Changes in follicular fluid steroids, insulin-like growth factors (IGF) and IGF-binding protein concentration, and proteolytic activity during equine follicular development


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ABSTRACT: The objective of the present study was to evaluate changes in equine follicular fluid insulin-like growth factor binding protein (IGFBP) proteolytic activity as well as steroid, IGF, and IGFBP concentrations during follicular development in the mare. Mares (n = 14) were classified as either in the follicular phase (n = 8) or luteal phase (n = 6). Follicles (n = 92) were categorized as small (6 to 15 mm; n = 54), medium (16 to 25 mm; n = 23), or large (> 25 mm; n = 15), and follicular fluid was collected. Estradiol and androstenedione levels in follicular fluid were greater (P < 0.05), and IGFBP-3 concentrations tended to be greater (P < 0.10) in large than in small or medium follicles, whereas IGFBP-2, -4, and -5 levels were less (P < 0.05) in large than in small or medium follicles. Estradiol and androstenedione concentrations were negatively correlated (P < 0.01) with IGFBP-2, -4, and -5 but not IGFBP-3 concentrations. To evaluate proteolysis of IGFBP, follicular fluid was incubated with human ¹²⁵I-labeled IGFBP-2, -3, and -5 and protein separated by 12% SDS-PAGE. Follicular fluid caused little or no proteolysis of ¹²⁵I-labeled IGFBP-2 or -3, and the small amount of proteolysis of IGFBP-2 and -3 did not differ (P > 0.10) among follicle classes. However, more ¹²⁵I-labeled IGFBP-5 was cleaved (P < 0.05) by follicular fluid from large follicles collected during the follicular phase than large follicles during the luteal phase, and small or medium follicles from follicular and luteal phase mares indicating that a protease to IGFBP-5 exists in estrogen-dominant equine follicles. This IGFBP-5 protease was inhibited by kallikrein/serine protease and metalloprotease inhibitors. We conclude that the tendency of estrogen-dominant follicles of mares to have greater levels of IGFBP-3 and lesser levels of IGFBP-2 does not appear to be due to differences in proteolysis, whereas changes in IGFBP-5 levels are likely due to changes in activity of a serine protease or metalloprotease. Changes in IGFBP may alter levels of bioavailable IGF that stimulate steroidogenesis and mitogenesis in developing mare follicles.

Key Words: Follicles, Insulin-like Growth Factor, Mares

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and/or proteolysis (Rajarm et al., 1997). During follicular growth in sheep (Besnard et al., 1996), swine (Besnard et al., 1997), and cattle (Spicer et al., 2001), proteolytic activity for IGFBP-4 and -5 increases dramatically with little or no change in proteolytic activity for IGFBP-3. Currently, exiguous information is available concerning the intrafollicular level of IGFBP and IGFBP proteolytic activity during folliculogenesis of the mare. We hypothesize that, in the mare, the largest follicle present during the follicular phase will contain greater IGFBP proteolytic activity and less amounts of IGFBP than the largest follicle present during the luteal phase. Therefore, the objective of this study was to determine if IGFBP proteolysis as well as steroid and IGFBP levels in follicular fluid change during follicular development in the mare.

**Materials and Methods**

**Biological Material**

Follicular fluid from follicles (n = 92) of 14 cyclic mares was collected separately using needles and syringes at an abattoir and centrifuged (200 × g for 5 to 7 min) to remove granulosa cells (Langhout et al., 1991), and follicular fluid was stored at −20°C. The mares were classified as either in the follicular (n = 8) or luteal (n = 6) phase based on gross ovarian morphology; ovaries with a viable (vasculature visible) corpus luteum were classified as being in the follicular phase (Ireland et al., 1980). The purpose of comparing luteal vs follicular phase follicles was to control for the physiologic state of the mare and to compare large atretic (luteal) vs large dominant (follicular) follicles (Gastal et al., 1997). External diameters of follicles were measured to categorize them as being small (6 to 15 mm), medium (16 to 25 mm), or large (> 25 mm) (Draincourt and Palmer, 1984).

**Radioimmunoassays (RIA)**

Concentrations of estradiol in follicular fluid were determined by RIA as previously described (Spicer and Enright, 1991). The intra- and interassay CV were 12.8 and 21.1%, respectively. Concentrations of androstenedione in follicular fluid were determined using a solid-phase RIA kit (ICN Biomedicals, Costa Mesa, CA) as previously described (Stewart et al., 1996). The intraassay CV was 15%. Concentrations of progesterone and estradiol-β in follicular fluid were determined by RIA as previously described (Spicer and Enright, 1991). The intra- and interassay CV were 13.3 and 6.7%, respectively. Concentrations of IGF-I in follicular fluid were determined by RIA as previously described (Spicer et al., 1981). Recombinant human IGFBP-3 (47,000 molecular weight) and IGFBP-2 (31,000 molecular weight) was obtained from Austral Biologicals (San Ramon, CA), and recombinant human IGFBP-5 (30,000 molecular weight) was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Two micrograms of IGFBP-2, -3, and -5 bands were scanned, and the resultant arbitrary densitometric units (ADU) for each IGFBP was used to calculate intergel CV, which averaged 28 ± 2% for eight gels.

**Insulin-Like Growth Factor Binding Protein Iodination**

The IGFBP in follicular fluid were analyzed by one-dimensional SDS-PAGE, as previously described (Stewart et al., 1996; Simpson et al., 1997). Briefly, 4 µL of follicular fluid was mixed with 21 µL of nonreducing denaturation buffer (BIORAD, Hercules, CA). The samples were heat-denatured (3 min at 100°C), subsequently centrifuged at 4657 × g for 3 min, and separated using 12% PAGE (12 or 15 lanes per gel) for about 18 to 20 h, at a constant current and varying voltage. Follicular fluid samples from two or three mares were run on each gel, and each gel contained samples from at least one follicular and luteal phase mare. Four gels were run at a time in a single electrophoresis chamber. In addition to follicular fluid samples, colored molecular mass markers (Sigma Chemical Co., St. Louis, MO) and 4 µL of bovine follicular fluid were used to identify band size and specific IGFBP. After separation, proteins in the gels were electrophoretically transferred to nitrocellulose paper (Midwest Scientific, St. Louis, MO) for 2.5 to 3.0 h and ligand-blotted overnight with 125I-IGF-II. Tris-buffered saline with 0.1% Tween was used to wash the nitrocellulose blots. The nitrocellulose blots were dried and exposed to X-ray film at −70°C for 12 d. X-ray films were then developed, and band intensity on autoradiographs was determined using scanning densitometry with a Molecular Analyst (BIORAD). Variation among gels was monitored via running the same mare follicular fluid pool on each gel. The IGFBP-2, -3, and -5 bands were scanned, and the resultant arbitrary densitometric units (ADU) for each IGFBP was used to calculate intergel CV, which averaged 28 ± 2% for eight gels.

**Insulin-Like Growth Factor Binding Protein Proteolysis**

A subset of 36 of 92 follicles were selected for IGFBP proteolysis assay based on: 1) the follicle with the greatest estradiol-17β concentration as well as the largest...
follicle was selected from each mare and 2) one small and one medium follicle, if present, was randomly selected from each mare. In addition to testing for differences in estrous cycle phase, size of follicle and high-vs low-estrogen follicles, this subset of follicles was selected in order to be able to run all samples at the same time (four gels) in the same electrophoresis chamber and hence minimize variation among gels. Each sample of follicular fluid (6 µL) was incubated at 37°C for 5 h with 60,000 cpm of either recombinant human 125I-labeled IGFBP-2, -3, or -5 in 20 µL of medium (Dulbecco’s modified Eagle medium: Ham’s F12, 1:1). For each IGFBP, all follicular fluid samples from three to four mares were placed in a single gel so that samples from all 14 mares were run in four gels at the same time in the same electrophoresis chamber. Proteolytic fragments of the various 125I-labeled IGFBP were separated by SDS-PAGE. Prior to electrophoresis, samples (26 µL plus 26 µL of nonreducing denaturation buffer) were boiled for 3 min to denature proteins and then added to the wells in the gel. In addition to samples, colored molecular mass markers (Sigma Chemical Co.) were used to identify band size. Gels were dried on a vacuum and exposed to X-ray film for 48 h. Amounts of the resultant breakdown products were quantified using scanning densitometry with a Molecular Analyst (BIORAD) and expressed as ADU. Because no discernible breakdown products were detected for 125I-IGFBP-2 and -3, degradation was expressed relative to control 125I-IGFBP-2 and -3 samples incubated without follicular fluid using the following equation: 100 – [(ADU of 125I-IGFBP-2 or -3 incubated with follicular fluid sample/ADU of 125I-IGFBP-2 or -3 incubated without follicular fluid) × 100].

Protease Inhibitors to Insulin-Like Growth Factor Binding Protein Degradation

Inhibition of follicular fluid protease activity to IGFBP was evaluated by incubating 60,000 cpm of recombinant human 125I-labeled IGFBP-5 in medium (20 µL, Dulbecco’s modified Eagle medium: Ham’s F12, 1:1) with 6 µL of follicular fluid (pooled from estrogen-active follicular-phase mare follicles) and 0, 0.01, or 0.10 mM of aprotinin (a kallikrein and serine protease inhibitor), 0.10 or 1.0 mM of either phenylmethylene-sulfonyl fluoride (PMSF) (a serine, cysteine, and thiolprotease inhibitor) (Boehringer Mannheim, Indianapolis, IN), 1,10-phenanthroline (a metalloprotease inhibitor), or doxycycline (a metalloprotease inhibitor), 0.001, 0.01, or 0.10 mM of cyclohexylacetyl-phe-arg-ser-val-gln amide (CAA) (a serine protease inhibitor) or 0.10, 1.0, or 5.0 mM of EDTA (a metalloprotease inhibitor) (Sigma Chemical Co.) for 5 h at 37°C. Each treatment was replicated two to three times. After the 5-h incubation, samples were separated by 12% SDS-PAGE, subjected to autoradiography, and resultant bands scanned as previously described. A total of four gels were run, and each gel contained one or more replicates of each treatment. Data (ADU of breakdown products) were expressed as percentage of control incubations in the absence of protease inhibitors.

Statistical Analyses

Follicular fluid hormone and IGFBP data as well as proteolysis data were analyzed using least squares ANOVA by the General Linear Models procedure of SAS (SAS Inst. Inc., Cary, NC). Main effects, consisting of follicle size (small, medium, large) and follicle phase (follicular, luteal) and their various interactions were analyzed (Ott, 1977). Data exhibiting heterogeneous variance (i.e., follicular fluid estradiol, 125 to 135-kDa, and 90 to 96-kDa proteins) were analyzed after transformation to ln (x + 1). Means were compared using Fisher’s protected LSD mean test if significant main effects were observed (Ott, 1977). Relationships among variables measured were evaluated by simple correlation analysis (Pearson correlation coefficients) (Ott, 1977). Means are presented as least squares means ± SE.

Results

A significant (P < 0.0001) main effect of follicle size, but not phase or size × phase interaction (P > 0.10) was observed with follicle diameter. Diameters averaged 33 ±1.0 mm, 17.8 ±0.70 mm, and 11.4 ± 0.48 mm, for large, medium, and small follicles, respectively.

Follicle size, phase of estrous cycle, or their interaction did not affect (P > 0.10) follicular fluid progesterone levels. Progesterone concentrations in follicular fluid averaged 33.4 ± 8.1 ng/mL, 23.8 ± 5.7 ng/mL and 30.7 ± 3.5 ng/mL, for small, medium, and large follicles, respectively.

Follicle size × phase interaction affected (P < 0.005) follicular fluid estradiol levels. Main effects of follicle size and phase were also significant (P < 0.005). Large, follicular phase follicles (1,425.1 ± 114.2 ng/mL) contained 3- to 72-fold greater (P < 0.05) levels of estradiol than large follicles in the luteal phase (473.3 ± 228.4 ng/mL), small follicles in the follicular (46.3 ± 77.6 ng/mL) or luteal phase (19.9 ± 74.8 ng/mL), and medium follicles in either the follicular (41.2 ± 98.9 ng/mL) or luteal phase (84.6 ± 149.5 ng/mL). Mean estradiol concentrations for small, medium, and large follicles are shown in Figure 1A.

A significant (P < 0.0005) main effect of follicle size, but not phase or size × phase interaction (P > 0.10) was observed for follicular fluid androstenedione levels. Large follicles had six- to eight-fold greater (P < 0.05) concentrations of androstenedione than small or medium follicles (Figure 1B).

Follicle size affected (P < 0.01) follicular fluid IGF-I concentrations, whereas phase of estrous cycle and size × phase had no effect (P > 0.10) on follicular fluid IGF-I concentrations. Large follicles contained 78 to 90% more (P < 0.05) IGF-I than did small or medium follicles.
Follicular fluid concentrations of estradiol (Panel A), androstenedione (Panel B), and insulin-like growth factor-I (Panel C) collected from small (6 to 15 mm; \( n = 54 \)), medium (16 to 25 mm; \( n = 23 \)), or large (>25 mm; \( n = 15 \)) follicles in mares.\(^{a,b}\)Within a panel, means without a common superscript differ \( (P < 0.05) \).

(Figure 1C). Phase of estrous cycle tended to affect \( (P < 0.10) \), whereas size and size × phase had no significant effect on follicular fluid IGF-II levels. Follicular phase follicles \( (67.7 \pm 3.0 \text{ ng/mL}) \) tended to have greater \( (P < 0.10) \) amounts of IGF-II than did luteal phase follicles \( (57.0 \pm 5.0 \text{ ng/mL}) \). To assess the relative proportion of IGF to IGFBP in follicular fluid, a ratio was calculated using the total amount of IGF-I and -II (sum of follicular fluid IGF-I and -II) divided by the total amount of IGFBP (sum of all ADU of IGFBP). This ratio measuring relative proportion of IGF to IGFBP levels was affected \( (P < 0.05) \) by size × phase of cycle such that large follicular phase follicles \( (1.96 \pm 0.23) \) had twofold \( (P < 0.05) \) greater relative proportion of IGF to IGFBP levels than did large luteal phase follicles \( (0.96 \pm 0.42) \) or medium \( (0.97 \pm 0.18) \) or small \( (0.89 \pm 0.13) \) luteal or follicular phase follicles.

Ligand blotting with \(^{125}\text{I}\) IGF-II revealed at least six forms of IGFBP present in follicular fluid: 125 to 135 kDa, 115 kDa, 90 to 96 kDa, 40 to 44 kDa (IGFBP-3), 34 kDa (IGFBP-2), 27 to 29 kDa (IGFBP-5), and 20 to 22 kDa (IGFBP-4) (Figure 2). Follicle size \( (P < 0.01) \) but not phase of cycle or size × phase interaction \( (P > 0.10) \) affected IGFBP-2 levels in follicular fluid. Concentrations of IGFBP-2 in small and medium follicles were 1.8- and 1.9-fold greater \( (P < 0.05) \) than in large follicles (Figure 3A). The interaction between follicle size × phase on follicular fluid IGFBP-3 levels approached significance \( (P < 0.10) \) (Figure 3B). Large luteal phase...
follicles had 60% greater ($P < 0.05$) IGFBP-3 concentrations than did large follicular phase follicles, and had 48, 57, and 60% greater ($P < 0.05$) concentrations of IGFBP-3 than did medium luteal, small follicular, and small luteal phase follicles, respectively (Figure 3B).

Follicle size × phase interaction affected ($P < 0.05$) IGFBP-4 concentrations in follicular fluid (Figure 3C). Concentrations of IGFBP-4 were 3.0- and 2.6-fold greater ($P < 0.05$) in small follicular phase and small luteal phase follicles than in both large follicular phase and medium luteal phase follicles, respectively. Medium follicular phase follicles had 3.3-fold greater ($P < 0.05$) concentrations of IGFBP-4 than both medium luteal phase and large follicular phase follicles (Figure 3C).

Follicle size × phase interaction affected ($P < 0.05$) IGFBP-5 concentrations in follicular fluid (Figure 3D). Main effect of follicle size was also significant ($P < 0.005$). Concentrations of IGFBP-5 in medium and small follicular phase follicles, and small luteal phase follicles were 5-, 4.1-, and 4-fold greater ($P < 0.05$) than in large follicular phase follicles, respectively. Concentrations of IGFBP-5 were 2.3- and 2.1-fold greater ($P < 0.05$) in medium follicular phase follicles than large luteal phase and medium luteal phase follicles, respectively (Figure 3D).

Follicle size tended ($P < 0.10$) to affect levels of the 115-kDa protein that bound $^{125}$I-IGF-II in follicular fluid. Small (0.69 ± 0.09 ADU) and medium (0.81 ± 0.14 ADU) follicles had 3.1- and 3.6-fold greater ($P < 0.05$) concentrations of the 115-kDa protein, respectively, than did large (0.23 ± 0.20 ADU) follicles. The 135 and 125-kDa proteins that bound $^{125}$I-IGF-II were arithmetically combined for analysis and labeled as 125 to 135-kDa protein. Follicle size × phase interaction affected ($P < 0.05$) 125 to 135-kDa protein concentrations in follicular fluid. Main effects of follicle size were also significant ($P < 0.05$). Medium follicular phase follicles (7.2 ± 1.06 ADU) had 1.9- and 2.6-fold greater ($P < 0.05$) concentrations of 125 to 135-kDa protein than did large follicular phase (3.7 ± 1.23 ADU) and small follicular phase follicles (2.6 ± 1.23 ADU). Medium follicular and luteal phase follicles (3.1 ± 1.39 ADU) as well as small luteal phase follicles (3.2 ± 1.22 ADU) had three- to several fold greater ($P < 0.05$) concentrations of 125 to 135-kDa protein than did large luteal phase follicles (0.6 ± 2.5 ADU). Large follicular phase (3.7 ± 1.3 ADU)
and large luteal phase (0.6 ± 2.5 ADU) follicles did not differ (P > 0.10).

The 96 and 90-kDa proteins that bound 125I-IGF-II were combined for analysis and labeled as 90 to 96-kDa protein. Follicle size (P < 0.05) and size × phase interaction (P < 0.10) affected the 90 to 96-kDa protein concentrations in follicular fluid. Medium follicular phase follicles (11.9 ± 2.4 ADU) had 3.2- and 2.6-fold greater (P < 0.05) concentration of 90 to 96-kDa protein than large luteal phase follicles (1.3 ± 5.5 ADU) and small follicular phase follicles (2.9 ± 2.1 ADU), respectively. Medium luteal phase follicles (10.2 ± 3.6 ADU) had 2.6-fold greater (P < 0.05) concentrations of the 90 to 96-kDa protein than did small follicular phase follicles (2.9 ± 2.1 ADU). Small luteal phase follicles (7.8 ± 2.0 ADU) had 2.1-fold greater (P < 0.05) concentrations of the 90 to 96-kDa protein than did small follicular phase follicles (2.9 ± 2.1 ADU). Large follicular phase (7.1 ± 2.9 ADU) vs large luteal phase (1.3 ± 5.5 ADU) follicles did not differ (P > 0.10).

Amount of 125I-labeled IGFBP-2 degraded (expressed relative to the control intact 125I-IGFBP-2 band after incubation without follicular fluid; large, 23.7 ± 4.2%; medium, 23.1 ± 4.6%; and small, 32.8 ± 3.0%) after 5 h incubation was not affected (P > 0.10) by follicle size, phase of cycle, or follicle size × phase interaction (Figure 4). Similarly, amount of 125I-labeled IGFBP-3 degraded (large, 18.4 ± 5.2%; medium, 21.2 ± 5.6%; and small, 25.4 ± 3.7%) was not affected (P > 0.10) by follicle size, phase of cycle, or follicle size × phase interaction. However, 125I-labeled IGFBP-5 was cleaved more by follicular fluid from large follicles collected during the follicular phase than during the luteal phase (Figure 5B). Intact IGFBP-5 migrated at 30 kDa, and breakdown products migrated at ca. 19, 16, and 14.5 kDa (Figure 5A). Because all three of the breakdown products (i.e., 19, 16, and 14.5 kDa) were influenced by follicle size and phase in a similar way, all three breakdown products were arithmetically combined, labeled as IGFBP-5 proteolytic activity and expressed as ADU per 6 μL (Figure 5B). A significant (P < 0.05) interaction was observed between follicle size and phase on the breakdown products of 125I-IGFBP-5 (Figure 5B). Large follicular phase follicles had two- to fourfold greater (P < 0.05) amounts of the 14.5- to 19-kDa breakdown bands than did all other follicle classes (Figure 5B).

Serine protease inhibitors CAA and aprotinin inhibited (P < 0.01) IGFBP-5 protease activity (Figure 6A). At 0.01 mM and 0.10 mM, CAA caused greater (P < 0.01) inhibition of IGFBP-5 protease activity than at 0.001 mM (Figure 6A). Also, PMSF at 1.0 mM had little or no effect on IGFBP-5 protease activity (Figure 6A). Aprotinin inhibited IGFBP-5 protease activity to an extent similar to that of CAA; the inhibition caused by 0.01 and 0.10 mM of aprotinin did not differ (P > 0.10) and 0.01 mM of CAA inhibited IGFBP-5 protease more effectively than 0.01 mM of aprotinin or PMSF (Figure 6A).

The metalloprotease inhibitors doxycycline, EDTA, and 1,10-phenanthroline inhibited (P < 0.01) IGFBP-5 protease activity (Figure 6B). Doxycycline at 1.0 mM caused greater (P < 0.01) inhibition of IGFBP-5 protease activity than at 0.10 mM, and at 1.0 mM, doxycycline inhibited IGFBP-5 protease more effectively than 1, 10-phenanthroline or EDTA (Figure 6B). Only 5.0 mM EDTA inhibited (P < 0.01) IGFBP-5 protease activity (Figure 6B). 1,10-Phenanthroline caused greater (P < 0.01) inhibition of IGFBP-5 protease activity at 1.0 mM than at 0.10 mM, and at 1.0 mM, 1, 10-phenanthroline inhibited IGFBP-5 protease more effectively than EDTA (Figure 6B).

Concentrations of estradiol-17β, androstenedione, IGF-I, and IGF-II in follicular fluid were negatively correlated (P < 0.05) with levels of IGFBP-2 and -5 but not with (P > 0.10) IGFBP-3 levels (Table 1). Levels of IGFBP-4 were negatively correlated (P < 0.05) with only follicular fluid estradiol-17β and IGF-I. Concentrations...
Figure 5. Panel A: Representative autoradiograph measuring proteolytic activity of mare follicular fluid to recombinant human $^{125}$I-labeled insulin-like growth factor binding protein (IGFBP)-5. Mare 8 was in the follicular phase, and mares 4, 9, and 10 were in the luteal phase. mm = diameter of follicles; C = control incubation of $^{125}$I-IGFBP-2 or -3 without follicular fluid; E$_2$ = estradiol concentration in follicular fluid. Note the association of estrogenic activity in the 30-mm follicle of mare 8 with degradation of $^{125}$I-IGFBP-5. Panel B: Mare follicular fluid protease activity to $^{125}$I-IGFBP-5 as measured by the amount of breakdown products expressed in arbitrary densitometric units per 6 µL of follicular fluid. Follicles were classified as small (6 to 15 mm; n = 17), medium (16 to 25 mm; n = 8) and large (>25 mm; n = 11). Interaction of size by group ($P < 0.05$). a,b Within a panel, means without a common superscript differ ($P < 0.05$).

of progesterone in follicular fluid were negatively correlated ($P < 0.05$) with IGFBP-3 and -5 but not IGFBP-2 or -4 in follicular fluid (Table 1). Follicle size (diameter) was negatively correlated ($P < 0.05$) with IGFBP-2 ($r = -0.40$), IGFBP-4 ($r = -0.27$), IGFBP-5 ($r = -0.43$) and positively correlated ($P < 0.05$) with estradiol-17$\beta$ ($r = 0.73$), androstenedione ($r = 0.52$), and IGF-I ($r = 0.49$). Concentrations of estradiol-17$\beta$ in follicular fluid were positively correlated with IGF-I ($r = 0.49$; $P < 0.0001$) and IGF-II ($r = 0.35$; $P < 0.01$).

Amount of IGFBP-5 proteolysis was positively correlated with follicular fluid estradiol-17$\beta$ concentrations ($P < 0.01$) and to a lesser extent ($P < 0.05$) with follicular

Figure 6. Inhibition of equine follicular fluid insulin-like growth factor binding protein (IGFBP)-5 protease activity. Pooled follicular fluid (6 µL) from large follicular phase mares was incubated for 5 h at 25°C with $^{125}$I-IGFBP-5 in the absence or presence of various amounts of protease inhibitors. Resultant breakdown products were separated by 12% PAGE and quantified. Data are expressed as percentage of control incubations in the absence of protease inhibitors. Panel A: serine protease inhibitors cyclohexylacetyl-phe-arg-ser-val-gln amide (CAA; black bars), phenylmethane-sulfonyl fluoride (PMSF; hatched bars), and aprotinin (open bars). Panel B: metalloprotease inhibitors doxycycline (black bars), EDTA (hatched bars), and 1,10-phenanthroline (1,10-Phen; open bars). a,b,c Within a panel, means without a common superscript differ ($P < 0.05$). Each mean represents two to three replicates.
fluid IGF-I and IGF-II concentrations and follicular diameter (Table 1). Amount of $^{125}$I-IGFBP-5 proteolytic activity was negatively correlated with IGFBP-2 ($r = -0.47; P < 0.05$) and IGFBP-5 ($r = -0.51, P < 0.01$) but not ($P > 0.05$) with IGFBP-3 ($r = -0.27$) or -4 ($r = -0.33$).

**Discussion**

Results of the present study revealed that: 1) more $^{125}$I-labeled IGFBP-5 was cleaved by equine follicular fluid from large follicles collected during the follicular phase than during the luteal phase, whereas proteolysis of $^{125}$I-labeled IGFBP-2 and -3 did not vary among follicle classes; 2) the amount of IGFBP-5 proteolysis was positively correlated with follicular fluid estradiol-17$\beta$, androstenedione, IGF-I, and IGF-II concentrations; 3) follicular fluid IGFBP-2, -4, -5 and a 125 to 135-kDa protein were less in large than in small or medium follicles, particularly during the follicular phase; 4) follicular fluid IGF-I, estradiol-17$\beta$ and androstenedione concentrations were greater in large than in small or medium follicles, whereas follicular fluid IGF-II concentrations tended to be greater in follicular phase vs luteal phase follicles; and 5) metalloprotease and serine protease inhibitors significantly reduced IGFBP-5 protease activity in a dose-dependent fashion.

For the first time, proteolysis of IGFBP-5 in mare follicular fluid has been evaluated. In particular, we found evidence for increased IGFBP-5 proteolysis in large follicular phase vs luteal phase follicles with no change in IGFBP-2 or -3 proteolysis with follicle size or estrous cycle stage. Previously, evidence for proteolysis of IGFBP-5 has been found in bovine (Stanko et al., 1994; Spicer et al., 2001), ovine (Besnard et al., 1996; Monget et al., 1996), and porcine (Besnard et al., 1997) follicular fluid. Specifically, IGFBP-5 proteolytic activity is greater (Spicer et al., 2001), and IGFBP-5 levels are less (Stewart et al., 1996; de la Sota et al., 1996; Funston et al., 1996) in follicular fluid of dominant follicles than in subordinate large or small follicles from cattle. Also in agreement with the present study, intrafollicular IGFBP-5 concentrations were higher in large nonovulatory and subordinate/atretic follicles of mares (Gerard and Monget, 1998). Previously, FSH has been reported to regulate IGFBP production and proteolysis by rat granulosa cells (Adashi et al., 1990; 1991; Fielder et al., 1993; Liu et al., 1993). Thus, changes in intraovarian levels of IGFBP-5 may be regulated by changes in local synthesis as well as proteolysis of IGFBP-5. Rat granulosa-cell IGFBP-5 protease is inhibited by IGF-I and IGF-II (Fielder et al., 1993; Liu et al., 1993). Because IGFBP-5 protease was positively correlated ($r = 0.4$ to $0.5$) with follicular fluid concentrations of IGF-I and -II, it is unlikely that IGF-I or -II inhibits this IGFBP-5 protease activity in mare follicles. Also, proteolysis of $^{125}$I-IGFBP-5 was positively correlated with follicular fluid estradiol-17$\beta$ ($r = 0.5$ to $0.7$) and androstenedione ($r = 0.2$ to $0.4$) levels in the present study. Whether IGF-I and -II directly activate IGFBP-5 protease activity in mare follicles or whether the effects are indirect via IGF-I induction of steroidogenesis (Spicer and Echternkamp, 1995) will require further study.

Consistent with the present study, others have reported IGFBP-2 levels are greater in small than in medium or large follicles in sheep (Spicer et al., 1995), pigs (Mondschein et al., 1991; Echternkamp et al., 1994b), and cattle (Echternkamp et al., 1994a; Stewart et al., 1996). Dominant follicles of cattle (Stewart et al., 1996; de la Sota et al., 1996; Funston et al., 1996) and mares (Gerard and Monget, 1998) contain less IGFBP-2 in follicular fluid than subordinate follicles, and atretic follicles contain more IGFBP-2 than do healthy follicles in sheep (Monget et al., 1993), cattle (Echternkamp et al., 1994a), and women (San Roman and Magoffin, 1993). Evidence of proteolysis of IGFBP-2 by follicular fluid has been reported for cattle (Stanko et al., 1994; Spicer et al., 2001), sheep (Besnard et al., 1996), and pigs (Besnard et al., 1997). We found that the amount of follicular fluid proteolysis of IGFBP-2 averaged less than $30\%$ and did not significantly differ among various mare follicles, and thus changes in IGFBP-2 levels are

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<th>Follicular Fluida</th>
<th>IGFβ-5 Protease Activityb</th>
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<tr>
<td>Diameter</td>
<td>$-0.40^{**}$ 0.18</td>
<td>$-0.27^{<strong>}$ $-0.43^{</strong>}$ 0.50$^*$</td>
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<tr>
<td>Estradiol-17$\beta$</td>
<td>$-0.46^{**}$ 0.01</td>
<td>$-0.26^{*}$ $-0.42^{<strong>}$ 0.63$^{</strong>}$</td>
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<td>Androstenedione</td>
<td>0.37$^{**}$ 0.02</td>
<td>$-0.20$ $-0.32^{**}$ 0.29</td>
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<tr>
<td>Progesterone</td>
<td>$-0.18$ $-0.24^{*}$</td>
<td>$-0.20$ $-0.33^{**}$ 0.21</td>
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<tr>
<td>IGF-I</td>
<td>$-0.73^{**}$ $-0.06$</td>
<td>$-0.40^{<strong>}$ $-0.37^{</strong>}$ 0.47$^*$</td>
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<tr>
<td>IGF-II</td>
<td>$-0.29^{**}$ $-0.16$</td>
<td>$-0.009$ $-0.24^{<em>}$ 0.46$^</em>$</td>
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Table 1. Simple Pierson correlation coefficients among follicle diameter, levels of insulin-like growth factor binding protein (IGFBP)-5 protease activity, estradiol-17$\beta$, androstenedione, progesterone, IGF-I, IGF-II, IGFBP-2, -3, -4, and -5 in equine follicular fluid.

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$^a$Follicular fluid IGFBP-2, -3, -4 and -5 correlations are based on n = 92.

$^b$IGFBP-5 protease activity correlations are based on n = 36.

$^*P < 0.05$

$^{**}P < 0.01$
likely a result of changes in local production of IGFBP-2 within mare follicles. This later notion awaits verification. Using this same proteolysis assay, we were able to detect IGFBP-2 proteolysis by pig uterine flushings (Geisert et al., 2001), indicating that our 125I-IGFBP-2 probe is capable of being proteolized. Because follicular fluid levels of estradiol-17β, androstenedione, IGF-I, and IGF-II were negatively correlated with follicular fluid IGFBP-2 levels in the present study, further research should focus on the role these hormones play in regulating follicular IGFBP-2 production in the mare.

Similar to the present study, IGFBP-3 was found to be the major IGFBP in follicular fluid of pigs (Howard and Ford, 1992; Echternkamp et al., 1994b), humans (Cataldo and Giudice, 1992; San Roman and Magoffin, 1993), and cattle (Echternkamp et al., 1994a; Funston et al., 1996; Stewart et al., 1996). In ewes, IGFBP-3 levels increase during follicular growth in some (Monget et al., 1993) but not other (Spicer et al., 1995) studies. Similarly, in pigs, IGFBP-3 levels increase during follicular growth in some (Grimes et al., 1994) but not other (Echternkamp et al., 1994b) studies. A recent study in mares indicates that follicular fluid IGFBP-3 levels do not vary with physiological status of the follicle (Gerard and Monget, 1998). In contrast, we found follicular fluid IGFBP-3 levels tended to be greater in large luteal phase follicles when compared to large follicular phase follicles, but were not different among any other classes of follicles. Reasons for the discrepancies among studies are unknown, but based on the present results, IGFBP-3 levels may be influenced by stage of cycle when follicles are collected. In contrast to studies in women (Giudice et al., 1990; Hossenlopp et al., 1990; Davies et al., 1991), pigs (Besnard et al., 1997), sheep (Besnard et al., 1996) and rats (Davenport et al., 1990), we found little or no evidence for IGFBP-3 protease activity in follicular fluid of mares (present study) or cattle (Spicer et al., 2001). Whether these differences are due to species differences or due to some other factor is unclear. Using this same proteolysis assay, we are able to detect IGFBP-3 proteolysis by pig uterine flushings (Geisert et al., 2001), indicating that our 125I-IGFBP-3 probe is capable of being proteolysed. Because the amount of follicular fluid proteolysis of IGFBP-3 averaged less than 25% and did not significantly differ among various mare follicles in the present study, the changes in follicular fluid IGFBP-3 observed in the mare are likely due to factors other than proteolysis such as changes in IGFBP-3 transudation from serum as suggested for cattle (Echternkamp et al., 1994a).

In pigs (Howard and Ford, 1992), humans (Cataldo and Giudice, 1992), and cattle (Echternkamp et al., 1994a; Stewart et al., 1996; Mihm et al., 2000), follicular fluid IGFBP-4 concentrations are undetectable in estrogen-active and dominant follicles. In atretic and late atretic follicles of sheep, the 22 to 24-kDa band (IGFBP-4) was found to be intense (Monget et al., 1993), whereas in estrogen-active preovulatory ewe follicles the 22 to 24-kDa band was undetectable using Western ligand blotting (Monget et al., 1993; Spicer et al., 1995). Although not evaluated in the present study, protease activity for IGFBP-4 has been reported in dominant estrogenic follicles of women (Chandraskher et al., 1995), mare (Mazerbourg et al., 2000), bovine (Mazerbourg et al., 2000; Spicer et al., 2001), porcine (Besnard et al., 1997; Mazerbourg et al., 2000), and ovine (Besnard et al., 1996; Mazerbourg et al., 1999) follicular fluid. Experiments using in situ hybridization have shown atresia to be characterized by an increase in IGFBP-4 gene expression in rat granulosa cells (Nakatani et al., 1991). Thus, as with IGFBP-2 and -5, intraovarian changes in IGFBP-4 levels may be regulated by changes in local synthesis as well as proteolysis of IGFBP-4. We found large follicular phase follicles and medium luteal phase follicles to have less IGFBP-4 concentrations than any other class of follicles. Because absolute levels of IGFBP-4 were minor compared with all other IGFBP in mare follicular fluid, and Gerard and Monget (1998) showed little or no change in IGFBP-4 levels (using 125I-IGF-II ligand blotting) during growth of the dominant follicle in mares, we chose not to evaluate its proteolysis by follicular fluid. Because IGFBP-4 has a greater affinity for IGF-I than for IGF-II (Spicer and Echternkamp, 1995), 125I-IGF-II rather than 125I-IGF-I used for ligand blotting procedures in the present and previous study may have reduced the ability to detect differences in follicular fluid IGFBP-4 concentrations among follicles. Whether these minor changes in follicular fluid IGFBP-4 concentrations found in the present study are due to changes in local synthesis or proteolysis will require further study.

Similar to a 96-kDa protein detected by 125I-IGF-I or -II ligand blotting in equine (Prosser and McLaren, 1992) and bovine (Simpson et al., 1997) serum, we found a 90 to 96-kDa protein that bound 125I-IGF-II in mare follicular fluid. As previous studies have suggested, this 90 to 96-kDa protein is most likely a proteolytic fragment of the soluble form of the IGF-II receptor (Wendland et al., 1989; Westlund et al., 1991). We also found at least two other high-molecular weight proteins that bound 125I-IGF-II in mare follicular fluid, a 115-kDa, and a 125 to 135-kDa protein. The 115- and 125 to 135-kDa proteins are likely soluble forms of the IGF type I receptor (Bhaumick et al., 1981; Pillion et al., 1988). Of interest, all of these high molecular weight proteins changed with follicular status, indicating that changes in IGF type I and II receptors may play a role in follicular development in the mare as suggested in cattle (Spicer et al., 1994; Stewart et al., 1996).

For the first time, protease inhibitors to IGFBP-5 proteolysis in mare follicular fluid has been evaluated. We found metalloprotease inhibitors such as doxycycline, EDTA, and 1,10-phenanthroline, as well as serine protease inhibitors CAA, PMSF, and aprotinin inhibited, in a dose-dependent way, IGFBP-5 protease activity, suggesting that the IGFBP-5 protease present in mare follicular fluid is a serine-metalloprotease. The protease(s) that degrades IGFBP-2, -4, and -5 found in...
swine follicles was inhibited by metalloprotease inhibitors but not serine protease inhibitors (Besnard et al., 1997). However, a serine protease inhibitor has been found to inhibit the degradation of IGFBP-3 in swine follicles (Grimes et al., 1994). The IGFBP protease that degrades IGFBP-3, -4, and -5 found in sheep follicular fluid is a metalloprotease (Besnard et al., 1996). In humans, the IGFBP protease that degrades IGFBP-4 is a metallo-serine protease (Chandrasekher et al., 1995). Thus, species differences may exist with regard to the specific type(s) of protease present in follicular fluid that degrades IGFBP.

Estradiol is the dominant steroid secreted by the preovulatory follicle of the mare at the beginning of estrus (Kenney et al., 1979; Meinecke et al., 1987; Spicer et al., 1991). As ovulation approaches, the follicular content of androstenedione (Meinecke et al., 1987), testosterone (Watson and Hinrichs, 1988; Spicer et al., 1991), and estradiol (Spicer et al., 1991) increases. In agreement with previous studies in mares (Kenney et al., 1979; Fay and Douglas, 1987; Spicer et al., 1991), we found large (>25 mm) follicles in the follicular phase to have threefold greater concentrations of estradiol-17β than large follicles in the luteal phase, and 17- to 72-fold greater amounts of estradiol than small or medium follicles in either the follicular or luteal phase. These results confirm our physiologic classification of the abattoir ovaries used in the present study. Moreover, we found that follicular fluid estradiol-17β and androstenedione levels were negatively correlated with levels of IGFBP-2, -4, and -5 but not IGFBP-3 in follicular fluid. Previous studies have also reported negative correlations between follicular fluid estradiol and low molecular weight IGFBP levels in cattle (Echternkamp et al., 1994a; Stewart et al., 1996), sheep (Spicer et al., 1995), and mares (Gerard and Monget, 1998). Whether estradiol-17β and androstenedione regulate IGFBP levels via changes in local production or changes in proteolysis will require further study.

Concentrations of IGF-I and -II in dominant and subdominant follicles did not differ in cattle (Stewart et al., 1996), yet follicular fluid concentrations of IGF-I increase (Spicer et al., 1988; Echternkamp et al., 1990; Spicer and Enright, 1991) and IGF-II decrease (Spicer and Echternkamp, 1995; Stewart et al., 1996) with follicle size in cattle. Similarly, we found follicular fluid IGF-I concentrations to be greater in large than in small or medium follicles of mares in the present study. However, follicular fluid IGF-II concentrations were not different among small, medium, and large follicles but tended to be greater in follicular phase vs luteal phase follicles of mares. Whether these changes in follicular fluid IGF-I and -II are due to changes in local production of these IGF will require further study. Regardless of the source of IGF-I and -II, the relative proportion of IGF to IGFBP concentrations was twofold greater in large estrogen-active follicular phase follicles than other follicle classes in the present study. This increase in bioavailable IGF-I and -II likely plays a key role in promoting follicular differentiation by further enhancing gonadotropin-induced action (Spicer and Echternkamp, 1995).

**Implications**

Intraovarian factors that regulate development of dominant follicles in mares are not well understood, and identifying these factors could lead to ways to enhance reproduction in the mare. In the present study, levels of estradiol increased in large follicles coincident with a reduction in levels of binding proteins for insulin-like growth factors and an increase in proteolysis of insulin-like growth factor-binding protein-5. Thus, increases in levels of follicular fluid hormones during the estrous cycle of the mare may modulate development of follicles and/or atresia by affecting the synthesis or proteolysis of insulin-like growth factor-binding proteins. Future research should focus on identifying the specific hormonal regulators of insulin-like growth factor-binding protein-5 protease activity in equine follicles.

**Literature Cited**


