J. Reprod. Fert. (1984) **72**, 503–507

Printed in Great Britain

Acid phosphatase and leucine aminopeptidase activity in the uterine flushings of non-pregnant and pregnant gilts*

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Summary. The activities of uteroferrin, measured as acid phosphatase (AP), and an aminoacylpeptidase (AA) were measured in uterine flushings collected from gilts on Days 6, 8, 10, 12, 14, 15, 16 and 18 of the oestrous cycle and pregnancy (N = 37). Changes in AP ($P < 0{\cdot}05$) were associated with day for both specific and total AP in non-pregnant and pregnant gilts. For pregnant and non-pregnant gilts, AP activity was greatest between Days 14 and 16 and then decreased to Day 18.

The AA specific activity increased ($P < 0{\cdot}01$) between Days 10 and 12 of the oestrous cycle and pregnancy, but neither effects of pregnancy nor day by pregnancy status interaction were detected. The AA total activity was greater for pregnant gilts ($P < 0{\cdot}01$). These data suggest an inhibitory effect of oestrogens of blastocyst origin on synthesis and/or secretion of uteroferrin, but not AA.

Introduction

Previous work from our laboratory has been concerned with analysis of the uterine environment of cyclic, pregnant and pseudopregnant pigs. Murray, Bazer, Wallace & Warnick (1972) found that uterine secretions collected from non-pregnant gilts between Days 12 and 16 of the oestrous cycle contained a purple-coloured basic glycoprotein, which was later shown by Schlosnagle, Bazer, Tsibris & Roberts (1974) to contain iron and possess acid phosphatase activity. Knight, Bazer & Wallace (1973) demonstrated that the synthesis and/or secretion of this protein was regulated by progesterone. It has been suggested by Roberts & Bazer (1980) that uteroferrin may play a critical role in the transport of iron from the sow to the fetus.

Murray *et al.* (1972) determined that proteins other than uteroferrin were unique to uterine flushings collected when peripheral progesterone concentrations were elevated. Roberts, Bazer, Baldwin & Pollard (1976) reported that uteroferrin, lysozyme, leucine aminopeptidase and a number of other proteases were secreted into the uterine lumen of pigs in increased amounts in response to progesterone. This response was not induced by oestrogen.

The objective of the present study was to compare amounts of total protein, uteroferrin, measured as acid phosphatase, and aminoacylpeptidase (aminopeptidase), in uterine flushings of pregnant and non-pregnant gilts. Uteroferrin was chosen because it represents the major progesterone-induced secretory protein of porcine uterine secretions which is taken-up and metabolized by the conceptus (Buhi, Ducsay, Bartol, Bazer & Roberts, 1983). The aminopeptidase, on the other hand, is a relatively minor protein component of uterine flushings which is probably released into the uterine lumen as a consequence of processing of the endometrial epithelial brush border associated with secretory activity (Mancarella, Basha, Mullins, Bazer & Roberts, 1981).

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Materials and Methods

Crossbred gilts of similar age, weight and genetic background were used. The 37 gilts were checked for oestrus at 12-h intervals with mature boars and randomly assigned to provide uterine flushings representing Day 6, 8, 10, 12, 14, 15, 16 or 18 of the oestrous cycle and pregnancy (Day 0 = day of onset of oestrus). Gilts were allowed a recovery period of one full oestrous cycle after collection of the initial uterine flushing and were then mated to mature boars at 12 and 24 h after detection of the next oestrus. Uterine flushings were then collected on the same days of pregnancy with the same gilts represented within each day of the oestrous cycle and pregnancy.

In preparation for surgery, animals were fasted for 24 h. At the time of surgery, anaesthesia was induced with a 5% solution of thiopentone sodium, given intravenously, and maintained with methoxyflurane. The reproductive tract was exposed by midventral laparotomy and uterine flushings obtained as described by Murray *et al.* (1972). Flushings from the two uterine horns were quantified for recovery volume, pooled, and stored on ice until centrifugation at 12 000 *g* for 20 min. The supernatant was stored at −70°C. Pregnant gilts were ovario-hysterectomized at the second surgery and the reproductive tract was stored on ice until thoroughly chilled. Sterile 0·33 M-NaCl was infused into each uterine horn, as previously described. For collections on Days 6, 8 and 10 of pregnancy, the flushing medium was retrieved from the uterine horns via the catheter. Collections on Days 12, 14, 15, 16 and 18 required that a section of the uterine horn near the tubo-uterine junction be excised to facilitate complete collection of all the components within the uterus, i.e. embryos and supportive membranes. Uterine flushings from pregnant gilts were handled as described above and pregnancy was confirmed by the presence of apparently normal conceptuses in the uterine flushings.

Acid phosphatase (*EC 3.1.3.2*). Acid phosphatase activity, which is due to the presence of uteroferrin in the lumen of gilts (Roberts & Bazer, 1980), was determined using *p*-nitrophenyl phosphate (12 mM) as substrate (Schlosnagle *et al.*, 1974). The substrate was prepared in 0·1 M-acetate buffer, pH 4·9, and the total reaction volume adjusted to 1·0 ml with buffer. At the end of a 10-min incubation period at 30°C, 3·0 ml 0·25 N-sodium hydroxide were added to terminate the reaction and absorbance was read at 410 nm. The amount of substrate hydrolysed was determined as μmol *p*-nitrophenol released/min.

α-Aminoacylpeptidase hydrolase, microsomal (*EC 3.4.11.2*). The activity of this aminopeptidase was determined by modification of the procedure described by Basha, Horst, Bazer & Roberts (1978) using the substrate L-leucyl-β-naphthylamide. A sample of uterine flushing (5–500 μl) was incubated with 0·5 ml substrate in 0·05 M-phosphate buffer, pH 7·4, for 60 min at 30°C and the reaction terminated by addition of 0·5 ml of 2·0 M-hydrochloric acid. Liberated β-naphthylamine was measured by the following steps: (1) 0·5 ml 0·2% (w/v) sodium nitrite was added to the reaction mixture and after 3 min the excess nitrite was decomposed by the addition of 1·0 ml ammonium sulphamate (0·5%, w/v); (2) after an additional 3 min, 2·0 ml *N*-(1-naphthyl) ethylene-diamine-dihydrochloric acid (0·05%, w/v) in 95% ethanol was added; and (3) the absorbance of the blue colour at 580 nm was measured after an incubation period of 45 min. One unit of enzyme activity is defined as the amount of enzyme required to release sufficient β-naphthylamine in 1 h to yield a diazotized product having an absorbance of 1·0 at 580 nm.

Statistical analysis. Data were analysed by least squares regression analysis (Harvey, 1976) to determine effects of day and status (non-pregnant *versus* pregnant) and day by status interaction. Variability amongst days was partitioned into single degrees of freedom by weighted least squares to detect curvilinearity in trends and differences in trends when a significant day by status interaction was indicated. In the analysis of these data, the term 'total' refers to the concentration of the substance measured multiplied by the recovery volume of the flushing. When the volume of flushing medium recovered was less than 100% (i.e. 40 ml) a value of 40 was used as the recovery volume, but when the recovery was greater than 100%, the actual volume collected was used.

Results

Protein

Total recoverable protein (Table 1) was greater overall ($P < 0.01$) for pregnant gilts, but for non-pregnant and pregnant gilts changes in total recoverable protein ($P < 0.01$) were affected by day of the oestrous cycle and pregnancy. Furthermore, there was a day by status interaction ($P < 0.01$) which indicated that the temporal changes in total recoverable protein were different for pregnant and non-pregnant gilts. For non-pregnant gilts, total recoverable protein increased between Day 6 and Day 16, i.e. during dioestrus, and then decreased to Day 18 in association with luteal regression and the beginning of pro-oestrus. For pregnant gilts, total recoverable protein increased between Days 6 and 16 and did not decline after Day 16 as for non-pregnant gilts.

Acid phosphatase activity

There were no differences ($P > 0.10$) between non-pregnant gilts with respect to total acid phosphatase or acid phosphatase specific activity in uterine flushings nor did the temporal changes in acid phosphatase differ due to status. For non-pregnant and pregnant gilts, day trends for total acid phosphatase and acid phosphatase specific activity were significant ($P < 0.05$) since values obtained after Day 12 were greater than those obtained between Days 6 and 12 (Table 1).

Aminopeptidase activity

The aminopeptidase total activity was greater ($P < 0.01$) for pregnant gilts. Changes within each status were affected ($P < 0.01$) by day and the pattern of change over the days studied was different ($P < 0.01$) for non-pregnant and pregnant gilts (Table 1).

Table 1. Changes in mean ± s.e.m. total protein, and total (units; see text) and specific (units/mg protein; see text) activity of acid phosphatase and aminopeptidase in uterine flushings from non-pregnant and pregnant gilts

Day	No. of gilts	Total protein (mg/ uterine flushings)*	Acid phosphatase†		Aminopeptidase‡	
			Total	Specific	Total	Specific
Non-pregnant gilts						
6	4	20 ± 6	2 ± 1	0·07 ± 0·04	88 ± 40	3 ± 1
8	5	31 ± 8	4 ± 1	0·13 ± 0·02	144 ± 55	4 ± 1
10	4	36 ± 10	31 ± 19	1·20 ± 0·80	192 ± 52	6 ± 2
12	6	29 ± 13	40 ± 16	1·30 ± 0·50	788 ± 381	26 ± 10
14	5	68 ± 12	230 ± 79	3·10 ± 2·00	2578 ± 842	25 ± 8
15	4	85 ± 17	748 ± 449	7·00 ± 3·00	1309 ± 427	20 ± 6
16	5	93 ± 22	736 ± 195	7·00 ± 2·00	1344 ± 104	17 ± 4
18	4	41 ± 19	302 ± 289	6·00 ± 2·00	1166 ± 291	38 ± 7
Pregnant gilts						
6	4	9 ± 2	4 ± 3	0·19 ± 0·07	252 ± 61	33 ± 9
8	5	34 ± 4	5 ± 1	0·15 ± 0·02	244 ± 35	7 ± 1
10	4	53 ± 19	6 ± 1	0·13 ± 0·03	284 ± 54	9 ± 2
12	5	35 ± 2	31 ± 16	0·90 ± 0·40	1023 ± 312	23 ± 6
14	3	123 ± 46	449 ± 203	3·00 ± 1·00	2476 ± 341	22 ± 1
15	4	117 ± 11	239 ± 59	2·00 ± 1·00	2305 ± 250	20 ± 3
16	4	152 ± 13	314 ± 258	2·00 ± 0·6	4395 ± 1161	28 ± 6
18	4	188 ± 26	119 ± 66	1·00 ± 0·4	5330 ± 720	31 ± 7

* Day, status and day by status effects ($P < 0.01$) detected.

† Day effects ($P < 0.05$) detected for pregnant and non-pregnant gilts.

‡ Day, status and day by status effects significant ($P < 0.01$) for total activity, but only of day ($P < 0.01$) were detected for specific activity.

For non-pregnant gilts, aminopeptidase total and specific activity increased ($P < 0.01$) from Days 6 to 14 and decreased during the period of luteal regression and pro-oestrus. Between Days 12 and 18 specific activity remained relatively constant, indicating that changes in aminopeptidase activity were in proportion to changes in total protein and neither status nor day-by-status interaction was significant.

For pregnant gilts, aminopeptidase total activity increased ($P < 0.01$) from Days 6 to 18 and status ($P < 0.01$) and day-by-status ($P < 0.01$) effects were detected due to continued increase in total aminopeptidase for pregnant gilts on Days 16 and 18. The day trends for total aminopeptidase and total protein were closely related ($r = 0.77$; $P < 0.01$) for pregnant and non-pregnant gilts.

Discussion

Changes in total recoverable protein were similar to those reported by Murray *et al.* (1972) which can be induced with progesterone in ovariectomized gilts (Knight *et al.*, 1973). The continued increase in total recoverable protein in pregnant gilts is a reflection of continued progesterone stimulation of endometrial secretory activity.

The general trend for total acid phosphatase and acid phosphatase specific activity for non-pregnant gilts, i.e. low activity before Day 12 and after Day 16 has been observed previously (Chen, Bazer, Gebhardt & Roberts, 1975; Frank, Bazer, Thatcher & Wilcox, 1977). Total acid phosphatase and acid phosphatase specific activity for pregnant gilts in this study followed a similar pattern, with an increase of about 15-fold in total activity between Days 12 and 14 followed by a decline. Acid phosphatase specific activity was highest on Day 12 for pregnant gilts rather than Days 15 and 16 for non-pregnant gilts. Oestrogens, injected (Frank *et al.*, 1977) or of conceptus origin (Geisert, Renegar, Thatcher, Roberts & Bazer, 1982), initiate the synchronized release of proteins from secretory vesicles in epithelial cells of the uterine glands. This synchronized release of secretory material, including acid phosphatase, which is not detected in non-pregnant gilts (Geisert *et al.*, 1982), may account for the higher acid phosphatase and protein in pregnant gilts on Day 14. The subsequent decline in pregnant gilts may reflect the selective uptake and metabolism of uteroferrin by the conceptus to meet its demands for iron. Uteroferrin has been observed in association with the yolk sac (Chen *et al.*, 1975) which is the major site of haematopoiesis at this stage in conceptus development. The trophectoderm is actively engaged in endocytosis of histotrophe (Fazleabas, Bazer & Roberts, 1982; Geisert *et al.*, 1982). Alternatively, the decline in acid phosphatase between Days 14 and 18 may reflect a decline in uteroferrin synthesis and/or secretion due to increasing production of oestrogen by the conceptuses. Knight, Bazer, Wallace & Wilcox (1974) and Basha, Bazer & Roberts (1979) determined that low levels of oestrogen are synergistic with progesterone in increasing uterine secretory activity, while high levels of oestrogen were inhibitory. Oestrogen concentrations in uterine flushings and maternal blood increase during the period of conceptus elongation and early placentation whereas progesterone declines (see Bazer, Geisert, Thatcher & Roberts, 1982). The ratio of progesterone to oestrogen, therefore, falls after Day 14 and does not increase again until after Day 30 of pregnancy (Robertson & King, 1974). The progesterone : oestrogen ratio is highest between Days 50 and 75 of pregnancy when uteroferrin production is maximal (Basha *et al.*, 1979).

The pattern of change in total aminopeptidase differs from that of total acid phosphatase during pregnancy even though both enzymes have been reported to be progesterone-induced (Roberts *et al.*, 1976). However, Mancarella *et al.* (1981) demonstrated that the soluble form of aminopeptidase in uterine flushings is very similar, if not identical, to that of the membrane brush border aminopeptidase of gut epithelium. Mancarella *et al.* (1981) suggested that the soluble form of aminopeptidase is present in uterine flushings as a result of proteolytic and non-proteolytic processing of epithelial cell membranes and that it is not a true secretory product. Total aminopeptidase increased in uterine flushings from pregnant gilts between Days 10 and 18. Denker

(1980) has also reported about a 60-fold increase in aminopeptidase activity in uterine secretion of rabbits between Days 2 and 5 of pregnancy followed by a decline to Days 6 to 8·5. Denker (1980) found that the proteinase inhibitor, aprotinin, did not inhibit blastocyst-induced depletion of the aminopeptidase from adjacent uterine epithelium. The increase in aminopeptidase activity in secretions of pregnant gilts and rabbits (Denker, 1980) probably reflects increases in membrane processing by secretory endometrium, whereas uteroferrin synthesis and/or secretion are induced by progesterone. The activities of acid phosphatase and aminopeptidase increase during dioestrus, but only acid phosphatase (uteroferrin) is affected by the antagonistic effects of oestrogen on its synthesis and/or secretion. The results of this study indicate the need to distinguish between proteins in the uterine lumen which increase coincidentally with increased secretory activity during dioestrus, e.g. aminopeptidase, and those for which synthesis and secretion are truly induced by progesterone, e.g. uteroferrin.

This research was supported by NIH Grant HD08560 and is published as Florida Agricultural Experiment Station Journal Series No. 5465.

References

Basha, S.M., Horst, M.N., Bazer, F.W. & Roberts, R.M. (1978) Peptidases from pig reproductive tract: purification and properties of aminopeptidases from uterine secretions, allantoic fluid and amniotic fluid. *Archs Biochem. Biophys.* **185**, 174–193.

Basha, S.M.M., Bazer, F.W. & Roberts, R.M. (1979) The secretion of a uterine specific, purple phosphatase by cultured explants of porcine endometrium. Dependency upon the state of pregnancy of the donor animal. *Biol. Reprod.* **20**, 431–441.

Bazer, F.W., Geisert, R.D., Thatcher, W.W. & Roberts, R.M. (1982) The establishment and maintenance of pregnancy. In *Control of Pig Reproduction*, pp. 227–252. Eds D. J. A. Cole & G. R. Foxcroft. Butterworth Scientific, London.

Buhi, W.C., Ducsay, C.A., Bartol, F.F., Bazer, F.W. & Roberts, R.M. (1983) A function of the allantoic sac in the metabolism of uteroferrin and maternal iron by the fetal pig. *Placenta* **4**, 455–470.

Chen, T.T., Bazer, F.W., Gebhardt, B.M. & Roberts, R.M. (1975) Uterine secretions in mammals: synthesis and placental transport of a purple acid phosphatase in pigs. *Biol. Reprod.* **13**, 304–313.

Denker, H.W. (1980) Endometrial arylamidase secretion: a sensitive parameter of steroid hormone action and embryo-maternal interrelationships in the rabbit. In *Steroid Induced Uterine Proteins*, pp. 121–132. Ed. M. Beato. Elsevier North-Holland Biomedical Press, Amsterdam.

Fazleabas, A.T., Bazer, F.W. & Roberts, R.M. (1982) Purification and properties of a progesterone-induced plasmin/trypsin inhibitor from uterine secretions of pigs and its immunocytochemical localization in the pregnant uterus. *J. biol. Chem.* **257**, 6886–6897.

Frank, M., Bazer, F.W., Thatcher, W.W. & Wilcox, C.J. (1977) A study of prostaglandin $F_{2\alpha}$ as the luteolysin in swine. III. Effects of estradiol valerate on prostaglandin F, progestins, estrone and estradiol concentrations in the utero-ovarian vein in nonpregnant gilts. *Prostaglandins* **14**, 1183–1196.

Geisert, R.D., Renegar, R.H., Thatcher, W.W., Roberts, R.M. & Bazer, F.W. (1982) Establishment of pregnancy in the pig. I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. *Biol. Reprod.* **27**, 925–939.

Harvey, W.R. (1976) Least-squares analysis of data with unequal subclass frequencies. U.S. Department of Agriculture, Animal Research Station Publication No. 20.

Knight, J.W., Bazer, F.W. & Wallace, H.D. (1973) Hormonal regulation of porcine uterine protein secretion. *J. Anim. Sci.* **36**, 546–553.

Knight, J.W., Bazer, F.W., Wallace, H.D. & Wilcox, C.J. (1974) Dose response relationships between exogenous progesterone and estradiol and porcine uterine protein secretions. *J. Anim. Sci.* **39**, 747–751.

Mancarella, D.A., Basha, S.M.M., Mullins, D.E., Bazer, F.W. & Roberts, R.M. (1981) Properties of a membrane associated aminopeptidase from the porcine uterus and its inhibition by polyamines. *Biol. Reprod.* **24**, 879–887.

Murray, F.A., Bazer, F.W., Wallace, H.D. & Warnick, A.C. (1972) Quantitative and qualitative variation in the secretion of protein by the porcine uterus during the estrous cycle. *Biol. Reprod.* **7**, 314–320.

Roberts, R.M. & Bazer, F.W. (1980) The properties, function and hormonal control of synthesis of uteroferrin, the purple protein of the pig uterus. In *Steroid Induced Uterine Proteins*, pp. 133–149. Ed. M. Beato. Elsevier North-Holland Biomedical Press, Amsterdam.

Roberts, R.M., Bazer, F.W., Baldwin, N. & Pollard, W.E. (1976) Progesterone induction of lysozyme and peptidase activities in the porcine uterus. *Archs Biochem. Biophys.* **177**, 499–507.

Robertson, H.A. & King, G.J. (1974) Plasma concentrations of progesterone, oestrone, oestradiol-17β and oestrone sulphate in the pig at implantation, during pregnancy and at parturition. *J. Reprod. Fert.* **40**, 133–141.

Schlosnagle, D.C., Bazer, F.W., Tsibris, J.C.M. & Roberts, R.M. (1974) An iron-containing phosphatase induced by progesterone in the uterine flushings of pigs. *J. biol. Chem.* **249**, 7574–7579.

Received 20 March 1984