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# Types I and IV Collagenolytic and Plasminogen Activator Activities in Preovulatory Ovarian Follicles

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During ovulation, enzymatic degradation of the extracellular matrix occurs within and around the graafian follicles. In this study, the activities of several different proteolytic enzymes were measured in the culture media of follicles taken from pregnant mare serum gonadotropin (PMSG)-primed immature rats. At 52 h after PMSG, the follicles were cultured for 2 to 15 h in media with or without human chorionic gonadotropin (hCG). Type I collagenase activity in hCG-stimulated follicles gradually increased within 6 h to 3.3-fold above that of the controls. Relatively pure populations of granulosa cells produced type I collagenase to a similar extent. Likewise, type IV collagenase increased 3.8-fold by 6 h after exposure of the follicles to hCG. In contrast, plasminogen activator activity increased by 3.9-fold at 2 h after hCG, but was negligible at 4, 6, and 15 h after incubation. These results suggest that plasminogen activator may activate both type I and type IV collagenase in hCG-stimulated ovulatory follicles.

**Key words: basement membrane, collagenase, type I collagen, type IV collagen, ovulation**

The ovarian follicles are surrounded by the inner and outer theca layers. The inner theca layer has little connective tissue and is separated from the granulosa cell layer by the basement membrane. The major collagenous component of the basement membrane is type IV collagen. A specific metalloproteinase, referred to as type IV collagenase, degrades type IV collagen into two major fragments and renders the molecule susceptible for further proteolysis [1,2]. Type IV collagen is, however, also partially degraded by several unspecific proteinases like leukocyte elastase and trypsin [3]. The outer theca layer is rich in connective tissue, its major fibrous components being the interstitial types I and III collagens. Both these collagen types are degraded by type I collagenase [4].

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Twenty years ago, Espey et al [5] showed that ovulation is preceded by local destruction of the connective tissue in the follicular wall. This finding led to a speculation that collagenolytic enzymes are responsible for this destruction of extracellular matrix and that luteinizing hormone (LH), which induces ovulation, somehow regulates these enzyme activities.

In recent years, a considerable amount of new information has accumulated on the destruction of collagen molecules in normal and pathological states and, also, in the process of ovulation [6,7]. The plasminogen activator system has been shown to be associated with collagenolysis in, for example, rheumatic synovial fluid [8]. There is also considerable evidence of a linkage between follicular rupture and the production of plasminogen activator by ovarian granulosa cells [9–15]. Due to the heterogenic structure of the ovaries, detailed information on hormonal regulation of collagenolytic activities and the possible interaction between the plasminogen activator system and collagenolysis can only be obtained from in vitro studies using isolated follicles.

In the present study, we have investigated gonadotropin regulation of collagenolytic activities in rat ovarian follicles. We incubated carefully isolated rat ovarian follicles synchronized with pregnant mare serum gonadotropin (PMSG)-priming in tissue culture conditions with or without human chorionic gonadotropin (hCG). This approach enabled the direct analysis of both types I and IV collagenolytic activities and the correlation of these activities to plasminogen activator activity. The incorporation of radioactive hydroxyproline as well as the activity of prolylhydroxylase were assayed to demonstrate whether quantitative changes in collagen biosynthesis occurred concomitantly with the destruction.

## MATERIALS AND METHODS

### Hormonal Treatment of the Animals and Isolation of Follicles

Immature 27-day-old Wistar rats were injected sc with 35 IU of PMSG (2,000 IU/mg, Diosynth, Oss, The Netherlands). After this treatment, the follicles reach the preovulatory stage within 52 h [16]. The animals were killed by decapitation 52 h after the injection, and the ovaries were removed and preovulatory follicles isolated under a preparation microscope. Special care was taken to ensure that the follicles would not rupture and that the surrounding connective tissue would be removed precisely. Some follicles were fixed in 10% neutral buffered formalin, processed in paraffin, and sectioned at 5 $\mu$m for H&E staining.

### Incubation Conditions

The isolated follicles were placed in 150–300 $\mu$l Dulbecco's modified Eagle's medium containing 50 $\mu$g/ml ascorbic acid with or without 1 mg/mL bovine serum albumin and incubated in 96-well microtiter plates in a humidified atmosphere containing 5% $CO_2$ for desired time periods in the absence or presence of 10 IU of hCG (10,000 IU/mg, Diosynth).

### Assay of Type I Collagenase Activity

Type I collagenase activity was measured using [$^3$H]proline-labeled collagen substrate prepared in freshly isolated chicken fibroblasts [17]. The assay was performed as described earlier [18], the cleavage products of the initial collagenase digestion being further degraded with a mixture of trypsin and chymotrypsin. The degradation products were separated from the remaining uncleaved collagen substrate by trichloroacetic acid precipitation. The trypsin activation of latent collagenase was always carried out using concentrations from 0.1 to 0.2 $\mu$g per 50 $\mu$l of medium [19] to confirm the optimal enzyme substrate ratio.

### Assay of Type IV Collagenolytic Activity

The type IV collagenolytic activity was assayed from cell culture medium proteins as described previously [20] by using soluble [$^3$H]proline-labeled type IV procollagen as a substrate [21]. Briefly, enzyme samples were activated with trypsin (10 $\mu$g/ml) followed by the addition of soyabean trypsin inhibitor (40 $\mu$g/ml), N-ethylmaleimide (4 mM), aprotinin (1,000 KIU/ml), and substrate (3,000 cpm). The reaction was carried out for 18 h at 35°C and was terminated by adding 20 $\mu$l of bovine serum albumin (1 mg/ml) and 100 $\mu$l of a solution containing 10% trichloroacetic acid and 5% tannic acid. The mixture was incubated on ice, and the undigested material was precipitated and removed by centrifugation at 5,000g for 15 min. Radioactivity in the supernatant was measured in a scintillation counter (Wallac, Turku, Finland).

### Assay of Total Collagen Production

The dissected follicles or granulosa cells were incubated as described above and metabolically labeled with [$^{14}$C]proline (10 $\mu$Ci/ml) for 6 h. After the labeling period, the medium of the granulosa cells was collected, proteinase inhibitors were added (25 mM sodium EDTA, 1 mM p-aminobenzamide, 1 mM phenylmethanesulfonyl-fluoride, and 10 mM N-ethylenmaleimide), and proteins were precipitated by adding ammonium sulphate to a final concentration of 390 mg/ml. After overnight stirring at 4°C, the precipitates were collected by centrifugation at 13,000g for 30 min. The precipitate was solubilized into 0.1 M Tris 0.4 M NaCl (pH 7.4) and dialyzed against this same buffer. The [$^{14}$C]hydroxyproline was assayed according to the method of Juva and Prockop [22] after hydrolysis at 116°C in 6 N HCl for 16 h. The procedure for [$^{14}$C]proline-labeled follicles was essentially the same, except that after the pulse, the follicles were homogenized in the incubation medium containing the protease inhibitors and frozen and thawed prior to ammonium sulphate precipitation.

### Assay of Procollagen Prolyl-4-Hydroxylase Activity

Both follicles and granulosa cells were homogenized after 6 h incubation in a cold solution containing 0.2 M NaCl, 0.1 M glycine, 0.1% (w/v) Triton X-100, 0.01% (w/v) soyabean trypsin inhibitor, and 0.02 M Tris-HCl buffer, pH adjusted to 7.5 at 4°C. The homogenates were centrifuged at 15,000g for 30 min at 4°C, and the enzyme activity of prolyl-4-hydroxylase was assayed from the supernatant, according to Tuderman et al [23].

### Assay of the Activity of the Plasminogen Activator and Determination of the Type of Plasminogen Activator

Plasminogen activator activities were measured from 50 $\mu$l of the culture media using highly purified plasminogen [24] and [$^{125}$I]-labeled fibrinogen as substrates, as previously described [25]. The results were first expressed as percentage of total releasable activity (obtained with a sample of trypsin) and then converted into Ploug units (PU) by plotting the values on a standard curve made with commercial urokinase (Leo Pharmaceutical, Ballerup, Denmark). Finally, the control PU values were expressed as 100%, and the hormonal effects were compared to this value.

The molecular weights of plasminogen activators were determined as follows: 50 $\mu$l of culture medium was applied onto a $NaDodSO_4$-polyacrylamide gradient gel [26]. After electrophoresis, the gels were thoroughly washed in 0.01 M Tris-HCl, pH 8.0, containing 2% Triton X-100 solution, essentially as previously described [27]. Finally, the washed gels were placed on an agarose-casein indicator gel containing plasminogen [28]. Parallel gels were always analyzed to exclude nonspecific proteolysis. The lytic zones in the indicator gels were finally photographed under indirect illumination.

### Determination of Caseinolytic Activity

Caseinolytic activity was assayed at pH 7.5 with a slight modification of Starkey and Barret [29] using azocasein as substrate.

### DNA and Protein Assays

The DNA contents were assayed by the fluorometric method of Brunk, Jones, and James [30]. Total protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

## RESULTS

### Histology of the Follicles

The histological section of a dissected follicle is shown in Figure 1. The follicles contain intact granulosa cell zone surrounded by theca layers. The follicles are virtually free of stromal connective tissue.

### Type I Collagenase Activity

Figure 2 shows the type I collagenase activity in the culture media at various time points both before and after trypsin activation of the latent enzyme form. When hCG was not present in the culture medium, the directly assayable, free collagenase activity increased only slightly from 2 to 6 h and was decreased at 15 h to a level below the 2-h value. Latent, trypsin-activatable enzyme activity reached its maximum at 4 h, decreasing thereafter to a low level by 15 h. The ratio of the total type I collagenase activity achieved after trypsin activation of the latent enzyme to the free directly assayable activity varied from 1.6:1 to 2.2:1, the ratio being lowest at 6 h. Both activities could be inhibited completely with 25 mM EDTA.

When hCG (10 IU) was present in the culture, the free collagenase activity increased from 2 to 6 h, reaching a maximum of 3.3-fold compared to control incubations. At 15 h, the activity fell to a level below the 2-h value. Also a clear enhancement of the total enzyme activity after trypsin activation was observed when hCG was present in the medium. The highest values could again be measured at 6 h, being about 2.6 times higher than those observed at the corresponding time point in the controls.

### Type IV Collagenolytic Activity

The type IV collagenolytic activity could only be assayed after trypsin activation of the media. Figure 3 shows the mean change in trypsin-activatable enzyme activity

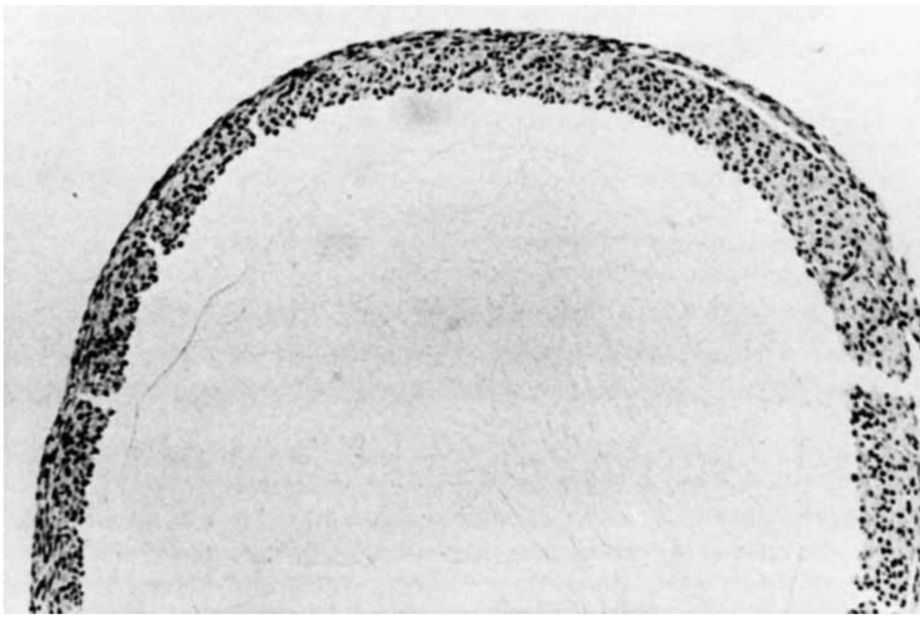

Fig. 1. Section (5 $\mu$m) of a dissected rat ovarian follicle used in in vitro incubations. H&E × 24.

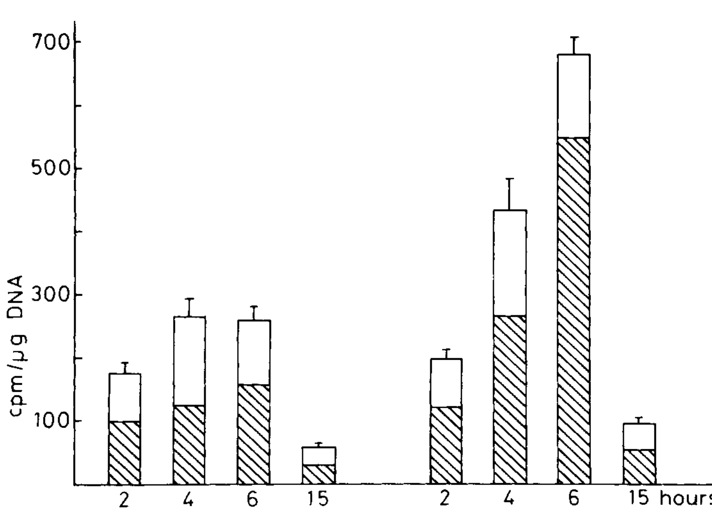


Fig. 2. Type I collagenase activity in the incubation media of rat ovarian follicles after various time periods. The hatched areas represent the directly assayable, free collagenase activity, and the open bars represent the activities detected after trypsin activation. Values are means and SEM of 10-15 determinations.

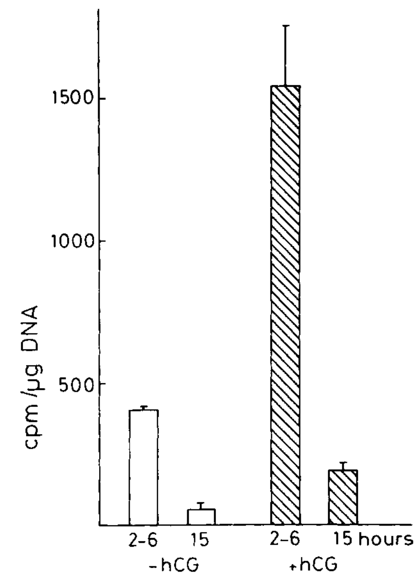


Fig. 3. Type IV collagenolytic activity detected in the incubation media of rat ovarian follicles after various time periods (2–6 h represent the pooled enzyme activities from media collected at 2, 4, and 6 h). All values represent activities detected after trypsin activation. The open bars represent activities observed in incubations without hCG and hatched bars represent activities observed in incubations with hCG. Values are means and SEM of 5–7 determinations.

in the incubation medium of the ovarian follicles. When hCG was present in the incubation, the enzyme activity was 3.8-fold higher at 2-6 h (pooled enzyme activity from media collected at 2, 4- and 6-h incubation periods) than in control incubations without hCG. Like the type I collagenase, the type IV collagenolytic activity clearly dropped at 15 h. No clear difference in the type IV collagenolytic activity was found regardless of whether the assay was performed from incubation medium or from homogenized follicles. The activity could always be inhibited by 20 mM EDTA.

### Caseinolytic Activity

To evaluate whether the increased collagenolytic activity observed in the presence of hCG in the culture media reflected only an increase in general proteolysis, the total crude caseinolytic activity was measured. The activity was stable throughout the 15-h incubation, increasing only by 10% from 2 to 6 h (data not shown). The addition of hCG had no effect on the caseinolytic activity.

### Plasminogen Activator Activity

In contrast to the nonspecific proteolysis, the plasminogen activator activity was markedly influenced by hCG. The highest activities of plasminogen activator were observed in the incubation medium after 2 h (Fig. 4). When hCG was present in the medium, the activities were almost 4 times higher than those observed in control incubations. Casein agarose indicator gels revealed two prominent plasminogen activator activities in the culture media. The most prominent one had a molecular weight of 65 kD and the other one 43 kD (Fig. 5), both comigrating precisely with the immunologically verified t-PA (65 kD) and PA (43 kD) secreted by rat seminiferous

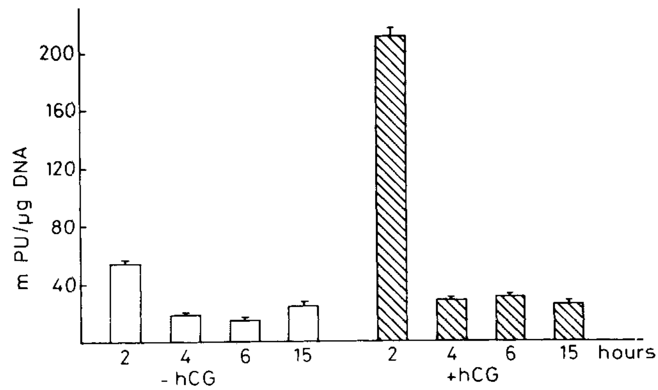


Fig. 4. Plasminogen activator activity detected in the incubation medium of rat ovarian follicles after various time periods. Open bars represent activities observed in incubations without hCG, hatched bars represent activities observed in incubations with hCG. Values are means and SEM of 4–5 determinations.

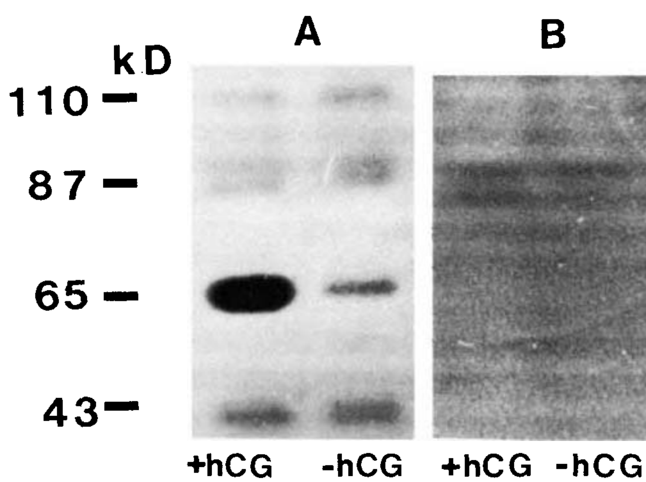


Fig. 5. Zymographic analysis of secreted plasminogen activator. A, with plasminogen; B, without plasminogen. Two prominent plasminogen activators were found in the 43 and 65 kD regions. In addition, some nonspecific activity was found at 87 kD and some small plasminogen-dependent activity in the 110 kD region. The hCG effect was mainly due to the induction of the 65 kD plasminogen activator. The development of the lysis zones in the casein overlay were observed for 3 days and photographed thereafter.

tubules in vitro [31]. Some nonspecific activity was observed in the 87 kD region. Slight activity was observed also in the 110 kD region. The addition of hCG into the medium seemed to influence predominantly the 65 kD activator.

## Collagen Biosynthesis in the Cultured Ovarian Follicles

The collagen biosynthesis in preovulatory rat ovarian follicles was also measured in vitro. The isolated follicles were incubated for 6 h in the presence of [$^{14}$C]proline, the time point corresponding to the maximal collagenolytic activity. No significant increase or decrease in the incorporation of [$^{14}$C]hydroxyproline could be detected in the presence of hCG (Table I). Also the [$^{14}$C]hydroxyproline: [$^{14}$C]proline

**TABLE I. Effect of hCG on Collagen Synthesis in Preovulatory Rat Ovarian Follicles. Isolated Follicles Were Incubated for 6 h in the Absence (Control) or Presence of hCG**

| | −hCG | +hCG | +hCG / −hCG |
|---|---|---|---|
| $^{14}$[C]hydroxyproline | 6.0 | 5.6 cpm/μg DNA | 0.93 |
| $\frac{14[C]\text{hydroxyproline}}{^{14}[C]\text{proline}} \times 100$ | 1.2% | 1.1% | 0.92 |
| Prolyl-4-hydroxylase activity | 21 | 26 cpm/μg DNA | 1.24 |

Values are means of 3–4 determinations.

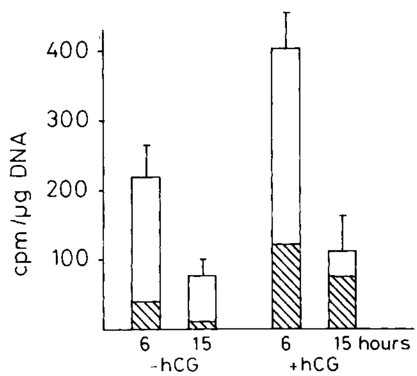


Fig. 6. Type I collagenase activities in granulosa cell incubations of rat ovaries. The hatched areas represent the directly assayable, free collagenase activity, and the open bars represent the activities observed after trypsin activation. The values represent mean of 5–7 determinations.

ratio remained unchanged. The activity of prolyl-4-hydroxylase, the first specific enzyme of collagen biosynthesis, was not affected by hCG stimulation either.

### Collagenolytic Activity in the Cultured Granulosa Cells

To find out whether the granulosa cells are responsible for the production of collagenolytic activities, the follicles of PMSG-primed rat ovaries were punctured and the granulosa cells squeezed into the culture medium. About $5 \times 10^5$ cells were applied to each incubation well, and both collagenolytic and collagen biosynthetic events were assayed.

After incubation for 6 h, hardly any free type I collagenase activity was detectable in the absence of hCG (Fig. 6). After activating the latent enzyme with trypsin, a significant amount of type I collagenase was detectable. When hCG was present, a clear increase (about 2-fold), both in the free and trypsin-activatable enzyme activities occurred (Fig. 6). As in the follicle incubations, the activity of type I collagenase clearly decreased from 6 h to 15 h.

When type IV collagenase was assayed, virtually no free or trypsin-activatable activity could be seen in the granulosa cell medium either at 6 or 15 h (Table II).

**TABLE II. Effect of hCG on Collagen Metabolism in Preovulatory Rat Ovarian Granulosa Cells. Isolated Granulosa Cells Were Incubated for 6 h in the Absence (Control) or Presence of hCG**

| | −hCG | +hcG | $\frac{+\text{hCG}}{-\text{hCG}}$ |
|---|---|---|---|
| Type IV collagenolytic activity | 40 | 46 cpm/$\mu$g DNA | 1.15 |
| $^{14}$[C]hydroxyproline | 264 | 266 cpm/$10^6$ cells | 1.01 |
| $\frac{14[\text{C}]\text{hydroxyproline}}{^{14}[\text{C}]\text{proline}} \times 100$ | 0.26% | 0.35% | 1.35 |
| Prolyl-4-hydroxylase activity | 130 | 288 cpm/$10^6$ cells | 2.2 |

## Collagen Biosynthesis in the Cultured Granulosa Cells

The granulosa cell suspension culture was incubated with [$^{14}$C]proline in a way analogous to the pulse experiments performed with follicles. Marked hydroxyproline incorporation was observed after incubation for 6 h, indicating collagen synthesis. However, hCG caused no further increase in the incorporation (Table II). The [$^{14}$C]hydroxyproline: [$^{14}$C]proline ratio was 0.26% without and 0.36% with hCG. These values suggest that the total collagen synthesis of the granulosa cells in vitro is about 1.0% of the total protein synthesis, the value being independent of hCG stimulation. This value is based on [$^{14}$C]proline and [$^{14}$C]hydroxyproline incorporation. In contrast, hCG caused a 2.2-fold increase of prolyl-4-hydroxylase activity in the granulosa cells after 6 h incubation (Table II).

## DISCUSSION

Local destruction of the extracellular matrix of the ovarian follicular wall is necessary for the rupture to occur in the follicles [5]. It is known that LH initiates the ovulatory process in all mammalian species. It has, therefore, been suggested that LH regulates the activity of the specific collagenolytic enzymes involved in follicular rupture.

The present work deals with the regulation of collagenolytic activities by human chorionic gonadotropin (hCG) in isolated rat preovulatory follicles in vitro. To avoid the problems of tissue inhibitors and high substrate affinity of the collagenase [4], an in vitro incubation method of the follicles was adopted. Moreover, specific and highly sensitive methods utilizing biosynthetically labeled collagen as substrates were applied for assaying both type I collagenase activity [18] and type IV collagenase activity [21].

We found that hCG caused a transient (maximum at 6 h) increase in type I collagenase activity, suggesting that this enzyme activity is regulated by luteinizing hormones and that it is involved in the destruction of the follicular wall. The relatively rapid changes in the activities of type I collagenase also suggest the relative speed of the cells to mobilize this enzyme activity, which might indicate a short half-life for type I collagenase, at least in rat ovarian follicles.

The question arises, how are the proenzymes activated at the cellular level? Several earlier reports suggest that plasminogen and the plasminogen activator system are involved in ovulation [9,14,15].

Among other proteinases, plasminogen activator via plasmin seems to have also a marked role in the activation of latent collagenase [8,36]. Also in our study, a marked elevation in the plasminogen activator activities was observed after hCG stimulation. The increase reached a maximal level at 4 h prior to the maximal activity of types I and IV collagenase. This result supports the hypothesis that the plasminogen activator system could act as an activator of collagenolysis in vivo in the cascade of events leading to ovulation. However, on the basis of our results, a causative relationship cannot be claimed. The results are in accordance with the recent results of Ny et al [37].

The finding that plasminogen activator activity dramatically decreased after 2 h incubation in the presence of hCG is of interest. One possible explanation is that the decrease in activity is a result of a plasminogen activator inhibitor, which could be induced in order to prevent excessive proteolysis. In fact, the 110 kD plasminogen activator observed here could represent a t-PA inhibitor complex, the molecular weight corresponding well with the inhibitor-bound t-PA described by Levin [38].

Two types of regulation of type I collagenase activity have been reported. The more common type is an activation of the proenzyme observed, for example, in certain pathological conditions of the skin [18, 32]. The other type of regulation is an increased synthesis of the enzyme as reported in a human skin disorder, a rare form of epidermolysis bullosa [33]. Since both the free and the trypsin-activatable enzyme of type I collagenase increased in response to hCG, both regulation mechanisms of the enzyme seem to exist in ovarian follicles.

To our knowledge, the type IV collagenase activity has not been previously found in ovarian follicles. This enzyme activity was more difficult to detect than type I collagenase activity, likely due to the very low enzyme content in the follicles. This might result from the fact that there is much less type IV collagen, located only in the basement membranes of the ovaries, than types I and III collagens [34], which occupy wide areas in the follicular wall and in the interfollicular space. Nevertheless, a distinct increase in type IV collagenolytic activity was detected in the presence of hCG. It is also interesting that the activity increased in a manner parallel to type I collagenase activity, reaching its maximum at 6 h. The increase in type IV collagenase activity towards ovulation is also demonstrated by our group [35] in human follicular fluid.

The enzyme activities were also assayed in isolated granulosa cells to investigate which cells in the follicles produce the collagenase activity. Eventually a similar increase in type I collagenase activity (as in the whole follicle cultures) was seen in granulosa cell cultures in the presence of hCG, the activity levels being clearly lower than in the whole follicles. This suggests that part of the type I collagenase activity is produced by the granulosa cells. No type IV collagenase activity could be detected in the granulosa cell cultures. This may be due either to the low level of this enzyme activity or to the presence of inhibitors in the granulosa cells.

To investigate whether the collagen biosynthesis is affected by hCG concomitantly with the collagenolysis, we measured also some parameters of collagen biosynthesis in this study. A low basic production of collagen was detected by [$^{14}$C]hydroxyproline incorporation in the whole follicles, but the production did not respond to hCG stimulation. Also, the granulosa cells showed a basic collagen production, in agreement with previous data obtained with bovine cells [39]. In contrast to the whole follicles, isolated granulosa cells responded to hCG stimulation with an increased prolyl-4-hydroxylase activity without an increase in [$^{14}$C]hydroxyproline incorporation. Slightly contradictory findings such as these have been reported in other systems as well, but a definitive explanation is still lacking [40].

In summary, these results suggest that both type I and type IV collagenases are under gonadotropin control in rat ovarian follicles and that both may be involved in the ovulatory process. Our results also support the idea that a plasminogen activator system may be responsible for activation of both type I and type IV collagenases. It also seems evident that the local destruction of the follicular wall in ovulation is not associated with marked changes in the collagen biosynthesis.

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