ANALYSIS OF ASPARTIC PROTEINASE ACTIVITY IN PORCINE OVARIES

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ABSTRACT

Background & Objectives: Tightly regulated proteolytic activity is vital in the mammalian ovary for follicular growth, ovulation, as well as for luteal formation and regression. Even though studies are reported on proteinases in ovary, investigations carried out on aspartic proteinases (AP) are scarce. Objectives of this study were to analyze the AP activity in the porcine ovaries with cycling features (CO) and without cycling features (NCO) and to analyze the AP activity at different stages of follicular phase and luteal phase.

Materials and Methods: Porcine ovaries (200) were collected from a private abattoir. Forty ovaries were grouped in to CO (n=20) and NCO (n=20), based on the presence or absence of corpora lutea. Remaining ovaries were used to aspirate follicular fluid from different stages of the follicular phase and to prepare luteal extracts from different stages of the luteal phase. AP activity (U/ ml) was determined using denatured haemoglobin at pH 3. Specific activity (U/ mg) was determined after estimating the total protein concentration. Results were analyzed using T-test and ANOVA.

Results: CO showed a significantly higher (p<0.001) mean AP activity (65.70 U/ ml) and a specific activity (4.26 U/ mg) compared to that of NCO (6.40 U/ ml and 0.53 U/ mg respectively). FF showed low activity (≤3 U/ml) and very low specific activity (<0.05 U/mg). A remarkably high AP activity was detected at the luteal phase with statistically significant differences (p<0.001) in AP activity among the four different luteal stages. Mean AP activities of the luteal extracts of CH, Mid CL, ECA and LCA were 26.94, 59.71, 205.73 and 78.85 U/ ml respectively.

Conclusions: AP activity was significantly higher in CO compared to that of NCO. AP activity at different stages of the follicular and luteal phases varied significantly. A remarkably high AP activity was observed in ECA.

Key words: Ovary, porcine, Aspartic proteinases, Corpus luteum

INTRODUCTION

Every protein in all forms of life undergoes proteolysis. In addition to the involvement in degradation, proteinases have equally or more important roles as efficient processing enzymes of many bioactive mediators such as cytokines, growth factor binding proteins and their receptors1. Limited and specific processing of bioactive mediators can lead to profound alterations in the cell behavior.

The highly selective and limited cleavage of specific protein substrates by proteinases is critical for a number of essential biological processes such as remodeling extracellular matrix proteins, modulating growth factor actions and controlling the levels of receptor proteins. Therefore, proteinases are critical determinants of
cellular proliferation, differentiation, migration, tissue remodeling and homeostasis. Female reproductive tract is unique in that rapid and extensive tissue remodeling is required throughout each estrus cycle. Furthermore, mammalian ovary requires tightly regulated proteolytic activity for follicular growth, ovulation, as well as for luteal formation and regression, hence to have regular cycles. Most of the studies conducted on proteinases involved in ovary are on matrix metalloproteinases (MMPs). Matrix-degrading proteinases, such as MMPs and their tissue inhibitors, were postulated to play critical roles in the remodeling of the extracellular matrix throughout the ovarian cycle. The existence of autocrine/paracrine modulators such as insulin-like growth factors (IGFs) in the ovary has been well documented in many species including the pig. IGF binding proteins (IGFBPs) and proteinases regulating the IGFBP concentration were detected in ovaries of many species including pigs. However, studies conducted on aspartic proteinases in ovary are limited.

Aspartic proteinases (AP) belong to the group acid proteinases which has acidic pH optima. pH range of aspartic proteinases is rather low compared to that of other classes of proteinases, which lies between 1.5 and 5.0. We observed variations of the AP activity between different batches of ovaries while working on AP in porcine ovaries. Hence, we hypothesized that AP activity vary during the ovarian cycle.

**OBJECTIVES**

Objectives of this study were to analyze the AP activity in the porcine ovaries in absence and presence of cycling features and to analyze the AP activity at different stages of follicular and luteal phases.

**METHODOLOGY**

Ovaries (n=200) from 6-8 months old pigs were obtained from a private abattoir at the time of the slaughter, within a period of one month. All the precautions were taken to minimize the loss of proteinase activity, from the collection of samples to the final assays.

**Collection of ovarian extracts from ovaries with and without cycling features**

Twenty large ovaries (6.53-7.97 g) with corpora lutea (CL) were used as cycling ovaries (CO). Twenty small ovaries (1.18-1.83 g) without CL were used as non-cycling ovaries (NCO) for the study (Figure 1). Each ovary was weighed and homogenized separately at 4°C in phosphate buffered saline (pH 7.5) (2 ml/ g of ovary). The resulting extracts were centrifuged to separate debris and the supernatants were used for the assays.

**Collection of follicular fluid**

Follicles were grouped into three groups according to their diameter, as small (< 3 mm), medium (3-5 mm) and large (> 5 mm) (Figure 1). Follicular fluid (FF) was aspirated from them separately. FF from several small follicles and a few medium follicles was pooled separately in order to obtain sufficient volumes for the assays. Follicular cells present in the FF were separated by centrifugation and the supernatant was used for the AP assay. Number of FF samples in each category were, small follicles n=22, medium follicles n=25 and large follicles n=25.
**Figure 1:** Ovaries with or without cycling features
NCO: Ovaries without CL, CO: Ovaries with CL. Arrows show follicles of various sizes- small (<3 mm), medium (3-5 mm) and large (>5 mm).

**Preparation of luteal extracts**
Luteal structures from four different stages representing very early to latter stages of the luteal phase were identified based on their gross morphology\(^{11}\). Those were (from early to late luteal phase) corpus haemorrhagicum (CH) (n=18), mid luteal corpora lutea (Mid CL) (n=30), early corpus albicans (ECA) (n=30) and late corpus albicans (LCA) (n=30) (Figure 2). Corresponding luteal structures were incised and homogenized separately in 50 mM phosphate buffered saline (pH 7.5). Resulting extracts were centrifuged to remove debris and the supernatants were used for the assays.

**Figure 2:** Different stages of luteal phase and their extracts
Arrows show CL of various stages-CH: Corpus haemorrhagicum, Mid CL: Mid luteal corpora lutea, ECA: Early corpus albicans, LCA: Late corpus albicans. Bottom panel: Luteal extracts.
Measurement of acid proteinase activity

AP activity was determined using the method of Athauda et al., 12. In brief, two tests and two controls were carried out for each sample. Test was prepared with tissue extract or follicular fluid (50 µl), formate buffer at pH 3.0 (50 µl), 2% denatured haemoglobin (400 µl) and incubated at 37°C for 45 minutes. Reaction was terminated by adding 5% (w/v) trichloroacetic acid (800 µl). Test blanks were prepared by adding the extract after terminating the AP assay reaction. The reaction mixture was then centrifuged and the absorbance of the supernatant was measured at 280 nm. An increase in absorbance of 1.0 at 280 nm per hour per ml of sample was defined as one unit (U) of proteolytic activity. Specific activity was expressed as the activity per mg of total protein (U/mg).

Measurement of total protein concentration

Total protein concentration of each extract was determined by the standard Bradford method with bovine serum albumin (10 µg/ml) as the standard.13 The estimations were carried out in triplicate with ovarian samples diluted with phosphate buffer.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE under denaturing conditions was used to analyze the gross pattern of proteins in the extracts obtained from CO, NCO, and CL and FF (Enduro vertical mini gel electrophoresis system).

Standard method of Laemelli was used.14 Protein bands were detected using Coomassie brilliant blue.

Analysis of data

Data are presented as mean ± SD. Significance of the differences in activity and specific activity between the two groups of ovaries were determined using T-Test (Statistical package-Minitab, version-30). Significance of the differences of AP activity in the three follicular groups and in the four luteal groups was analyzed separately using ANOVA.

RESULTS

AP activity of the cycling and non cycling ovaries

AP activity (U/ml) and specific activity (U/mg) of the ovarian extracts obtained from CO and NCO are shown in table 1.

AP activity was detected in both CO and NCO. CO showed a significantly higher AP (p<0.001) activity and a specific activity (p<0.001) compared to that of NCO. On average a 10.3 fold higher AP activity and 8.0 fold higher specific activity were observed in CO, compared to that of NCO. Even though, AP activity was strikingly different between the two groups of ovaries, total protein concentrations and protein band patterns obtained with the SDS-PAGE were fairly similar (Table 1, Figure 3).

Table 1. Activity and the specific activity of AP in the two groups of ovaries

<table>
<thead>
<tr>
<th>Ovary type</th>
<th>Weight (g) Mean ± SD</th>
<th>AP Activity (U/ml) Mean ± SD</th>
<th>Total Protein (mg/ml) Mean ± SD</th>
<th>Specific AP activity (U/mg) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>7.14 ± 0.45</td>
<td>65.70 ± 14.54</td>
<td>15.45 ± 0.31</td>
<td>4.26 ± 0.97</td>
</tr>
<tr>
<td>NCO</td>
<td>1.50 ± 0.20</td>
<td>6.40 ± 0.32</td>
<td>12.92 ± 2.88</td>
<td>0.53 ± 0.18</td>
</tr>
</tbody>
</table>

CO: cycling ovaries (n=20)  NCO: Non cycling ovaries (n=20)
Figure 3: Gross pattern of the proteins of ovaries with or without cyclic features
NCO: ovaries without CL, CO: Ovaries with CL. SDS-PAGE was carried out with 12% gels. Approximate molecular weights (KDa) of protein bands are shown.

AP activity of follicular fluid from different stages of follicles
AP activity (U/ml) and specific activity (U/mg) of the FF of small, medium and large follicles are shown in table 2. Results indicate significantly higher (p < 0.001) AP activity in FF collected from small follicles compared to that of large follicles. Gross pattern of the proteins and the concentrations of proteins between FF obtained from small, medium and late follicles did not show a clear difference (Figure 4 and Table 2).

Figure 4: Gross pattern of the proteins of FF obtained from different stages of the follicular phase
S: Small follicles, M: Medium follicles, L: large follicles. SDS-PAGE was carried out with 12% gels. Approximate molecular weights (KDa) of protein bands are shown.

Table 2. Activity and the specific activity of AP in different stages of follicles

<table>
<thead>
<tr>
<th>Follicle size</th>
<th>AP Activity (U/ml) Mean ± SD</th>
<th>Total Protein (mg/ml) Mean ± SD</th>
<th>Specific AP activity (U/mg) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
<td>3.02 ± 0.58</td>
<td>62.69 ± 4.56</td>
<td>0.048 ± 0.03</td>
</tr>
<tr>
<td>Medium</td>
<td>2.02 ± 0.59</td>
<td>65.43 ± 3.47</td>
<td>0.031 ± 0.02</td>
</tr>
<tr>
<td>Large</td>
<td>1.84 ± 0.77</td>
<td>66.72 ± 6.52</td>
<td>0.028 ± 0.02</td>
</tr>
</tbody>
</table>

Small follicles (n=22), medium follicles (n=25) and large follicles (n=25) were used.

AP activity of luteal extracts from different stages of luteal phase
AP activity and specific activity (U/mg) of luteal extracts of CH, Mid CL, ECA and LCA are shown in table 3.

A remarkably high AP activity was detected at the luteal phase, even at the very early stages. Furthermore, statistically significant differences of the AP activity were found among the four different groups investigated (p< 0.001). Within the luteal phase, lowest AP activity was seen in the CH. In comparison to CH, 2.2 fold higher AP activity was observed at the mid luteal phase (Mid CL) and 7.6 fold higher AP activity was observed at the early regression phase (ECA).
Towards the later part of regression (LCA) there was a significant decline of AP activity compared to that of ECA. Concentrations of total proteins and the gross pattern of the proteins in four stages of CL are shown in Table 3 and Figure 5 respectively.

Table 3. Activity and the specific activity of AP in different stages of luteal phase

<table>
<thead>
<tr>
<th>Stage of Luteal phase</th>
<th>AP Activity (U/ml) Mean ± SD</th>
<th>Total Protein (mg/ml) Mean ± SD</th>
<th>Specific AP activity (U/mg) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH</td>
<td>26.94 ± 8.45</td>
<td>41.45 ± 4.56</td>
<td>0.65 ± 0.27</td>
</tr>
<tr>
<td>Mid CL</td>
<td>59.71 ± 11.89</td>
<td>16.05 ± 1.24</td>
<td>3.72 ± 0.88</td>
</tr>
<tr>
<td>ECA</td>
<td>205.73 ± 17.96</td>
<td>19.12 ± 2.45</td>
<td>10.76 ± 1.69</td>
</tr>
<tr>
<td>LCA</td>
<td>78.85 ± 28.93</td>
<td>9.95 ± 0.93</td>
<td>7.92 ± 1.71</td>
</tr>
</tbody>
</table>

CH: Corpus haemorrhagicum (n=18), Mid CL: Mid luteal corpora lutea (n=30), ECA: Early corpus albicans (n=30), LCA: Late corpus albicans (n=30)

Figure 5: Gross pattern of the proteins of CL extracts from different stages of the luteal phase

CH: Corpus haemorrhagicum, Mid CL: Mid luteal corpora lutea, ECA: Early corpus albicans, LCA: Late corpus albicans

Figure 5: Gross pattern of the proteins of CL extracts from different stages of the luteal phase

CH: Corpus haemorrhagicum, Mid CL: Mid luteal corpora lutea, ECA: Early corpus albicans, LCA: Late corpus albicans

DISCUSSION

Gross anatomical differences were observed in the ovarian structures in the two groups of ovaries used in this study, where CO were large and consisted of various stages of CL and developing follicles. NCO were small and did not consist of CL or large follicles, even though numerous small follicles were present.

Aspartic proteinases investigated in our study showed an optimum pH of 3.0 with lower activity up to pH 5.0 during the optimization of the AP activity assay (results not shown). Acidic microenvironments required for AP activity have been demonstrated in cells and tissues such as bone, stomach, hepatocytes, inflammatory sites, secretary granules and tumors. Several mechanisms contributing to an acidic microenvironment were identified in tissues including ovary. Local acidification needed for the activity of well known AP, cathepsin D was found to be mainly driven by the Na⁺/H⁺ exchanger family and H⁺/ATPase. Hypovascularity, hypoxia and anaerobic glycolysis were observed in the ovary, during late folliculogenesis and early CL development. Vasoconstriction, detachment of vascular endothelial cells, and occlusion of blood vessels are early events of CL regression which results in a diminished oxygen supply to the luteal parenchyma and lactic acid accumulation. H⁺-pumps, which actively extrude H⁺, are present and active in endothelial cells and macrophages which are predominant cell types in the CL.

According to the results of our study, the AP activity was markedly higher in the CO even though, the total protein concentration of the two groups of ovaries was quite similar. We postulate that AP activity increases in the ovary, after attaining estrous cycling. This increase may be due to increased AP activity in CL or the larger follicles or both as those structures were found only in CO. However, all CO did not consist of large follicles even though all those ovaries had CL of one or several stages, suggesting that the likely source of high AP activity found in CO is the CL.
In the second part of the study, we observed a very high AP activity during the luteal phase, compared to that of follicular phase. This increase was highest in the early regression (ECA). Therefore CL seems to be contributing to the majority of increase in AP activity found in CO. Erdmann et al., demonstrated a similar increase in cathepsin D levels between early and late luteal stages in the bovine CL.

Even though, AP activity in the FF was lower, there was detectable activity and these proteinases may have a regulatory role on follicular functions. Comparatively high AP activity observed in the FF of small follicles may have come from a general increase occurring during early stages of follicles or an increase seen in a group of follicles which are in a different destination, such as growth or atresia. We have not analyzed the AP activity in follicular cells. Protein concentrations in FF were comparable with the findings of the other studies and were higher than those of CL extracts.

The development and regression of CL is characterized by intense angiogenesis, angioregression and extracellular matrix remodeling. Extracellular matrix provides an environment for cell migration, division, differentiation and anchorage. Turnover of extracellular matrix is regulated by proteases and the role of MMPs and their inhibitors in the ovary is demonstrated. Cyclic changes observed in MMP activity during luteal phase of different species have been reviewed.

Aspartic proteinases are very likely to play an important role in regulating ovarian functions and most probably the luteal function. We postulate that these functions may include regulation of blood supply, modulation of concentration of growth factors, degradation of proteins and regulation of apoptosis as those are some of the known functions of the well known AP, cathepsin D in tissues including bovine CL.

CL is one of the few organs in the adult body that exhibits extensive angiogenesis and angioregression under physiological conditions. Breakdown of blood vessels and loss of endothelial cells are inherent features of CL regression. Five to ten grams of luteal tissue grow and disappear at each ovarian cycle in sow. Increase synthesis of cathepsin D and its release by lysosomes occur in many instances of cell destruction.

Release of angiogenic fragments by cathepsin mediated processing of prolactin was demonstrated in variety of tissues of mice. Ciereszko et al., showed the stimulation of progesterone production in porcine early corpora lutea in vitro by prolactin. Expression of prolactin and their receptors in bovine CL was demonstrated previously. Erdmann et al., demonstrated the product of cathepsin mediated prolactin cleavage inhibiting the growth of CL-derived endothelial cells. These findings suggest the involvement of cathepsin D mediated fragmentation of prolactin in angioregression during luteolysis. Role of cathepsin D in apoptosis was demonstrated in fibroblasts and endothelial cells.

Whether the AP activity detected in the present study is due to cathepsin D or a different AP need to be investigated. Further studies are necessary to identify the role of AP in porcine ovary. Based on the published information and marked increases in AP activity observed in our study during early regression of CL, we postulate that AP activity increased during this period, activates angioregression and apoptosis of the CL.

CONCLUSION
AP activity was significantly higher in CO, compared to that of NCO. AP activities at different stages of the follicular and luteal phases of the porcine ovarian cycle vary significantly. A remarkably high AP activity was observed towards early regression phase of the corpus luteum. Further studies are necessary to clarify the role of AP in the ovary.

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