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**Mechanisms Involved in
Follicular Rupture in Mammals**

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ABSTRACT

The main objectives of the proposed research were to reveal some of the basic physiological and biochemical mechanisms underlying follicular rupture. The involvement of the following processes and factors was examined: collagenolysis, plasminogen activator (PA), lipoxxygenase products and changes in follicular hemodynamics. The essential role of the plasmin activating system and of collagenase in follicular rupture at ovulation has been demonstrated by temporal increase in these activities following the ovulatory stimulus and pharmacological blockage of follicular rupture by their inhibitors. Furthermore, the specific stimulation of only the tissue type plasminogen activator (t-PA) and its production primarily in the granulosa cell compartment of the follicle was shown. Evidence for participation of the PA in activation of ovarian collagenolysis was presented.

The involvement of the lipoxxygenase pathway products of arachidonic acid, in addition to the previously known participation of cyclooxygenase metabolites, in follicular rupture was demonstrated. They seem to be involved in (i) the mediation of LH/hCG activation of ovarian collagenolysis at a site posterior to the plasminogen activator; (ii) increase in vascular permeability.

This study performed using rat preovulatory follicles laid the foundations for examining proteolytic changes in the follicular wall of farm animals. Unravelling some of the basis mechanisms involved in follicular rupture may disclose additional site(s) susceptible to experimental monitoring with increase in animal fecundity.

OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

The main objective of this project was to reveal basic physiological and biochemical mechanisms underlying the process of follicular rupture - which is an essential component of ovulation.

Specifically, the following issues were examined and their involvement in follicular rupture assessed:

- a. Collagen breakdown and collagenase activity in periovulatory follicles.
- b. The activity of proteolytic enzymes and especially of plasminogen activator (PA) and their hormonal control.
- c. Correlations of changes in steroidogenesis, collagenolysis and PA activity.
- d. Role of metabolites of arachidonic acid and especially of the lipoxxygenase pathway.
- e. Changes in follicular microcirculation during the periovulatory period.

DESCRIPTION OF THE RESEARCH RESULTS

(1) Collagenolysis and Ovulation

By administration of L-(5-³H) proline, we have labeled ovarian and follicular collagen and followed collagenolytic activity by separation of ³H-hydroxyproline (³H-Hyp) from acid hydrolyzates of ovarian tissue by HPLC.

The level of ovarian and follicular ^3H -Hyp decreased by about 40% on the afternoon of proestrus or after exogenous stimulation of ovulation by hCG, and this decrease was abolished by blocking the surge of gonadotropins with Nembutal. To verify that the observed reduction in ^3H -Hyp was due to the action of a typical collagenase, the collagenous fraction was prepared from ovarian tissue and from preovulatory follicles prior to and after the ovulatory stimulus. The extracts were treated with trypsin (25 min, 25°C, 0.01 mg/ml) plasmin and p-amino-phenyl-mercuric acetate (APMA) to fully activate the collagenase extracted along with collagen. Both, enzymatic and chemical activation of collagenase in vitro resulted in degradation of collagen. This degradation could be inhibited by cysteine and EDTA, both are classic inhibitors of mammalian collagenases. The activity of ovarian collagenase increased within 3 h after hCG-stimulation, peaked at 5-fold 6 h after hCG and declined afterwards. Administration of cysteine (0.001 - 0.01 mmole) into the bursal cavity of proestrous rats blocked ovulation and breakdown of ovarian collagen in a dose-dependent manner. Cysteine effectively inhibited ovulation even when injected 7 h after the hCG stimulus. These results were summarized in our publication in Endocrinology (Reich et al., 1985a).

(2) Establishing in vitro Assays for Collagenase.

Since in farm animals it is impossible to utilize the approach described in the previous section, in vitro assays for endogenous collagenase activity have been attempted. The work on this issue has been examined vigorously by the collaborating group of Prof. LeMaire in Miami and published by them (Curry et al., 1985, 1986). We have tested their extraction procedure, as well as

that of Wolf and Wirl (Eur. J. Biochem. 121, 623, 1982), in combination with in vitro assay for collagenase developed in the Department of Connective Tissue Research in the Max-Planck Institute, Martinsried and both extraction procedures proved equally effective and demonstrated an increase in collagenase activity by hCG, as reported by the Miami group (Reich et al., 1987).

(3) Plasminogen Activator (PA) and Ovulation.

We have examined activity of PA in intact rat follicles. LH (NIH-LH-S23) was 5-fold more potent than FSH (NIH-FSH-S14), and purified ovine LH and FSH were equally potent in enhancing follicular PA activity. Furthermore, injection into the ovarian bursa of proestrous rats of ϵ -amino-caproic acid (CA) and benzamidine (0.05-0.25 mmole), inhibitors of serine proteases, including PA and plasmin, resulted in a dose-dependent inhibition of ovulation without causing changes discernible by histological examination of the ovaries. Whereas steroids did not change basal follicular PA production in culture, addition of estradiol-17 β (E₂; 1 μ g/ml) but not progesterone (P) or testosterone (T), further enhanced LH-stimulated PA. Aminoglutethimide phosphate (10⁻³M) and 17 β -formamidoandrost-4-en-3-one inhibited LH-induced increase in follicular PA and this inhibition was reversed by addition of E₂ (Reich et al., 1985b).

(4) Intrafollicular Distribution of Plasminogen Activators and their Hormonal Regulation in vitro.

Two different well characterized molecular types of PA have been identified in mammals, the urokinase type (u-PA) and the tissue type (t-PA). The two types are products of different genes and known to have different affinities to substrates. In our previous work (Reich et al., 1985b) we have demonstrated that LH stimulates PA production in vitro in the Graafian follicle and this LH-stimulated PA production is enhanced by estradiol 17 β (E₂). In the present work we examined the relative contribution of Graafian follicle components, i.e. granulosa and theca cells, to the production of PA types and their regulation by gonadotropins.

Explanted preovulatory follicles produced in vitro both, urokinase type (u-PA) and tissue type (t-PA), activators. Upon gonadotropin stimulation a highly significant increase in t-PA, but not in u-PA was observed. Separation of the follicle into granulosa cells and residual tissue, mainly theca, revealed that both compartments produce both types of PA. The granulosa compartment was found to produce 80-90% of the total follicular PA activity. Gonadotropins stimulated predominantly t-PA. Most of the gonadotropin-enhanced PA activity produced by granulosa cells was secreted into the culture medium whereas that from thecal origin remained in the tissue. Likewise, in whole follicles only about 10% of PA was secreted into the medium (Reich et al., 1985b).

Gonadotropin-induced PA in vitro, was reduced by inhibitors of steroidogenesis. This inhibition was overcome by the addition of estradiol-17 β . The

inhibition of steroidogenesis affected predominantly the t-PA type of PA. In conclusion, the granulosa cells contribute most of the follicular PA activity, and t-PA is predominantly enhanced by gonadotropin and estrogen. It seems, therefore, that t-PA is the activator involved in the processes leading to follicular rupture (Reich et al., 1986).

(5) Ovarian Metabolism of Arachidonic Acid

Our previous studies (Reich et al., 1983) have suggested the involvement of both cyclooxygenase and lipoxygenase products of arachidonic acid in follicular rupture at ovulation. We have estimated lipoxygenase activity by a chemiluminescence assay using unlabeled arachidonic acid as substrate in the presence of luminol and by conversion of ^{14}C -arachidonic acid into lipoxygenase products which were separated and identified by HPLC. Both approaches demonstrated lipoxygenase activity in whole ovarian homogenate as well as in homogenate of preovulatory Graafian follicles. Within 6 h after LH administration in vivo, lipoxygenase activity increased 2-fold in whole ovarian homogenate and 5-fold in the follicular homogenate. Inclusion of reduced glutathione in the incubation mixture shifted the HPLC separated products from hydroperoxides to leukotrienes. Inclusion of lipoxygenase inhibitors abolished almost completely lipoxygenase activity while inhibitors of cyclooxygenase had no effect on the measured activity. Affinity-chromatography of ovarian homogenate on linoleylaminoethyl-sepharose column enriched arachidonic acid metabolizing enzymes, i.e. cyclooxygenase and lipoxygenases. Our recent studies suggest the existence of 12- and 15-lipoxygenase, in addition to the previously demonstrated 5-lipoxygenase in ovarian and follicular tissue (Reich et al., 1985c,

1987). Furthermore, by the use of RIA's we observed an increase in 5,12,15 HETE's and LTC₄/D₄/E₄ levels in ovaries and follicles after hCG treatment of rats with hCG (Abisogun et al., in preparation).

(6) The Mechanism(s) of GnRH- and LH-Induced Steroidogenesis, Oocyte Maturation and Plasminogen Activator in Preovulatory Rat Follicles.

Recent studies revealed that in addition to the cAMP-mediated action of gonadotropins on the follicle, GnRH, without measurable involvement of adenylate cyclase, induces prostaglandin production, progestin accumulation and ovulation. Studies on the mechanism of GnRH action suggested that inositol phospholipid turnover, calcium mobilization and alteration in cellular arachidonic acid metabolism may be involved in intracellular mediation of GnRH action. In this study we compared the effects of inhibitors of arachidonic acid metabolism i.e. cyclooxygenase and lipoxygenase on GnRH- and LH-stimulated steroid accumulation, plasminogen activator production and oocyte maturation. The inhibitors used in this study were indomethacin (10 μ M), a cyclooxygenase inhibitor and nordihydroguaiaretic acid, caffeic acid, esculetin and AA861 (100 μ M), inhibitors of lipoxygenases. All the inhibitors used, attenuated GnRH-induced rise in progestin (progesterone + 20 α -hydroxyprogesterone) accumulation by approximately 50% during the first 6 h of incubation, while they had no effect on the same when induced by LH. The inhibitory effect on GnRH-induced steroid accumulation was persistent, in some cases, even during further 18 h of incubation. Zymography and immunabsorbtion revealed that both, LH and GnRH specifically enhanced the tissue type plasminogen activator (PA) in preovulatory follicles in vitro. Inhibitors of cyclo-

oxygenase and of lipoxxygenase inhibited GnRH-induced PA production but not that induced by LH during a 6 h culture period. This inhibition was transient since after 24 h of culture a recovery in PA production was observed. GnRH-induced meiotic maturation appears to be mediated by lipoxxygenase derived metabolites since only inhibitors of lipoxxygenase, but not indomethacin, attenuated oocyte maturation. Exogenous phospholipase C and 1-oleyl-2-acetyl-glycerol, compounds which simulate GnRH effects, induced progesterone and PA production in preovulatory follicles. Addition of these compounds attenuated LH-induced progesterone and PA accumulation. The results presented are compatible with the notion that GnRH action on the ovary is mediated via phospholipid turnover, and that AA metabolites are essential mediators of GnRH action on the ovary (Abisogun et al., 1987).

(7) Ovarian Collagenolysis: the Role of Products of AA Metabolism.

Using L(5-³H)proline incorporated into ovarian collagen, collagenolysis was measured as previously described (Reich et al., 1985a). Using this method, ovarian collagenolytic activity was enhanced $\approx$ 2-fold in the hOG treated group (Table 1).

Addition of indomethacin or nordihydroguaiaretic acid (NDGA) (inhibitors of cyclooxygenase and lipoxxygenase) to ovarian extracts did not affect the collagenolytic activity in vitro (Table 1B). Likewise, indomethacin did not reduce collagenase activity extracted from PMSG-hOG-treated rat ovaries tested after activation in vitro of the enzyme by APMA (Curry et al., 1985, 1986). These results seem to indicate that inhibitors of arachidonic acid metabolism do not affect activity of native ovarian collagenase. By contrast, when indomethacin

or NDGA were administered to PMSG-treated immature rats concomitantly with hCG, ovarian collagenolysis in vivo was significantly reduced (Table 1A). Thus, arachidonic acid metabolites seem to play an essential role in gonadotropic activation of ovarian collagenolysis, required for follicular rupture at ovulation (Abisogun et al., in preparation).

Table 1. Effect of eicosanoid synthesis inhibitors on ovarian collagenase activity in vitro. The inhibitor of cyclooxygenase indomethacin (INDO), or of lipoxygenase nordihydroguaiaretic acid (NDGA), were either administered into the ovarian bursa concomitantly with injection of hCG (A) or added to ovarian extracts obtained 3-9 h after hCG administration (B). In both cases, endogenous collagenase activity was assayed in vitro during a 48 h incubation period of the ovarian ^3H -proline-labeled collagen extract.

	Collagenase activity (% of ^3H -Hyp solubilized, Mean $\pm$ SEM)	(n)
Control	2.67 $\pm$ 0.17	(14)
hCG (3-9 h)	4.50 $\pm$ 0.38	(36)
A. hCG (3-9 h)		
+ INDO (0.5 mg/bursa, <u>in vivo</u>)	1.30 $\pm$ 0.60	(22)
hCG (3-9 h)		
+ NDGA (0.5 mg/bursa, <u>in vivo</u>)	0.52 $\pm$ 0.17	(18)
B. hCG (3-9 h)		
+ INDO (10^{-5}M <u>in vitro</u>)	4.60 $\pm$ 1.50	(12)
hCG (3-9 h)		
+ NDGA (10^{-4}M <u>in vitro</u>)	7.51 $\pm$ 1.77	(18)

(8) Changes in Ovarian Hemodynamics: the Role of Arachidonic Acid Metabolites

The preovulatory surge of LH causes hyperemic changes in ovarian vasculature.

We have tested the effect of ovulation-blocking doses of inhibitors of arachidonic acid metabolism on ovarian vascular changes induced by hCG. Thus the action of inhibitors of cyclooxygenase and lipoxygenase (i) on ovarian uptake of marker proteins as a measure of capillary permeability and (ii) ovarian blood flow by radiolabeled microspheres. Ovarian ^{125}I -BSA uptake was significantly enhanced by hCG ($p < 0.001$) at all time intervals examined, from basal control values of 100% to 150% by 3 hours, 210% by 6 hours and 213% at 9 hours. After administration of any of the arachidonic acid metabolism inhibitors reduced ^{125}I -BSA uptake to the range of 101-135% of control by 3 hours after hCG, 112-168% by 6 hours and 124-163% by 9 hours.

Blood flow was measured 1.5, 6 and 9 hours after injection of hCG to rats. Ovarian blood flow increased significantly by 1.5 h ($p < 0.01$), remaining elevated at 6 h ($p < 0.05$), slightly decreasing, though not statistically significantly, by 9 hours after hCG. Similar changes were observed in isolated preovulatory follicles. Inhibitors of arachidonic acid metabolism did not attenuate the action of hCG on ovarian or follicular blood flow. Likewise, morphometric analysis of follicular capillary area revealed an increase within 3 and 6 hours after hCG administration. Inhibitors of arachidonic acid metabolism did not attenuate hCG action on follicular capillaries. Our data suggest, therefore, that one way in which eicosanoids are involved in follicular

rupture is by their modulation of capillary permeability as revealed by uptake of the protein marker albumin (Abisogun et al., 1987).

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COOPERATION AND RESEARCH ACHIEVEMENTS

The cooperation included planning and evaluation of results.

The description of the research results attests to progress in our understanding of follicular rupture in each of the areas aimed at in the proposal. As usual, our study generated more specific questions than those which we succeeded in solving. However, the use of bovine and ovine follicles obtained from an abattoir led to equivocal results due to the high variability in the developmental stage and atresia of the follicles. Therefore, the approaches established in this study using rat follicles, should be applied to well-defined periovulatory bovine follicles in order to establish the role of proteolytic enzymes in the ovulation in cattle. An application based on these results has been submitted to EARD (No. I-1253-87C).

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