Kallikrein gene expression in the gonadotrophin-stimulated rat ovary

A M Holland, J K Findlay and J A Clements

Introduction

The complex process of mammalian ovulation requires the cyclical growth of the follicle, and maturation and expulsion of the oocyte followed by formation of the corpus luteum. The physiological and endocrinological changes occurring throughout this fundamental process have been well understood for many years, but it is only relatively recently that some of the mechanisms underlying these changes have been elucidated. A number of factors involved locally in the ovulatory process have been identified, including tissue remodelling enzymes, vasoactive peptides and growth factors (Skinner & Parrot 1994, Bhoola et al. 1989, Gao et al. 1986, 1989, Tanaka et al. 1989). These studies, which used biochemical assays to detect enzyme activity, lacked the specificity and sensitivity necessary to characterise conclusively the activity of the individual KLK gene family members. In this study, we have used a gene-specific RT-PCR/Southern hybridisation strategy to delineate the expression patterns of six of the individual KLK genes expressed in the rat ovary (rKLK1–3 and rKLK7–9). We have identified three broad patterns of expression in the eCG/hCG-stimulated ovary in which there is either a post–eCG increase/pre-ovulatory decrease in rKLK expression (rKLK1, rKLK3), a peri-ovulatory decrease in expression (rKLK2, rKLK8) or a relatively unchanged pattern of expression (rKLK7, rKLK9). In addition to clarifying the earlier biochemical studies, these findings support a differential role for the individual KLKs in the ovulatory process.

Abstract

The kallikreins (KLKs) are a highly conserved multi-gene family of serine proteases that are expressed in a wide variety of tissues and act on a diverse range of substrates. KLK-like enzyme activity has variously been reported to increase or decrease during the period leading up to ovulation in the equine chorionic gonadotrophin (eCG)-primed, human chorionic gonadotrophin (hCG)-stimulated immature rat ovary. These earlier studies, which used biochemical assays to detect enzyme activity, lacked the specificity and sensitivity necessary to characterise conclusively the activity of the individual KLK gene family members. In this study, we have used a gene-specific RT-PCR/Southern hybridisation strategy to delineate the expression of the oocyte followed by formation of the corpus luteum. The physiological and endocrinological changes occurring throughout this fundamental process have been well understood for many years, but it is only recently that some of the mechanisms underlying these changes have been elucidated. A number of factors involved locally in the ovulatory process have been identified, including tissue remodelling enzymes, vasoactive peptides and growth factors (Skinner & Parrot 1994, Bhoola et al. 1989, Gao et al. 1986, 1989, Tanaka et al. 1989). These studies, which used biochemical assays to detect enzyme activity, lacked the specificity and sensitivity necessary to characterise conclusively the activity of the individual KLK gene family members. In this study, we have used a gene-specific RT-PCR/Southern hybridisation strategy to delineate the expression patterns of six of the individual KLK genes expressed in the rat ovary (rKLK1–3 and rKLK7–9). We have identified three broad patterns of expression in the eCG/hCG-stimulated ovary in which there is either a post–eCG increase/pre-ovulatory decrease in rKLK expression (rKLK1, rKLK3), a peri-ovulatory decrease in expression (rKLK2, rKLK8) or a relatively unchanged pattern of expression (rKLK7, rKLK9). In addition to clarifying the earlier biochemical studies, these findings support a differential role for the individual KLKs in the ovulatory process.

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The rat KLK gene family consists of 13 genes, of which three are pseudogenes (Clements 1989). We have previously demonstrated that six members of the rat KLK gene family (rKLK1, rKLK3, rKLK7, rKLK8, rKLK9 and rKLK12) are expressed in the rat ovary (Clements et al. 1995). In this study, we have used a sensitive and specific semi-quantitative RT-PCR/Southern hybridisation strategy to delineate the expression patterns of the individual rKLK gene family members in the eCG-primed, hCG-stimulated immature rat model of ovulation in order to relate the previous enzymatic data (Espey et al. 1986, 1989, Gao et al. 1992) to the expression of specific kallikrein genes. We have also used a semi-quantitative RT-PCR analysis of luteinising hormone receptor (LH-R) and follicle stimulating hormone receptor (FSH-R) expression, and analysis of luteinising hormone receptor (LH-R) and follicle-stimulating hormone receptor (FSH-R) expression, to confirm that used by Camp et al. (1991), to confirm the efficacy of the eCG/hCG treatment.

Materials and Methods

Animals/drug treatments

Twenty-one to 23-day-old immature female Sprague–Dawley rats were kept under a 12 h darkness:12 h light cycle; rat chow and water were available ad libitum. The immature rats, n=4 per group, were injected s.c. with 10 IU eCG (eCG; Folligon, Intervet, NSW, Australia) in saline or saline alone (control group) at 0830 h. After 48 h, 10 IU hCG (Chorulon, Intervet, NSW, Australia) was injected s.c. In this model, previously validated in our laboratory (J McMaster & J K Findlay, unpublished observations; Clements et al. 1995), the number of ova shed per ovary ranges from 17 to 40, and ovulation occurs between 10 and 12 hours after hCG administration. Animals were killed at 0, 2, 4, 8, 10, 12, 16, 20 or 30 h after hCG administration and tissues collected. This experiment was performed on two separate occasions. These experiments were approved by the Monash Medical Centre Animal Ethics Committee and performed in accordance with the National Health and Medical Research Council of Australia guidelines on animal experimentation.

Semi-quantitative RT-PCR

RNA was prepared essentially according to the procedure of Chomczynski & Sacchi (1987), except that the RNA was finally precipitated with 2 vol 4·5 M sodium acetate (pH 7) at −20 °C for 1 h to eliminate any further residual DNA. First-strand cDNA was synthesised as follows: 500 ng ovarian total RNA was heated to 65 °C for 5 min before addition of a reaction mix containing 1 × PCR buffer (50 mM KCl; 10 mM Tris, pH 8·0; 0·1% TritonX-100), 6 mM MgCl2; 1 mM each dATP, dCTP, dTTP, dGTP; 25 pmol p(dT)15; 20 U RNASin (Promega) and 5 U AMV-RT (Boehringer-Mannheim, Nunawading, Victoria, Australia) in a final volume of 20 µl. Reactions were incubated at 42 °C for 60 min. The enzyme was then denatured by heating to 95 °C for 3 min, before adding the cDNA to the PCR reaction.

Semi-quantitative PCR conditions were established for both the LH and FSH receptor and kallikrein genes using a modified procedure based on our previous study (Clements et al. 1995) and the method established by Camp et al. (1991). The latter method has become widely used in semi-quantitative RT-PCR studies (Orly et al. 1996, Nemeth et al. 1998, Ronan-Fuhrman et al. 1998).

PCR analysis of KLK multigene family expression

Primers were designed to highly conserved common sequences in exons 2 (sense primer: 5′ACTGGAATT CGAATTCCGAACCCTGGCAAGT3′) and 4 (anti-sense primer: 5′GCTAGGATCCATGTTCCACACA CTGGAGATC3′) of the rat KLK genes such that all gene family members were amplified in the same reaction to give a PCR product of 426 bp (Clements et al. 1995). A second set of primers (sense 5′TCTCATCTCGGG CTCACACTG3′; antisense: 5′AGAAGATGGTGTGCT TCATTGC3′) (Cole et al. 1989) was used to amplify a 228 bp PCR product from the ubiquitously expressed β2-microglobulin gene as an internal control to correct for any loading differences between samples. PCR conditions were optimised by determining the appropriate number of cycles of amplification and amount of RNA required for both the kallikrein and control PCR products to be amplified in the linear reaction range (data not shown). Optimal PCR conditions using 25 pmol universal KLK primers were: 4 µl cDNA (an equivalent of 100 ng total RNA) amplified at 95 °C for 1 min, 50 °C for 2 min, 72 °C for 3 min over 30 cycles. The β2-microglobulin sense and antisense primers (25 pmol) were added to the reactions after the seventh cycle of amplification, such that β2- microglobulin products were amplified for 22 cycles only.

Southern blot analysis

Fifteen microlitres of the PCR products were electrophoresed and Southern blotted by standard procedures. Blots were hybridised with either a cRNA probe (KLK1 or β2-microglobulin cDNA) at 50 °C overnight or gene-specific oligonucleotide probes for rKLK1, rKLK3, rKLK7, rKLK8, rKLK9 or rKLK12 as previously
described (Clements et al. 1995) at 37 °C overnight. Blots were washed at a stringency of 0·1 × SSC, 0·1% SDS at 50 °C (for cRNA probes) or 2 × SSC, 0·1% SDS at 37 °C (for oligo probes) before autoradiography. Hybridised membranes were also scanned on a Fuji phosphoimager system and analysed using the MacBas image analysis software program. The final KLK expression patterns, as assessed by densitometry, were normalised relative to each individual β2-microglobulin expression level and plotted as corrected integrated optical density as a function of treatment. Statistical analysis was performed by one-way analysis of variance (ANOVA) using Prism (GraphPad Software Inc., San Diego, CA, USA).

PCR analysis of LH-R and FSH-R

A modified version of the semi-quantitative RT-PCR assay for the expression of LH-R and FSH-R established by Camp et al. (1991) was used in these studies. Reaction conditions were optimised for the appropriate amount of RNA and number of amplification cycles as for the kallikrein semi-quantitative RT-PCR assay. Briefly, oligonucleotide primer pairs designed to the LH-R (sense: 5‘GAGCTGACAGCTCTATACAAAG3’; antisense: 5‘CCACCGAGGCTTAACTCAC3’), FSH-R (sense 5‘ACCTTGTGTCTCATCAAGC3’; antisense: 5‘GAAA CCTCATCGCTACCCAC3’) (primer sequences kindly provided by Dr K Mayo) and β2-microglobulin genes (in place of the ribosomal protein RPL-19 internal standard used by Camp et al. (1991)) were used to amplify PCR products of 419 bp (LH-R), 311 bp (FSH-R) and 228 bp (β2-microglobulin). LH- and FSH-R products were amplified for 28 cycles of 95 °C for 1 min, 65 °C for 2 min, 72 °C for 3 min followed by extension at 72 °C for 10 min, and β2-microglobulin products were amplified in the same reactions for 24 cycles.

Radiolabelled α-[32P]dCTP was incorporated into the PCR to enable direct detection of PCR products. An aliquot (5µl) of the RT-PCR product was electrophoresed on a 6% denaturing polyacrylamide gel. Vacuum-dried polyacrylamide gels were then scanned on a Fuji phosphoimager system and analysed as described above.

Results

Confirmation of the hormonal status of the gonadotrophin-stimulated rat model of ovulation

The patterns of expression of the LH-R and FSH-R were determined in the two separate experiments in order to confirm the hormonal status of the animals and to validate the semi-quantitative assay approach by direct comparison with the earlier findings of Camp et al. (1991) (Fig. 1). The results of one of these experiments is shown in Fig. 1; however, both animal experiments gave similar results and showed the expected patterns of expression of these two gonadotrophin receptor mRNAs, consistent with those observed by Camp et al. (1991). The level of LH-R expression was increased after eCG administration, peaking at 2 h after hCG administration in both experiments before mRNA levels decreased to the expected basal values by 12 h post-hCG, around the time of ovulation, as was described previously (Camp et al. 1991). As expected, FSH-R expression did not change as markedly as that of the LH-R, with a moderate increase in mRNA levels after eCG administration, before decreasing to a stable low level of expression at around 12 h post-hCG.

Characterisation of KLK gene expression

The results obtained in the two animal experiments were essentially the same; the results of one experiment are shown in Figs 2 and 3. Initially, RT-PCR products were probed with a rKLK1 cRNA probe to detect the expression pattern of all rKLK genes (Fig. 2). Although the Southern blots show some intergroup variation and variation in the levels of β2-microglobulin mRNA expression, there is a clear pattern of KLK expression that is also apparent in the β2-microglobulin normalised KLK expression levels illustrated in the chart in Fig. 2. KLK expression patterns, although variable early in the experimental period, showed a decrease in rKLK mRNA levels after eCG administration, followed by an increase briefly at 2 h after hCG administration, before continuing to decrease throughout the pre-ovulatory period until just before the time of ovulation at around 10 h. The rKLK mRNA levels then remained at this significantly lower value (compared with control groups) until 30 h after hCG treatment. The patterns of expression of the individual rKLK genes were subsequently delineated by hybridisation of the RT-PCR products with gene-specific oligonucleotides. Although there was a considerable difference in the response of rKLK1 and rKLK3 to eCG treatment, (note different scales in Fig. 3), the patterns of gene expression observed for rKLK1 and rKLK3 (Fig. 3) after hCG stimulation were similar to the trend observed for the overall expression of the rKLK genes as detected with the rKLK1 cRNA probe (Fig. 2), with a post-hCG increase in expression followed by a significant decrease in rKLK1 and rKLK3 mRNA levels by the time of ovulation.

The patterns of expression observed for rKLK2, rKLK7, rKLK8 and rKLK9 (Fig. 3) were less clear than those described above for rKLK1 and rKLK3, with considerable intra-group variation observed for all of these genes throughout the ovulatory period. Low levels of expression were observed for both rKLK2 and rKLK8 throughout the pre- and post-ovulatory periods, before rKLK2 expression was observed to increase significantly around 30 h after hCG administration (Fig. 3). A notable point in the relatively stable patterns of expression of rKLK2 and rKLK was their respective levels of peri-ovulatory gene
expression. rKLK2 expression decreased by 4–8 h post-hCG administration, prior to an apparent increase in expression by 10 h post-hCG, whereas rKLK8 mRNA was undetectable 10 h after hCG stimulation, before an upregulation of expression by 12 h post-hCG. Both these observations, although not significant (P > 0.05), were consistent and reproducible.

The patterns of expression of both rKLK7 and rKLK9 were both characterised by relatively steady-state patterns of expression after eCG priming and hCG stimulation. The decrease in the level of rKLK9 expression at 12 h post-hCG was consistent and reproducible, although it did not reach a level of significance compared with pre-ovulatory values (P > 0.05; Fig. 3).

Discussion

This study has used a sensitive, semi-quantitative RT-PCR assay to delineate the ovarian expression patterns of six members of the highly conserved KLK multi-gene family in the peri-ovulatory period of the rat. In using the eCG/hCG rat model of ovulation, we have first shown that the expression patterns of the LH-R and FSH-R are similar to those reported by Camp et al. (1991) and confirm the hormonal status of the animals, the physiological validity of the model and, importantly, demonstrate the reproducibility of the semi-quantitative RT-PCR approach.

Apart from the studies by Espey and Gao, in which kallikrein-like enzyme activity has previously been shown to be regulated in the gonadotrophin-stimulated rat ovary (Espey et al. 1989, Gao et al. 1992), the present study is the first attempt to determine regulatory changes in expression, at the mRNA level, of KLK gene family members in this model, and it has been able to elucidate further the patterns of expression of the individual rKLK genes. We have identified three broad patterns of expression across the rKLK gene family characterised by (i) a post-eCG increase/pre-ovulatory decrease in expression (rKLK1,
rKLK3); (ii) a peri-ovulatory decrease, post-ovulatory increase in expression (rKLK2 and rKLK8) or (iii) a level of expression relatively unchanged by gonadotrophin stimulation (rKLK7, rKLK9).

After delineation of the patterns of expression of individual KLK genes, we found that the pattern of kallikrein enzyme activity detected in the previous study by Gao et al. (1992), in which kallikrein-like enzyme activity was shown to peak at around the time of ovulation. This suggests that the study by Gao et al. (1992), which used the more specific substrate, Pro-Phe-Arg-methylcoumarylamide, was in fact detecting true rKLK1 enzyme activity. The reactivity of multiple members of the KLK gene family with the chromogenic substrate S2266, used by Espey et al. (1986) – which was able to be hydrolysed by not only rKLK1 but also rKLK2, rKLK7 and rKLK9.
Figure 3 Expression patterns of individual rKLK gene family members in the gonadotrophin-stimulated rat ovary. RNA was obtained from the ovaries of sham-treated controls (saline), and after 48 h from animals treated with hCG for 0 (eCG), 2 (hCG-2), 4 (hCG-4), 8 (hCG-8), 10 (hCG-10), 12 (hCG-12), 16 (hCG-16), 20 (hCG-20) or 30 h (hCG-30) and subjected to semi-quantitative RT-PCR as described in the text. Expression levels of rKLK1, rKLK2, rKLK3, rKLK7, rKLK8 and rKLK9 were determined by hybridisation to gene-specific oligonucleotides, phosphorimage analysis and normalisation to β2-microglobulin expression levels. Arrows indicate the time of ovulation, which occurs at 10–12 h after hCG treatment in this model. Results are shown as corrected integrated optical density (IOD) (mean ± S.E.M.) as a function of treatment. Statistical analyses for rKLK1, hCG2 compared with hCG-10, -12, -16, -20 and -30; rKLK2, hCG-30 compared with all groups; rKLK3, hCG-2 compared with hCG-4, -8, -10, -12, 16, -20 and -30; rKLK8, saline compared with hCG-2, -4, -8, -20 and -30; rKLK9, hCG-12 compared with hCG-30: *P<0.05, **P<0.01, ***P<0.001.
(Berg et al. 1992, Wang et al. 1992) – is presumably responsible for the different pattern of enzyme activity reported by Espey et al. (1986), although it is unclear from the present study which specific rKLK gene(s) were responsible for the pattern of expression observed by Espey et al. (1986). Clearly, biochemical assays for kallikrein-like enzyme activity will remain severely limited without highly specific enzyme substrates for each enzyme encoded by the rKLK genes.

Bradykinin, the effector peptide generated by the action of tissue kallikrein (rKLK1) on its primary substrate low-molecular-weight kininogen, has been shown to stimulate the ovulatory process in in vitro perfused rat and rabbit ovaries (Hellberg et al. 1991, Yoshimura et al. 1994) and is also well characterised for its promotion of vascular permeability at sites of inflammation, leading to oedema (Bhoola et al. 1992). As ovulation has been likened to an inflammatory response, and as the kallikrein-kinin system has also been more recently implicated as having a role in ovarian hyperstimulation syndrome – another inflammatory-like condition of the ovary (Kobayashi et al. 1998, Ujioka et al. 1998) – it is possible that bradykinin plays a similar role in the pre-ovulatory follicle. The increased level of rKLK1 expression after hCG stimulation of the immature rat would thus support such a role for this enzyme. The decrease in rKLK1 expression in the peri-ovulatory period may also be indicative of a down-regulation of the vasodilatory role of bradykinin in the post-ovulatory follicle.

As the substrates of other genes detected in this study (rKLK3, rKLK7 and rKLK8) are as yet uncharacterised, it is not clear what their potential role(s) may be, although the pre- and peri-ovulatory patterns of expression of rKLK3 and rKLK8 are particularly intriguing and warrant further investigation. Substrates of rKLK2 and rKLK9 have been determined in vitro which allows some speculation as to their potential roles in the process of ovulation.

Tatin (encoded by rKLK2), is able to process angiotensinogen to angiotensin II, in vitro. Angiotensin is believed to play numerous roles in the pre- and post-ovulatory follicle, including vascularisation of the early follicle, maturation of the oocyte, and regulation of stromal, thecal, granulosa and luteal cell growth and proliferation (Andrade-Gordon et al. 1991, Yoshimura 1997). As rKLK2 levels are not increased at this time, tonin may not be a physiological activator of angiotensin II in the rat ovary and thus it is possible that angiotensin is activated by other factors, such as tissue plasminogen activator in this tissue (Yoshimura 1997). The marked increase in expression of rKLK2 at 20–30 h after hCG administration may be indicative of a role for this enzyme in the process of luteolysis, particularly in the formation of the vasculature associated with the luteinised granulosa cells (Hillier 1994), although this has yet to be determined.

rKLK9 is known to encode the potent vasoconstrictive agent submandibular enzymatic vasoconstrictor (Yamaguchi et al. 1991). Although expression of this gene throughout the pre-ovulatory period was highly variable, there was a consistent decrease in the level of its expression at 12–16 h after hCG stimulation. It is therefore possible to speculate that an increased level of rKLK9 expression in the pre-ovulatory period, and the subsequent decrease at around 12 h post-hCG, may reflect a role for this enzyme in the control of local blood flow (perhaps in concert with bradykinin and angiotensin II) at around the time of ovulation.

In conclusion, we have identified and characterised the patterns of expression of six members of the highly conserved KLK gene family (rKLK1, rKLK2, rKLK3, rKLK7, rKLK8, and rKLK 9) throughout the ovulatory process in an ovulatory rat model. It is likely that tissue kallikrein (encoded by rKLK1) does play a role in ovulation; however, further studies are required to determine the sites of action, the cell types expressing these enzymes, and the physiological substrates of all of these KLK enzymes in the ovary. These findings will help us to understand the events controlling the regulation of expression of the members of the KLK gene family and further elucidate the roles of these enzymes in the process of ovulation and ovarian function.

Acknowledgements

The authors are indebted to Associate Professor Peter Fuller for guidance and helpful discussion of this work, Julie McMaster for technical assistance and Sue Panckridge for assistance with figures. The financial support of Prince Henry’s Institute of Medical Research (AMH), the National Health and Medical Research Council of Australia (J K F: No. 983212; J A C: No. 950859) is gratefully acknowledged.

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Tsafiri A & Reich R 1999 Molecular aspects of mammalian ovulation. 1–11.


Received 7 February 2001
Accepted 1 March 2001