Effects of genistein on the periovulatory expression of messenger ribonucleic acid for matrix metalloproteinases and tissue inhibitors of metalloproteinases in the rat ovary

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The matrix metalloproteinases (MMPs) play critical roles in the ovulatory process. Their expression and activity, together with those of the endogenous tissue inhibitors of metalloproteinases (TIMPs), are stimulated by LH. The LH surge initiates a cascade of events resulting in ovulation and formation of the corpus luteum via activation of protein kinases A and C, as well as tyrosine kinases. *In vitro* perfused rat ovaries were untreated, or treated with LH (0.2 µg ml⁻¹) plus 0.2 mmol 3-isobutyl-1-methylxanthine I⁻¹ with 0, 10 or 100 µmol genistein I⁻¹ (an inhibitor of tyrosine kinases) to assess whether tyrosine kinases are mediators of the LH-stimulated increase in ovarian expression of the MMPs and TIMPs. After 10 h of perfusion, ovaries were collected and frozen until RNA isolation. Northern and RNase protection analyses were used to

measure mRNA encoding collagenase 3, gelatinases A and B, and TIMPs-1, -2 and -3. Treatment with LH plus 3-isobutyl-1-methylxanthine resulted in a two- and fivefold increase in mRNA encoding collagenase 3 and TIMP-1, respectively ($P < 0.05$). Treatment with 100 µmol genistein I⁻¹ blocked the LH-stimulated increase in collagenase 3 (0.012 ± 0.002 versus 0.028 ± 0.005 relative units for 100 µmol genistein I⁻¹ versus LH; $P < 0.05$), whereas neither dose of genistein affected LH-induced TIMP-1 expression. LH alone or with genistein did not alter the expression of mRNA encoding TIMP-2 and TIMP-3, or mRNA encoding gelatinases A and B. These data indicate that tyrosine kinases play a role in the LH-induced tissue remodelling required for ovulation by mediating the LH-stimulated expression of collagenase 3.

Introduction

Ovulation requires degradation of the apical follicle wall and dissolution of the granulosa and epithelial cell basement membranes. Matrix metalloproteinases (MMPs), a family of proteinases comprising the gelatinases, collagenases, stromelysins, and membrane-type MMPs (MT-MMPs; for review, see Matrisian, 1990; Nagase and Woessner, 1999), are capable of breaking down basement membranes and the extracellular matrix. Various members of this family are regulated by the preovulatory gonadotrophin surge and have been shown to play a critical role in the process of ovulation (Fukumoto *et al.*, 1981; Reich *et al.*, 1985; Murdoch *et al.*, 1986; Yoshimura *et al.*, 1987; Balbín *et al.*, 1996; Hulboy *et al.*, 1997). Administration of hCG to eCG-primed rats increases the activity of ovarian proteoglycanase, gelatinase (Curry *et al.*, 1992) and collagenase (Curry *et al.*, 1985; Hirsch *et al.*, 1993), and treatment with hCG increases collagenase

activity in cultured rat follicles (Palotie *et al.*, 1987). In ewes, collagenase activity increases after a GnRH-induced gonadotrophin surge (Murdoch *et al.*, 1986), and gelatinase A activity in follicular fluid increases after hCG administration (Russell *et al.*, 1995). Inhibition of MMP activity with exogenous chemical inhibitors blocks ovulation both *in vitro* (Brännström *et al.*, 1988; Butler *et al.*, 1991) and *in vivo* (Reich *et al.*, 1985), further supporting a critical role for MMPs in the ovulatory process.

The activity of MMPs is regulated, in part, by tissue inhibitors of metalloproteinases (TIMPs). Four TIMPs (TIMP-1–4) have been identified to date and they inhibit MMP activity by forming non-covalent bonds with the MMPs in a 1:1 molar ratio. TIMPs bind to active and latent forms of MMPs: TIMP-1 associates with the latent form of gelatinase B (92 kDa gelatinase; MMP-9), while TIMP-2 and TIMP-4 complex with the latent form of gelatinase A (72 kDa gelatinase; MMP-2; Bigg *et al.*, 1997; Hulboy *et al.*, 1997). In addition to their role as inhibitors of MMPs, TIMP-1 and TIMP-2 act as growth factors (Hayakawa *et al.*, 1992; Chesler *et al.*, 1995; Nemeth *et al.*, 1996; Yamashita *et al.*, 1996) and are anti-angiogenic

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agents (Takigawa *et al.*, 1990; Murphy *et al.*, 1993; Johnson *et al.*, 1994). TIMP-1 has also been shown to regulate steroidogenesis in cultured ovarian cells (Boujrad *et al.*, 1995; Nothnick *et al.*, 1997).

TIMP expression is similar to that of MMPs in that it is influenced by the preovulatory gonadotrophin surge. The amount of mRNA for TIMP-1 (Mann *et al.*, 1993) and TIMP-like activity (Zhu and Woessner, 1991) increase in response to the LH surge and by treating cultured rat granulosa cells with LH (Mann *et al.*, 1991). The expression of mRNA for TIMP-3 also increases after LH administration to cultured rat granulosa cells (Nothnick and Curry, 1996).

The dynamic cascade of events set in motion by the LH surge is mediated by the protein kinase A and protein kinase C pathways (Davis *et al.*, 1986; Morris and Richards, 1995), as well as tyrosine kinases (TKs) (Wong and Richards, 1992; Morris and Richards, 1993). LH stimulates the expression of P450 side-chain cleavage, prostaglandin endoperoxide synthase 2 (Wong and Richards, 1992; Morris and Richards, 1993), and activation of tissue plasminogen activator (Shimamoto *et al.*, 1998) in ovarian cells, in part, by activating TKs. In other tissues, TKs are involved in regulating the expression of mRNA for collagenase 1, stromelysin 1 (Li and Zafarullah, 1998), and TIMP-3 (Su *et al.*, 1998), as well as activating gelatinase A (Li *et al.*, 1997). However, the role of TKs in mediating MMP and TIMP activity in the ovary is unknown. The present study used genistein, an inhibitor of TKs, to test the hypothesis that TKs mediate the LH-stimulated changes in ovarian expression of gelatinases A and B, collagenase 3, and TIMPs 1–3.

Materials and Methods

In vitro ovarian perfusion

Immature female Sprague–Dawley rats (B&K Universal, Sollentuna) were housed under controlled conditions with lights on from 05:00 to 19:00 h with *ad libitum* access to food and water. At 28 days of age, the rats were administered 20 iu eCG (Sigma Chemical Co., St Louis, MO) s.c. at 09:00 h. Forty-eight hours after eCG, rats were anaesthetized with a combination of ketamine (Park Davis, Barcelona; 40 mg kg⁻¹) and xylazine (Bayer, Leverkusen; 6.5 mg kg⁻¹); and then heparin sulphate (Lovens Ballerup; 300 iu) was injected i.v. Laparotomy was performed and the right ovary was isolated surgically as described by Brännström *et al.* (1987) and Koos *et al.* (1984). All experiments were approved by the animal ethics committee of Göteborg University.

Perfusions were performed in a recirculating system with 30 ml M199 with Earle's salts (Gibco, Gaithersburg, MD) supplemented with gentamicin (Biological Industries, Kibbutz Beit Haemek; 50 µg ml⁻¹), insulin (Novo, Bagsvaerd; 0.02 iu ml⁻¹), 4% bovine serum albumin (Boehringer Mannheim, Mannheim), and 0.026 mol sodium bicarbonate l⁻¹ as described by Koos *et al.* (1984), Brännström *et al.* (1987) and Matousek *et al.* (1999).

Ovaries were perfused approximately 30–60 min before the administration of the following treatments: control (no treatment; *n* = 4), or 0.2 µg LH ml⁻¹ (ovine LH NIADDK-oLH-26) and 0.2 mmol 3-isobutyl-1-methylxanthine l⁻¹ (IBMX; Sigma) with 0 (*n* = 8), 10 (*n* = 4) or 100 µmol genistein l⁻¹ (*n* = 13; Sigma). The perfusions were continued for 10 h, after which time the ovaries were collected, frozen in liquid nitrogen, and stored at –70°C for later isolation of RNA.

RNase protection assay

Total RNA was isolated from perfused ovaries using TRIZOL reagent (Gibco BRL, Rockville, MD) and quantified by spectrophotometry. RNase protection assays were used to measure mRNA for collagenase because its expression is too low to be detected using northern blot analysis. Rat cDNAs for collagenase 3 (MMP-13; a generous gift from L. Matrisian, Vanderbilt University, Nashville, TN) and mouse ribosomal protein L32 (kindly provided by O.K. Park-Sarge, University of Kentucky, Lexington, KY) were linearized with XhoI and EcoRI, respectively. Antisense riboprobes were transcribed using Ambion's MAXIScript kit (Ambion Inc., Austin, TX), [α -³²P]UTP (10 mCi ml⁻¹; DuPont New England Nuclear, Boston, MA) and T7 RNA polymerase.

RNase protection assays were carried out according to Ambion's RPA II kit instructions. Samples of total RNA (6 µg) were hybridized for 15–18 h at 50°C with excess radiolabelled antisense riboprobe (2 × 10⁵ c.p.m.). Loading variation among samples was standardized by including L32 riboprobe in all hybridization reactions. Protected RNA fragments were analysed by electrophoresis through a 5% acrylamide–8 mol urea l⁻¹ gel. Quantification of band intensity in the gels was determined from an imaging plate using a phosphor-imager (Molecular Dynamics, Sunnyvale, CA). The band intensity of mRNA for collagenase 3 was normalized to the corresponding band for L32 per sample.

Northern blot analysis

Twenty micrograms of total RNA were subjected to electrophoresis through a 1% agarose gel containing 2.2 mol formaldehyde l⁻¹, and then transferred to a Nytran membrane (Schleicher and Schuell, Keene, NH) according to the manufacturer's instructions. ³²P-labelled cDNA probes corresponding to mouse TIMPs 1–3 and gelatinases A and B were synthesized as described by Nothnick *et al.* (1996, 1997). After probing for the TIMPs and MMPs, membranes were hybridized with a cDNA probe for rat ribosomal 18S. Expression of mRNA for the TIMPs and MMPs was normalized to the relative expression of the 18S transcript per sample.

Statistical analysis

Because of the inherent variation among animals, sample values that were greater than 3 SD from the group

Fig. 1. (a) Representative RNase protection assay demonstrating the protected fragments of mRNA for collagenase 3 and ribosomal protein L32 in 6 μg total RNA from individual *in vitro* perfused rat ovaries. (b) Relative abundance (mean $\pm$ SEM) of mRNA for collagenase 3 in 6 μg total RNA isolated from *in vitro* perfused rat ovaries. Relative abundance as calculated by correcting for the amount of L32 within each sample. Gen: genistein; IBMX: 3-isobutyl-1-methylxanthine. Bars with different superscripts are significantly different ($P < 0.05$).

mean were not included in statistical analyses. Data were subjected to Levene's test for homogeneity of variance. Log or square root transformations were performed on data that were determined to have heterogeneous variances. Effects of treatment on expression of mRNA for the MMPs and TIMPs were tested for by one-way ANOVA. *Post hoc* comparisons were made using Fisher's least significant difference procedure. A $P < 0.05$ denoted a significant difference.

Results

The administration of LH to *in vitro* perfused rat ovaries stimulated a twofold increase in mRNA encoding collagenase 3 ($P < 0.05$; Fig. 1). Treatment with 10 μmol genistein l⁻¹ resulted in expression of mRNA for collagenase 3 that did not differ from that of LH-treated ovaries. However, the administration of 100 μmol genistein l⁻¹ blocked the LH-stimulated increase in expression of mRNA for collagenase 3 (0.028 ± 0.005 versus 0.012 ± 0.001 relative units

Fig. 2. (a) Representative northern blot for tissue inhibitors of metalloproteinase 1 (TIMP-1) and ribosomal 18S mRNA in 20 μg total RNA isolated from individual *in vitro* perfused rat ovaries. (b) Relative abundance (mean $\pm$ SEM) of mRNA for TIMP-1 in *in vitro* perfused rat ovaries. Relative abundance as calculated by correcting for the amount of 18S within each sample. Gen: genistein; IBMX: 3-isobutyl-1-methylxanthine. Bars with different superscripts are significantly different ($P < 0.05$).

for LH versus 100 μmol genistein l⁻¹ treatments, respectively; $P < 0.05$).

Treatment of *in vitro* perfused ovaries with LH resulted in a significant increase in the expression of mRNA for TIMP-1 (1.8 ± 0.6 versus 6.3 ± 1.6 relative units for control and LH treatments, respectively; Fig. 2). There was no effect of genistein treatment on the LH-stimulated mRNA for TIMP-1, although the administration of 100 μmol genistein l⁻¹ reduced mRNA expression for TIMP-1 to a value intermediate between that from control and that from LH-treated ovaries.

There was a trend towards an increase in mRNA for gelatinase A in response to LH, although this was not significant (Fig. 3). Genistein had no effect on the expression of gelatinase A. There was no effect of treatment on the expression of mRNA for gelatinase B or TIMP-2 (data not shown). The treatment of perfused ovaries with LH did not

Fig. 3. (a) Representative northern blot for gelatinase A and ribosomal 18S mRNA in 20 µg total RNA isolated from individual *in vitro* perfused rat ovaries. (b) Relative abundance (mean $\pm$ SEM) of mRNA for gelatinase A in *in vitro* perfused ovaries. Relative abundance as calculated by correcting for the amount of 18S within each sample. Gen: genistein; IBMX: 3-isobutyl-1-methylxanthine.

alter the amount of mRNA encoding TIMP-3. The expression of mRNA encoding TIMP-3 in ovaries treated with either 10 or 100 µmol genistein l⁻¹ did not differ from that in control ovaries (data not shown).

Discussion

Treatment of *in vitro* perfused rat ovaries with genistein to block the activity of TKs reduces ovulation efficiency without affecting steroid production, prostaglandin production, or the activity of plasminogen activator (Matousek *et al.*, 1999). Ovulation was also blocked when tyrophostin, another inhibitor of TKs, was administered to eCG-primed rats before the injection of hCG (Shimamoto *et al.*, 1998). In the present study, genistein was used to assess the role of TKs in LH-stimulated changes in ovarian expression of TIMPs 1–3, collagenase 3 and the gelatinases.

The present survey of mRNA expression of MMPs critical to the ovulatory process revealed that the adminis-

tration of genistein blocked the LH-stimulated increase in mRNA encoding collagenase-3. The dose of genistein that prevented the LH-induced increase in collagenase 3 also inhibited ovulation (Matousek *et al.*, 1999). Taken together, these data can be interpreted to show that the decrease in ovulation efficiency resulting from inhibition of TKs may be due to a decrease in collagenase 3 resulting in inadequate degradation of the follicle wall.

Because genistein is a phyto-oestrogen, another mechanism by which genistein may exert its effect on the ovary is via binding to oestrogen receptors. There are two types of oestradiol receptor, oestrogen receptor α and oestrogen receptor β , with genistein showing greater binding affinity for oestrogen receptor β (Kuiper *et al.*, 1997). In the ovary, oestrogen receptor β is present in granulosa cells of developing and mature follicles (Kuiper *et al.*, 1996; Byers *et al.*, 1997). Both the oestrogen receptor β mRNA (Byers *et al.*, 1997) and protein (Fitzpatrick *et al.*, 1999) are reduced markedly in response to the LH surge. Therefore, it is unlikely that the decrease in LH-stimulated mRNA for collagenase 3 seen after genistein treatment in the present study resulted from genistein binding to oestrogen receptor β in granulosa cells.

Tyrosine kinases play a role in the expression of COX-2 (Wong and Richards, 1992; Morris and Richards, 1993), and genistein is capable of inhibiting the expression and activity of COX-2 (Akarasereenont *et al.*, 1994; Mutoh *et al.*, 2000). However, the reduction in ovulation efficiency (Matousek *et al.*, 1999) and decrease in LH-stimulated collagenase 3 mRNA expression (present study) in response to genistein treatment most likely do not result from a decrease in prostaglandin production. Findings in support of this conclusion come from a study showing that the dose of genistein that inhibited ovulation did not effect prostaglandin production (Matousek *et al.*, 1999), and Espey *et al.*, (1991) have reported that ovulation can occur uncompromised even when prostaglandin synthesis is reduced by 80–90%. These data indicate that, by blocking TK action, ovulation is affected by some mechanism other than the inhibition of prostaglandin production.

In the present study, it was surprising that the expression of mRNA for gelatinase A did not increase in response to LH treatment since other studies have reported an increase in both mRNA (Reich *et al.*, 1991; Curry *et al.*, 2000) and activity (Curry *et al.*, 1992) during the periovulatory period. This finding may be explained by the fact that, although LH plus IBMX stimulated a 2.5-fold increase in the expression of mRNA encoding gelatinase A, fewer oocytes are released in the *in vitro* perfused ovary model than in the eCG/hCG model. This decrease in the number of ruptured follicles may result in a corresponding diminution of gelatinase A mRNA expression.

LH/hCG-induced expression of collagenase 3 has been observed using RNase protection assays (Curry *et al.*, 2000; present study). These observations contrast with those of other investigators who were unable to identify collagenase 3 in ovaries from eCG-primed rats up to 72 h

after administration of hCG (Balbín *et al.*, 1996), or in follicles from adult cyclic rats (Liu *et al.*, 1999). These differences in the expression of mRNA for collagenase 3 most likely reflect the greater sensitivity of the RNase protection assay compared with the northern analysis used in the other studies.

From the present study, it appears that TKs do not play a major role in LH-mediated TIMP expression during the periovulatory period. The lack of an effect of genistein on the LH-stimulated expression of mRNA for TIMP-1 indicates that this species of mRNA is regulated primarily by a second messenger system that does not depend on activation of TKs. These findings are in agreement with the presence of a cAMP response element in the upstream promoter region of the TIMP-1 gene (Edwards *et al.*, 1992; O-K. Park-Sarge, personal communication). TIMP-2 tends to be constitutively expressed and its expression does not appear to change in response to the LH surge (Inderdeo *et al.*, 1996; Nothnick *et al.*, 1997; Smith *et al.*, 1995; Hägglund *et al.*, 1999; Curry *et al.*, 2000; present study). The expression of mRNA for TIMP-3 was not affected by genistein treatment, indicating that it is regulated by factors other than TKs. This contention is supported by the finding that cultured granulosa cells treated with the progesterone receptor antagonist, mifepristone, expressed less mRNA for TIMP-3 compared with controls (Veloudis *et al.*, 1998). Taken together, these data indicate that the expression of TIMP-3 may be influenced by progesterone rather than the activity of TKs.

The finding that TKs may be involved in the LH-stimulated increase in mRNA for collagenase 3 does not indicate whether this is a direct effect of LH on TKs, or if LH stimulates other factors that are responsible for activating TKs. Potential candidates for such intermediary factors are growth factors and cytokines. For example, interleukins are capable of activating TKs (D'Arcangelo *et al.*, 2000; Tominaga *et al.*, 2000), concentrations of interleukins increase in association with the gonadotrophin surge (Chen *et al.*, 1995) and have been implicated as modulators of the ovulatory process (for reviews, see Brännström *et al.*, 1993; Norman and Brännström, 1996; Tsafirri and Reich, 1999). Other factors that activate TKs, such as vascular endothelial growth factor and colony stimulating factor 1, also increase in response to the gonadotrophin surge and may play a role in the periovulatory period (Koos, 1995; Christenson and Stouffer, 1997; Shinetugs *et al.*, 1999).

The dose of genistein required to inhibit ovulation (Matousek *et al.*, 1999) and the expression of mRNA for collagenase 3 (100 $\mu\text{mol l}^{-1}$; present study) is similar to doses used in other *in vitro* studies, as discussed by Matousek *et al.* (1999). In this *in vitro* perfusion system, some compounds have to be present in the micromolar range to produce an effect because of the dilution due to the adsorption of added compounds to the tubing material and perfusion apparatus, as well as the fact that compounds added exogenously have to perfuse into the organ from the vasculature (Brännström *et al.*, 1993; Matousek *et al.*, 1999).

It has been well documented that LH uses various second messenger systems to effect cellular events (Davis *et al.*, 1986; Morris and Richards, 1995; Herrlich *et al.*, 1996; Flores *et al.*, 1998). However, the targets of LH-stimulated second messenger activity are not as well known. The findings of the present study illustrate the importance of the LH-stimulated activity of TKs in the ovulatory process by indicating that they may mediate MMP expression and activity, thereby regulating the dissolution of the follicle wall and extrusion of the oocyte.

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