Inhibition of ovulation by tyrosine kinase inhibitors in the *in vitro* perfused rat ovary

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Protein tyrosine kinase activity, leading to tyrosine phosphorylation of the intracellular domains of receptors or non-receptor proteins, is an important feature of downstream signalling after receptor binding of a variety of factors, such as growth factors and cytokines. Since several members of these classes of paracrine–autocrine mediator may be involved in the intraovarian events of ovulation, the present study was designed to evaluate the effect of protein tyrosine kinase inhibition on the *in vitro* perfused rat ovary. Immature rats were primed with 20 iu pregnant mares' serum gonadotrophin 48 h before surgical isolation of the right ovary with connecting vasculature. The ovary was placed in a perfusion system for either 10 h, to examine ovarian concentrations of the established ovulatory mediators plasminogen activator, prostaglandins E_2 and $F_{2\alpha}$, or for 20 h, enabling a complete ovulatory process to occur *in vitro*. Ovulation was induced by ovine LH ($0.2 \mu\text{g ml}^{-1}$) in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.2 mmol l^{-1}) and the effects of two different protein tyrosine kinase inhibitors, genistein and tyrphostin A25, were studied. Unstimulated control ovaries did not ovulate and showed low secretion of progesterone and oestradiol. Addition of LH + 3-isobutyl-1-methylxanthine resulted in a marked stimulation of steroid release, and ovulations occurred in all ovaries (9.0 ± 0.9 ; mean $\pm$ SEM). The protein tyrosine kinase inhibitors, genistein and tyrphostin A25, significantly inhibited ovulation at the higher concentrations tested (3.0 ± 0.3 at $100 \mu\text{mol l}^{-1}$ genistein; 5.8 ± 1.0 at $500 \mu\text{mol l}^{-1}$ tyrphostin A25) but no effect was seen at lower concentrations. The presence of genistein and tyrphostin A25 at any concentration used did not significantly decrease the LH + 3-isobutyl-1-methylxanthine-induced progesterone or oestradiol concentrations. The intraovarian concentrations of plasminogen activator activity, and prostaglandin E_2 and $F_{2\alpha}$ were not altered by the presence of genistein ($100 \mu\text{mol l}^{-1}$). In conclusion, the results of the present study indicate that protein tyrosine kinase signalling pathways are integral parts of the mammalian ovulatory process but do not involve actions on the synthesis of steroids, plasminogen activator or prostaglandins.

Introduction

Mammalian ovulation involves complex intraovarian mechanisms that are regulated by a number of autocrine and paracrine factors (Brännström *et al.*, 1996). Binding of LH to its receptor induces the expression of several genes or activates enzyme systems to increase the intrafollicular concentrations of mediators which, in a redundant fashion, control the continued ovulatory process. The LH-induced signal to cause ovulation seems to be transduced primarily by the cAMP–protein kinase A (Brännström *et al.*, 1987a) and phosphoinositol–protein kinase C (Kaufmann *et al.*, 1992) second messenger systems. Several mediators of the growth factor and cytokine families may be of importance in the cascade leading to oocyte meiosis, follicle rupture and

luteinization. Several of these mediators are dependent on tyrosine phosphorylation. Among these mediators, epidermal growth factor (EGF) seems to be involved in the stimulation of oocyte meiosis and cumulus cell expansion (Downs, 1989). Inhibitory effects of EGF on the ovulatory mechanism may also exist, since administration of EGF to the *in vitro* perfused rabbit ovary results in a significant reduction in ovulatory efficiency (Endo *et al.*, 1992). EGF possesses intrinsic protein tyrosine kinase (PTK) activity (Smith *et al.*, 1993) and there are several PTK interaction sites on the intracellular domain of the EGF receptor (Seedorf, 1995). Platelet-derived growth factor (PDGF) enhances plasminogen activator (PA) activity in granulosa cells but not in theca cells of the ovulating follicle in domestic hens (Tilly and Johnson, 1990). Another factor of this family, vascular epidermal growth factor (VEGF), may be involved in control of angiogenesis and permeability changes at ovulation (Koos, 1993).

Inflammatory changes are active in ovulation and several of these processes may be controlled by cytokines (Brännström *et al.*, 1993a, 1994), such as interleukin (IL) 1, IL-6 and granulocyte macrophage colony stimulating factor (GM-CSF). The expression of IL-1 in rat theca interstitial cells is induced by LH (Hurwitz *et al.*, 1991), and IL-1 induces ovulation *in vitro* (Brännström *et al.*, 1993b). Furthermore, a regulatory effect of colony stimulating factor 1 (CSF-1) on rate of ovulation has been indicated (Cohen *et al.*, 1997). The intracellular signalling pathways for these cytokines are reported to involve activity of non-receptor PTK (Tadagatsu, 1995), which would lead to enhanced downstream signal transductions (Schaffhausen, 1995).

The aim of the present study was to investigate the possible role of PTK in the mechanism controlling the ovulatory process by use of genistein, which is a potent noncompetitive inhibitor (Akiyama and Ogawara, 1991) of soluble and membrane-bound PTK (Tadagatsu, 1995). Genistein is an isoflavone phyto-oestrogen that has both oestrogenic and anti-oestrogenic effects (Wähle *et al.*, 1995), as well as other non-specific effects (Grunicke, 1991). In view of these non-PTK related effects by genistein, a second PTK inhibitor (tyrphostin A25) was also used. A well characterized method involving isolated rat ovaries during *in vitro* perfusion (Brännström, 1993a) was used to exclude systemic effects of PTK inhibition on the ovulatory process.

Materials and Methods

Chemicals and hormones

Ovine luteinizing hormone (NIADDK-oLH-26) was kindly provided by the NIADDK and National Hormone and Pituitary Program (Rockville, MD). Pregnant mares' serum gonadotrophin (PMSG), genistein, tyrphostin A25 and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma Chemical Company (St Louis, MO); ketamine was obtained from Park Davis (Barcelona); xylazine was obtained from Bayer (Leverkusen); medium M199 was obtained from GIBCO (Geithersburg, MD); gentamycin sulphate was obtained from Biological Industries (Kibbutz Beit Haemek); bovine serum albumin (BSA; fraction V) was obtained from Boehringer Mannheim (Mannheim); insulin was obtained from Novo (Bagsvaerd) and heparin was obtained from Lövens (Ballerup).

Animals and surgical procedure

Sprague-Dawley rats (Beekey, Stockholm) were housed under controlled conditions with light on from 05:00 to 19:00 h and fed water and food *ad libitum*. At 09:00 h on day 28 of age, the rats were injected s.c. with 20 iu PMSG. This treatment induces follicular growth, and follicles of the preovulatory stage acquired 48 h later. On the morning of day 30 of age, the rats were anaesthetized with ketamine: xylazine (67:13 mg kg⁻¹ bodyweight) i.p. and were then given 300 iu heparin i.v. through a femoral vein. The operation procedure is described in detail by Koos *et al.* (1984). Briefly, a laparotomy was performed and the caudal

abdominal parts of the aorta and the vena cava were cannulated in a retrograde direction. All the connecting vessels, except the right ovarian vein and artery, were ligated and severed. At this stage, 2–5 ml saline was injected through the aorta cannule to flush the blood from the ovarian tissue. At the end of the surgical procedure, the aorta and the vena cava were ligated and cut cranially to the level of renal vessels and caudally to cannulation. The bursa was gently opened before the placement of the ovary in the perfusion chamber to enable ovulated oocytes to sediment to the bottom of a beaker, which was placed inside the perfusion chamber.

Perfusion

The procedure and experimental set up is shown schematically (Fig. 1). The perfusions were performed as described by Koos *et al.* (1984) with minor modifications (Brännström *et al.*, 1987b). The perfusion pressure was maintained at 80 mmHg and resulted in an average flow of 0.9 ml min⁻¹. The ovaries were perfused initially for 1 h before any agents were added, to allow metabolic stabilization of the tissue. Only ovarian preparations exhibiting flow rates between 0.7 and 1.3 ml min⁻¹ at 80 mmHg perfusion pressure were used to exclude specimens with vascular leakage or clotted vascular beds. The perfusion medium was 30 ml M199 with Earl's salts supplemented with insulin (0.02 iu ml⁻¹), gentamycin sulphate (50 µg ml⁻¹), 4% BSA and 0.026 mol sodium bicarbonate l⁻¹. A pH of 7.4 was maintained when the perfusion medium was continuously gassed with 5% CO₂ and 95% O₂. Genistein and tyrphostin A25 were diluted in 1% (v/v) DMSO as stock solutions. Aliquot volumes (1 ml) of the stock solutions were added to the perfusion medium 30 min before LH (0.2 µg ml⁻¹) and IBMX (0.2 mmol l⁻¹) administration to get final concentrations of 10 or 100 µmol genistein l⁻¹ and 100 or 500 µmol tyrphostin A25 l⁻¹. Maximum volume (1 ml) of diluent was added to unstimulated and LH + IBMX control perfusions. Four to six ovaries were perfused simultaneously in identical perfusion systems. Samples (1 ml) of perfusion medium were withdrawn at different time points throughout the perfusion period and stored at -70°C for later analysis. The same volume of fresh medium was added to the perfusion medium after each sampling, to keep a constant volume in the system. One set of ovaries was perfused for 20 h to study ovulation rate and steroidogenesis.

Another set of ovaries was perfused for 10 h to study the concentrations of the ovulatory mediators of prostaglandin (PG) E₂, PGF_{2α} and PA activity after LH + IBMX and 100 µmol genistein l⁻¹. After perfusion, each ovary was divided into two sections and snap frozen to -70°C until evaluation.

Ovulation rate

Oocytes were collected from the bottom of the beaker inserted in the perfusion chamber after 20 h perfusion. The number of ovulated oocytes was counted under a stereomicroscope, with the observer unaware of the experimental data.

Fig. 1. Schematic figure of the perfusion apparatus. The ovary is contained in the perfusion chamber. Perfusion medium is circulated as indicated by the arrows. Oxygenation takes place when the medium is passing down along the inside of the oxygenation reservoir. Pressure is monitored by the Hg manometer.

Steroid assay

Samples were kept frozen (-70°C) until analysis. Progesterone and oestradiol concentrations in samples of perfusion medium were analysed by radioimmunoassay technique using specific antisera purchased from Immuno-Chemicals (Tumba). The validity controls have been described by Hillensjö *et al.* (1984). The standard curves were calculated from eight standards (6.25–800 pg per tube) using a logit-log transformation.

PGE_2 , $\text{PGF}_{2\alpha}$ and PA assay

The ovarian tissue perfused for 10 h was homogenized (glass-glass homogenizer; 5000 r.p.m. 30 s) in 1 ml buffer (0.1 mol acetate buffer l^{-1} at pH 4.5 for PGE_2 and $\text{PGF}_{2\alpha}$

Fig. 2. Effects of genistein and tyrphostin A25 on ovulation rate in the *in vitro* perfused rat ovary. Asterisks indicate values significantly lower than those of the LH + 3-isobutyl-1-methylxanthine (IBMX) group (* $P < 0.05$; ** $P < 0.01$).

assays, and 0.05 mol Tris buffer l^{-1} at pH 8.0 for PA assay). After centrifugation at 10 000 g for 20 min, the supernatants were transferred to propylene tubes and PGE_2 and $\text{PGF}_{2\alpha}$ concentrations were analysed by enzyme immunoassay kit (RPN222; TRK900 Amersham, Bucks).

The procedure for PA activity assay was essentially as described for ovarian PA activity evaluation (Espesby *et al.*, 1985).

Statistical analysis

The results are presented as the mean $\pm$ SEM. Statistical differences regarding ovulation rate and steroid concentrations at each time point were calculated by the Kruskal-Wallis test followed by the Mann-Whitney U test. A value of $P < 0.05$ was considered significant for individual comparisons.

Results

Ovulation rate

The number of ovulations for the different experimental groups are shown (Fig. 2). In the unstimulated control group ($n = 6$), no ovulation occurred, whereas addition of LH + IBMX ($n = 8$) resulted in ovulations from all perfused ovaries (9.0 ± 0.9 ; mean $\pm$ SEM). Administration of 10 μmol genistein l^{-1} ($n = 6$) did not significantly change the LH + IBMX-induced ovulation rate (8.8 ± 1.3). The ovulation rate was significantly reduced ($P < 0.01$) by the administration of a tenfold higher concentration of genistein (3.0 ± 0.3 ; $n = 5$) and the higher concentration of tyrphostin A25 (5.8 ± 1.0 at 500 $\mu\text{mol l}^{-1}$; $n = 4$).

Steroid concentrations

The concentrations of progesterone and oestradiol in the perfusion medium, evaluated at five different time points throughout the perfusions are presented (Figs 3 and 4).

Fig. 3. Progesterone concentrations in the medium used to perfuse rat ovaries *in vitro*. Each symbol represents the mean of values ($\pm$ SEM) at each time point during perfusion. Significantly lower concentrations of progesterone were detected in the unstimulated control group compared with those of the other groups at 3 and 8 h (a: significantly lower ($P < 0.01$) than all other groups; b: lower than the 10 μ mol genistein I^{-1} group ($P < 0.05$); c: lower than the 100 μ mol genistein I^{-1} group ($P < 0.01$)). There were no differences among the three treatment groups at any time point. ○: control; ●: LH + 3-isobutyl-1-methylxanthine (IBMX); □: LH + IBMX + 10 μ mol genistein I^{-1} ; ■: LH + IBMX + 100 μ mol genistein I^{-1} .

Unstimulated control ovaries showed lower secretion of progesterone and oestradiol than the LH + IBMX-stimulated groups, with significantly lower concentrations of progesterone at 3 h than in all other groups and, at 8 h, than in the genistein-treated groups (Fig. 3). Oestradiol concentrations in the control group were significantly lower than they were in the LH + IBMX group at 1, 3 and 8 h (Fig. 4). The presence of genistein or tyrphostin A25 (data not shown) did not significantly change the progesterone or oestradiol concentrations compared with those in the LH + IBMX groups at any time point (Figs 3 and 4).

Intraovarian concentrations of PA, PGE₂ and PGF_{2 α}

The concentrations of PA activity in the ovary after 10 h of perfusion with LH + IBMX were 2.43 ± 0.38 iu mg^{-1} wet ovarian mass. Genistein exposure did not significantly ($P > 0.05$) alter the concentrations of PA activity (3.60 ± 0.74). The concentrations of PGE₂ and PGF_{2 α} were 1.13 ± 0.08 and 0.33 ± 0.02 , respectively. These concentrations were not significantly ($P > 0.05$) altered by genistein treatment (1.08 ± 0.32 and 0.25 ± 0.05 ng mg^{-1} wet ovarian mass, respectively).

Discussion

The ovulatory process bears many similarities to an inflammatory reaction (Espey, 1980) and among many

Fig. 4. Oestradiol concentrations in the medium used to perfuse rat ovaries *in vitro*. Each symbol represents the mean values ($\pm$ SEM) at each time point during perfusion. Significantly lower concentrations of oestradiol were detected in the unstimulated group compared with those in the LH + 3-isobutyl-1-methylxanthine (IBMX) group and 10 μ mol genistein I^{-1} group (a: significantly lower than LH + IBMX + 10 μ mol genistein I^{-1} group ($P < 0.01$ – 0.05); b: significantly lower than LH + IBMX group and the 10 μ mol genistein I^{-1} group ($P < 0.05$)). There were no significant differences among the three treatment groups at any time point. ○: control; ●: LH + IBMX; □: LH + IBMX + 10 μ mol genistein I^{-1} ; ■: LH + IBMX + 100 μ mol genistein I^{-1} .

mediators of inflammation, several of the recently discovered cytokines may actively regulate ovulation by inducing intrafollicular production of ovulation-associated mediators, such as progesterone (Brännström *et al.*, 1993a), collagenase (Hurwitz *et al.*, 1993), prostaglandins (Kokia *et al.*, 1992) and nitric oxide (Ben-Shlomo *et al.*, 1994). While it was first recognized that growth factors were likely to be involved mostly in early stages of follicular development, it is now accepted that several members of this family of substances also regulate ovarian and follicular functions at the preovulatory stage (Koos, 1993; Mulherin and Schomberg, 1993). Several of the cytokines and growth factors involved in intraovarian regulation use either receptor or intracellular non-receptor tyrosine kinase pathways for their intracellular signalling.

The present study demonstrates for the first time that the PTK pathway is involved in the intraovarian regulation of mammalian ovulation. The presence in the perfusion medium of either of two structurally different PTK inhibitors at the concentrations of 100 and 500 μ mol I^{-1} , respectively, significantly reduced the LH-induced ovulatory response. This inhibition of ovulation did not seem to affect the PA or prostaglandin systems.

The concentrations at which these PTK inhibitors inhibited ovulation are relatively high. However, other studies also performed *in vitro* on tissue from the female genital tract, revealed inhibitory effects of genistein at similar concentrations. In isolated myometrial tissue from pregnant

rats, genistein decreased the L-type calcium current and the half-maximal inhibition of voltage-dependent concentration was obtained at $50 \mu\text{mol l}^{-1}$ (Kusaka and Sperelakis, 1995). In nonreproductive tissue, such as vascular smooth muscle tissue, a dose-dependent inhibition of contraction was seen, with maximum inhibition obtained at $100 \mu\text{mol l}^{-1}$ genistein (Filipeanu *et al.*, 1995). The effective inhibition of PTK activity at a concentration of about $100 \mu\text{mol l}^{-1}$ was demonstrated by monitoring the phosphorylation of the EGF receptor in a cell line in the presence of various concentrations of genistein (Akiyama and Ogawara, 1991). Tyrphostin A25 inhibits PTK over a similar or slightly higher concentration range to genistein, as has been demonstrated in aortic endothelial cells (Yano *et al.*, 1996).

Although the half-maximal effects of both genistein and tyrphostin A25 seem to be similar, the present study showed that genistein effectively inhibited ovulation at $100 \mu\text{mol l}^{-1}$, whereas tyrphostin A25 was ineffective at this concentration. The relatively high concentrations of both PTK inhibitors added to obtain inhibition of ovulation in this specific perfusion system are in accordance with earlier studies in which, in some cases, drugs needed to be present in the micromolar range (Brännström, 1993b). The reason for this dependence of relatively high concentrations could be adsorption of compounds to the tubing material and the fact that the perfused ovary represents an intact organ, to which the compounds have to be transported by diffusion from the vascular bed to the target cells. A difference in these two factors between the two compounds may explain why fivefold higher concentrations of tyrphostin A25 had to be used. Nevertheless, the fact that PTK inhibitors had no negative effects on steroid release, prostaglandin concentrations and PA activity indicates that their effects on ovulation were not the result of non-specific cytotoxicity.

The presence of genistein or tyrphostin A25 at either concentration examined in the present study did not significantly change the LH + IBMX-stimulated steroid release. Earlier studies in rats indicated that the pre-ovulatory production of oestradiol was of no importance for ovulation (Morioka *et al.*, 1988) but that progesterone was essential (Brännström and Janson, 1988). The fact that steroid output was not negatively affected by genistein or tyrphostin A25 treatment indicates that the PTK pathway is not directly involved in steroid regulation in the rat pre-ovulatory follicle and that the ovulation-inhibiting effect of PTK inhibitor is not related to the inhibition of progesterone synthesis. In previous experiments, another tyrosine kinase inhibitor, tyrphostin AG18, has been shown to inhibit the FSH-induced gene expression of the three key steroidogenic enzymes, cholesterol side-chain cleavage cytochrome 450, 3β -hydroxysteroid dehydrogenase, and aromatase cytochrome 450, in rat granulosa cells in a differentiation stage before the preovulatory stage (Orly *et al.*, 1994). The inhibition of these steroidogenic enzymes by PTK inhibitors was shown to be less pronounced in granulosa cells of the preovulatory stage and in luteinized granulosa cells (Orly *et al.*, 1996). However, the oestradiol and progesterone concentration profiles in this recirculated perfusion media were similar, with an initial sharp increase up to 4 h and then a plateau. These profiles indicate a selective and marked decrease in oestrogen

synthesis after 4 h of the ovulatory process, which is in line with conditions *in vivo* where the blood concentrations of oestradiol decrease 4–6 h after the LH surge (Goff and Henderson 1979). Oestradiol is not adsorbed to the walls of this perfusion system and constant concentrations in the media indicate that ovarian secretion is minimal (Bruning *et al.*, 1981). In contrast, progesterone is rapidly adsorbed, with a $t_{1/2}$ of 2.9 h of exogenously added progesterone (Brännström *et al.*, 1987b). In the present study, progesterone concentrations in the $100 \mu\text{mol l}^{-1}$ genistein group did not decrease at the same rate as in the other LH + IBMX-stimulated groups. However, there was no significant difference in progesterone concentration among these groups at any time point.

In the present study, the intraovarian concentrations of PGE_2 and $\text{PGF}_{2\alpha}$ were measured when maximal. Prostaglandins have been implicated as important mediators in the process of follicular rupture, since inhibitors of prostaglandin synthesis depress the ovulatory rate *in vivo* in several species, including humans (Killich and Elstein, 1987). The intraovarian role of prostaglandins in this process was substantiated by the detection of an LH-induced increase in the intrafollicular prostaglandin concentration (LeMaire *et al.*, 1975) and the finding that prostaglandin synthesis blockers inhibit follicular rupture in the isolated *in vitro* perfused ovary (Hamada *et al.*, 1977; Sogn *et al.*, 1987). Since a PTK inhibitor has been shown to block both LH- and GnRH-mediated induction of cyclooxygenase 2 in cultured granulosa cells (Morris and Richards, 1993), it was assumed that at least part of the inhibitory action on ovulation of the PTK inhibitors in the present study was due to decreased prostaglandin production. However, the unchanged prostaglandin concentrations within the ovary observed in the present study indicate another mechanism for the PTK inhibition of ovulation.

Plasminogen activators, especially tissue type PA (tPA) appear to play an important role in the ovulatory process (Beers *et al.*, 1975). Plasminogen activator is secreted from the granulosa cells and the PA gene activity is enhanced around the time of the LH surge (Shimamoto *et al.*, 1993), and growth factors, such as EGF, have been implicated as regulators of PA expression (Galway *et al.*, 1989). In the present study, the 10 h perfusion showed no significant difference in intraovarian PA concentration when the gonadotrophin-stimulated ovaries and those also treated with genistein were compared. These findings indicate that the PTK pathways in the ovulatory process do not involve the PA system.

Exogenously added EGF decreases ovulatory efficiency in the *in vitro* perfused rabbit ovary (Endo *et al.*, 1992). This finding is somewhat in contrast to those of the present study, in which a presumed inhibition of EGF-mediated effects by PTK inhibition also resulted in a decreased ovulatory response. This discrepancy may be explained by reported species differences between the rabbit and rat in the regulation of ovulation (Brännström and Janson, 1988) or by an EGF-mediated negative regulation of the ovulatory process in rats that was not detected in the present study, in which it is likely that several important PTK mediated pathways were inhibited.

In conclusion, this study demonstrates that PTK is

involved critically in the intraovarian events of follicular rupture but does not influence the luteinization process in terms of progesterone secretion. The action of PTK does not involve the prostaglandin or PA systems.

The authors would like to thank the National Hormone and Pituitary Program of the NIDDK for the generous gift of LH. The study was supported by grants from the Swedish Medical Research Council (11607 to MB), Medical Faculty of Göteborg University, Göteborg Medical Society and Hjalmar Svensson Research Foundation.

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