The role of tyrosine kinase in gonadotropin-induced ovulation in the rat ovary

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Abstract

Objectives: Our purpose was to elucidate the involvement of the tyrosine kinase pathway in gonadotropin-induced ovulation in the rat ovary.

Study design: We investigated the effect of a tyrosine kinase inhibitor, tyrphostin, on the rat ovulatory process *in vivo* and *in vitro*.

Methods: In cultured rat granulosa cells, the effect of tyrphostin on LH-, dibutyl cyclic AMP ((Bu)₂cAMP)- or forskolin-stimulated tissue type plasminogen activator (tPA) activities was examined by using a fibrin autography technique. In an *in vivo* system, tyrphostin was injected into the bursal cavity of the ovary in pregnant mare serum gonadotropin-treated rats, just before human chorionic gonadotropin administration. After 24 h, the number of oocytes in the oviduct was counted and the tyrphostin-treated ovaries were examined histologically.

Results: Tyrphostin inhibited LH-stimulated tPA activity but did not affect (Bu)₂cAMP- or forskolin-stimulated ones. In an *in vivo* study, tyrphostin suppressed oocyte release dose-dependently. Histological observations revealed that tyrphostin-treated ovaries contained many large unruptured follicles and a few corpora lutea.

Conclusion: This study suggests that the suppressive effect of tyrphostin on ovulation may be partly due to tPA activity inhibition in the granulosa cells via the suppression of tyrosine kinase activity. Additionally, tyrosine kinase phosphorylation may be involved in gonadotropin-activated signaling systems in the rat ovulatory process.

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Introduction

Luteinizing hormone (LH)/chorionic gonadotropin (CG) regulates ovarian functions, including functional differentiation of granulosa cells, ovulation and corpus luteum formation (1). It is well established that cAMP-dependent protein kinase acts as an intracellular signaling transduction system of LH/human CG (hCG) actions (2). LH also increases phosphoinositide turnover and activates protein kinase C that may modulate gonadal functions (2–4). In recombinant LH receptor expressing L-cells, higher concentrations of LH could lead to stimulation of phospholipase C and result in the formation of inositol phosphate and the elevation in Ca²⁺ (5). We previously reported that a protein kinase C inhibitor, H-7, could suppress gonadotropin-induced ovulation (6). Recently, it was reported that a tyrosine kinase inhibitor suppressed follicle-stimulating hormone (FSH)-dependent differentiation in cultured granulosa cells (7, 8). Costrici *et al.* (9) have reported that FSH effects on aromatase activity involve the activation of cytosolic soluble protein tyrosine kinases

in human granulosa cells. According to Morris & Richards (10), the luteinization of granulosa cells that is stimulated by the ovulatory surge of LH is dependent on both the increased intracellular cAMP and the activation of tyrosine kinases. Taking these findings together, it is possible that LH stimulates multiple intracellular signaling systems.

Tissue type plasminogen activator (tPA), which has been shown to be secreted by granulosa cells, may play an important role in the ovulatory process (6, 11–13). In the ovary, tPA activity has been modulated by gonadotropins (14), cAMP derivatives (15), gonadotropin-releasing hormone (GnRH) (16), phorbol ester (17), and epidermal growth factor (EGF) (18). These factors presumably act through different intracellular signaling systems. Since it is well known that EGF stimulates tyrosine kinases, it is suggested that the activation of tyrosine kinases may be involved in the induction of tPA activity in granulosa cells.

In the present study, to elucidate the involvement of the tyrosine kinase pathway in gonadotropin-induced ovulation, we have studied the effect of a tyrosine kinase

inhibitor, tyrphostin, on LH-stimulated tPA activity in cultured rat granulosa cells. Furthermore, we have studied the *in vivo* effect of tyrphostin on oocyte release in pregnant mare serum gonadotropin (PMSG)-treated rats by using the ovarian intrabursal injection technique.

Materials and methods

Hormones and reagents

McCoy's 5a medium, penicillin–streptomycin solution, and trypan blue stain were obtained from Gibco (Santa Clara, CA, USA). Ovine LH (oLH) (NIH-oLH-26; 2.3 U/mg; FSH contamination <0.5% by weight) was obtained from the National Hormone and Pituitary Distribution Program (National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA). Halothane was purchased from Hoechst Japan Co. (Tokyo, Japan). Tyrphostin, dibutyl cAMP ((Bu)₂cAMP), forskolin (FSK), GnRH, mouse EGF, thrombin, androstenedione, PMSG, and hCG were purchased from Sigma Chemical Co. (St Louis, MO, USA). Fibrinogen was purchased from Calbiochem (La Jolla, CA, USA). Ammonium persulfate, *N,N*-methylene bis-acrylamide, sodium dodecyl sulfate (SDS), and Coomassie Brilliant Blue were purchased from Bio-Rad (Richmond, CA, USA). Seaplaque agarose was purchased from FMC Co. (Rockland, ME, USA). Glu-plasminogen was obtained from Bio-Pool Co. (Umeå, Sweden).

Animals

Intact immature female Sprague–Dawley rats (21 days old) were purchased from Kiwa Laboratory Animals Co. Ltd (Wakayama, Japan). Animals were housed three to five per cage with free access to food and water and with lights on from 0600–2000 h.

Granulosa cell culture

The animals (23 days old) were injected with 20 IU PMSG to initiate follicular development. After 48 h, the rats were killed by cervical dislocation. The ovaries were removed and were punctured with a 26 gauge needle to obtain granulosa cells. After washing, the cells were stained with 0.25% trypan blue, and viable cells were counted with a hemocytometer. The average viability of the cells was approximately 90%. The cells (5×10^5 viable cells per well) were cultured in Falcon 24-multiwell culture plates (Falcon Plastics, Los Angeles, CA, USA) containing 0.5 ml McCoy's 5a medium (modified, without serum) supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin sulfate, and 10^{-7} mol/l androstenedione. The cells were cultured in a humidified 95% air–5% CO₂ incubator at 37 °C. The cells were cultured for 48 h

in the presence or absence of tyrphostin at concentrations of 0.3–100 µmol/l with oLH (30 ng/ml), mouse EGF (30 ng/ml), GnRH (30 ng/ml), (Bu)₂cAMP (5 mmol/l), or FSK (10^{-6} mol/l). Fresh tyrphostin was added at 24 h intervals. Tyrphostin was replenished after 24 h because the cells degraded the reagent. At the end of culture, conditioned media were removed and stored at –40 °C in the presence of 0.01% Tween-80 until assayed for tPA activity. Assessment of cell viability by trypan blue exclusion after the culture with tyrphostin (0.3–100 µmol/l) demonstrated no overt toxicity (data not shown).

SDS-PAGE and fibrin autography technique

Samples (100 µl) of conditioned medium were adjusted to 2.5% SDS and 0.0125% Coomassie Brilliant Blue and fractionated by SDS-PAGE. SDS-polyacrylamide slab gels were prepared according to the method of Laemmli (19). Electrophoresis was at 50 V for approximately 16 h. tPA activity was assayed by the fibrin autography method (13). After electrophoresis, gels were washed twice in 2.5% (v/v) Triton X-100 in distilled water for 45 min to remove SDS. The fibrin-agarose indicator gel was prepared by combining plasminogen (8 µg/ml) as the zymogen, as well as fibrinogen (2.4 mg/ml) and thrombin (0.5 U/ml) to allow the formation of fibrin as the substrate for plasmin. The electrophoresed SDS slab gel was placed onto the fibrin-agarose indicator gel and incubated at 37 °C in a humidified chamber until clear lytic zones were formed, indicating the presence of tPA activity. All experiments using the fibrin autography technique were repeated four times, and a photographic record of representative experiments using darkfield illumination is presented. tPA activity was estimated semiquantitatively by measuring the areas of the lytic zones (height × width), and these results were expressed as the relative ratios between different groups by setting the enzyme level in the control group at 1.0.

Intrabursal injection technique and oocyte retrieval

At 27 days after birth, the female rats were injected intraperitoneally with 20 IU PMSG at 0900 h to stimulate follicle development. After 48 h (0900 h on day 29), the rats were anesthetized with halothane. Fur was shaved from the right dorsolateral flank, and the shaved area was rinsed with ethanol. An approximately 1 cm incision was made through the skin and muscle layers, followed by a 5 mm cut into the peritoneum. The fat pad containing the right ovary and oviduct was gently pulled from the abdominal cavity. Forty microliters of tyrphostin dissolved in PBS or vehicle were injected into the bursal cavity of the ovary with a 30 gauge needle attached to a Hamilton syringe (Hamilton Co., Reno, NV, USA). The ovary was gently replaced in the

peritoneal cavity, and the abdominal wall was closed (20). The left ovary received no treatment and served as a control. After the completion of these procedures, the animals were immediately injected intraperitoneally with 10 IU hCG to induce the ovulation. After 24 h, the animals were killed by cervical dislocation. The ovaries and oviducts were removed from each animal and examined with a dissecting microscope. The oocyte-cumulus mass was removed by making a small incision into the tubal wall through which the mass was extruded. Further flushing of the oviduct and the uterus did not reveal the presence of any additional oocytes. The number of oocytes removed from each side was recorded. Statistical analysis was performed by Student's *t*-test.

Histological examinations

Treated and untreated ovaries were fixed in Bouin's solution. The tissue was then dehydrated, embedded in paraffin, and serially sectioned at 4 μ m. The sections were mounted on glass slides and stained with hematoxylin and eosin and photographed under a microscope.

Results

Specificity of effect of tyrphostin on tPA activity in rat granulosa cells

Granulosa cells were obtained from PMSG-treated rats and cultured for 48 h in medium alone (control) or in media with mouse EGF (30 ng/ml), or GnRH (30 ng/ml) without or with tyrphostin (30 μ mol/l). EGF, which

Figure 1 Specific effect of tyrphostin on tPA activity in cultured rat granulosa cells. Granulosa cells were cultured for 48 h in medium alone (C) or in media with EGF (30 ng/ml) or GnRH (30 ng/ml) with or without tyrphostin (30 μ mol/l). Conditioned media (100 μ l) were analyzed for tPA activity by SDS-PAGE and the fibrin autography method. The photographic record of one representative experiment.

Figure 2 Effects of tyrphostin on LH-, FSK- and cAMP-stimulated tPA activity in cultured rat granulosa cells. Granulosa cells were cultured for 48 h in medium alone (C) or in media with LH (30 ng/ml), FSK (10^{-6} mol/l), or (Bu) $_2$ cAMP (5 mmol/l) with or without tyrphostin (30 μ mol/l). Conditioned media were analyzed by SDS-PAGE and the fibrin autography method. The photographic record of one representative experiment.

activates tyrosine kinases as an intracellular signaling system, enlarged the lytic area compared with control and tyrphostin reduced the EGF-stimulated lytic area. GnRH, whose intracellular signaling system is the protein kinase C pathway, also enlarged the lytic area, whereas tyrphostin could not affect the GnRH-enlarged lytic area (Fig. 1).

We also studied the effect of tyrphostin on (Bu) $_2$ cAMP- or FSK-stimulated tPA activity in cultured rat granulosa cells in order to examine whether tyrphostin affected the protein kinase A pathway. Although (Bu) $_2$ cAMP (5 mmol/l) or FSK (10^{-6} mol/l) increased tPA activity, 30 μ mol/l tyrphostin did not inhibit the (Bu) $_2$ cAMP- or FSK-stimulated tPA activity (Fig. 2). These data indicated that tyrphostin specifically inhibited the tyrosine kinase pathway.

Effect of tyrphostin on LH-induced tPA activity

We examined the effect of tyrphostin on oLH-induced tPA activity. Figure 3A shows one representative photographic record of the four separate examinations. oLH markedly enlarged the lytic area compared with control, and tyrphostin (0.3–100 μ mol/l) dose-dependently reduced oLH-stimulated tPA activity. Urokinase type plasminogen activator activity was not affected in any treatment groups (data not shown). Figure 3B shows the results of semiquantitative analysis of the four different examinations on tPA activity. Setting the tPA activity in the control group at 1.0, oLH stimulated tPA activity approximately 2.5-fold as much as control, whereas tyrphostin inhibited the LH-stimulated tPA activity in a dose-dependent manner. Tyrphostin alone did not affect the tPA activity (data not shown).

Figure 3 Dose-dependent inhibition of LH-stimulated tPA activity by tyrphostin in cultured rat granulosa cells. Granulosa cells were cultured for 48 h in medium alone (c) or in media containing LH (30 ng/ml) or increasing doses of tyrphostin (0–100 μmol/l). Conditioned media were analyzed for tPA activity by the fibrin autography method after SDS-PAGE. (A) The photographic record of one representative experiment. (B) Results shown represent the mean $\pm$ S.E.M. of the four separate experiments, setting the tPA activity level in the control group at 1.0.

We examined the time course of the effect of tyrphostin on oLH-stimulated tPA activity. oLH stimulated tPA activity by 6 h of culture. The inhibitory effect of tyrphostin on the LH-stimulated tPA activity was found by 6 h of culture and the effect was maintained at 12 h of culture (Fig. 4).

Figure 4 Time course of the effect of tyrphostin on LH-stimulated tPA activity. Rat granulosa cells were cultured in medium alone (C) or in media containing LH (30 ng/ml) with or without tyrphostin (30 μmol/l). Cultures were terminated at 3, 6, or 12 h. Conditioned media were analyzed for tPA activity by the fibrin autography method after SDS-PAGE. The photographic record of one representative experiment.

Effect of tyrphostin on ovulation

PMSG-treated immature rats were injected with tyrphostin into the ovarian bursal space. Table 1 shows the number of released oocytes from the treated and untreated ovaries in the different dose groups. Injection of 300 or 3000 μmol/l tyrphostin significantly decreased the number of released oocytes from the treated ovaries compared with that from the contralateral untreated ovaries. PBS alone did not affect the number of released oocytes. The percent inhibition was expressed as the difference in the number of released oocytes between the treated and untreated ovaries divided by the total number of released oocytes from the untreated ovary.

Table 1 Effects of tyrphostin on PMSG–hCG induced ovulation. Results are means $\pm$ S.E.M.

Treatment	No. of oocytes/ovary ($\pm$ S.E.M.)		Inhibition (% $\pm$ S.E.M.)
	Untreated	Treated	
PBS	25.3 $\pm$ 3.7	23.8 $\pm$ 3.6	7.9 $\pm$ 7.1
Tyrphostin (μmol/l)			
30	24.2 $\pm$ 3.2	22.3 $\pm$ 3.7	10.7 $\pm$ 6.6
300	22.7 $\pm$ 3.5	15.5 $\pm$ 2.6*	31.6 $\pm$ 3.6**
3000	28.5 $\pm$ 3.1	15.5 $\pm$ 1.6*	44.8 $\pm$ 3.9**

Forty microliters of PBS or tyrphostin (30–3000 μmol/l) were injected unilaterally into the ovarian bursa ($n = 6$ rats) at the time of hCG administration. The number of released oocytes from treated and untreated control ovaries are shown separately. Percent inhibitions are also shown. Percent inhibition is expressed as the difference in the number of oocytes released between the treated and untreated ovaries divided by the total number of oocytes on the untreated side. * $P < 0.01$, when compared with untreated ovary. ** $P < 0.01$, when compared with PBS-injected rats.

Administration of 300 or 3000 $\mu\text{mol/l}$ tyrphostin significantly inhibited ovulation compared with the PBS-treated group.

Histological observations

Intrabursal injections did not cause any obvious pathological changes in the ovaries, such as vascular thrombosis, infarcts, or hemorrhage. The ovaries treated with PBS contained many corpora lutea and a few large preovulatory follicles (Fig. 5A). In contrast, the ovaries treated with 3000 $\mu\text{mol/l}$ tyrphostin contained a few corpora lutea and many large unruptured follicles (Fig. 5B). In these large unruptured follicles, the oocytes with normal meiotic maturation were entrapped within the follicular antrum. Figure 5C shows one representative luteinized unruptured follicle in a tyrphostin (3000 $\mu\text{mol/l}$)-treated ovary. The oocytes were entrapped in the antrum, the basal lamina separating the granulosa cells and the theca interna was disintegrated and there were no signs of disintegration of the tunica albuginea.

Discussion

In the present study, we investigated a possible involvement of tyrosine kinase phosphorylation in the ovulatory process *in vitro* and *in vivo*. Although quercetin and genistein block not only a variety of tyrosine kinases but also other protein kinases, including cAMP-dependent protein kinases and protein kinase C, tyrphostin seems to be a selective and nontoxic protein tyrosine kinase inhibitor which competes for the substrate site of protein tyrosine kinases and not with ATP (21, 22). Therefore, we used tyrphostin as a tyrosine kinase inhibitor. In cultured rat granulosa cells, tyrphostin could inhibit EGF-stimulated tPA activity, whereas tyrphostin did not have any effects on cAMP-, FSK-, or GnRH-stimulated tPA activity. Taking these findings together, tyrphostin can selectively inhibit tyrosine kinases in rat granulosa cells.

In this study, tyrphostin inhibited LH-stimulated tPA activity in cultured rat granulosa cells in a dose-dependent manner. It is indicated that tyrosine kinase phosphorylation might be involved in LH action

Figure 5 Representative photomicrographs of rat ovaries after intrabursal injections. (A) Low-power photomicrograph after injection of PBS. The corpora lutea are observed (magnification $\times 40$). (B) Low-power photomicrograph after injection of tyrphostin (3000 $\mu\text{mol/l}$). Some large unruptured follicles and no corpus luteum are observed (magnification $\times 40$). (C) High-power photomicrograph after injection of tyrphostin (3000 $\mu\text{mol/l}$). A luteinized unruptured follicle is present (magnification $\times 100$).

for tPA activity induction in the granulosa cells. On the other hand, it is suggested that in the granulosa cells, the tyrosine kinase activation by LH may be an indirect effect via some factors such as EGF and insulin-like growth factor-I (IGF-I) whose intracellular signaling system is tyrosine kinase phosphorylation. According to LaPolt *et al.* (23), EGF stimulates tPA activity in rat granulosa cells but IGF-I has no effect on tPA activity. Feng *et al.* (24) reported that LH/hCG reduced EGF-binding sites in rat granulosa cells. In the present time-course study, both the stimulatory effect of LH on tPA activity and the inhibitory effect of tyrphostin on LH-stimulated tPA activity occurred at the same time and there was no time lag with these effects. Therefore, the stimulation of tPA activity by LH does not seem to be an indirect effect mediated via EGF and IGF-I. Davis (25) has demonstrated that the intracellular C-terminal domain and the three putative intracellular loops of the transmembrane domain of the LH/CG receptor contain numerous motifs possessing serine, threonine, and tyrosine residues, suggesting the potential role for modulation of receptor function by phosphorylation, serine–threonine protein kinases, and tyrosine kinases. Therefore, it is suggested that the effect of tyrphostin on LH-stimulated tPA activity might be partly due to the inhibition of LH-involved tyrosine kinase phosphorylation.

According to the previous studies on ovulation, tumor-promoting phorbol ester (26) and GnRH (27) can induce ovulation without gonadotropins. Some authors have reported that oocyte maturation and gonadotropin-induced follicular rupture may be mediated via a cAMP-dependent pathway (28, 29). On the other hand, Hosoi *et al.* (30) have reported that cAMP fails to induce ovulation in the absence of gonadotropins. Moreover, a tyrosine kinase inhibitor blocks LH induction of luteinization and of prostaglandin endoperoxide synthase-2 expression in granulosa cells, which is one of the markers of ovulation (10, 31). These data suggest a primary role for cAMP, a supportive but essential role for protein kinase C, and an obligatory role for tyrosine kinases acting in the cascade of luteinization and ovulation. Together with these findings, multiple signal transduction systems seem to be involved in the ovulatory process. To examine the involvement of a tyrosine kinase activation in hCG-induced ovulation *in vivo*, tyrphostin was intrabursally injected into PMSG-treated rats. Tyrphostin suppressed hCG-induced ovulation in a dose-related manner, suggesting that tyrosine kinase phosphorylation may be involved in the ovulatory process.

Since the actual ovarian concentration of tyrphostin is unknown, it is difficult to relate concentration of tyrphostin in the *in vivo* studies to those *in vitro*. In the histological examination, ovaries treated with tyrphostin (3000 $\mu\text{mol/l}$) did not have any pathological changes such as vascular thrombosis, infarcts, or hemorrhage. Therefore, the effect of tyrphostin does

not seem to be pharmacological within the doses used in this study. Although tyrphostin-treated ovaries had healthy follicles and a few normally ruptured follicles and corpora lutea, they also had many large unruptured follicles with normally maturing oocytes histologically. In some of these unruptured follicles, the basal lamina separating the granulosa cells and the theca interna were disintegrated, while the tunica albuginea was intact. These findings are consistent with the reports of Tsafirri *et al.* (32) that disintegration of the apical theca externa and tunica albuginea was dependent on tPA. It is suggested that tyrphostin may not affect folliculogenesis and oocyte maturation and may inhibit the dissolution of theca externa and tunica albuginea. On the other hand, Morris & Richards (10) have shown that in the presence of an ovulatory dose of LH, the luteinization of the granulosa cells was blocked by a tyrosine kinase inhibitor, AG18. In our *in vivo* study, even the ovaries treated with high dose of tyrphostin had luteinized granulosa cells morphologically. Although the reason for this discrepancy is unknown, some factors other than tyrosine kinases may also be involved in luteinization mechanism.

In summary, the results of our *in vitro* and *in vivo* studies suggest that the suppressive effect of tyrphostin on hCG-induced ovulation might be partly due to the inhibition of tPA activity in the granulosa cells via the block of tyrosine kinase phosphorylations. In the ovulatory process, gonadotropin action might be mediated not only by a cAMP-dependent pathway and protein kinase C activation but also by tyrosine kinase activation. The mechanism of gonadotropin-related tyrosine kinase activation in the ovulatory process is not yet clear and further studies will be required.

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References

- 1 Brännström M & Janson PO. The biochemistry of ovulation. In *Ovarian Endocrinology*, pp 132–166. Ed. SG Hillier. Oxford: Blackwell Scientific Publications, 1991.
- 2 Leung PCK & Steele GL. Intracellular signaling in the gonads. *Endocrine Reviews* 1992 13 476–498.
- 3 Davis JS, West LA & Farese RV. Effects of luteinizing hormone on phosphoinositide metabolism in rat granulosa cells. *Journal of Biological Chemistry* 1984 259 15028–15034.
- 4 Davis JS, Weakland LL, West LA & Farese RV. Luteinizing hormone stimulates the formation of inositol trisphosphate and cyclic AMP in rat granulosa cells: evidence for phospholipase C generated second messengers in the action of luteinizing hormone. *Biochemical Journal* 1986 238 597–604.
- 5 Gudermann T, Birnbaumer M & Birnbaumer L. Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and Ca^{2+} mobilization: studies with the cloned murine luteinizing hormone receptor expressed in L cells. *Journal of Biological Chemistry* 1992 267 4479–4488.

- 6 Shimamoto T, Yamoto M & Nakano R. Possible involvement of protein kinase C in gonadotropin-induced ovulation in the rat ovary. *Endocrinology* 1993 **133** 2127–2132.
- 7 Gomberg-Malool S, Ziv R, Re'em Y, Posner I, Levitzki A & Orly J. Tyrphostins inhibit follicle-stimulating hormone-mediated functions in cultured rat ovarian granulosa cells. *Endocrinology* 1993 **132** 362–370.
- 8 Orly J, Rei Z, Greenberg NM & Richards JS. Tyrosine kinase inhibitor AG18 arrests follicle-stimulating hormone-induced granulosa cell differentiation: Use of reverse transcriptase-polymerase chain reaction assay for multiple messenger ribonucleic acids. *Endocrinology* 1994 **134** 2336–2346.
- 9 Costrici N, Elberg G, Lunenfeld B, Pariente C, Dor J, Kanety H & Karasik A. A cytosolic protein tyrosine kinase activity is induced by follicle stimulating hormone and insulin like growth factor-I in human granulosa cells. *Endocrinology* 1995 **136** 4705–4708.
- 10 Morris JK & Richards JS. Hormone induction of luteinization and prostaglandin endoperoxide synthase-2 involves multiple cellular signaling pathways. *Endocrinology* 1993 **133** 770–779.
- 11 Beers WH. Follicular plasminogen and plasminogen activator and the effect of plasmin on ovarian follicle wall. *Cell* 1975 **6** 379–386.
- 12 Beers WH, Strickland S & Reich E. Ovarian plasminogen activator: relationship to ovulation and hormonal regulation. *Cell* 1975 **6** 387–394.
- 13 Ny T, Bjersing L, Hsueh AJW & Loskutoff DJ. Cultured granulosa cells produce two plasminogen activators and an antiactivator, each regulated differently by gonadotropins. *Endocrinology* 1985 **116** 1666–1668.
- 14 Wang C & Leung A. Gonadotropins regulate plasminogen activator production by rat granulosa cells. *Endocrinology* 1983 **112** 1201–1207.
- 15 Strickland S & Beers WH. Studies on the role of plasminogen activator in ovulation: *in vitro* response of granulosa cells to gonadotropins, cyclic nucleotides, and prostaglandins. *Journal of Biological Chemistry* 1976 **251** 5694–5702.
- 16 Ny T, Liu Y-X, Ohlsson M, Jones PBC & Hsueh AJW. Regulation of tissue-type plasminogen activator activity and messenger RNA levels by gonadotropin-releasing hormone in cultured rat granulosa cells and cumulus-oocyte complexes. *Journal of Biological Chemistry* 1987 **262** 11790–11793.
- 17 Ohlsson M, Hsueh AJW & Ny T. Hormonal regulation of tissue-type plasminogen activator messenger ribonucleic acid levels in rat granulosa cells: mechanisms of induction by follicle-stimulating hormone and gonadotropin releasing hormone. *Molecular Endocrinology* 1988 **2** 854–861.
- 18 Galway AB, Oikawa M, Ny T & Hsueh AJW. Epidermal growth factor stimulates tissue plasminogen activator activity and messenger ribonucleic acid levels in cultured rat granulosa cells: mediation by pathways independent of protein kinases-A and -C. *Endocrinology* 1989 **125** 126–135.
- 19 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970 **227** 680–685.
- 20 Martin GG, Talbot P & Pendergrass P. An intrabursal injection procedure for the *in vivo* study of ovulation in hamsters. *Journal of Experimental Zoology* 1981 **216** 461–468.
- 21 Gazit A, Yaish P, Gilon C & Levitzki A. Tyrphostins I: synthesis and biological activity of protein tyrosine kinase inhibitors. *Journal of Medicinal Chemistry* 1989 **32** 2344–2352.
- 22 Levitzki A. Tyrphostins – potential antiproliferative agents and novel molecular tools. *Biochemical Pharmacology* 1990 **40** 913–918.
- 23 LaPolt PS, Yamoto M, Veljkovic M, Sincich C, Ny T, Tsafiriri A & Hsueh AJW. Basic fibroblast growth factor induction of granulosa cell tissue-type plasminogen activator expression and oocyte maturation: potential role as a paracrine ovarian hormone. *Endocrinology* 1990 **127** 2357–2363.
- 24 Feng P, Knecht M & Catt K. Hormonal control of epidermal growth factor receptors by gonadotropins during granulosa cell differentiation. *Endocrinology* 1987 **120** 1121–1126.
- 25 Davis JS. Mechanisms of hormone action: luteinizing hormone receptors and second-messenger pathways. *Current Opinion in Obstetrics and Gynecology* 1994 **6** 254–261.
- 26 Kaufman G, Dharmarajan AM, Takehara Y, Cropp CS & Wallach EE. The role of protein kinase-C in gonadotropin-induced ovulation in the *in vitro* perfused rabbit ovary. *Endocrinology* 1992 **131** 1804–1809.
- 27 Hsueh AJW, Liu Y-X, Cajander S, Peng X-R, Dahl K, Kristensen P & Ny T. Gonadotropin-releasing hormone induces ovulation in hypophysectomized rats: studies on ovarian tissue-type plasminogen activator activity, messenger ribonucleic acid content, and cellular localization. *Endocrinology* 1988 **122** 1486–1495.
- 28 Hillensjö T, Ekholm C & Åhrén K. Role of cyclic AMP in oocyte maturation and glycolysis in the pre-ovulatory rat follicle. *Acta Endocrinologica* 1978 **87** 377–388.
- 29 Brännström M, Koos RD, LeMaire WJ & Janson PO. Cyclic adenosine 3',5'-monophosphate-induced ovulation in the perfused rat ovary and its mediation by prostaglandins. *Biology of Reproduction* 1987 **37** 1047–1053.
- 30 Hosoi Y, Yoshimura Y, Atlas SJ, Adachi T & Wallach EE. Effects of dibutyryl cyclic AMP on oocyte maturation and ovulation in the perfused rabbit ovary. *Journal of Reproduction and Fertility* 1989 **85** 405–411.
- 31 Wong WYL & Richards JS. Induction of prostaglandin H synthase in rat preovulatory follicles by gonadotropin-releasing hormone. *Endocrinology* 1992 **130** 3512–3521.
- 32 Tsafiriri A, Bicsak TA, Cajander SB, Ny T & Hsueh AJW. Suppression of ovulation rate by antibodies to tissue-type plasminogen activator and α_2 -antiplasmin. *Endocrinology* 1989 **124** 415–421.

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