Luteinization and proteolysis in ovarian follicles of Meishan and Large White gilts during the preovulatory period

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This experiment was conducted to determine why follicles luteinize faster in the Meishan breed than in the Large White breed of pig. Follicles were recovered during the late follicular phase from ovaries of both breeds before and after administration of hCG given to mimic the LH surge. First, the patterns of cholesterol transporters (high and low density lipoproteins: HDL and LDL) were compared. Cholesterol transporters detected in follicular fluid consisted of HDL only. Similar amounts of Apolipoprotein A-I were found in all samples. There was no obvious breed effect on minor lipoproteins found in the HDL-rich fraction, and this pattern was altered similarly by hCG in the two breeds. The LDL-rich samples of serum from both breeds contained similar amounts of protein. Second, three steroidogenic enzymes, adrenodoxin, 17 α -hydroxylase-lyase (P450_{17 α}) and 3 β -hydroxysteroid-dehydrogenase (3 β -HSD) were detected by immunohistochemistry and quantified by image analysis on sections of the two largest follicles. Before hCG treatment, theca interna cells demonstrated immunoreactivities for adrenodoxin (strong), P450_{17 α} and 3 β -HSD (very strong), whereas granulosa cells displayed immunoreactivities for adrenodoxin only. After hCG treatment, the localization of the enzymes was unchanged but the staining intensity of adrenodoxin on granulosa cells and 3 β -HSD on theca cells increased ($P < 0.01$ and $P < 0.05$, respectively). Breed effects were detected for the amounts of adrenodoxin in theca cells (Meishan > Large White; $P < 0.05$) and of 17 α -hydroxylase (Large White > Meishan, $P < 0.01$). Breed $\times$ treatment interactions were never detected. Finally, gelatinases, plasminogen activator, plasminogen activator inhibitor, tissue inhibitors of metalloproteases (TIMP-1 and TIMP-2) were visualized by direct or reverse zymography or western blotting. Whatever the stage relative to LH administration, follicular fluid from Large White gilts contained more TIMP-1, and TIMP-2 ($P < 0.02$ and $P < 0.01$, respectively). No breed effect was detected for the amounts of gelatinases and plasminogen activator inhibitor 1. However, for these parameters, a significant breed $\times$ time interaction was obvious, as the Meishan follicles had a greater response to hCG ($P < 0.01$). Since proteolysis plays a key role in the bioavailability of growth factors such as insulin-like growth factor 1, fibroblast growth factor and transforming growth factor β , which have the ability to alter gonadotrophin-induced progesterone production in pigs, the differences observed in its control in the present study may explain, at least in part, the different patterns of luteinization observed in Meishan and Large White follicles.

Introduction

Chinese Meishan pigs are more prolific than European Large White pigs (Legault and Caritez, 1983; Bolet *et al.*, 1986). This appears to be largely due to an altered embryonic growth and improved embryonic development and survival (Bazer *et al.*, 1988, 1991; Simmen *et al.*, 1989; Wilmot *et al.*, 1992; Anderson *et al.*, 1993), although additional alterations of

ovulation rate have been identified in some studies (Ashworth *et al.*, 1992; Haley and Lee, 1993). However, the mechanisms involved in these differences have not been fully clarified.

A positive relationship between progesterone secretion during the early luteal phase and embryo survival in pigs has been demonstrated (Pharazyn *et al.*, 1991; Einarsson and Rojittikhun, 1993; Zak *et al.*, 1997). Plasma progesterone concentrations increase earlier after the LH surge in Meishan than they do in Large White gilts (Hunter *et al.*, 1996) and

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follicular luteinization after hCG administration at the preovulatory stage proceeds faster in Meishan than it does in Large White gilts (Downey and Driancourt, 1994). Because this earlier progesterone increase may play a key role in the superior embryo survival of Meishan gilts, the possible mechanisms involved in follicular luteinization were compared between Meishan and Large White breeds in the present study.

A design identical to that described by Downey and Driancourt (1994), in which follicular progesterone output 16 h after an hCG challenge was fivefold higher in Meishan than it was in Large White gilts, was used to assess the contribution of three candidates (that is, the lipoprotein supply to follicles, the changes in amounts and localization of steroidogenic enzymes induced by hCG and hCG-induced follicular proteolysis) to this altered luteinization process. Lipoproteins are the main source of cholesterol for follicular steroidogenesis (Gore Langton and Armstrong, 1988). Furthermore, high density lipoproteins (HDL) as well as low density lipoproteins (LDL) have the ability to affect progesterone secretion by swine granulosa cells (Veldhuis *et al.*, 1984; Rajkumar *et al.*, 1989). Hence, an increased hCG induced lipoprotein supply to Meishan follicles may explain the faster progesterone increase in this breed. This hypothesis was addressed in the first part of the present study.

Follicular steroidogenesis in Meishan and Large White gilts has been described previously on the basis of concentrations of steroids in follicular fluid or follicle incubation media, or from cell culture studies (Hunter *et al.*, 1993, 1994; Downey and Driancourt, 1994). However, owing to the dynamic nature of steroidogenesis, these approaches have failed to identify why breed differences occur. Available information in swine on the tissue specific localization of steroidogenic enzymes (3β -HSD: theca; 17α hydroxylase: theca; P450 side chain cleavage (P450_{scc}): theca and granulosa) (Conley *et al.*, 1995; Meduri *et al.*, 1996) suggests that the faster progesterone increase observed in Meishan gilts after hCG treatment could be generated either by the induction by hCG of 3β -HSD among granulosa cells in this breed or to an overall alteration in the amounts of the enzymes involved in progesterone production (P450_{scc}, 3β HSD) or utilization (17α hydroxylase). This hypothesis was explored in the second part of the study.

Since there is no evidence for differences in follicular sensitivity to LH among breeds (Biggs *et al.*, 1993), autocrine and paracrine regulations of LH action on follicular steroidogenesis may differ between the two breeds studied here. At least three growth factors (insulin-like growth factor I (IGF-I): Urban *et al.*, 1990; Caubo *et al.*, 1989; fibroblast growth factor (FGF): Biswas *et al.*, 1988; transforming growth factor β (TGF β): Kubota *et al.*, 1994) have the ability to alter gonadotrophin-induced progesterone secretion in pigs. However, to be active, these growth factors require proteolysis from their binding proteins (IGF-I) or release by proteolysis from the extracellular matrix (FGF) or activation by proteolysis from a latent form (TGF β). Some of the factors involved in the LH-induced proteolytic cascade have been characterized in pig follicles (Politis *et al.*, 1990; Smith *et al.*, 1994) but it is unknown whether proteolysis after an LH

surge proceeds similarly in Meishan and Large White follicles. This was the last aim of the present study.

Materials and Methods

Animals and experimental design

After they had completed at least three oestrous cycles after puberty, gilts from the Meishan and Large White breeds were given an 18 day altrenogest (Regumate, Roussel Uclaf, Romainville) treatment to synchronize them. Hemi-ovariectomy was conducted under general anaesthesia induced by sodium thiopentone (Specia, Rhone Poulenc Rorer, Paris) and maintained by halothane in oxygen during the mid-late follicular phase (at day 4–5 after cessation of Regumate administration) (Downey and Driancourt, 1994). An i.m. injection of 500 iu hCG (Chorulon, Intervet, Angers) to mimic the LH surge was given 8 h after surgery, and the second ovary was removed at slaughter 16 h later, a time when early luteinization of the ovulatory follicles has been shown to have started (Downey and Driancourt, 1994).

The experiment involved 16 gilts (eight Meishan and eight Large White) in two replicates. At the time of slaughter, the respective ages of the gilts were 242 ± 1.6 days and 270 ± 1.0 (replicates 1 and 2) as opposed to 216 ± 0.2 and 197 ± 0.3 days (replicates 1 and 2) for Large White and Meishan breeds, respectively.

Lipoprotein characterization

Follicular fluid from the third and fourth largest follicles of each gilt was used (replicate 1: five gilts per breed). Samples were mixed to create pools of follicular fluid of a specific breed (Meishan or Large White) at a specific time (pre- or post-hCG) to obtain enough material for ultracentrifugation. Serum from the preovulatory period of each breed was also obtained.

One millilitre pools of follicular fluid, as well as 3 ml pools of serum, were fractionated by ultracentrifugation in a density gradient according to the procedure described by Hermier *et al.*, (1985). Ultracentrifugation was performed in a Beckman SW 41 rotor and a Beckman L8-55 ultracentrifuge at 200 000 g for 48 h at 15°C. After centrifugation, density gradients were fractionated by aspiration of 0.5 ml fractions with a narrow bore pipette. Phospholipid concentration was determined in each fraction of the serum pools (but not of the follicular fluid pools owing to limited amounts of material) using an enzymatic kit (Bio Mérieux, Marcy L'Etoile) (Takayama *et al.*, 1977) to characterize the distribution of lipoproteins along the density gradient (Hermier *et al.*, 1985). Because the phospholipid content of lipoproteins is relatively constant (20–25%), phospholipid concentrations in lipoprotein subfractions are good indicators of the relative proportions of the major lipoprotein classes and of their density limits.

After determination of the lipoprotein profile in serum, the LDL- and HDL-rich fractions were identified. This profile was used to identify fractions of follicular fluid to be pooled

to form putative HDL- and LDL-rich fractions of follicular fluid. All fractions were then dialysed extensively in Spectrapor tubing (Spectrum Medical Industries, Los Angeles, CA) for 48 h at 4°C against a solution containing 0.05 mol NaCl l⁻¹, 0.005 mol NH₄ HCO₃ l⁻¹, 0.04% (v/v) EDTA and 0.01% (v/v) sodium azide at pH 7.4. After quantification of the protein concentrations (Lowry *et al.*, 1951), LDL- and HDL-rich samples from serum and follicular fluids of both lines were collected before or after hCG administration and, since they contained similar amounts of proteins, they were subjected to one-dimensional electrophoresis on 4% gel (LDL) or a 3–22% acrylamide gradient (HDL). Slab gels were then stained with silver according to the method of Henkeshoven and Dernick (1985).

Immunohistochemistry

Immunohistochemistry was conducted on the two largest follicles of each ovary (replicate 1: five gilts per breed) after they had been excised intact from the ovaries. Each specimen was coated with Tissue-Tek OCT cryoprotectant embedding medium (Miles Inc., Napperville, IL) and frozen in liquid nitrogen. Frozen sections (6 µm) were cut serially at -20°C with an ultramicrotome. They were air dried for 30 min, fixed in cold acetone (-20°C) for 5 min and allowed to dry for a further 20 min. The sections were wrapped in cellulose and stored at -20°C. Furthermore, one section of each follicle was stained with Feulgen and pycnotic bodies were counted to check that all follicles sectioned were healthy.

Immunostaining was performed according to the method described by Meduri *et al.* (1996). This method is based on the formation of a streptavidin-biotin complex using the Vectastain ABC kit (Vector Laboratory, Burlingame, CA). Sections were rinsed in 10 mmol PBS l⁻¹ (pH 7.2) and incubated at room temperature for 20 min with sheep serum diluted at 1:20 in PBS containing 5% (w/v) BSA, to block nonspecific background staining. Sections were then incubated overnight at 4°C in a moist chamber with rabbit polyclonal antibodies directed against bovine adrenodoxin (1:3000), guinea-pig 17α-hydroxylase-lyase (P450_{17α}, 1:7000) and 3β-hydroxysteroid dehydrogenase (3β-HSD) from bovine adrenal cortex (1:4000). Negative control sections were incubated with normal sheep serum (1:50). After rinsing three times for 3 min, sections were treated with biotinylated sheep anti-rabbit IgG (1:200) for 30 min and with avidin-peroxidase conjugate (1:100) for 45 min at room temperature. The chromogen used was amino-ethylcarbazole (Sigma Chemicals, St Louis, MO) in acetate buffer containing 0.02% (v/v) hydrogen peroxide. After counterstaining with Mayer's haematoxylin, the sections were examined under a light microscope (Leitz Diaplan).

Primary antibodies were kindly provided by E. M. Chambaz, unité INSERM 244, Centre d'études nucléaires, Grenoble France (anti 3β-HSD), S. Takemori and S. Kominani, Faculty of Integrated Arts and Sciences, Hiroshima City, Japan (anti-P450_{17α}) and I. Hanukoglu, Weizmann Institute of Science, Rehovot, Israël (anti-adrenodoxin).

Immunostaining for each enzyme was conducted on all samples simultaneously and its intensity was quantified by

densitometry using an image analysis system (Samba TM 2005, ALCATEL TITN, Meylan). The system was calibrated to have constant parameters for light (that is, intensity and area of light beam) throughout the experiment. Absorbance was determined in fields completely occupied by cells in the theca interna and in the granulosa of one section per follicle at 20 different 45 µm² locations to quantify the intensity of staining (Huet *et al.*, 1997). Absorbance was expressed in arbitrary units. P450_{17α} and 3β-HSD stainings were not homogeneous in theca interna cells. Therefore, the percentage of stained surface was determined in addition to absorbance.

Comparisons among groups were performed by analysis of variance using the general linear model procedure of SAS. A split-plot design was used with breed, animal tested within breed, time (before hCG versus after hCG) and breed × time as sources of variation. The effect of breed was tested using animal within breed as the error term, and the effects of time and breed × time were tested using the residual error term. When the influence of time was significant, a new analysis was performed within each breed, with time and animal as main effects and with the diameter of each follicle as a covariate.

Study of follicular proteolysis

This study involved the three largest follicles of each ovary obtained in the second replicate of the experiment (*n* = 3 gilts per breed). Gelatinases were demonstrated by zymography. A modified zymography was also used to assess the presence of plasminogen activator. Reverse zymography was used to demonstrate TIMP-1 and TIMP-2. Finally, the plasminogen activator inhibitor 1 (PAI-1) was demonstrated by western blot analysis.

Zymography

Samples (5 µl) of follicular fluid of each follicle were assessed by zymography for gelatinase activity. SDS-PAGE was used with the addition of 1 mg gelatin ml⁻¹ to a 10% resolving gel (Fisher and Werb, 1990) and was achieved under non-reducing conditions. At the completion of migration, the gels were washed in 2.5% (v/v) Triton-X100 twice for 30 min, rinsed thoroughly in water and then incubated overnight in buffer (50 mmol Tris-HCl l⁻¹, 5 mmol CaCl₂ l⁻¹; pH 7.8). Gelatinase activity was visualized as a clear band against a blue background after staining in Coomassie blue and destaining in 30% methanol: 10% acetic acid in water.

Modified zymography for plasminogen activator

The same procedure as described above was used, except that the resolving gel contained both casein (1 mg ml⁻¹) and plasminogen (13 µg ml⁻¹). Under these conditions, plasminogen is cleaved by plasminogen activators, yielding plasmin which then degrades the casein (Fisher and Werb,

1990). The areas containing enzyme are clear against a blue background. Again, all samples (5 μ l per lane) were run on an individual basis.

Reverse zymography

Follicular fluids were analysed for the presence of TIMP, on mini gels, of 1 μ l samples by zymography after SDS-PAGE (10% gel containing 1 mg gelatin ml⁻¹ and a metalloprotease (MMP) preparation) under non-reducing conditions. A kit purchased from University Technologies International (Calgary, Alberta) was used for this purpose. After washing in a solution of 2.5% Triton-X100, 50 mmol Tris l⁻¹ (pH 7.5) and 5 mmol CaCl₂ l⁻¹, the gel was incubated for 24 h in the same buffer without Triton-X100. The gel was stained with Coomassie blue and the presence of TIMP was visualized by the presence of dark bands on a clearer background. Standards (provided with the kit) helped to establish the presence of TIMP in the samples.

Western blotting

A subsample (from the two largest follicles before LH and the two largest follicles after LH) of follicular fluid of each gilt was used. Follicular fluid samples (5 μ l) were fractionated with SDS-PAGE under reducing conditions. Proteins were electrotransferred onto a nitrocellulose filter (0.2 μ m pore size) overnight at 4°C. After electrotransfer, filters were incubated for 2 h at room temperature with 20 mmol Tris-buffered saline (TBS) l⁻¹ (pH 7.6) containing 10% non-fat dry milk powder (NFDMP) and 0.2% (v/v) Tween 20 to saturate non-specific binding sites. Thereafter, the filters were incubated for 1 h at 37°C with the primary antibody (rabbit anti-human PAI-1, American Diagnostica, final dilution 1:750) for 2 h at 37°C in TBS containing 5% NFDMP. This procedure was followed by two washes in TBS containing 10% NFDMP and 0.2% Tween 20. Filters were then incubated for 1 h at 37°C in TBS containing 5% NFDMP and peroxidase-labelled goat anti-rabbit IgG (final dilution 1:1000) (Sanofi Pasteur, Paris). The signal was revealed by enhanced chemiluminescence (ECL) (Amersham, Les Ulis).

Image and data analysis

Dark bands present on the reverse zymographs and on the blots were scanned and the magnitude of the signal in each lane was qualified by image analysis (Kepler Software, Large Scale Biology, Rockville, TN) as described by Driancourt *et al.* (1996). The signal obtained on the zymograms for gelatinases and for plasminogen activator were white. Scanning and qualification were achieved as described above, except that the amounts of signal (which were negative because the bands were white) are presented as absolute values.

All data regarding proteolysis were analysed by ANOVA for breed and time relative to hCG effects as well as breed $\times$ time interactions. Gilt within breed was entered in

the model and breed effects were tested using the later residual.

Results

Follicular features

The average (mean of the three largest follicles) follicular diameters (pooled over the two replicates) of the Large White follicles were 6.3 ± 0.2 and 7.2 ± 0.2 mm before and after hCG administration, respectively. The corresponding values for Meishan follicles were 6.1 ± 0.1 and 6.6 ± 0.1 mm before and after hCG administration, respectively (Large White > Meishan after hCG administration; $P < 0.05$). None of the gilts in the groups had initiated their LH surge before hCG administration, as evidenced by circulating concentrations of 1.2 ± 0.04 and 1.2 ± 0.13 ng ml⁻¹ (Large White and Meishan gilts, respectively) measured in the LH assay described and validated by Camous *et al.* (1985).

Lipoprotein characterization in follicular fluid and serum

The serum lipoprotein profile deduced from the determination of phospholipids in each fraction from Meishan and Large White serum allowed identification of LDL and HDL (the two major peaks) on the basis of density (Fig. 1). For electrophoresis, fractions were pooled as follows: LDL-rich fraction (fractions 1–9: density < 1.055 arbitrary units) and HDL-rich fraction (fractions 12–16: 1.075 < density < 1.145 arbitrary units). The last phospholipid peak (fractions 19–22: density > 1.180 arbitrary units), of which lipid moiety consisted almost exclusively of phospholipids and contained mostly serum proteins, was not studied. Detectable amounts of proteins could be measured in the LDL-rich fraction of serum samples (0.235 and 0.241 mg ml⁻¹ in Meishan and Large White serum, respectively), but not in the follicular fluid samples, whatever the time of sampling and the breed. The resolution of the LDL from serum on one-dimensional gels showed that amounts of Apolipoprotein B were similar between the two breeds (data not shown). Protein concentrations in the HDL-rich fraction of serum were 0.554 and 0.574 mg ml⁻¹ in Meishan and Large White females, respectively. In both breeds, hCG administration induced a clear decrease in the protein concentrations of the HDL-rich fraction of follicular fluid (Meishan before hCG: 0.685 mg ml⁻¹; Meishan after hCG: 0.302 mg ml⁻¹; Large White before hCG: 0.723 mg ml⁻¹; Large White after hCG: 0.370 mg ml⁻¹). The HDL-rich samples were submitted to one-dimensional PAGE and silver staining (Fig. 2). In all HDL-rich samples, a major band corresponding to the molecular weight of Apolipoprotein AI was detected at 28 kDa, the intensity of which was similar in all samples. An additional major band was detected with a molecular mass typical of Apolipoprotein AIV (46 kDa) in serum samples and as a doublet of 43–45 kDa in follicular fluid samples. There was no breed effect on the amounts of proteins corresponding to this doublet but hCG administration appeared to increase the amounts of the 45 kDa band and to

Fig. 1. Serum lipoprotein profile deduced from determination of phospholipid concentrations in successive fractions of a density gradient from serum of Meishan (□) and Large White (●) gilts. Fraction 1 is the top fraction containing very low density lipoproteins.

decrease that of the 43 kDa band in both breeds (Fig. 2). The 43 kDa band was missing in the serum samples. Additional proteins affected by hCG administration (but not by breed) were found at 23 kDa and 11 kDa, the latter corresponding to the molecular mass of Apolipoprotein Cs. Apolipoprotein E (molecular mass, 34 kDa) was not detected in any sample.

Immunohistochemistry

Before hCG treatment, theca interna cells demonstrated immunoreactivities for adrenodoxin (strong), P450_{17α} and 3β-HSD (very strong) whereas granulosa cells displayed immunoreactivities for adrenodoxin only (Fig. 3). After hCG treatment, localization of the enzymes was unchanged. Quantitative estimates of the amounts of each enzyme were obtained after image analysis and compared (Tables 1 and 2). No breed × time interaction was obvious for any enzyme in granulosa or theca cells (Tables 1 and 2). Meishan follicles displayed higher amounts of adrenodoxin in theca cells and, to a smaller extent, in granulosa cells than did Large White follicles ($P < 0.05$) (Table 1). However, amounts of P450_{17α} were significantly increased in Large White compared with Meishan follicles, irrespective of the time relative to hCG administration (Table 2). Administration of hCG altered the amounts of adrenodoxin in granulosa cells ($P < 0.01$) and of 3β-HSD in theca cells ($P < 0.05$; Tables 1 and 2).

Study of follicular proteolysis

When follicular fluid samples were loaded onto an acrylamide gel containing gelatin (zymography), a clear signal at 92 kDa was present in all samples. Significant amounts of other gelatin-degrading proteases at lower

Fig. 2. One-dimensional separation (3–22% gradient) of the lipoproteins contained in the high density lipoprotein rich fractions of the serum and follicular fluid of Meishan and Large White breeds of gilt before and after administration of hCG. Molecular mass markers (kDa) are shown on the left. Lane 1, high density lipoproteins (HDLs) from Meishan serum; lane 2, HDLs from Large White serum; lane 3, HDLs from Meishan follicular fluid collected before hCG administration; lane 4, HDLs from Meishan follicular fluid collected after hCG administration; lane 5, HDLs from Large White follicular fluid collected before hCG administration; lane 6, HDLs from Large White follicular fluid collected after hCG administration. For comparison, HDLs from human serum are shown in lane 7.

molecular mass could not be visualized in samples of specific groups. Analysis of the magnitude of the absolute value of the signal by ANOVA revealed a significant effect of hCG administration ($P < 0.01$) and a significant breed × time interaction ($P < 0.01$), as the amounts of gelatinase increased sevenfold after hCG administration in Meishan follicles, while increasing only 1.5-fold in Large White follicles (Fig. 4).

When follicular fluid samples were loaded on an acrylamide gel containing casein and plasminogen to detect plasminogen activator, a single clear signal of 80 kDa resulted. Analysis of the magnitude of the signal by ANOVA demonstrated no significant main effects but an interaction between breed and hCG ($P < 0.01$). Follicular fluid from Large White follicles contained twice the amounts of plasminogen activator as that of Meishan follicles before hCG administration, but this difference was reduced after hCG administration (Fig. 5).

TIMP-1 and TIMP-2 were identified by reverse zymog-

Fig. 3. Patterns of immunoreactive (a) adrenodoxin, (b) P450_{17α}, (c) P450 aromatase, and (d) 3β-HSD observed before hCG administration on follicles from Large White gilts. The localization of these enzymes was similar in follicles from Meishan gilts. Scale bars represent 100 μm.

raphy. In most of the samples of Large White follicular fluid and in part of the samples of Meishan follicular fluid, two bands (at 29 kDa and at 24 kDa) were detected. Inclusion of TIMP standards in the gel established that the bands observed were TIMP-1 and TIMP-2, respectively. Statistical analysis of the data demonstrated a significant breed effect ($P < 0.02$) and a significant ($P < 0.05$) breed $\times$ time interaction (Fig. 6) for TIMP-1, since TIMP-1 amounts increased after hCG in Large White follicles, while remaining low and steady in Meishan follicles after hCG administration (Fig. 6). A significant effect ($P < 0.01$) of the breed and a significant breed $\times$ time interaction ($P < 0.01$) were detected for TIMP-2. TIMP-2 concentrations were higher in Large White follicles, and an increase after hCG was noted only in Meishan follicles.

Plasminogen activator inhibitor 1 in follicular fluid was identified and quantified by western blot. After chemiluminescence (ECL) revelation, a single band was obvious on the films (data not shown). Quantification of the signal demonstrated a significant breed $\times$ hCG administration interaction ($P < 0.01$), as the induction of PAI-1 by hCG was larger in Meishan follicles compared with Large White follicles.

Discussion

The aim of this study was to compare the effects of an LH surge (mimicked by hCG injection) on the final preovulatory maturation of follicles belonging to Meishan and Large White gilts to determine which mechanisms are involved in the earlier luteinization of Meishan compared with Large White females. Three possible hypotheses for this faster luteinization that relate to the cholesterol supply to follicles, to the amounts of steroidogenic enzymes and to the mechanisms controlling follicular proteolysis were studied in the present work.

Whether cholesterol supply to follicles is affected by breed or time relative to hCG has not been clarified before. Lipoproteins constitute the most abundant source of cholesterol for steroidogenesis (Gore Langton and Armstrong, 1988). Both LDL and HDL appear to be able to modulate progesterone secretion of swine granulosa cells *in vitro* (Veldhuis *et al.*, 1984; Rajkumar *et al.*, 1989). However, since LDL do not gain access to follicular fluid (Chang *et al.*, 1976; Veldhuis *et al.*, 1984), only HDL are likely to be involved in the control of progesterone secretion by granulosa cells *in vivo*. In contrast, because the theca layer is

Table 1. Mean absorbance, expressed in arbitrary units (mean $\pm$ SEM), of adrenodoxin staining in theca interna cells and granulosa cells of large follicles of Large White and Meishan gilts before and after hCG treatment

Treatment	Breed	Adrenodoxin	
		Theca interna cells	Granulosa cells
Before hCG	Large White	36.0 $\pm$ 4.4	29.6 $\pm$ 2.8
	Meishan	60.2 $\pm$ 12.9	40.8 $\pm$ 4.8
After hCG	Large White	43.9 $\pm$ 14.3	39.5 $\pm$ 11.6
	Meishan	56.5 $\pm$ 7.2	50.1 $\pm$ 6.4
Significance ^a		Breed* Treatment NS B $\times$ T NS	Breed NS Treatment** B $\times$ T NS

^aStatistical significance of breed, hCG treatment or the interaction breed $\times$ treatment (B $\times$ T) indicated by ** $P < 0.01$; * $P < 0.05$ and NS $P > 0.10$.

highly vascularized, both LDL and HDL from serum may affect steroidogenesis by theca cells *in vivo*. There was no breed difference in the pattern of LDL in serum visualized after ultracentrifugation and one-dimensional electrophoresis. One-dimensional patterns of the HDL-rich fractions were compared between the two breeds at specific times relative to hCG. The protein profile of the follicular fluid samples was close to that reported by Veldhuis *et al.* (1984). Apolipoprotein AI and a doublet at 43–45 kDa were the main components of the HDL-rich fraction in both breeds. The 43 kDa band was present in follicular fluid but was not detected in the serum samples. Its origin (possibly by proteolysis) requires further investigation. In contrast to the situation in rats (Nicosia *et al.*, 1992), ovarian production of Apolipoprotein E was not detected. The ratio between the amounts of the two forms of the 43–45 kDa doublet appeared to be modified by hCG administration. However, this effect was similar in the two breeds. Other low molecular mass bands were also modified by hCG but not by breed. In addition, it was noted that the amounts of proteins present in the HDL-rich fraction of follicular fluid of both breeds

were reduced by hCG administration, which may reduce the cholesterol supply to luteinizing granulosa cells. Alternatively, this reduction in the HDL-rich fraction could be a consequence of increased binding of HDL to follicular cells after hCG administration. In any case, the similarities between breeds in the patterns of HDL in follicular fluid and serum, and in the amounts of HDL and LDL in serum, do not support the hypothesis that a different cholesterol supply to follicles plays a major role in generating the faster luteinization found in Meishan follicles.

It has not been investigated previously whether amounts of steroidogenic enzymes differ between Meishan and Large White follicles before or after an ovulatory hCG signal. Extensive information is available on steroidogenesis in pig follicles based on follicular fluid concentrations (Foxcroft and Hunter, 1985; Hunter *et al.*, 1993) or cell culture studies (Evans *et al.*, 1981; Stoklosowa *et al.*, 1982; Tsang *et al.*, 1985; Hunter *et al.*, 1994). Furthermore, data from northern blots on mRNAs for steroidogenic enzymes (Conley *et al.*, 1994; Guthrie *et al.*, 1994) have largely confirmed this information. Comparison of the amounts of specific steroidogenic enzymes in Meishan and Large White follicles using semi-quantitative immunohistochemistry has not been undertaken previously. Image analysis of ovarian sections of sheep (Huet *et al.*, 1997) using the same software proved useful for quantitating steroidogenic enzymes in follicular walls. Because heterogeneity within the granulosa (Lahtemaki *et al.*, 1982) and theca layers, indicated by the use of morphological or functional (LH receptors) markers (Meduri *et al.*, 1992), has been reported in gilts, an approach retaining this heterogeneity (that is, immunohistochemistry on intact follicles) was selected. The location of P450_{17 α} and 3 β -HSD in large follicles was similar to that described by Conley *et al.* (1995) and Meduri *et al.* (1996). The location of the enzymes studied was similar in both Meishan and Large White gilts, whereas the staining intensity of adrenodoxin and P450_{17 α} differed slightly between breeds. The lower P450_{17 α} content in Meishan gilts is in agreement with the lower basal testosterone production observed by Downey and Driancourt (1994) in preovulatory follicles. Adrenodoxin belongs to the enzymatic complex catalysing the conversion of cholesterol to pregnenolone, which is the first and rate-

Table 2. Percentage of stained surface and mean absorbance, expressed in arbitrary units, of 17 α -hydroxylase-lyase (P450_{17 α}) and 3 β -hydroxysteroid-dehydrogenase (3 β -HSD) (mean $\pm$ SEM) in theca interna cells of large follicles of Large White and Meishan gilts, before and after hCG treatment

Treatment	Breed	P450 _{17α}		3 β -HSD	
		Staining percentage	Absorbance	Staining percentage	Absorbance
Before hCG	Large White	51.5 $\pm$ 23.8	81.3 $\pm$ 5.2	34.8 $\pm$ 13.5	52.2 $\pm$ 2.8
	Meishan	35.8 $\pm$ 16.2	76.4 $\pm$ 2.6	30.7 $\pm$ 23.0	53.2 $\pm$ 3.2
After hCG	Large White	69.5 $\pm$ 17.5	88.2 $\pm$ 7.6	57.0 $\pm$ 8.7	59.7 $\pm$ 4.5
	Meishan	48.5 $\pm$ 10.9	78.1 $\pm$ 2.6	43.8 $\pm$ 3.7	54.6 $\pm$ 4.2
Significance ^a		Breed [†] Treatment NS B $\times$ T NS	Breed** Treatment NS B $\times$ T NS	Breed NS Treatment* B $\times$ T NS	Breed NS Treatment* B $\times$ T NS

^aStatistical significance of breed, hCG treatment or the interaction breed $\times$ treatment (B $\times$ T) indicated by ** $P < 0.01$; * $P < 0.05$; [†] $P < 0.10$ and NS $P > 0.10$.

Fig. 4. Visualization of gelatinases in the follicular fluid of follicles from Large White and Meishan gilts before (a) and after (b) hCG administration. Molecular mass markers are shown on the left. (a) Lanes 1–6 contain 5 μ l Meishan follicular fluid, while lanes 7–12 contain 5 μ l Large White follicular fluid, obtained before hCG administration. (b) Lanes 1–6 contain 5 μ l Meishan follicular fluid, while lanes 7–13 contain 5 μ l Large White follicular fluid, obtained after hCG administration. (c) Densitometry analysis of all samples. ■, Large White before hCG; ▨, Large White after hCG; □, Meishan before hCG; ▩, Meishan after hCG.

limiting step in the biosynthesis of steroid hormones. The greater amount of adrenodoxin in Meishan large follicles may contribute to the provision of more substrate for steroidogenesis. Since P450_{17 α} is less abundant in Meishan

Fig. 5. Visualization of tissue-type plasminogen activator (tPA) in the follicular fluid of Large White and Meishan gilts before and after hCG administration. Molecular mass markers are shown on the left. The arrow indicates the band presumed to be tPA revealed by casein zymography. (a) Lanes 1–6 contain 5 μ l of Meishan follicular fluid, while lanes 7–12 contain 5 μ l Large White follicular fluid, obtained before hCG. (b) Lanes 1–6 contain 5 μ l Meishan follicular fluid, while lanes 7–13 contain 5 μ l Large White follicular fluid, obtained after hCG. (c) Densitometry analysis of all samples. ■, Large White before hCG; ▨, Large White after hCG; □, Meishan before hCG; ▩, Meishan after hCG.

large follicles, pregnenolone may be converted to progesterone preferentially by 3 β -HSD, resulting in a greater production of progesterone in Meishan gilts. However, the present data do not show any breed $\times$ time interaction for

Fig. 6. (a) Visualization of tissue inhibitor of metalloproteinase 1 (TIMP-1) and TIMP-2 in the follicular fluid of Large White and Meishan gilts before and after hCG administration. Lanes 1–6, 1 μ l Large White follicular fluid, obtained after hCG; lanes 7 and 8, 1 μ l Large White follicular fluid, obtained before hCG; lane 9 (STD) TIMP standards; lanes 10 and 11, 1 μ l Meishan follicular fluid before hCG; lanes 12–14, 1 μ l Meishan follicular fluid after hCG. Densitometry analysis of all samples for (b) TIMP-1 and (c) TIMP-2. ■, Large White before hCG; ▨, Large White after hCG; □, Meishan before hCG; ▤, Meishan after hCG.

these enzymes. The present study also demonstrated that, after hCG administration, the adrenodoxin immunoreactivity of granulosa cells and the 3β -HSD immunoreactivity of theca cells were increased in Meishan and Large White gilts. In conclusion, these results do not support the hypothesis that the faster luteinization of Meishan follicles after an LH–hCG surge is related to differences in steroidogenic enzymes.

Since there are no differences between Meishan and Large White breeds in follicular sensitivity to LH (Biggs *et al.*, 1993), it may be that autocrine–paracrine regulations of LH-induced steroidogenesis by growth factors after LH administration differ between the two breeds. Since the bioavailability of growth factors commonly requires proteolysis (Flaumenhaft and Rifkin, 1992), the control of

follicular proteolysis in the two breeds was explored. Follicular proteolysis involves two families of proteases (plasminogen activators and collagenases) and two families of inhibitors (plasminogen activator inhibitor and TIMPs) (Matrisian, 1992). Most of the members of these families were compared in Meishan and Large White follicles before and after hCG administration. Earlier observations in pig follicular fluid (Politis *et al.*, 1990) showed that tissue-type plasminogen activator (tPA) and PAI-1 were present in follicular fluid from large follicles and were modulated differently by LH administration. In addition, Smith *et al.* (1994) demonstrated that TIMP-1 was also present in follicular fluid and that the amounts of a TIMP-1-like protein could be increased by LH. The present study extends these earlier findings by providing a comprehensive approach of the proteolytic cascade and by comparing it between Meishan and Large White follicles.

The present study also confirmed several earlier findings regarding the plasmin generation system. First, the main plasminogen activator in pig follicular fluid was shown to be tPA (Politis *et al.*, 1990) by the presence of a lytic band at 80 kDa, the molecular mass of tPA. In contrast, no signal at a lower molecular mass corresponding to urokinase-type plasminogen activator (uPA) was present in either breed. Second, the plasmin generation system appeared to be sensitive to hCG, as reported by Politics *et al.* (1990). Although this was not obvious for tPA, owing to a short interval between hCG administration and sampling of follicular fluid, PAI-1 appeared to be affected by hCG. The induction of PAI-1 by hCG was more marked in Meishan follicles than in Large White follicles. Overall, the higher amounts of tPA found in Large White follicular fluid, together with the relative inability of hCG to induce PAI-1 in this breed (as opposed to what happens in Meishan follicles) may result in a higher supply of plasmin in Large White follicular fluid, particularly after hCG. Since plasmin is known to activate latent TGF β (Lyons *et al.*, 1990), and since TGF β inhibits progesterone synthesis (Kubota *et al.*, 1994), the end result may be a limitation of luteinization in Large White follicles.

A number of new findings regarding MMPs and TIMPs were made in the present study, extending earlier reports (Smith *et al.*, 1994). First, a high molecular mass gelatinase (at 92 kDa), possibly MMP-9, was shown to be the major MMP present in follicular fluid from large healthy follicles. No other lower molecular mass gelatinases, typical of atretic follicles (Garcia *et al.*, 1997) could be observed. As reported in sheep (Russel *et al.*, 1995), the amounts of the 92 kDa gelatinase were increased after hCG administration. The hCG increase in the 92 kDa gelatinase was sevenfold stronger in Meishan follicles than in Large White follicles. Whether similar changes occur within the theca–granulosa cell layers remains to be established. Second, neither TIMP-2 nor TIMP-1 were increased in follicular fluid 16 h after hCG compared with the pre-injection amounts. While this is the first report of TIMP-2 amounts in pig follicular fluid, the lack of induction of TIMP-2 by hCG is in agreement with an earlier report in sheep (Russel *et al.*, 1995). The lack of increase in amounts of TIMP-1 after hCG administration contrasts with another report in pigs (Smith *et al.*, 1994). A shorter time lag between hCG injection and ovariectomy in the present

study (16 h versus 24 h) may explain this discrepancy. Third, clear breed differences in the changes in the amounts of TIMP-1 and TIMP-2 were observed both before and after hCG administration. Whatever the stage relative to hCG administration, Meishan follicles contained very limited amounts of TIMP-1 and TIMP-2. The interpretation of these data is complicated by the fact that the amounts of TIMP-1 and TIMP-2 present in follicular fluid may not indicate accurately the amounts actually present within the follicular cells. However, our present working hypothesis is as follows. Since TIMP-1 and TIMP-2 bind and inactivate matrix metalloproteases (Matrisian, 1992), the lower amounts of these inhibitors in Meishan follicles are likely to increase the bioactivity of matrix MMPs in Meishan follicles. Since hCG has a stronger stimulatory effect on the amounts of matrix MMPs in Meishan follicles, high amounts of bioactive matrix MMPs may be present in Meishan follicular fluid after hCG administration. The physiological consequences of this increased MMP may be an increased availability of IGF-I after IGF binding protein (IGFBP) proteolysis (Fowlkes *et al.*, 1994). The resulting high amounts of IGF-I released from IGFBP would then stimulate progesterone production by ovarian cells more efficiently in Meishan follicles (Caubo *et al.*, 1989; Urban *et al.*, 1990).

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