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Hormonal Regulation of Tissue Inhibitors of Metalloproteinases During Follicular Development in the Rat Ovary

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Tissue inhibitors of metalloproteinases (TIMPs) are members of a multigene family of proteinase inhibitors that regulate the activity of metalloproteinases. To test the hypothesis that TIMPs regulate connective tissue remodeling during follicular development, rats were injected with PMSG (20 IU, sc), and ovaries and serum were collected at the time of pregnant mare serum gonadotropin (PMSG) administration (0 h) and at 6, 12, 24, 36, and 48 h later for analysis of TIMP expression, metalloproteinase inhibitor activity, and steroidogenesis. Serum estradiol levels increased from 20.9 pg/mL at 0 h to 461 pg/mL at 48 h. Northern analysis was performed for analysis of TIMP-1, TIMP-2, and TIMP-3 expression ($N = 4$). For TIMP-1, PMSG stimulated a 2.4- to 2.5-fold increase in TIMP-1 mRNA at 6 and 12 h compared to ovaries collected at the time of PMSG administration (i.e., 0 h control). TIMP-1 mRNA returned to control levels within 24 h and remained unchanged through 48 h. In contrast to TIMP-1, TIMP-3 mRNA decreased by approx 2.5-fold at 6 h following PMSG administration, and expression remained decreased through 48 h. For TIMP-2, the expression of the 3.5-kb transcript decreased at 24 h after PMSG, whereas expression of the 1 kb transcript was unchanged. There was no change in metalloproteinase inhibitor activity in whole ovarian extracts between 0 and 36 h. However, there was an increase in inhibitor activity at 48 h. These findings are the first demonstration of hormonal regulation of TIMPs during the follicular phase. The differential regulation of the TIMPs by gonadotropins, for example, an increase in TIMP-1 and a concomitant decrease in TIMP-3 expression, may reflect different roles, sites of action, or enzyme specificity for the inhibitors as the follicle grows.

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Introduction

Matrix metalloproteinases (MMPs) are a family of related proteolytic enzymes that degrade extracellular matrix proteins, such as collagen, laminin, and fibronectin, thereby playing an important role in connective tissue remodeling throughout the body (Woessner, 1991). This class of enzymes includes collagenase, gelatinases, stromelysins, and other enzymes that are involved in extracellular matrix remodeling. Metalloproteinase inhibitors regulate the activity of matrix metalloproteinases in both serum and tissue. The predominant serum-borne metalloproteinase inhibitors include the macroglobulins, such as α_2-macroglobulin (Birkedal-Hansen et al., 1993). The large size of α_2-macroglobulin (720 kDa) prevents it from readily crossing the basement membrane of the capillary epithelium, so its action is primarily limited to serum-borne substrates. However there is evidence that it is produced locally by the corpus luteum (Gaddy-Kurten et al., 1989) and regulated by prolactin (Dajee et al., 1996).

The second class of metalloproteinase inhibitors include the tissue inhibitors of metalloproteinases (TIMPs), which are members of a family of inhibitors that in addition to their ability to inhibit metalloproteinases, share common characteristics, such as conservation of gene structure, conformational similarities, including 12 cysteine residues assumed to form six disulfide bonds, which are important for the overall protein structure, and a highly conserved N-terminal domain functionally critical for metalloproteinase inhibition (Apte et al., 1995). Three distinct TIMPs (TIMP-1, TIMP-2, and TIMP-3) have been identified based on their molecular weight and biological action, but differ in their regulation, enzyme specificity, and mode of action (Matrisian, 1990). TIMP-1 is a 28 kDa secreted glycoprotein capable of binding to all forms of metalloproteinases,

as well as the latent form of the 92-kDa gelatinase (also known as gelatinase B; Wilhelm et al., 1989). TIMP-2 is also a secreted inhibitor, has been shown to be differentially regulated from TIMP-1, and proposed to act selectively on different MMPs (Leco et al., 1992; Roswit et al., 1992). For example, TIMP-2 has a high affinity for the latent and active forms of the 72-kDa gelatinase known as gelatinase A (Stetler-Stevenson et al., 1989). Unlike TIMP-1 or TIMP-2, TIMP-3 is a 21-kDa protein that is secreted and then bound to the extracellular matrix. TIMP-3 has been suggested to act as an additional regulatory stop point for MMP action (Pavloff et al., 1992; Leco et al., 1994).

Metalloproteinase inhibitors are present in the ovaries of numerous species, and have been postulated to play a regulatory role in ovarian connective tissue remodeling associated with ovulation and luteal function (Zhu and Woessner, 1991; Waterhouse et al., 1993; Smith et al., 1994, 1995; Nothnick et al., 1995). For example, TIMP-1 mRNA is abundant in periovulatory rat, ovine, and human ovaries and in preovulatory human and rat granulosa cells (Mann et al., 1991; Rapp et al., 1991; Zhu and Woessner, 1991; Smith et al., 1994). In fact, TIMP-1 has been reported to be one of the most abundant mRNAs present in preovulatory human granulosa cells (Rapp et al., 1991). In concord with TIMP-1 expression, a TIMP-1-like protein has been identified in human follicular fluid (Curry et al., 1988), rat ovaries (Zhu and Woessner, 1991), and rat granulosa cell-culture media (Mann et al., 1991). The levels of ovarian TIMP-1 mRNA and inhibitor activity increase following a luteinizing hormone and/or human chorionic gonadotropin stimulus (Mann et al., 1991). Collectively, these findings demonstrate that TIMP-1 is present in the ovary and is stimulated by the events associated with the preovulatory LH surge. The function of periovulatory TIMPs has been postulated to maintain proteolytic homeostasis and provide localized control of extracellular degradation associated with follicular rupture (Curry et al., 1988; Mann et al., 1991; Zhu and Woessner, 1991).

The presence of metalloproteinase inhibitors in murine (Waterhouse et al., 1993), rat (Zhu and Woessner, 1991; Nothnick et al., 1995), and ovine luteal tissue (Smith et al., 1994, 1995) has led to the suggestion that TIMPs function in extracellular matrix remodeling associated with luteal formation and regression. Nothnick and coworkers (1995) reported that TIMP-1 mRNA and inhibitor activity are elevated during early luteal changes associated with pseudopregnancy in the rat, i.e., 1–2 d after hCG administration. During luteal maintenance, there is an increase in TIMP-3 mRNA levels that persists through the period of luteal regression when TIMP-1 expression and inhibitor activity increases (Nothnick et al., 1995). These observations support the postulate for a crucial role of metalloproteinase inhibitors in the regulation of corpus luteum formation and regression.

Although there is substantial evidence for metalloproteinases and their inhibitors in the events associated with the periovulatory and luteal periods, the role of TIMPs in follicular development has not been examined. Since follicular growth may also require extensive changes in the extracellular matrix, we hypothesized that follicular growth occurs in association with changes in metalloproteinases and their inhibitors, and that these changes are under hormonal regulation. In support of such a theory is the report by Pellicer and colleagues (1988) that inhibition of serine protease activity, a separate proteolytic pathway, early in follicular development leads to impaired follicular growth and decreased ovulation. To test the hypothesis that follicular growth occurs in association with changes in metalloproteinase inhibitors, we designed an experiment to induce follicular development in immature rats, and measured TIMP mRNA expression and metalloproteinase inhibitor activity during this period of ovarian connective tissue remodeling.

Table 1
Effect of PMSG Administration on Ovarian Weight and Serum Estradiol Concentrations[a]

Hours after PMSG	Ovarian weight, mg ± SEM	Estradiol, pg/mL ± SEM
0	8.5 ± 0.39	20.9 ± 2.7
6	9.3 ± 0.59	18.2 ± 1.0
12	9.4 ± 0.87	14.7 ± 0.8
24	13.6 ± 1.26*	29.2 ± 14.6
36	23.0 ± 1.84*	126.3 ± 20.2*
48	27.1 ± 1.88*	461.0 ± 154.2*

[a]Animals were necropsied at the indicated times after PMSG administration. Serum was collected for determination of estradiol concentrations. Ovaries were removed and weighed before being processed for Northern analysis or extraction for determination of metalloproteinase inhibitor activity as described in the Materials and Methods. Values represent the mean ± SEM and an * represents a significant difference ($p < 0.05$) from the 0 h time-point ($N = 4$).

Results

Hormonal Stimulation of Ovarian Follicular Development

There was an increase in ovarian weight after pregnant mare serum gonadotropin (PMSG) treatment, which reached a maximum at 48 h after PMSG stimulation (Table 1). Ovaries collected at the time of PMSG administration, 24 h, and 48 h after PMSG administration were fixed and stained for histologic examination to confirm the hormonal stimulation of follicular development. Ovaries obtained at the time of PMSG administration contained multiple small follicles. In marked contrast, ovaries obtained 48 h after PMSG administration were larger than the 0 h ovaries and contained numerous antral follicles (Fig. 1).

To confirm further the induction of follicular growth, serum estradiol levels were analyzed. Estradiol levels did not change significantly through the first 36 h, but increased significantly at 48 h to reach a peak of 461 pg/mL (Table 1, $N = 4$).

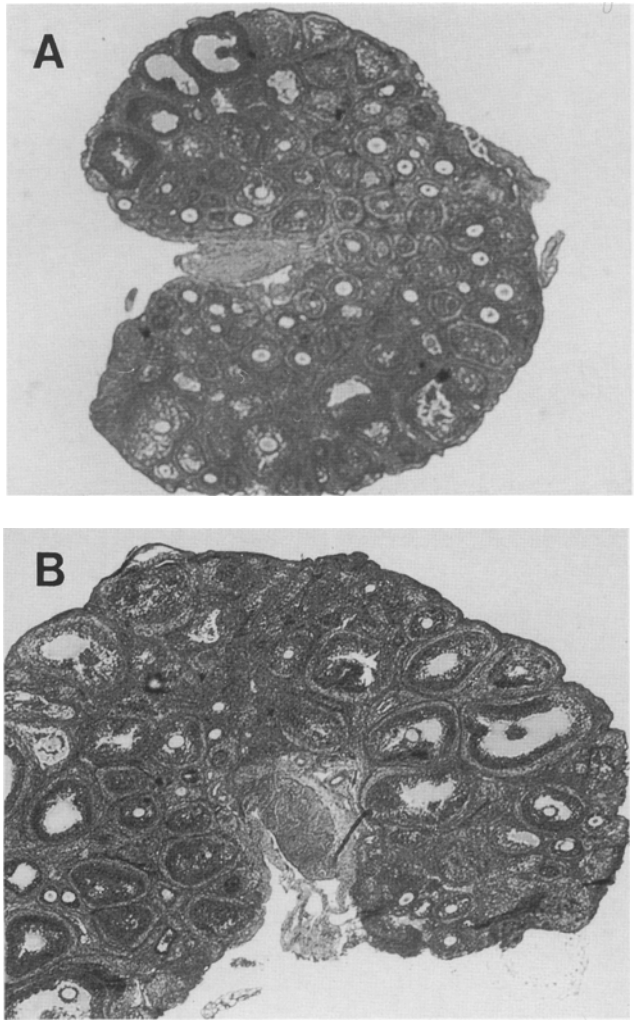

Fig. 1. Histological evaluation of follicular growth. Ovaries were removed after PMSG treatment, fixed, sectioned, and stained as described in Materials and Methods. **(A)** Photomicrograph of an ovary collected 24 h after PMSG treatment. **(B)** Photomicrograph of an ovary collected 48 h after PMSG treatment. Magnification is 155×.

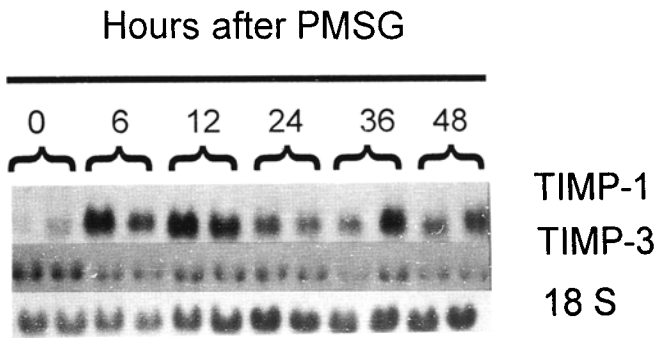

Fig. 2. Northern analysis of TIMP expression during follicular development. Ovaries were collected at the indicated times after PMSG administration, and total RNA was isolated. RNA samples (20 μg/lane) were hybridized with mouse cDNA probes for TIMP-1 and TIMP-3. TIMP-1 and TIMP-3 were hybridized concomitantly, and demonstrated a single transcript for TIMP-1 of approx 900 bp and a single transcript for TIMP-3 of approx 4500 bp. The blot was allowed to decay and then probed for the 18S ribosomal RNA. The figure is representative of a total of two experiments with four separate observations per time-point ($N = 4$).

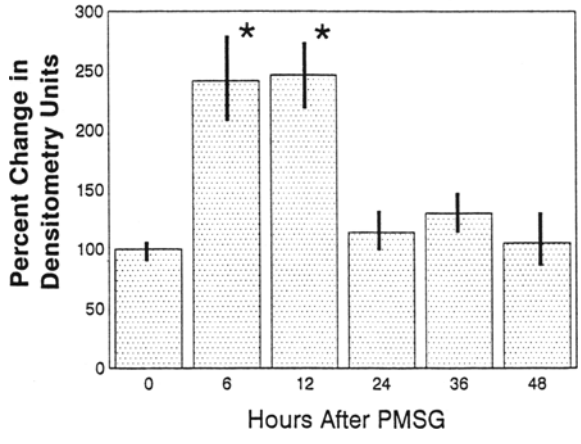

Fig. 3. Expression of TIMP-1 mRNA after PMSG administration. TIMP-1 mRNA levels were normalized to the 18S ribosomal RNA and expressed as the percent change ± SEM from the 0 h time-point, which has arbitrarily been set = 100%. An * represents a significant difference ($p < 0.05$) from the 0 h time-point ($N = 4$).

Northern Analysis

Northern analysis was performed on four samples at each time-point during follicular development. TIMP-1 demonstrated a single transcript of approx 900 bp and TIMP-3 exhibited a single transcript of approx 4500 bp (Fig. 2). Following PMSG treatment, there was a 240 and 250% increase in TIMP-1 expression at 6 and 12 h, respectively (Fig. 3), compared to ovaries collected at the time of PMSG administration (i.e., 0 h control). TIMP-1 mRNA returned to control levels (i.e., 0 h) within 24 h and remained unchanged through 48 h after gonadotropin stimulation. In contrast to the initial stimulation of TIMP-1, TIMP-3 mRNA at 6 h decreased to approx 40% of the 0 h value following PMSG administration, and expression remained decreased through 48 h (Fig. 4). For TIMP-2, two transcripts of 3500 and 1000 bp were observed (Fig. 5). The expression of the 3.5-kb transcript (upper) decreased at 24 h after PMSG, but was not different from control levels at 36 and 48 h (Fig. 6). For the 1-kb transcript (lower) of TIMP-2, ANOVA demonstrated no statistically significant differences between the time-points, although there was a trend toward an increase in expression of the lower transcript at 6 h after hormonal treatment (Fig. 6). Northern analysis of the TIMPs from the animals not receiving gonadotropin demonstrated no significant change in either TIMP-1, TIMP-2, or TIMP-3 mRNA levels at 0, 6, 12, or 24 h after their control litter mates received PMSG ($N = 2$, data not shown).

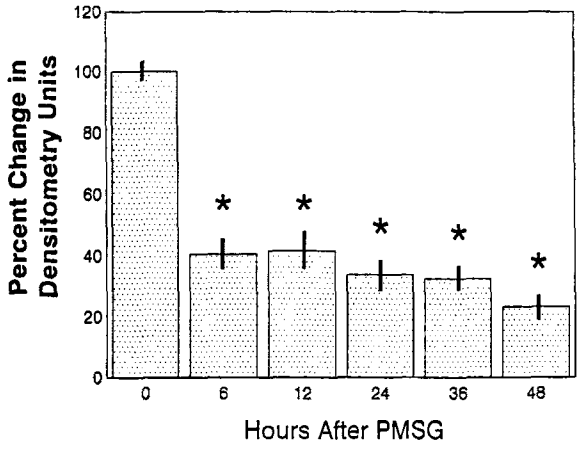

Fig. 4. Expression of TIMP-3 mRNA after PMSG administration. TIMP-3 mRNA levels were normalized to the 18S ribosomal RNA and expressed as the percent change ± SEM from the 0 h time-point, which has arbitrarily been set = 100%. An * represents a significant difference ($p < 0.05$) from the 0 h time-point ($N = 4$).

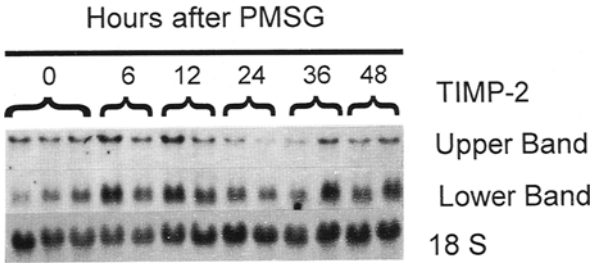

Fig. 5. Northern analysis of TIMP-2 during follicular development. The TIMP-1 blot in Fig. 2 was stripped and probed for TIMP-2. Two transcripts of approx 3500 bp (upper) and 1000 bp (lower) were demonstrated. The blot was allowed to decay and then probed for the 18S ribosomal RNA. The figure is representative of a total of two experiments with four separate observations per time-point ($N = 4$).

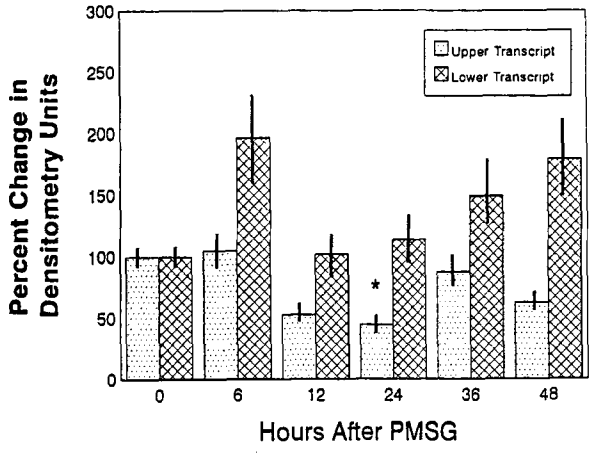

Fig. 6. Expression of TIMP-2 mRNA after PMSG administration. The two transcripts for TIMP-2 were normalized to the 18S ribosomal RNA and expressed as the percent change ± SEM from the 0 h time-point, which has arbitrarily been set = 100%. An * represents a significant difference ($p < 0.05$) from the 0 h time-point ($N = 4$).

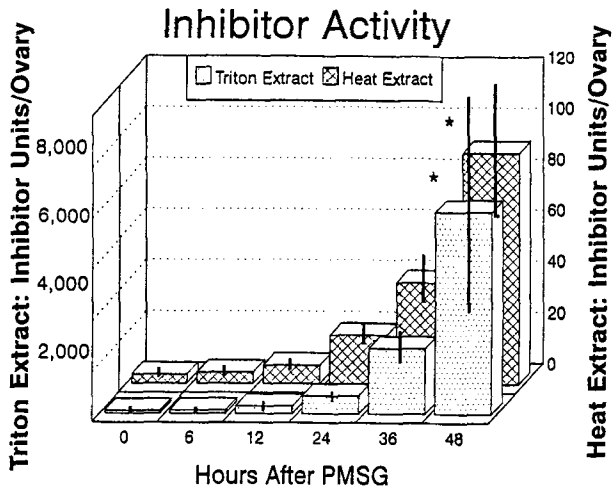

Fig. 7. Metalloproteinase inhibitor activity in the heat and triton extracts from ovaries collected after PMSG treatment. Ovaries were collected at the indicated times after PMSG administration. Inhibitors were extracted and assayed as described in the Materials and Methods. Inhibitor activity is expressed as inhibitor units, which represent the percent inhibition in the 100-µL aliquot extracted sample normalized to the amount of protein in the sample for both the triton and heat supernatants. The triton group is shown on the left axis and the heat group on the right axis. An * represents a significant difference ($p < 0.05$) from the 0 h time-point in the heat or triton group ($N = 3$).

Metalloproteinase Inhibitor Assay

Results from the metalloproteinase inhibitor assay are expressed as inhibitor units, which represent the percent inhibition normalized to the amount of protein in the sample for both the triton and heat supernatants. The triton extract contained approx 50- to 75-fold more inhibitor activity than the corresponding heat extract at each time-point. There was no significant change in inhibitor activity between 0 and 36 h in either the triton or heat groups, but there was a significant increase in inhibitor activity in both groups at 48 h after PMSG treatment (Fig. 7; $N = 3$).

Discussion

The present findings demonstrate for the first time that specific tissue inhibitors of metalloproteinases are under hormonal regulation during follicular development in the rat ovary. We illustrate that after PMSG administration, serum estradiol, follicular development, and ovarian weight increase. Associated with the hormonal induction of follicular growth, there is an increase in metalloproteinase inhibitor activity and differential regulation of the various TIMPs. Gonadotropin stimulation increases expression of TIMP-1, which is found in the extracellular space, while concomitantly decreasing the matrix bound inhibitor TIMP-3. The importance of these findings is that follicular growth requires remodeling of the ovarian extracellular matrix, an intuitive idea that to date has not been investigated.

During follicular growth, there is extensive cellular proliferation, neovascularization, and changes in steroidogenesis as the follicle progresses from the primary stage through the secondary and tertiary phases to reach the large graafian follicle (Greenwald and Roy, 1994). We hypothesized that the proliferation and growth of the follicle, as well as the associated changes in vascularization, must also be accompanied by changes in the extracellular matrix, which provides functional and structural support for the follicle. Remodeling of the extracellular matrix in other tissues occurs through the coordinated action of a family of proteolytic enzymes known as matrix metalloproteinases and their associated inhibitors. The present study demonstrates that there is gonadotropin regulation of the metalloproteinase inhibitors. Specifically, PMSG stimulates an increase in the expression of TIMP-1, an inhibitor secreted and found in the extracellular fluid. In contrast, expression of the matrix-bound inhibitor TIMP-3 is decreased by gonadotropin administration. Such differential regulation of TIMP expression (i.e., stimulation of the secreted inhibitor and inhibition of the matrix-bound inhibitor) may reflect the varied physiological roles of the inhibitors during extracellular remodeling associated with follicular growth. It is possible that the extent and location of connective tissue remodeling vary during follicular development. For example, the decrease in TIMP-3 expression may decrease the inhibitor present in the extracellular matrix associated with the theca and surrounding fibroblasts, thereby allowing metalloproteinases to degrade the connective tissue for neovascularization or follicular growth. Stimulation of the secreted inhibitor TIMP-1 may ensure that any metalloproteinase activity not associated with follicular remodeling would be prevented. Alternatively, the secreted inhibitor may be in a different ovarian compartment, such as the granulosa cell layer, than the matrix bound TIMP-3 and act to protect the integrity of the granulosa cell basement membrane as progressive proliferation of the granulosa cells occurs. Support for such a postulate is the observation that granulosa cells contain an abundance of TIMP-1 mRNA (Rapp et al., 1991), produce metalloproteinase inhibitor activity, specifically a TIMP-like protein (Mann et al., 1991), and that follicular fluid contains TIMP-1-like activity (Curry et al., 1988). Our findings that PMSG stimulates inhibitors during follicular growth would appear paradoxical in that one would anticipate a decrease in inhibitor activity as enzyme activity increased. However, parallel regulation of both enzyme and inhibitor has been observed in numerous tissues, including the ovary, such as LH stimulation of ovulation and formation of the corpus luteum (Mann et al., 1991; Nothnick et al., 1995). Such concomitant regulation has been proposed to provide proteolytic homeostasis and maintain localized control of extracellular degradation.

Further evidence that regulation of TIMP expression occurs through the action of PMSG is evident from our observation in litter-matched animals not treated with PMSG. Without gonadotropin stimulation, there was no change in mRNA expression of any of the TIMPs, indicating that the change in TIMP mRNA expression was not an age-dependent phenomenon. Whether the gonadotropin-induced regulation is a direct effect or whether this effect is mediated through other gonadotropin-induced messenger systems, such as prostaglandins or steroidogenesis, remains to be determined. However, the basal levels of estradiol up to 36 h after PMSG administration would suggest that estradiol does not play a role in the initial regulation of follicular expression of the TIMPs.

The triton and heat extracts contain the two different classes of inhibitors present in the ovary: serum-borne and tissue-derived inhibitors. The triton extract contains high levels of the macroglobulin-type inhibitors (Zhu and Woessner, 1991). The increase in inhibitor activity in this extract probably reflects the increase in serum-borne inhibitors owing to an increased vascularization of the growing follicular population, whereas the heat extract contains predominately the tissue-derived inhibitors (Zhu and Woessner, 1991). We would speculate that the increase in inhibitor activity in this extract probably is indicative of increased levels of gonadotropin-stimulated TIMP-1-related activity, since TIMP-3 is predominately bound to the extracellular matrix and extraction is difficult.

Although metalloproteinase inhibitors were initially identified on the basis of their ability to regulate proteolysis, the TIMPs have recently been proposed to be multifunctional, acting as growth factors and stimulators of steroidogenesis. For example, the TIMPs have been suggested to act as growth factors based on reports that TIMP-1 promotes embryo growth and development (Satoh et al., 1994), has erythroid-potentiating action (Docherty et al.,1985), and stimulates endothelial cell growth in a variety of cells (Hayakawa et al., 1992). Thus, it is quite possible that the TIMPs may be involved in cellular proliferation and neovascularization during follicular growth, in addition to controlling follicular proteolysis. The ability of TIMPs to influence cellular differentiation is apparent from the recent report that TIMP-3 is associated with the recruitment of quiescent cells into the cell cycle (Wick et al., 1995) and that a TIMP-1-like protein stimulates ovarian steroidogenesis (Boujrad et al., 1995). Also of interest is the discovery of a 124-base homology in the 5'-noncoding region of TIMP-1 with the steroidogenic acute regulatory protein (StAR), an acute controller for the transfer of cholesterol to the inner mitochondrial membrane, suggesting possible common regulatory mechanisms between TIMP expression and regulation of steroidogenesis (Hartung et al., 1995). Thus, the TIMPs may have multifunctional activities during follicular growth.

In summary, the present study documents for the first time that TIMP mRNA expression is hormonally regulated during ovarian follicular development. Although this study

does not elucidate the reason why there is differential regulation of the TIMPs, we hypothesize that different sites of cellular origin, different modes of action, or differing enzyme specificities for TIMP-1 and TIMP-3 may, in part, be responsible.

Materials and Methods

Materials

PMSG was purchased from Sigma Chemical Co. (St. Louis, MO). Azocoll was obtained from Calbiochem (La Jolla, CA). Nylon membranes (Nytran) for Northern analysis were purchased from Schleicher and Schuell (Keene, NH). [α-^{32}P] dCTP was acquired from New England Nuclear (Boston, MA). Coat-A-Count kits for radioimmunoassay of estradiol were purchased from Diagnostic Products Corporation (Los Angeles, CA).

Animals

Immature Sprague-Dawley female rats, 18-d-old, were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN) and kept in environmentally controlled conditions under the supervision of a licensed veterinarian. All animal procedures for these experiments were approved by the University of Kentucky Institutional Animal Care and Use Committee. Rats were maintained on a 14:10 light:dark cycle, and provided water and rat chow ad libitum. Between 9 and 10 AM on day 23, rats were injected sc with 20 IU of PMSG to induce follicular development. The animals were sacrificed at the time of PMSG administration (0 h) and 6, 12, 24, 36, and 48 h after injection of PMSG. In order to verify that changes in TIMP-1, TIMP-2, and TIMP-3 mRNA expression were owing to hormonal induction and not merely a function of normal ovarian development, a control group of animals that did not receive PMSG was sacrificed at 0, 6, 12, and 24 h after their corresponding litter mates were injected with gonadotropin. After sacrifice, the ovaries were removed, cleaned of adnexa, weighed, and processed for either determination of TIMP mRNA expression, measurement of metalloproteinase inhibitor activity, or examination of ovarian histology as described below. Serum was collected for measurement of estradiol by radioimmunoassay. Representative sections of ovaries from each time-point were stained with hematoxylin and eosin for examination of follicular development by light microscopy.

Northern Analysis

RNA was isolated from the ovarian samples by the method of Chomczynski and Sacchi (1987) using an acid guanidinium thiocyanate-phenol-chloroform extraction procedure with the following modifications. Ovaries were homogenized in a denaturing solution of 4*M* guanidinium thiocyanate, 25 m*M* sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1*M* β-mercaptoethanol (1 mL/100 mg tissue). After transfer of the homogenate to fresh polypropylene tubes, 0.1 vol of 2.0*M* sodium acetate (pH 4.0) was added, and the samples were briefly vortexed. Water-saturated phenol (1 vol) and 0.2 vol of a chloroform:isoamyl alcohol mixture (49:1) were sequentially added with brief vortexing after addition of each reagent. The samples were placed on ice for 15 min and then reprecipitated with ethanol using standard procedures. RNA samples (20 μg/lane) were electrophoresed at 30 V through a 1.0% agarose gel containing 2.2*M* formaldehyde for 18 h. After electrophoresis, the RNA was transferred and fixed to a nylon membrane (Nytran) by baking at 80°C for 30 min. Plasmids for murine TIMP-1, TIMP-2, and TIMP-3 were obtained from D. Edwards (Calgary, Alberta), and cDNA probes were prepared by isolation of excised fragments and random primer labeling to a specific activity of approx 1.0×10^9 disintegrations per minute (dpm) using [α-^{32}P] dCTP. The membranes were hybridized for 18 h at 42°C and washed at 42°C in 2X standard saline citrate (SSC = 0.3*M* NaCl, 30 m*M* citric acid) + 0.1% (w/v) SDS followed by a wash at 65°C in 0.2X SSC + 0.1% SDS according to the recommendations of the manufacturer. Membranes were hybridized concomitantly with TIMP-1 and TIMP-3, allowed to decay, hybridized with TIMP-2, and then hybridized with the 18S probe. The resulting blots were visualized by autoradiography and analyzed with an LKB Ultrascan XL laser densitometer (LKB Instruments, Rockville, MD) to calculate the relative mRNA content. TIMP expression was then normalized to the 18S ribosomal mRNA to account for slight differences in sample loading or transfer to the Nytran membrane. Relative mRNA content encoding each normalized TIMP value was expressed as arbitrary units that were calculated by setting the control at 0 h value = 100% and expressing each other transcript as a percentage from the 0 h value (i.e., 100%).

Metalloproteinase Inhibitor Assay

MMP inhibitors were extracted from ovaries as previously described (Zhu and Woessner, 1991). The ovarian tissue was homogenized in 10 m*M* $CaCl_2$ and 0.25% Triton, and then centrifuged at 10,000*g* for 30 min. The supernatant was decanted, and it was referred to as the triton group. The pellet from the first centrifugation was resuspended in 100 m*M* $CaCl_2$, 50 m*M* Tris, and 150 m*M* NaCl at pH 7.5, heated at 60°C for 6 min and centrifuged at 17,500*g* for 30 min. The supernatant was decanted, and it was referred to as the heat group. Both the heat and triton groups were dialyzed overnight in assay buffer (50 m*M* Tris-HCl, 10 m*M* $CaCl_2$, 0.2*M* NaCl, 0.05% Na azide, and 0.167% Brij 35, pH 7.5) and then assayed with a colorimetric metalloproteinase inhibitor assay as routinely performed in our laboratory (Mann et al., 1991). This assay is based on the inhibition of a uterine neutral metalloproteinase, matrilysin, by metalloproteinase inhibitors in the ovarian extracts (Woessner and Taplin, 1988). To perform the assay, a volume of uterine matrilysin sufficient to yield a change in

absorbance of approx 0.2 (15 µL) was mixed with the ovarian sample and brought to a volume of 400 µL with assay buffer. This mixture was preincubated for 1 h at 37°C to allow the inhibitors present in the sample to interact with the uterine matrilysin. Azocoll (1 mg) suspended in 850 µL of assay buffer was added to each sample and incubated for 18 h at 37°C in a reciprocating water bath. The inhibition of uterine matrilysin activity from inhibitors present in the sample was quantitated on a Beckman DU-64 spectrophotometer (Beckman Instruments, Fullerton, CA) at 520 nm and expressed as the percent inhibition of total matrilysin activity. To correct for variation in ovarian weights and the amount of protein in each sample, the samples are expressed as inhibitor units, which represents the percent inhibition in the 100-µL aliquot extracted sample normalized to the amount of protein in the sample as determined by Bradford assay (Bradford, 1976). Buffer blanks (400 µL assay buffer and Azocoll suspension) were assayed to measure nonspecific degradation of the Azocoll.

Estradiol Radioimmunoassay

Serum 17β-estradiol levels were determined by radioimmunoassay with Coat-A-Count kits (Diagnostic Products Corp., Los Angeles, CA). The Coat-A-Count kits are direct, solid phase I^{125} RIA kits. Serum (100 µL) was incubated with antibody for 3 h. Samples were decanted, counted with a γ-counter, and estradiol concentration was determined. The sensitivity of the assay was 6 pg/mL. Intraassay and interassay coefficients of variation were 5.4 and 9.7%, respectively.

Ovarian Histology

Ovaries from each of the time-points after PMSG treatment were processed for histological examination of follicular development. Ovaries were fixed in 10% neutral buffered formalin, processed for paraffin embedding, and sectioned at 8 µm. Tissues were stained with hematoxylin and eosin.

Statistical Analysis

Analysis of variance was used to test for differences in TIMP expression and metalloproteinase activity. The data for the 0 h time-points were normalized to a value of 100%. Data for all other time-points were expressed relative to the 0 h time-point. If significance was obtained with ANOVA, post-hoc analysis with the Dunnett Multiple Comparisons procedure was used to examine group differences.

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