while indomethacin-treated samples had 0.134 ± 0.004 KU/ml extract.

Comparison of ovarian kallikrein activity with ovarian PA activity

Extracts from the ovaries of 6 additional rats (taken at 12 h after hCG administration) were pooled into one homogeneous solution, and aliquants were assayed for kallikrein or PA activity in triplicate at different pH values ranging from 6.8 to 8.6. The results show that the optimum pH for kallikrein activity was at pH 7.4, and the optimum for PA activity was at pH 8.0 (Fig. 3).

For further comparison of kallikrein and PA activities, aliquants of individual ovarian extracts from 5 rats (killed at 12 h after hCG) were assayed for both enzymes in the presence of 5 or 500 μ g SBTI/ml reaction mixture. The control level of $48.3 \pm 5.6 \times 10^{-3}$ KU/mg protein kallikrein

Fig. 2. Effect of s.c. injections of indomethacin on ovarian kallikrein activity at 12 h after hCG administration. Values are means $\pm$ s.e. for 8 rats.

Fig. 3. Comparison of effect of pH on ovarian kallikrein activity versus ovarian PA activity. Values are means $\pm$ s.e. for triplicate assays.

activity decreased insignificantly to $40.9 \pm 3.7 \times 10^{-3}$ and $38.1 \pm 8.2 \times 10^{-3}$ KU/mg protein when mixed with 5 and 500 μ g/ml SBTI, respectively. In contrast, the control level of 877 ± 52 IU PA activity/mg protein decreased significantly ($P < 0.001$) to 185 ± 48 and 67 ± 16 IU/mg protein when mixed with 5 and 500 mg/ml SBTI, respectively.

Discussion

The present measurement of an increase in kallikrein-like activity in ovulating ovaries provides further support for the hypothesis that the biochemical events of ovulation resemble an acute inflammatory reaction. The results also indicate that enzymes like kallikrein might be contributing (along with collagenase and PA) to the proteolytic degradation of the connective tissue elements in ovulatory follicles. Collectively, the information suggests that ovulation may depend on the activation of a proteolytic 'cascade' comparable to the cascade of enzyme activity in inflammatory responses (Kaplan *et al.*, 1981; Marceau *et al.*, 1983).

Kallikreins are distinguished from other serine proteases by their ability to cleave specifically kallidin (lys-bradykinin) from kininogen (Fuller & Funder, 1986). There are two types, plasma kallikrein and glandular kallikrein, with the latter being the subject of this study. Kallikreins are generated at sites of inflammation where abnormal surfaces activate Hageman factor, which cleaves pre-kallikrein to its active form (Solomkin & Simmons, 1983; Marceau *et al.*, 1983). The kallikreins then generate kallidin and kinins which lead to the vascular responses that are characteristic of inflamed tissues. Glandular kallikrein is inactivated within minutes after it is released into the circulation by binding to plasma protease inhibitors of the α_1 -antitrypsin and α_2 -macroglobulin type (Rabito *et al.*, 1985).

The present data show that ovarian kallikrein activity is different from ovarian PA activity in at least two respects: (1) the pH optima for the two enzymes are different, and (2) SBTI has a greater inhibitory effect on PA activity. Regarding this latter difference, the results concur with a report that SBTI has little effect on glandular kallikrein activity from pancreatic and synovial tissues (Sharma *et al.*, 1983), while it inhibits PA activity from fibroblasts (Quigley, 1979) and skeletal muscle cells (Festoff *et al.*, 1982). In this respect ovarian PA activity is more like plasma kallikrein, which is also inhibited by SBTI (Sharma *et al.*, 1983; Tans *et al.*, 1987).

At doses of 0.3 mg/rat and higher, the cyclooxygenase inhibitor indomethacin reduced ovarian kallikrein activity. This suggests that the kallikrein activity might be dependent on ovarian prostanoid synthesis. However, indomethacin at doses of only 0.03 and 0.1 mg/rat strongly inhibited ovarian prostaglandin synthesis (Espey *et al.*, 1988, 1989) and yet these same doses did not significantly affect ovarian kallikrein activity. Furthermore, the low dose of 0.03 mg/rat did not inhibit ovulation, and 0.1 mg/rat only marginally reduced the number of ova that were released (Espey *et al.*, 1988, 1989). Therefore, on the basis of the responses to graded doses of indomethacin, it appears that the ovulation rate is more closely correlated to ovarian kallikrein activity than to ovarian PG levels. The results also suggest that indomethacin may reduce ovarian kallikrein activity by some mechanism other than cyclooxygenase inhibition.

One of the expected actions of ovarian kallikrein would be to generate kinins and cause local vasodilatation and greater vascular permeability (Marceau *et al.*, 1983; Espey *et al.*, 1986a). By this action, kallikrein might contribute to the ovarian hyperaemia which develops during ovulation (Tanaka *et al.*, 1989). The kallikrein-kinin system is also known to activate phospholipase A₂ and promote the synthesis of prostanoids (Sharma *et al.*, 1983; Morita *et al.*, 1984; Fuller & Funder, 1986). Therefore ovarian kallikrein may be responsible, at least in part, for the well known increase in ovarian prostaglandins during ovulation.

Finally kallikrein is regulated by androgens in the submaxillary and prostate glands, by oestrogens in the anterior pituitary, and possibly by mineralocorticoids in the kidney (Fuller & Funder, 1986). There is therefore circumstantial evidence to suggest that there may be a relationship between ovarian kallikrein activity and the well known changes in ovarian steroidogenic activity

during ovulation. In any event, the present results suggest that glandular kallikrein contributes to the inflammatory-like changes which occur in ovulatory follicles of mammals.

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References

- Amundsen, E., Putter, J., Friberger, P., Knos, M., Larsbraten M. & Claeson, G. (1979) Methods of the determination of glandular kallikrein by means of a chromogenic tripeptide substrate. In *Kinins II: Biochemistry, Pathophysiology and Clinical Aspects*, pp. 83–95. Ed. M. Fujii. Plenum Publishing Corp., New York.
- Curry, T.E., Jr, Dean, D.D., Woessner, J.F., Jr & LeMaire, W.J. (1985) The extraction of a tissue collagenase associated with ovulation in the rat. *Biol. Reprod.* **33**, 981–991.
- Espey, L.L. (1980) Ovulation as an inflammatory reaction—A hypothesis. *Biol. Reprod.* **22**, 73–106.
- Espey, L.L. & Coons, P.J. (1976) Factors which influence ovulatory degradation of rabbit ovarian follicles. *Biol. Reprod.* **14**, 233–245.
- Espey, L.L., Shimada, H., Okamura, H. & Mori, T. (1985) Effect of various agents on ovarian plasminogen activator activity during ovulation in pregnant mare's serum gonadotropin-primed immature rats. *Biol. Reprod.* **32**, 1087–1094.
- Espey, L.L., Miller, D.H. & Margolius, H.S. (1986a) Ovarian increase in kinin-generating capacity in PMSG/hCG-primed immature rat. *Am. J. Physiol.* **251**, E362–E365.
- Espey, L.L., Norris, C. & Saphire, D. (1986b) Effect of time and dose of indomethacin on follicular prostaglandins and ovulation in the rabbit. *Endocrinology* **119**, 746–754.
- Espey, L.L., Kohda, H., Mori, T. & Okamura, H. (1988) Rat ovarian prostaglandin levels and ovulation as indicators of the strength of non-steroidal anti-inflammatory drugs. *Prostaglandins* **36**, 875–879.
- Espey, L.L., Tanaka, N. & Okamura, H. (1989) Increase in ovarian leukotrienes during hormonally-induced ovulation in the rat. *Am. J. Physiol.* **256**, E753–E759.
- Festoff, B.W., Patterson, M.R. & Romstedt, K. (1982) Plasminogen activator: the major secreted neutral protease of cultured skeletal muscle cells. *J. Cell. Physiol.* **110**, 190–195.
- Fuller, P.J. & Funder, J.W. (1986) The cellular physiology of glandular kallikrein. *Kidney Int.* **29**, 953–964.
- Kaplan, A.P., Ghebrehwet, B., Silverberg, M. & Sealey, J.E. (1981) The intrinsic coagulation-kinin pathway, complement cascades, plasma renin-angiotensin system, and their interrelationships. *Critical Rev. Immunol.* **3**, 75–93.
- Marceau, F., Lussier, A., Regoli, D. & Giroud, J.P. (1983) Pharmacology of kinins: their relevance to tissue injury and inflammation. *Gen. Pharmacol.* **14**, 209–229.
- Morita, I., Kanayasu, T. & Murota, S.-I. (1984) Kallikrein stimulates prostacyclin production in bovine vascular endothelial cells. *Biochim. Biophys. Acta* **792**, 304–309.
- Orczyk, G.P. & Behrman, H.R. (1972) Ovulation blockade by aspirin or indomethacin—*In vivo* evidence for a role of prostaglandin in gonadotrophin secretion. *Prostaglandins* **1**, 2–20.
- Quigley, J.P. (1979) Phorbol ester-induced morphological changes in transformed chick fibroblasts: evidence for direct catalytic involvement of plasminogen activator. *Cell* **17**, 131–141.
- Rabito, S.F., Seto, M., Maitra, S.R. & Carretero, O.A. (1985) Clearance and metabolism of glandular kallikrein in the rat. *Am. J. Physiol.* **248**, E664–E668.
- Reich, R., Kohen, F., Slager, R. & Tsafiriri, A. (1985) Ovarian lipoxygenase activity and its regulation by gonadotropin in the rat. *Prostaglandins* **30**, 581–590.
- Sharma, J.N., Zeitlin, I.J., Deodhar, S.D. & Buchanan, W.W. (1983) Detection of kallikrein-like activity in inflamed synovial tissue. *Arch. Int. Pharmacodyn.* **262**, 279–286.
- Smith, C. & Perks, A.M. (1983) Changes in plasma kininogen levels in rats before ovulation, and after treatment with luteinizing hormone and oestradiol-17 β . *Acta endocr., Copenh.* **104**, 123–128.
- Solomkin, J.S. & Simmons, R.L. (1983) Cellular and subcellular mediators of acute inflammation. *Surg. Clin. N. Am.* **63**, 225–243.
- Strickland, S. & Beers, W.H. (1976) Studies on the role of plasminogen activator in ovulation. *J. biol. Chem.* **251**, 5694–5702.
- Tanaka, N., Espey, L.L. & Okamura, H. (1989) Increase in ovarian blood volume during ovulation in the gonadotropin-primed immature rat. *Biol. Reprod.* **40**, 762–768.
- Tans, G., Rosing, J., Berrettini, M., Lammle, B. & Griffin, J.H. (1987) Autoactivation of human plasma prekallikrein. *J. biol. Chem.* **262**, 11 308–11 314.
- Yoshimura, Y., Espey, L., Hosoi, Y., Adachi, T., Atlas, S.J., Ghodgaonkar, R.B., Dubin, N.H. & Wallach, E.E. (1988) The effects of bradykinin on ovulation and prostaglandin production by the perfused rabbit ovary. *Endocrinology* **122**, 2540–2546.

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