Research article

Activation of the kinin system in the ovary during ovulation: Role of endogenous progesterone

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Abstract

Background: Previous work by our group and others has implicated a role for kinins in the ovulatory process. The purpose of the present study was to elucidate whether endogenous progesterone, which is an intraovarian regulator of ovulation, might be responsible for induction of the kinin system in the ovary during ovulation. The gonadotropin-primed immature rat was used as the experimental model, and the role of endogenous progesterone was explored using the antiprogestin, RU486.

Results: The results of the study revealed that RU486 treatment, as expected, significantly attenuated ovulation. Activity of the kinin-generating enzyme, kallikrein, was elevated in the ovary in control animals prior to ovulation with peak values observed at 4 h post hCG, only to fall to low levels at 10 h, with a recovery at 20 h post hCG. RU486 treatment had no significant effect on ovarian kallikrein activity as compared to the control group. Total ovarian kininogen levels in control animals increased significantly at 12–14 h after hCG – coinciding with initiation of ovulation. Thereafter, ovarian kininogen levels fell to low levels at 20 h, only to show a rebound from 24–38 h post-hCG. RU486 treatment had no significant effect on the rise of total ovarian kininogen levels from 12–14 h after hCG; however, from 30–40 h post hCG, RU486-treated animals had significantly higher total ovarian kininogen levels versus control animals, suggesting that endogenous progesterone may act to restrain elevations of kininogens in the post-ovulatory ovary. This robust elevation of ovarian kininogen levels by RU486 was found to be primarily due to an increase in T-kininogen, which is a potent cysteine protease inhibitor.

Conclusions: Taken as a whole, these results suggest that endogenous progesterone does not regulate kallikrein activity or kininogens prior to ovulation, but may provide a restraining effect on T-kininogen levels in the post-ovulatory ovary.

Background

The kininogen-kallikrein-kinin system is well established to be important in inflammatory processes due to its actions to induce vasodilation, prostaglandin biosynthesis, and tissue remodeling through regulation of proteases [1–3]. There are two types of kinins, B-kinin and T-kinin, which are products of cleavage of the substrates B-kininogen and T-kininogen, respectively [[1,2], for review]. The enzyme kallikrein cleaves B-kininogen to B-kinin, while the enzyme T-kininogenase cleaves T-kininogen to T-kinin [3,4]. B-kinin and T-kinin have similar vasodilation and prostaglandin regulatory actions, and are metabolized by the enzyme, angiotensin-converting enzyme [1,2]. T-kininogen, in addition to being a substrate for T-kinin production, is a potent cysteine protease inhibitor and has a role in tissue remodeling [5,6].

Due to its role in inflammatory processes and tissue remodeling functions, a number of investigators have focused on the kinin system as a possible important mediator in the ovulatory process. Along these lines, our group and others have demonstrated that kallikrein activity and kininogen levels in the ovary increase preceding and at the time of ovulation in the gonadotropin-primed immature rat [7–9]. A functional role for this activation of the kinin system was suggested by the fact that kinin agonists have been shown to induce ovulation, while conversely; kinin antagonists inhibit ovulation [10,11]. Additionally, work by Holland et. al. has shown that kallikrein (rKLK-1) gene expression increases in the ovary preceding ovulation [9]. While evidence is mounting supporting a role for the kinin system in ovulation, the precise regulators of this system in the ovary remains unclear. In our previous study, we noticed that the elevation of kallikrein activity and kininogens in the ovary preceding and during ovulation was paralleled by an increase in ovarian progesterone concentrations [7]. Since progesterone has been demonstrated to be an important intraovarian regulator of the ovulatory process [12,13], the aim of the present study was to determine whether endogenous progesterone is responsible for activation of the kinin system in the ovary during ovulation. The potent antiprogestin compound, RU486 was used to accomplish this aim.

Results

As shown in Figure 1, PMSG-hCG induced ovulation in control rats with peak ova released from the ovary from 24 h to 34 h after hCG administration. RU486 treatment significantly inhibited ovulation at 20 h and 30 h (p < 0.03) versus controls. Ovulation also appeared lower in RU486-treated rats at 24 and 34 h after hCG as compared to controls; however, due to variability this effect was not significant at these time points. Ovarian estradiol levels in control rats were high at 0 h only to fall to basal levels from 10 h to 38 h (Fig. 2). RU486 treatment caused a slight but significant elevation in ovarian estradiol levels at 30 h compared to control rats (p < 0.05). Nevertheless, even at this time point, ovarian estradiol levels were close to basal levels. Ovarian progesterone levels rose rapidly to reach a peak at 12 h in control animals, followed by a fall at 20 h to 38 h to low levels which, however, were still higher than the initial 0 h levels (Fig 2). RU486-treated rats had significantly attenuated peak progesterone levels at 12 h compared to control animals (p < 0.01). Figure 3 demonstrates that ovarian kallikrein activity in control rats was elevated prior to ovulation with peak values at 4 h, only to fall to low levels at 10 h, with a recovery at 20 h. RU486 treatment had no significant effect on ovarian kallikrein activity as compared to controls. As shown in Figure 4, ovarian total kininogen levels in control animals rose in a gradual manner from 0 h to 12 h to reach peak levels at 12 h, a time that coincides with the beginning of ovulation. Kininogen levels then fell to low levels at 20 h only to show a rebound from 24 to 38 h. RU486 treatment had no effect on the rise of ovarian total kininogen levels from 0 to 10 h; however, from 30–38 h RU486-treated rats had significantly elevated ovarian total kininogen levels compared to control rats (Fig 4). As illustrated in Figure 5, T-kininogen is the major type of kininogen in the postovulatory ovary and the elevation of total kininogen levels by RU486 from 30–38 h was primarily due to an elevation of T-kininogen levels at all time points. B-kininogen levels, on the other hand, did show a small but significant decrease at 30 h, followed by a significant increase at 34 h in RU486-treated rats versus controls.

Discussion

The present study demonstrates that treatment with the antiprogestin compound, RU486, significantly attenuates ovulation in PMSG-hCG-treated immature rats. This finding is in agreement with previous reports in the literature and is consistent with the hypothesis that progesterone has a direct role in ovulation [12,14]. Along these lines, the progesterone receptor knockout mouse has been shown to develop follicles to the ovulatory stage, but follicle rupture does not occur in these animals despite administration of a superovulatory dose of exogenous gonadotropins [12]. The precise genes/proteins regulated by progesterone in the ovary to modulate ovulation remains unclear. This study investigated the possible role that endogenous progesterone may have in regulation of the kinin system, which has been implicated as a mediator of the ovulatory process. Several interesting observations were yielded by the study. First, even though ovulation was lowered by RU486 treatment, activation of the kinin system as reflected by the elevation in ovarian kallikrein activity and kininogen levels prior to and during ovulation was not significantly affected. This suggests that progesterone may not be responsible for the elevation in kinin system activity in the ovary observed prior to and at
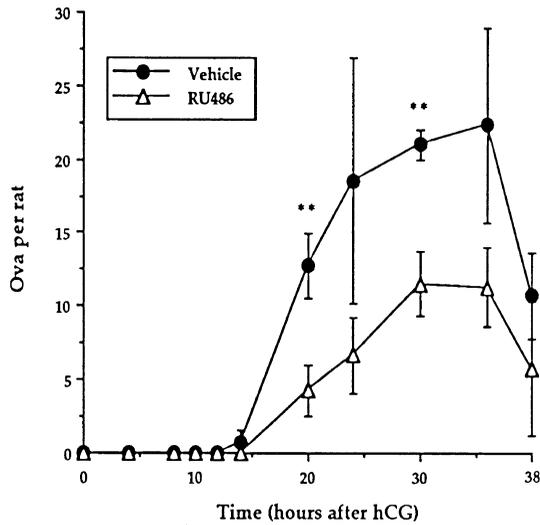
Figure 1
Effect of the antiprogestin RU486 upon ovulation rate in PMSG-hCG-primed immature rats. Twenty-three-day-old female rats were primed with 10 IU of PMSG and 48 h later ovulation was induced with hCG (10 IU). Either vehicle or RU486 (10 mg/kg) was administered 30 minutes prior to hCG. n = 6 rats per group. ** $p < 0.03$ vs. vehicle.

Figure 2
Effect of RU486 on ovarian steroid levels in the PMSG-hCG-primed immature rat. The model is the same as described in Figure 1. * $p < 0.05$ vs. Vehicle; ** $P < 0.01$ vs. Vehicle.

the time of ovulation, and that progesterone's modulatory influence in the ovulatory process may be mediated through some system other than the kinin system. Our finding of a lack of significant effect of RU486 on ovarian kallikrein activity during ovulation is somewhat at variance with the report of Tanaka et al. [15]. These investigators suggested a role for progesterone in the regulation of ovarian kallikrein activity based on studies using epostane, a progesterone synthesis inhibitor. In these studies, epostane treatment inhibited ovulation and ovarian kallikrein activity. It should be pointed out however that epostane, in addition to inhibiting progesterone synthesis, has also been shown to strongly inhibit synthesis of 17β-estradiol, testosterone and 4-androstene-3,17-dione in the gonadotropin-stimulated immature rat ovary, so it is not truly specific for inhibition of progesterone synthesis [16]. It should also be pointed out that the temporal pattern for elevation of kallikrein activity differs between the two groups. We see an early peak elevation from 0–4 h followed by a fall to low levels at 10 h and another smaller increase at 10–12 h. Tanaka et. al. see no early increase and their peak levels occur at 12–14 h post-hCG. These differences may reflect different assay specificities as the chromogenic substrates used in their study (D-Val-
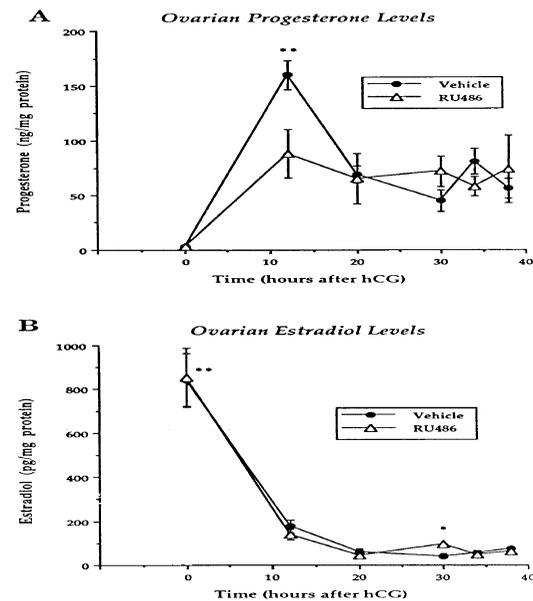
Leu-Arg-paranitroanilide) and ours (Pro-Phe-Arg-methyl-coumarylamide) are different. The substrate differences could be important because different kallikreins have been shown to have different substrate specificity [17]. The rat kallikrein gene family consists of 13 genes, of which six are expressed in the ovary (rKLK1, rKLK3, rKLK7, rKLK8, rKLK9 and rKLK12) [9]. In an attempt to explain the disparate temporal pattern between the two groups, Holland et. al. examined the gene expression pattern of all six kallikrein genes in the ovary in the same gonadotropin-primed immature rat model used by both groups. They found that only one gene is increased prior to ovulation, rKLK1 or true kallikrein [9]. They also noted that the pattern of gene expression for rKLK1 mirrored the temporal pattern for kallikrein activity observed in our study rather than that reported by Tanaka et. al. (e.g. it was elevated from 0–2 h after hCG) [9]. This led the authors to suggest that our substrate, Pro-Phe-Arg-methyl-coumarylamide is a more specific substrate and is detecting true rKLK1 enzyme activity. We cannot rule out the possibility that the activity of another kallikrein isoform, which is more specifically recognized by the substrate used by Tanaka et. al., changes after hCG, which could explain the temporal pattern observed by Tanaka et. al. and the apparent progesterone regulation they observed. However, Holland et. al. did not find any other kallikrein gene elevation prior to ovulation which would support this possibility
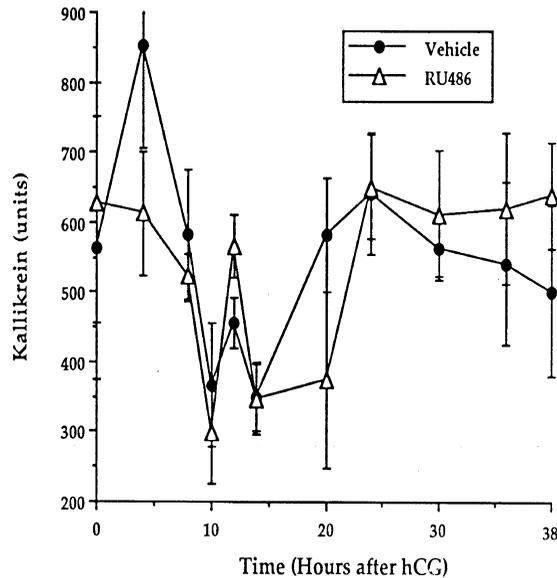
Figure 3
Effect of RU486 on ovarian kallikrein activity in the PMSG-hCG-primed immature rat. The model is the same as described in Figure 1. There is no statistical difference between the groups.
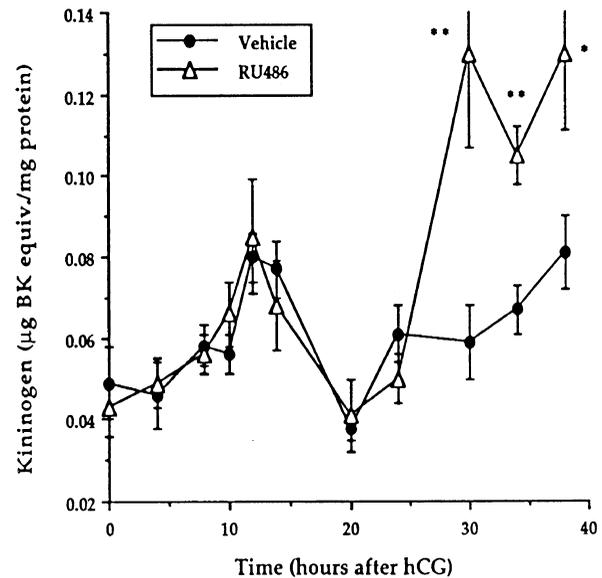

Figure 4
Effect of RU486 on total ovarian kininogen levels in the PMSG-hCG-primed immature rat. The model is the same as described in Figure 1. * $p < 0.05$ vs. Vehicle; ** $p < 0.01$ vs. Vehicle.

[9]. Finally, we did not assess for changes in one other component of the kinin system, the bradykinin receptor. The bradykinin B2 receptor has been demonstrated to be localized in the ovary in theca and granulosa cells [18], and thus regulation by progesterone at this level of the kinin signaling system could be possible and cannot be excluded.

A second interesting observation from our study was that in the post-ovulatory ovary, endogenous progesterone might actually be acting to *restrain* kininogen elevations. This suggestion is based on the finding that after ovulation, RU486-treated rats exhibited a significant robust elevation of total ovarian kininogen levels as compared to control animals. This elevation was principally due to a RU486-induced increase in T-kininogen levels in the ovary at 30–38 h post hCG. While the function of T-kininogen in the post-ovulatory ovary is not known, T-kininogen can be cleaved to yield the vasodilatory compound, T-kinin, and in its uncleaved state, it is a potent cysteine protease inhibitor [1,2,5,6]. An important class of cysteine proteases in the ovary that T-kininogen may regulate is cathepsins. Cathepsins degrade type I and IV collagen, fibronectin and laminin, and have been implicated to play a role in stimulation of steroidogenesis and degradation of extracellular matrix – events that would be occurring in the post-ovulatory ovary as the corpus luteum is forming

[13,17–20]. In support of a possible cathepsin cysteine protease regulatory function for T-kininogen in the ovary, we previously reported that a strong inverse correlation exists between T-kininogen levels and cathepsin activity in the gonadotropin-stimulated immature rat ovary (e.g. as T-kininogen levels increased, cathepsin activity decreased) [7]. We believe that T-kininogen is primarily serving a cysteine protease regulatory function in the ovary, as we have been unable to detect T-kininogenase, the enzyme that cleaves T-kininogen to T-kinin, in the ovary [7].

Conclusions
In conclusion, the current results do not provide support for a role for endogenous progesterone in activation of the kallikrein-kinin system in the ovary preceding ovulation. Thus, progesterone modulation of ovulation most likely is mediated by a system other than the kinin system. Intriguingly, the results suggest that endogenous progesterone may actually be more important in regulating the kinin system in the post-ovulatory ovary. Of significant interest, this post-ovulatory regulation was not observed on kallikrein activity and only minor effects were observed on B-kininogen, which together represent the classical kinin pathway. Rather, the effect was observed specifically on the newest member of the kinin/kininogen family, T-kininogen. Since we have been unable to detect T-kininogenase activity in the ovary, we believe that T-kininogen is
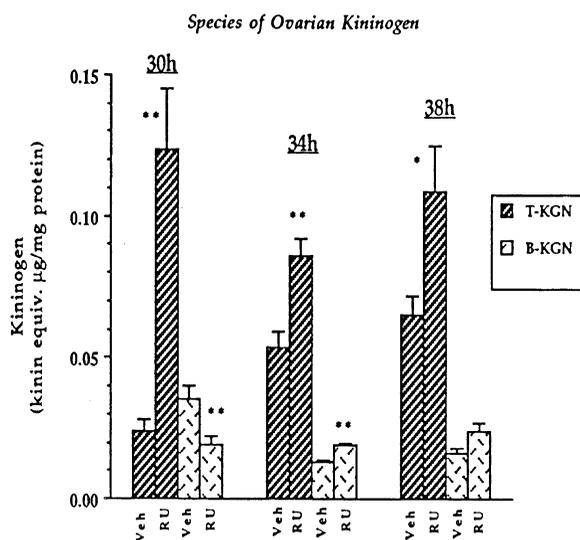
Figure 5
Species of kininogens in the ovary after RU486 treatment in the PMSG-hCG-primed immature rat. The model is the same as described in Figure 1. *p < 0.05 vs. Vehicle; ** p < 0.01 vs. Vehicle.

functioning primarily as a cysteine protease inhibitor in the ovary. Thus, endogenous progesterone restrainment of T-kininogen in the post-ovulatory ovary could be a mechanism for modulation of cysteine protease activity in the post-ovulatory ovary, thereby facilitating the extensive tissue remodeling that is known to occur after ovulation. Additional studies are underway in our laboratory to further explore this possibility.

Materials and Methods
Animals
Immature 23-day-old female Sprague Dawley rats (Holtzman, Madison, WI) were given PMSG at 0800 h. Forty-eight hours later, the animals were given hCG (10 IU, s.c. in saline; Sigma, St. Louis, MO) to induce ovulation. Thirty minutes prior to hCG injection, either vehicle or the antiprogestin RU486 (10 mg/kg in ethylene glycol ip; Roussel UCLAF, Romanville, France) was administered. In the PMSG-hCG induction model, mature follicles first begin to ovulate at 12–15 h, with peak number of ova appearing in the oviducts at 24–30 h [7]. To access the role of the kinin system in the ovulatory process, groups of animals were killed at various time points after hCG treatment; the ovaries were removed to determine ovulation and to measure the various components of the kininogen-kallikrein-kinin system. After removal, the ovaries were cleaned of fat, snap frozen in liquid nitrogen, and stored

at -70°C until the various assays were performed. All protocols involving animal use in this study were approved by our Institutional committee for the care and use of animals in research (CAURE).

Determination of ovulation
To determine ovulation, the animals were killed by decapitation and the oviducts were removed, pressed between two microscope slides, and examined under a microscope for the presence of ova as described previously [7].

Preparation of Tissue Homogenate
Ovaries excised from each animal were homogenized using a Dyna-Mix (Fisher Scientific, Pittsburg, PA) in 400 µl of 50 mM ice-cold PBS, pH 7.4. The homogenates were centrifuged at 4°C at 20,000 × g for 20 min. The supernatants were then assayed as described below.

Determination of Kininogens
Measurements of kininogen levels were performed as described previously by our laboratory using the protocols described by Barlas et al. [7,21]. *Total kininogens.* Plasma (100 µl) or tissue homogenate (100 µl) was incubated with 900 µl of 0.03 N HCl at 37°C for 15 min to destroy kininase activity. After neutralization with 25 µl of 1 M NaOH, 25 µl of 5 mg/ml trypsin was added (final concentration of trypsin was 1 mg/ml) and incubated at 37°C for 1 h to liberate kinins from the kininogens. The reaction was stopped by heating at 100°C for 10 min. The liberated kinins were quantified according to the RIA method of Greenbaum and Okamoto [2]. An aliquot of the sample (100 µl) was incubated at 4°C with 400 µl of 125I-labeled bradykinin (BK) and 100 µl of BK polycolonal antibodies, which were raised from the rabbit and able to recognize all kinins. After 2–24 h, 1% of bovine gamma globulin and 25% polyethylene glycol were added, respectively, to separate bound and free kinins. Radioactivity was counted by a gamma counter. Total kininogen was expressed as µg/mg protein of BK equivalents.

T-kininogen
After trypsin digestion, plasma was treated with an equal volume of 30 % trifluoroacetic acid (TFA). The supernatant, after centrifugation for 10 min at 3,000 rpm, was applied to a C-18 extraction column previously primed with 5 ml methanol and 5 ml of 1% TFA. After an initial wash with 1% TFA, the kinins were eluted with 1.5 ml of 50% acetonitrile in 1 % TFA. The elute was evaporated to dryness on a Speed Vac centrifuge, redissolved in 40 µl of distilled water, and applied to an HPLC column equilibrated with a mixture of 18% acetonitrile and 82% triethylammonium formate (v/v), pH 4.0. Fractions (14 ml/2 min) were collected and subjected to RIA. T-kinin (µg kinin equiv./mg protein) = [Total kinins released by trypsin] × %T-kinin in total kinin eluted. BK-kininogen was estimat-

ed by subtracting the amounts of T-kinin released from T-kininogen by trypsin from the amount of kinins released for total kininogen.

Assay of Kallikrein

Kallikrein activity was measured as described previously by our laboratory [7,22]. Briefly, tissue homogenate was incubated with a substrate, Pro-Phe-Arg-methylcoumary-lamide (MCA), in 0.1 M Tris-HCl buffer containing 0.15 M NaCl., pH 8.0. The initial hydrolysis of the substrate for the first 5 min is recorded. One unit of enzyme activity of kallikrein releases amino methylcoumarin (AMC) from the substrate at a rate of 1 nM/5 min. The hydrolysis of AMC was measured using a fluorescence spectrophotometer with excitation at 370 nm and emission at 460 nm.

Statistical Analysis

The results given in the text are expressed as means ± SEM. Six rats were used per group and the experiments were repeated three times. The differences between experimental groups were analyzed using the student *t*-test; $p < 0.05$ was considered significant.

Authors' Contributions

DWB conceived of the study and participated in its design, coordination, and analysis, and in the treatment of animals with hCG and measurement of ovulation. LMG participated in the measurement of kallikrein activity and kininogens and the interpretation of data. VBM participated in the conceptualization and interpretation of data and in the measurement of steroid levels. XXG participated in the design of the study, interpretation and analysis of data, measurement of kallikrein and T-kininogenase activity and kininogen levels.

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