Proteolytic degradation of IGF-binding protein (IGFBP)-2 in equine ovarian follicles: involvement of pregnancy-associated plasma protein-A (PAPP-A) and association with dominant but not subordinated follicles

N Gérard, T Delpuech, C Oxvig¹, M T Overgaard¹ and P Monget

INRA, UMR Physiologie de la Reproduction et des Comportements 6073, (INRA/CNRS/Université) 37380 Nouzilly, France

¹Department of Molecular Biology, University of Aarhus, 8000 Aarhus C, Denmark

(Requests for offprints should be addressed to P Monget; Email: monget@tours.inra.fr)

(M T Overgaard is now at Department of Gynecology and Obstetrics, Stanford University Medical Center, Stanford, CA 94305-5317, USA)

Abstract

In the ovary of mammalian species, terminal follicular growth is accompanied by a decrease in intrafollicular levels of IGF-binding protein-2 (IGFBP-2) and IGFBP-4. The decrease in IGFBP-4 levels is essentially due to an increase in proteolytic cleavage by intrafollicular pregnancy-associated plasma protein-A (PAPP-A) in growing healthy follicles. The decrease in IGFBP-2 levels is partly due to a decrease in mRNA expression by follicular cells. In addition, we have recently shown that IGFBP-2 is also proteolytically cleaved by PAPP-A in bovine and porcine growing follicles. In the present work, we showed that follicular fluid from late dominant equine follicles (35 mm diameter) contains a proteolytic activity against IGFBP-2. First follicular fluid from dominant follicles contained lower levels of native IGFBP-2 than the corresponding serum, as assessed by Western ligand blotting. In contrast, immunoblotting experiments showed much higher levels of a 12 kDa proteolytic fragment in dominant follicular fluid than in the serum. Moreover, equine dominant follicular fluid was able to induce proteolysis of exogenous recombinant bovine (rb)IGFBP-2, this degradation being dose-dependently enhanced by IGFs. The proteolytic activity against IGFBP-2 in equine follicles was partially immunoneutralized by a polyclonal antibody raised against human PAPP-A. Moreover, cleav-

age of rbIGFBP-2 by equine follicular fluid was dose-dependently inhibited by a peptide derived from the heparin-binding domain of IGFBP-5, as well as by peptides derived from other heparin-binding domain-containing proteins such as connective tissue growth factor, vitronectin and heparin-interacting protein, previously shown to inhibit PAPP-A. Finally, the proteolytic activity was very low in subordinate follicles, was high in both early (25 mm diameter) and late (35 mm diameter) dominant follicles, and was slightly lower in preovulatory follicles recovered 35 h after human chorionic gonadotropin (hCG) treatment.

Overall, these data show that in the equine ovary, the selection of dominant follicles is associated with an increase of the proteolytic degradation of IGFBP-2 by PAPP-A, as for IGFBP-4, and potentially other protease(s), probably contributing to the increase in IGF bioavailability. In atretic subordinate follicles, the decrease in the proteolytic degradation of IGFBP-2, probably due in part to a direct inhibition by peptides containing heparin-binding domains, contributes to the increase in IGFBP-2 levels and the decrease in IGF bioavailability. The expression of PAPP-A and IGFBP-2 mRNA during folliculogenesis remain to be investigated in the mare.

Journal of Endocrinology (2004) **182**, 457–466

Introduction

In mammalian species, ovarian follicular growth is characterized by a decrease in insulin-like growth factor-binding protein 2 (IGFBP-2) and IGFBP-4 intrafollicular levels, leading to an increase in IGF bioavailability and an increase in follicle-stimulating hormone (FSH) responsiveness of granulosa cells (Monget *et al.* 1996). The decrease in IGFBP-4 levels is essentially due to an increase in

intrafollicular proteolytic activity in ovine, bovine, porcine, equine and human species (Chandrasekher *et al.* 1995, Besnard *et al.* 1996b, 1997, Spicer *et al.* 2001). In preovulatory follicles from these species, the IGFBP-4 protease has been identified as pregnancy-associated plasma protein-A (PAPP-A) (Mazerbourg *et al.* 2001). The increase in IGFBP-4 proteolytic degradation during follicular growth is partly due to an increase in PAPP-A mRNA expression. Indeed, PAPP-A mRNA was shown

to be expressed in healthy growing but not atretic human follicles (Hourvitz *et al.* 2000), and to be closely correlated with aromatase and luteinizing hormone (LH)-receptor expression in bovine and porcine granulosa cells (Mazerbourg *et al.* 2001). Moreover, IGFBP-4 degradation by PAPP-A was shown to be enhanced by IGF-I and IGF-II, and inhibited by heparin-binding domain (HBD)-containing peptides (Mazerbourg *et al.* 1999, 2000, 2001). Recently, we have shown that this inhibition is due to a direct interaction of HBD peptides with PAPP-A (Monget *et al.* 2003).

During follicular growth, the decrease in IGFBP-2 levels is partly due to a decrease in mRNA expression by follicular cells in ovine, bovine and porcine ovaries (Samaras *et al.* 1992, Besnard *et al.* 1996a, Armstrong *et al.* 1998, Liu *et al.* 2000). Moreover, we have shown in the ewe, and more recently in the cow and the sow, that this decrease is also partly due to an increase in IGFBP-2 proteolytic degradation by PAPP-A (Besnard *et al.* 1996b, 1997, Monget *et al.* 2003), suggesting a well-conserved mechanism in mammalian species. In contrast, Bridges *et al.* (2002) recently claimed that equine preovulatory follicles did not contain any proteolytic activity degrading IGFBP-2, suggesting a species-specific mechanism in the horse.

The aim of the present work was to test whether (1) equine preovulatory follicles contain proteolytic activity degrading IGFBP-2, (2) PAPP-A is involved in IGFBP-2 degradation in preovulatory follicles of this species and (3) proteolytic cleavage of IGFBP-2 is associated with the selection of dominant follicles in the mare.

Materials and Methods

Materials

Détomidine and prifinium bromide (Prifinial) were obtained from Smithkline & French (Courbevoie, France) and Vetoquinol (Lure, France) respectively. Mixtencilline, penicillin and dihydrostreptomycin were obtained from Rhône-Mérieux (Lyon, France). IGF-I and IGF-II were generous gifts from Drs H H Peter and A Hinnen (Ciba-Geigy, Basel, Switzerland). Recombinant human (rh)IGFBP-4 was expressed in yeast and purified as previously described (Kiefer *et al.* 1992). Recombinant bovine (rb)IGFBP-2 was from GroPep (Adelaide, Australia). rhPAPP-A was expressed in HEK 293 cells as previously described (Overgaard *et al.* 2000). The 18-amino-acid P5 peptide (201 RKGfYKRKQCRPSKGR KR218) from the human C-terminal domain of IGFBP-5 was purchased from Agrobio (Orléans, France). The 18-amino-acid PA5 (130 KAEAVKKDRKKLT QSKF143) peptide from human IGFBP-5 and the 18-amino-acid P3 peptide (215 KKGfYKKKQCRPSK GRKR232) from human IGFBP-3 were a generous gift from R S Bar (Iowa City, IA, USA). Synthetic peptide

spanning the C-terminal region of human connective tissue growth factor (CTGF or IGFBP-related protein-2) CTGF247–260 (EENIKKGKKCIRTP) was obtained from D R Brigstock (Colombus, OH, USA) (Brigstock *et al.* 1997). The synthetic peptide containing the HBD of human heparin/heparan sulfate-interacting protein (HIP) (CRPKAKAKAKAKD QTK) was kindly provided by Dr D D Carson (Houston, TX, USA). Synthetic peptides derived from the HBD of human vitronectin, VN1 (341 APRPSLAKKQRFR HR355) and VN3 (357 RKGfYR-SQRGHSRGR370), were kindly provided by Dr K T Preissner (Bad Nauheim, Germany). Rabbit polyclonal antiserum against bovine IGFBP-2 was generously donated by Dr J Closset (University of Liège, Belgium). This antibody showed less than 1% of cross-reactivity with IGFBP-1, IGFBP-3, IGFBP-4 and IGFBP-5 (data not shown). Anti-rabbit IgG antibodies coupled to horseradish peroxidase were purchased from Dako (Trappes, France). Rabbit polyclonal anti-PAPP-A/pro major basic protein (MBP) was raised against PAPP-A/proMBP purified from pregnancy serum (Oxvig *et al.* 1994). Nitrocellulose membranes were purchased from Schleicher and Schuell (Ecqueville, France) and the enhanced chemiluminescence detection system for immunoblots was obtained from Amersham.

Collection of follicular fluids and steroid content

All procedures were approved by the Agricultural Agency and Scientific Research Agency (approval number A37801 and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching). Twelve cyclic Welsh pony mares were treated with 125 µg prostaglandin F2α analog (Estrumate) during the midluteal phase to induce luteolysis. Ovarian activity was then assessed by routine transrectal ultrasonic imaging (Aloka 210 with a 5 Mhz linear probe; Société Bernard, Nantes, France), as previously described (Gérard & Monget 1998). Follicle diameter was estimated by averaging two cross-sectional measures of follicles, and follicular morphology was judged by the presence or absence of echogenic dots in the follicular antrum. To induce ovulation, an i.v. injection of 20 mg crude equine gonadotropin (CEG) was given when the largest follicle reached 33–35 mm diameter, at the end of the follicular phase. Follicular fluid was aspirated by transvaginal ultrasound-guided follicular puncture with a 7.5 MHz sectorial probe (Kretz; Sofrased, Truchtersheim, France) coupled to a sterile single lumen needle (60 cm long, 0.8–1.8 mm outer diameter; Thiébaud Frères, Jouvenex Margencel, France) as previously described (Duchamp *et al.* 1995, Okolsky *et al.* 1995). Healthy follicles were aspirated either at the growing dominant stage when the diameter was 25 mm (early dominance stage (ED), *n*=4), or 33–35 mm (late dominance stage (LD), *n*=5), or at the preovulatory stage

(PO, >35 mm diameter, 34 h after CEG injection, $n=4$). Subordinate follicles with diameters larger than 15 mm in the presence of a PO follicle ($n=5$) were also aspirated. Follicular fluid samples were then immediately centrifuged and stored at -20°C until 17β -estradiol and progesterone radioimmunoassays (Belin *et al.* 2000).

Before each puncture session, mares were sedated by a single injection of 0.2 ml détomidine i.v. (Domosedan, 1 mg/100 kg body weight (BW)). Prifinium bromide (Prifinial) was injected (15 ml i.v., 45 mg/100 kg BW) to ensure rectal relaxation. After puncture sessions, the mares were injected with antibiotics (20 ml i.m. mixtencilline; 1 600 000 IU penicillin/100 kg BW and 1.3 g dihydrostreptomycin/100 kg BW).

Cleavage of IGFBP-2 by follicular fluid

Characterization of IGFBP-2 proteolytic degradation was made on follicular fluid from early and late dominant equine follicles. Briefly, 2.5 μl equine follicular fluid were incubated in a solution of 20 mM Tris (pH 7.6) containing 137 mM NaCl (TBS) and 0.1% BSA with IGFBP-2, with or without IGF-I or IGF-II for 20 h at 37°C (final volume, 10 μl). In some experiments, the synthetic peptide P5 was added to the incubation medium. At the end of the incubation time, samples were analyzed by Western ligand blotting (WLB) or immunoblotting.

Western ligand blotting (WLB) IGF-II was iodinated by the iodogen method and purified by Sephadex G-50 chromatography by using a 0.1 M ammonium acetate elution buffer. WLB was performed as previously described (Monget *et al.* 1993). Samples were submitted to electrophoresis on a 12% SDS-polyacrylamide gel under non-reducing conditions. The proteins were then electrotransferred onto nitrocellulose filters (0.2 μm pore size) overnight at 4°C . Filters were treated successively with PBS (0.01 M, pH 7.4) containing 0.1% Nodinet P-40, 0.5% gelatin and 0.1% Tween-20, then incubated overnight at 4°C with ^{125}I -labelled IGF-II in a solution containing 0.03 M NaH_2PO_4 , 500 $\mu\text{l/l}$ Tween-20, 200 mg/l protamine sulfate, 200 mg/l NaNO_3 , and 3.72 g/l EDTA (pH 7.4). Afterwards, filters were washed with PBS containing 0.1% Tween-20, air-dried and exposed to Hyperfilm MP (Amersham) with an intensifying screen at -70°C or to a phosphor screen for quantification.

Immunoblotting After electrophoresis and electrotransfer of proteins, as described for WLB, nitrocellulose filters were treated for 2 h at room temperature with TBS containing 10% non-fat dry milk (NFDM) and 0.2% Tween-20 to saturate non-specific sites. Thereafter, filters were incubated for overnight at 4°C in TBS containing 5% NFDM, 0.05% Tween-20 and antibodies against IGFBP-2 (final dilution 1/10 000). Once washed in TBS

containing 1% NFDM and 0.2% Tween-20, nitrocellulose filters were incubated for 1 h at 37°C with an anti-rabbit or anti-mouse IgG antibody coupled to horseradish peroxidase (final dilution 1/4000). After washing, the signal was revealed by chemiluminescence detection.

Immunoneutralization of IGFBP-2 proteolytic degradation For immunoneutralization, 2 μl follicular fluid were incubated in TBS containing 0.1% BSA, 75 ng IGFBP-2 and different dilutions of rabbit polyclonal antibody raised against PAPP-A, or non-specific rabbit IgG or glycerol for 20 h at 37°C (final volume, 10 μl), as previously described (Mazerbourg *et al.* 2001). At the end of the incubation time, samples were analyzed by WLB and immunoblotting.

Quantification of WLB Western ligand blots were quantified by a phosphorimager (Storm/Image Quant, Molecular Dynamics). Quantification was performed as previously described (Mazerbourg *et al.* 1999). Briefly, the amount of radiolabeled IGF-II bound to each IGFBP was expressed as the integrated optical density (IOD) of the corresponding band, expressed in arbitrary units. The extent of IGFBP-2 degradation by follicular fluid was determined as the difference $I-20 - I37$, where $I-20$ is the IOD of the IGFBP band from samples not incubated, and $I37$ is the IOD of the IGFBP band from samples incubated at 37°C . The percentage of IGFBP proteolysis inhibition was expressed as a ratio: $[(I - I37) \times 100] / (I-20 - I37)$, where I is the IOD of the IGFBP band from samples incubated at 37°C in the presence of PAPP-A polyclonal antibody or synthetic peptides.

Statistical analysis

All experimental data are expressed as means $\pm$ S.E.M. Statistical comparisons of means were made using one-way ANOVA for the effects of concentrations of P5 peptide on IGFBP-2 levels quantified from the blots. One-way analysis of variance followed by Tukey's or Newmann-Keuls test was performed to test differences between different amounts of P5 peptide. Means of inhibition of IGFBP-2 proteolytic degradation by peptides P3, CTGF, HIP, VN1 and VN3 were compared with 0 (absence of inhibition of IGFBP-2 degradation without peptides) by a paired *t*-test. In the case of heterogeneity of variance, the Mann-Whitney or the Kruskal-Wallis test were used to compare means between groups. Differences with $P>0.05$ were not considered significant.

Results

Identification of IGFBP-2 proteolytic fragments in follicular fluid from equine preovulatory follicles

In the mare, previous experiments have shown by WLB that serum contained higher levels of IGFBP-2 and

IGFBP-4 than the corresponding preovulatory follicles (Gérard & Monget 1998, Bridges *et al.* 2002). Immunoblotting experiments confirmed that the native 32 kDa form of IGFBP-2 was present at higher levels in serum (Fig. 1A, lanes 1, 3 and 5) than in the corresponding dominant follicles (Fig. 1A, lanes 2, 4 and 6). Interestingly, a 12 kDa proteolytic fragment was clearly visualized in dominant follicular fluids. Overexposure of autoradiographs revealed two other faint bands of 23 and 17 kDa in dominant follicular fluids (Fig. 1B, lanes 2, 4, 6 and 8). The 12 kDa proteolytic fragment exhibited the same apparent molecular weight as the smaller proteolytic fragment of IGFBP-2 present in porcine (Fig. 1C, lane 2 vs lane 3), as well as bovine and ovine preovulatory follicular fluids (data not shown). This suggested the presence of a proteolytic activity against IGFBP-2 in equine dominant follicles, as shown previously in the cow and sow (Besnard *et al.* 1997, Monget *et al.* 2003).

Characterization of IGFBP-2 proteolytic degradation in equine dominant follicular fluid

As assessed by WLB, overnight incubation of equine dominant follicular fluid with exogenous rbIGFBP-2 resulted in a partial proteolytic cleavage and the appearance of a faint 23 kDa band (Fig. 2A, lane 2 vs lane 1; Fig. 2B). Cleavage of exogenous rbIGFBP-2 was enhanced in the presence of an excess of IGF-II, and inhibited by polyclonal antibody raised against human PAPP-A (Fig. 2A, lanes 3 and 4; Fig. 2B). Rabbit IgG displayed no effect on IGFBP-2 degradation in equine follicular fluid (Fig. 2B). As for the proteolysis of IGFBP-4, IGFBP-2 cleavage in equine follicular fluid was maximal at pH 7.4–7.6, was partially inhibited at pH 8.5 and was completely inhibited at pH 6 (data not shown). IGFBP-2 proteolytic degradation in equine follicular fluid was shown to be inhibited by EDTA and 1,10 phenanthroline but not clearly by other protease inhibitors, as previously shown for IGFBP-2 and IGFBP-4 proteolysis in other species (Besnard *et al.* 1996a, 1997, Monget *et al.* 2003). As assessed by immunoblotting, exogenous rbIGFBP-2 cleavage by equine dominant follicular fluid resulted in the appearance of two 23 and 12 kDa proteolytic fragments, degradation being partially inhibited by the antibody raised against human PAPP-A (Fig. 3). The 23 and 12 kDa proteolytic fragments showed a similar apparent molecular weight as endogenous 23 and 12 kDa proteolytic fragments of IGFBP-2 present in porcine preovulatory follicular fluid (Fig. 3, lane 6).

Exogenous rbIGFBP-2 degradation was dose-dependently enhanced by IGFs, IGF-II being most efficient and Long-R3 (LR3)-IGF-I being less efficient than IGF-I (Fig. 4). As for the intrafollicular cleavage of IGFBP-4 by PAPP-A, cleavage of IGFBP-2 by equine dominant follicular fluid was dose-dependently inhibited by the presence of HBD-containing peptides derived from IGFBP-5 (P5 peptide; Fig. 5A and B). Similar inhibition

was observed with heparin-binding peptides contained within IGFBP-3, CTGF, VN and HIP, but not with the basic amino acid-rich PA5 peptide, derived from IGFBP-5, that is not able to bind heparin (Fig. 6).

Proteolytic activity of IGFBP-2 is associated with the selection of dominant follicles in the mare

Steroid levels in follicular fluids recovered from early dominant follicles (25 mm diameter), late dominant follicles (35 mm diameter), preovulatory follicles recovered 35 h after CEG injection and subordinate follicles from the cohort are shown in Table 1. Proteolytic activity was significantly lower in subordinate follicles compared with early and late dominant follicles (Fig. 7; $P < 0.001$). The activity tended to decrease in follicles recovered 35 h after gonadotropin treatment.

Discussion

We have previously shown that ovarian follicular growth is accompanied by an increase in IGFBP-4 proteolytic degradation by PAPP-A in bovine, ovine, porcine and equine species (Besnard *et al.* 1996b, 1997, Mazerbourg *et al.* 2001). We have also shown that follicular growth is accompanied by an increase in IGFBP-2 degradation in ovine and porcine species (Besnard *et al.* 1996b, 1997), and that PAPP-A is responsible for the degradation of IGFBP-2 in bovine and porcine dominant follicles (Monget *et al.* 2003). In the present work, we show that equine follicular fluid from equine dominant follicles contains significant levels of an endogenous 12 kDa proteolytic fragment of IGFBP-2, in contrast to the corresponding serum. Moreover, follicular fluid from dominant but not subordinate equine follicles contains a proteolytic activity against IGFBP-2. As in bovine and porcine species, IGFBP-2 proteolysis was dose-dependently enhanced by IGFs and inhibited by a peptide derived from the HBD of IGFBP-5. Finally, the polyclonal antibody against human PAPP-A was able to partially immunoneutralize the intrafollicular proteolytic activity degrading IGFBP-2 in the mare.

In the present immunoblotting experiments, using our antibody against bovine IGFBP-2, we were able to visualize the 12 kDa fragment, but could only poorly visualize the putative 23 kDa, endogenous proteolytic fragment of IGFBP-2 in equine preovulatory follicles, in contrast to bovine and porcine species. One can hypothesize that it is due to the poor cross-reactivity of this antibody with the equine IGFBP-2 23 kDa proteolytic fragment, and that this fragment is actually present in equine follicles. Indeed, incubation of exogenous rbIGFBP-2 with equine follicular fluid generated both 12 and 23 kDa fragments, as assessed by immunoblotting using the same antibody. Moreover, our results have

Figure 1 Detection of native IGFBP-2 and proteolytic fragments in equine follicular fluid (2.5 µl) from late dominant follicles (35 mm diameter) and the corresponding serum by immunoblotting. (A) Short exposure of the autoradiograph. Follicular fluid from dominant follicles (lanes 2, 4 and 6) contained lower levels of native 32 kDa IGFBP-2 and higher levels of 12 kDa proteolytic fragments than the corresponding serum (lanes 1, 3 and 5). (B) Long exposure of the autoradiograph. Follicular fluid from dominant follicles (lanes 2, 4, 6 and 8) also contained faint bands migrating at 17 and 23 kDa, probably corresponding to other proteolytic fragments of IGFBP-2. Lanes 1, 3, 5 and 7: corresponding serum. (C) The 12 kDa proteolytic fragment present in follicular fluid from dominant equine follicles exhibited the same apparent molecular weight as the smaller proteolytic fragment of IGFBP-2 present in porcine preovulatory follicles (lane 2 vs lane 3). Lane 1: corresponding equine serum.

Figure 2 IGFBP-2 cleavage in equine dominant follicles. (A) WLB analysis; 2.5 µl follicular fluid from equine dominant follicles were incubated for 20 h at 37 °C with 75 ng IGFBP-2, with (lanes 3 and 4) 50 ng IGF-II, in the absence (lanes 2 and 3) or presence (lane 4) of antibody against PAPP-A (0.6 µg) in a final volume of 10 µl. At the end of the incubation reaction mixtures were subjected to WLB. Lane 1: samples stored at -20 °C before WLB. Exogenous rbIGFBP-2 degradation was enhanced in the presence of IGF-II, and inhibited by polyclonal antibody raised against human PAPP-A (lane 3 vs lane 4). (B) Quantitative analysis of WLB; results are obtained as the means ± S.E.M. on five late dominant follicles. **P*<0.05 and ***P*<0.01 in comparison with samples not incubated (column 1). \$, lane 3 vs lane 4, *P*<0.05. Note that degradation of IGFBP-2 is not completely abolished by the antibody against PAPP-A. Non-immune IgG was without effect on IGFBP-2 cleavage.

shown that the proteolytic degradation of rbIGFBP-2 by equine preovulatory follicular fluid was not completely abolished by the antibody against PAPP-A, suggesting that the antibody against human PAPP-A that we have used

only partially inhibits equine PAPP-A, due to poor cross-reactivity. Alternatively, other intrafollicular proteases could be implicated in IGFBP-2 degradation. Of note, a faint band migrating at 17 kDa was frequently

Figure 3 IGFBP-2 degradation in equine dominant follicles – immunoblotting analysis; 2.5 µl follicular fluid from equine dominant follicles were incubated for 20 h at 37 °C with 75 ng IGFBP-2, without (lanes 2 and 3) or with (lanes 4 and 5) 50 ng IGF-II, in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of antibody against PAPP-A (0.6 µg) in a final volume of 10 µl. At the end of the incubation, reaction mixtures were subjected to immunoblotting. Lane 1: samples stored at -20 °C before immunoblot analysis. Exogenous rbIGFBP-2 degradation was enhanced in the presence of IGF-II, and partially inhibited by polyclonal antibody raised against human PAPP-A (lane 3 vs lane 4). Note the presence of a poorly defined non-specific band of 20–25 kDa in the samples containing the antibody against PAPP-A (lanes 3 and 5), as observed in our previous study (Monget *et al.* 2003). Lane 6: 2.5 µl follicular fluid from porcine preovulatory follicle.

Figure 4 Dose-dependent effect of IGF-I, IGF-II and LR3-IGF-I on IGFBP-2 cleavage by dominant equine follicular fluid – quantitative analysis of WLB; 2.5 µl follicular fluid from equine dominant follicles were incubated for 20 h at 37 °C with 75 ng IGFBP-2 and increasing concentrations of IGF-I (■), IGF-II (□) or LR3-IGF-I (△). Results are obtained as the means ± S.E.M. of four late dominant follicular fluids.

observed in follicular fluid after overexposure of immunoblots (Fig. 1B).

We have shown that addition of IGFs dose-dependently enhances the cleavage of IGFBP-2 by equine follicular fluid. This enhancing effect is probably due to a conformational change of the substrate after binding IGFs rather than a direct activation of PAPP-A activity. Indeed, as in our previous studies (Monget *et al.* 2003), LR3-IGF-I was

less efficient than IGF-I or IGF-II in enhancing IGFBP-2 cleavage by equine follicular fluid. This is consistent with the mechanism shown for IGF enhancement of the IGFBP-4 cleavage by rhPAPP-A (Laursen *et al.* 2001).

As with IGFBP-4 cleavage (Mazerbourg *et al.* 1999, 2000), we have shown that the cleavage of IGFBP-2 is inhibited by HBD-containing peptides like P5. Our recent Biacore analysis showed that the P5 peptide binds to PAPP-A with a high affinity, but not to IGFBP-2 (Monget *et al.* 2003). Therefore, this inhibition is probably due to a direct interaction of the P5 peptide with PAPP-A rather than with IGFBP-2. In ovine and bovine species, we can suggest that the high increase in IGFBP-5 levels in late atretic follicles (Monget *et al.* 1993, Echterkamp *et al.* 1994) might participate in the decrease in PAPP-A activity by direct interaction with PAPP-A via the heparin-binding domain encompassing the P5 peptide. In contrast to ruminant species, Western ligand blot experiments suggest that in the mare, IGFBP-5 is not elevated in atretic follicles (Gérard & Monget 1998), in concurrence with other species like human or pig, suggesting that other HBD-containing proteins could modulate PAPP-A activity in atretic follicles in these species. In particular, the degradation of IGFBP-2 by follicular fluid from equine dominant follicles was inhibited by HBD peptides from CTGF, VN and HIP. Interestingly, it has been shown recently in the pig that the expression of CTGF mRNAs in granulosa cells is inversely regulated during ovarian follicular maturation *in vivo* (Wandji *et al.* 2000). Moreover *in vitro*, FSH and human chorionic gonadotropin are able

Figure 5 Dose-dependent effect of the IGFBP-5-derived HBP (P5) on IGFBP-2 cleavage by equine dominant follicular fluid. (A) Immunoblot analysis; 2.5 µl follicular fluid from equine dominant follicle were incubated for 20 h at 37 °C with 75 ng IGFBP-2 without (lane 2) or with (lanes 3–7) 50 ng IGF-II, in the absence (lanes 2 and 3) or in the presence (lanes 4–7) of increasing concentrations of P5 peptide in a final volume of 10 µl. Lane 1: sample stored at – 20 °C. At the end of the incubation, reaction mixtures were subjected to WLB (for quantification) and immunoblotting. Note the endogenous 12 kDa proteolytic fragment present in the follicular fluid (lanes 1 and 7). (B) Quantitative analysis of autoradiographs. Results are shown as the means ± S.E.M. of four late dominant follicular fluids. a vs b and b vs c, $P < 0.05$; a vs c, $P < 0.01$.

Figure 6 Effects of different helix bundle proteins (HBPs) on IGFBP-4 degradation by equine follicular fluid; 2.5 µl follicular fluid from equine dominant follicle was incubated for 20 h at 37 °C with 75 ng IGFBP-2 and 5 µg P3, 5 µg CTGF, 10 µg VN1 (VN343–355), 5 µg VN3 (VN357–370), 5 µg of HIP or 10 µg PA5 peptides, in a final volume of 10 µl. At the end of the incubation, samples were subjected to WLB. Experimental data are expressed as the means ± S.E.M. of four late dominant follicular fluids. **P*<0.05 and ***P*<0.01 in comparison with samples incubated without peptide.

to inhibit CTGF expression by rat and human granulosa cells respectively (Harlow *et al.* 2002, Liu *et al.* 2002). This suggests that in immature follicles from non-ruminant species, the presence of CTGF or other HBD-containing proteins in follicular fluid could participate in the inhibition of PAPP-A proteolytic activity.

Recently, Bridges *et al.* (2002) claimed that equine preovulatory follicles contained proteolytic activity against IGFBP-4 but not IGFBP-2. The reason for the discrepancy with the present work is unknown. It could be due to lower sensitivity of the radiolabeled IGFBP-2 used by these authors to study the intrafollicular proteolytic activity of PAPP-A. In particular, in our hands, iodinated IGFBP-2, in contrast to native IGFBP-2, was not cleaved by dominant equine follicular fluid, and only poorly by rhPAPP-A. Of note, in the absence of IGF-II, proteolytic

Figure 7 Proteolytic activity degrading IGFBP-2 in equine follicles – effect of follicle status; 2.5 µl follicular fluid was incubated for 20 h at 37 °C with 75 ng IGFBP-2 with or without IGF-II. Subord., subordinate follicles; ED, early dominant follicles (25 mm diameter); LD, late dominant follicles (35 mm diameter); PO, follicular fluid recovered from preovulatory follicles, 35 h after CEG administration. a vs b and b vs c, *P*<0.05; a vs c, *P*<0.001.

cleavage of exogenous IGFBP-2 was low in some follicular fluids and was only clearly visualized by immunoblotting, by using our very sensitive antibody raised against bovine IGFBP-2.

The intrafollicular proteolysis of IGFBP-2 by PAPP-A is a well-conserved mechanism in preovulatory follicles in mammalian species, suggesting that this proteolysis has dramatic consequences on follicular maturation. Interestingly, and as previously observed in the sheep and the sow, proteolytic cleavage of IGFBP-2 was shown here to be associated with the selection of dominant follicles in the mare. Intrafollicular cleavage of IGFBP-2 directly participates in the increase in bioavailable IGFs that might further stimulate granulosa cell proliferation and steroidogenesis, as previously shown (Monniaux & Pisselet 1992). This hypothesis is reinforced by the recent data from Ginther *et al.* (2004) who showed that *in vivo* intrafollicular injections of IGF-I in the mare are able to support the selection and the ovulation of dominant follicles. Also, it is possible that IGFBP-2 proteolytic fragments have IGF-independent effects on follicular cells, as demonstrated for IGFBP-3 and IGFBP-5 fragments (Ferry *et al.* 1999). Recently, we have shown that changes in intrafollicular IGFBP-2 levels, due to changes in mRNA expression and

Table 1 Concentrations of steroids in equine follicular fluids (number of samples in parentheses)

	17β-estradiol (ng/ml)	Progesterone (ng/ml)
Early dominant	1239.7 ± 213.9 (n=4)	62.6 ± 12.7 (n=4)
Late dominant	2480.2 ± 196.4 (n=5)	120.2 ± 15.1 (n=5)
Preovulatory	2281 ± 391.5 (n=4)	198 ± 55.6 (n=4)
Subordinate	89.2 ± 33.5 (n=5)	17.7 ± 7.8 (n=5)

proteolytic cleavage, are involved in the regulation of intrafollicular IGFBP-4 proteolytic cleavage (Mazerbourg *et al.* 1999, 2000). Interestingly, recent data suggest that the regulation of intrafollicular IGFBP-4 proteolytic cleavage is involved in the establishment of ovarian follicular dominance (Rivera *et al.* 2001, Rivera & Fortune 2003). Therefore, one might hypothesize that in growing healthy follicles, the decrease in IGFBP-2 and IGFBP-4 levels, as well as the increase in PAPP-A activity, participates in the increase in IGF bioavailability and the selection of the dominant follicle. In contrast, in early atretic follicles, the decrease in IGFBP-2 cleavage, due to the decrease in PAPP-A expression, participates in the decrease in IGFBP-4 proteolytic cleavage, the decrease in IGF bioavailability and the degeneration of subordinate follicles of the cohort.

Overall, these data show that in the equine ovary, as for IGFBP-4, the selection of dominant follicles is associated with the proteolytic cleavage of IGFBP-2 by PAPP-A and potentially other protease(s), probably participating in the increase in IGF bioavailability. In atretic subordinate follicles, the decrease in proteolytic cleavage of IGFBP-2, participates in the increase in IGFBP-2 levels and the decrease in IGF bioavailability. Studies on expression of PAPP-A and IGFBP-2 mRNA during folliculogenesis remain to be investigated in this species.

Acknowledgements

We acknowledge Dr Jean Closset for the gift of the rabbit antibody raised against bovine IGFBP-2. We thank Claire Chabrux and Claudine Pisselet for excellent technical assistance. We thank Francis Paulmier and Guy Duchamp and their technical staff for animal management

Funding

This work was supported by the Institut National de la Recherche Agronomique, by the Haras Nationaux, by the FARO (Fonds d'Aide à la Recherche Organon) institution and by a grant from the Région Centre.

References

- Armstrong DG, Baxter G, Gutierrez CG, Hogg CO, Glazyrin AL, Campbell BK, Bramley TA & Webb R 1998 Insulin-like growth factor binding protein -2 and -4 messenger ribonucleic acid expression in bovine ovarian follicles: effect of gonadotropins and developmental status. *Endocrinology* **139** 2146–2154.
- Belin F, Goudet G, Duchamp G & Gerard N 2000 Intrafollicular concentrations of steroids and steroidogenic enzymes in relation to follicular development in the mare. *Biology of Reproduction* **62** 1335–1343.
- Besnard N, Pisselet C, Monniaux D, Locatelli A, Benne F, Gasser F, Hately F & Monget P 1996a Expression of insulin-like growth

factor binding protein-2, -4, and -5 messenger ribonucleic acids in the ovine ovary: localization and changes during growth and atresia of antral follicles. *Biology of Reproduction* **55** 1356–1367.

- Besnard N, Pisselet C, Zapf J, Hornebeck W, Monniaux D & Monget P 1996b Proteolytic activity is involved in changes in intrafollicular insulin-like growth factor-binding protein levels during growth and atresia of ovine ovarian follicles. *Endocrinology* **137** 1599–1607.
- Besnard N, Pisselet C, Monniaux D & Monget P 1997 Proteolytic activity degrading insulin-like growth factor-binding protein-2, -3, 4 and -5 in healthy growing and atretic follicles in the pig ovary. *Biology of Reproduction* **56** 1050–1058.
- Bridges TS, Davidson TR, Chamberlain CS, Geisert RD & Spicer LJ 2002 Changes in follicular fluid steroids, insulin-like growth factors (IGF) and IGF-binding protein concentration, and proteolytic activity during equine follicular development. *Journal of Animal Science* **80** 179–190.
- Brigstock DR, Steffen CL, Kim GY, Vegunta RK, Diehl JR & Harding PA 1997 Purification and characterization of novel heparin-binding growth factors in uterine secretory fluids. *Journal of Biological Chemistry* **272** 20275–20282.
- Chandrasekhar YA, Van Dessel HJHM, Fauser BCJM & Giudice LC 1995 Estrogen- but not androgen-dominant human ovarian follicular fluid contains an insulin-like growth factor binding protein-4 protease. *Journal of Clinical Endocrinology and Metabolism* **80** 2734–2739.
- Duchamp G, Bézard J & Palmer E 1995 Oocyte yield and the consequences of puncture of all follicles larger than 8 millimeters in mares. In *Equine Reproduction VI*, pp 233–241. Eds DC Sharp & FW Bazer. Madison, WI: Society for the Study of Reproduction.
- Echternkamp SE, Howard HJ, Roberts AJ, Grizzle J & Wise T 1994 Relationships among concentrations of steroids, insulin-like growth factor-I and insulin-like growth factor binding proteins in ovarian follicular fluid of beef cattle. *Biology of Reproduction* **51** 971–978.
- Ferry RJ Jr, Katz LE, Grimberg A, Cohen P & Weinzimer SA 1999 Cellular actions of insulin-like growth factor binding proteins. *Hormone Metabolism Research* **31** 192–202.
- Gérard N & Monget P 1998 Intrafollicular insulin-like growth factor binding protein levels in equine ovarian follicles during preovulatory maturation and regression. *Biology of Reproduction* **58** 1508–1514.
- Ginther OJ, Bergfelt DR, Beg MA, Meira C & Kot K 2004 *In vivo* effects of an intrafollicular injection of insulin-like growth factor 1 on the mechanism of follicle deviation in heifers and mares. *Biology of Reproduction* **70** 99–105.
- Harlow CR, Davidson L, Burns KH, Yan C, Matzuk MM & Hillier SG 2002 FSH and TGF-beta superfamily members regulate granulosa cell connective tissue growth factor gene expression *in vitro* and *in vivo*. *Endocrinology* **43** 3316–3325.
- Hourvitz A, Widger AE, Teixeira FL, Chang RJ, Adashi EY & Erickson GF 2000 Pregnancy-associated plasma protein-A gene expression in human ovaries is restricted to healthy follicles and corpora lutea. *Journal of Clinical Endocrinology and Metabolism* **85** 4916–4919.
- Kiefer MC, Schmid C, Waldvogel M, Schlöpfer I, Futo E, Masiarz FR, Green K, Barr PJ & Zapf J 1992 Characterization of recombinant human insulin-like growth factor binding proteins 4, 5, and 6 produced in yeast. *Journal of Biological Chemistry* **267** 12692–12699.
- Laursen LS, Overgaard MT, Soe R, Boldt HB, Sottrup-Jensen L, Giudice LC, Conover CA & Oxvig C 2001 Pregnancy-associated plasma protein-A (PAPP-A) cleaves insulin-like growth factor binding protein (IGFBP)-5 independent of IGF: implications for the mechanism of IGFBP-4 proteolysis by PAPP-A. *FEBS Letters* **504** 36–40.
- Liu J, Koenigsfeld AT, Cantley TC, Boyd CK, Kobayashi Y & Lucy MC 2000 Growth and the initiation of steroidogenesis in porcine follicles are associated with unique patterns of gene expression for individual components of the ovarian insulin-like growth factor system. *Biology of Reproduction* **63** 942–952.

- Liu J, Kosma VM, Vanttinen T, Hyden-Granskog C & Voutilainen R 2002 Gonadotrophins inhibit the expression of insulin-like growth factor binding protein-related protein-2 mRNA in cultured human granulosa-luteal cells. *Molecular Human Reproduction* **8** 136–141.
- Mazerbourg S, Zapf J, Bar RS, Brigstock DR, Lalou C, Binoux M & Monget P 1999 Insulin-like growth factor binding protein-4 proteolytic degradation in ovine preovulatory follicles: Studies of underlying mechanism. *Endocrinology* **140** 4175–4184.
- Mazerbourg S, Zapf J, Bar RS, Brigstock DR & Monget P 2000 Insulin-like growth factor binding protein-4 proteolytic degradation in bovine, equine and porcine preovulatory follicles: regulation by IGFs and heparin-binding domain-containing peptides. *Biology of Reproduction* **63** 390–400.
- Mazerbourg S, Overgaard MT, Oxvig C, Christiansen M, Conover CA, Laurendeau I, Vidaud M, Tosser-Klopp G, Zapf J & Monget P 2001 Pregnancy associated plasma protein-A (PAPP-A) in ovine, bovine, porcine and equine ovarian follicles: involvement in IGFBP-4 proteolytic degradation and mRNA expression during follicular development. *Endocrinology* **142** 5243–5253.
- Monget P, Monniaux D, Pisselet C & Durand P 1993 Changes in insulin-like growth factor-I (IGF-I), IGF-II, and their binding proteins during growth and atresia of ovine ovarian follicles. *Endocrinology* **132** 1438–1446.
- Monget P, Besnard N, Huet C, Pisselet C & Monniaux D 1996 Insulin-like growth factor-binding proteins and ovarian folliculogenesis. *Hormone Research* **45** 211–217.
- Monget P, Mazerbourg S, Delpuech T, Maurel M-C, Manière S, Zapf J, Lalmanach G, Oxvig C & Overgaard M 2003 Pregnancy associated plasma protein-A (PAPP-A) is responsible for IGFBP-2 proteolytic degradation in bovine and porcine preovulatory follicles as well as in human pregnant serum. *Biology of Reproduction* **68** 77–86.
- Monniaux D & Pisselet C 1992 Control of proliferation and differentiation of ovine granulosa cells by insulin-like growth factor-I and follicle-stimulating hormone *in vitro*. *Biology of Reproduction* **46** 109–119.
- Okolsky A, Bézard J, Duchamp G, Driancourt MA, Goudet G & Palmer E 1995 Successive puncture of the dominant follicle followed by ovulation and fertilization: a new model for the study of follicular maturation in the mare. In *Equine Reproduction VI*, pp 385–392. Eds DC Sharp & FW Bazer. Madison, WI: Society for the Study of Reproduction.
- Overgaard MT, Haaning J, Boldt HB, Olsen IM, Laursen LS, Christiansen M, Gleich GJ, Sottrup-Jensen L, Conover CA & Oxvig C 2000 Expression of recombinant human pregnancy-associated plasma protein-A and identification of the proform of eosinophil major basic protein as its physiological inhibitor. *Journal of Biological Chemistry* **275** 31128–31133.
- Oxvig C, Sand O, Kristensen T, Kristensen L & Sottrup-Jensen L 1994 Isolation and characterization of circulating complex between human pregnancy associated plasma protein-A and proform of eosinophil major basic protein. *Biochimica et Biophysica Acta* **1201** 415–423.
- Rivera GM & Fortune JE 2003 Proteolysis of insulin-like growth factor binding proteins -4 and -5 in bovine follicular fluid: implications for ovarian follicular selection and dominance. *Endocrinology* **144** 2977–2987.
- Rivera GM, Chandrasekhar YA, Evans ACO, Giudice LC & Fortune JE 2001 A potential role for insulin-like growth factor binding-protein-4 proteolysis in the establishment of ovarian follicular dominance in cattle. *Biology of Reproduction* **65** 102–111.
- Samaras SE, Hagen DR, Shimasaki S, Ling N & Hammond JM 1992 Expression of insulin-like growth factor-binding protein-2 and -3 messenger ribonucleic acid in the porcine ovary: localization and physiological changes. *Endocrinology* **130** 2739–2744.
- Spicer LJ, Chamberlain CS & Morgan GL 2001 Proteolysis of insulin-like growth factor binding proteins during preovulatory follicular development in cattle. *Domestic Animal Endocrinology* **21** 1–15.
- Wandji SA, Gadsby JE, Barber JA & Hammond JM 2000 Messenger ribonucleic acids for MAC25 and connective tissue growth factor (CTGF) are inversely regulated during folliculogenesis and early luteogenesis. *Endocrinology* **141** 2648–2657.

Received 23 April 2004

Accepted 28 May 2004