Mammary involution in ewes: Changes in milk plasminogen and plasmin

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ABSTRACT

Multiparous Border Leicester x Romney ewes were weaned early (day 35 of lactation), milked to empty the mammary gland and again milked 18 h later (day 0 of experiment) to establish baseline milk yields and concentrations in milk of the proteolytic enzyme plasmin and its zymogen plasminogen. They were then divided into 3 groups to be milked 3 (n=5), 8 (n=6) or 15 (n=5) days after the day 0 milking to determine changes in concentrations and yields of plasmin and plasminogen in mammary gland secretions during involution. Yields (mean±SE) on days 0 (groups 1+2+3), 3 (group 1), 8 (group 2) and 15 (group 3) were : secretion (g), 785±31.2 v. 598±94.8 v. 478±86.8 v. 51±66.6; plasminogen (μg), 7.8±2.2 v. 78.7±11.1 v. 22.4±4.6 v. 3.9±2.1; and plasmin (μg), 11.5±2.38 v. 148.5±8.00 v. 51.6±7.92 v. 4.9±2.32. Corresponding concentrations in the secretions were (μg/ml): plasminogen, 0.01±0.002 v. 0.15±0.027 v. 0.046±0.005 v. 0.061±0.007; and plasmin, 0.015±0.006 v. 0.275±0.027 v. 0.118±0.011 v. 0.083±0.009. There was a linear decline in the volume of secretions from day 0 to day 15. In contrast there was a transient increase (15 fold) in plasminogen and plasmin yields by 3 days after milking. These data suggest that any role of plasmin in mammary gland involution is likely to be limited to the first week after early weaning.

Keywords: Early weaning; milk; plasmin; plasminogen; involution.

INTRODUCTION

Plasmin (EC3.4.21.7) is a proteolytic enzyme which appears to pass from blood into the mammary gland primarily as its zymogen, plasminogen, and is then converted to plasmin by specific plasminogen activators (Ossowski *et al.*, 1979; Busso *et al.*, 1989). Plasmin activity in milk increases towards the end of lactation (Politis *et al.*, 1989a, 1989b; Benslimane *et al.*, 1990; Politis *et al.*, 1990; Bastian *et al.*, 1991). The proteolytic activity of plasmin, and its increased activity in milk at the end of lactation, have led to the suggestion that it may have a role in mammary gland involution at weaning or the cessation of milking (Hedkvist *et al.*, 1989; Politis *et al.*, 1989a). However, we have recently shown that, when cows are milked once daily for seven days, yields of plasmin in milk decrease despite a significant increase in the yields of plasminogen (Knutson *et al.*, 1993). The objective of this study was to examine the changes in plasminogen and plasmin in mammary secretions during acute regression initiated by early weaning (as opposed to a more gradual response to a short period of once daily milking).

MATERIALS AND METHODS

The study involved 16 multiparous Border Leicester x Romney ewes (13 single- and 3 twin- rearing) in which mammary involution was initiated by weaning the lambs at day 35 of lactation. Immediately after weaning, the ewes were hand-milked (after injection of 1 IU oxytocin into the jugular vein) to completely empty each gland. Approximately 18 h later, the ewes were again milked to provide baseline data on milk volumes and plasmin/plasminogen concentrations. They were then divided at random into three groups to be milked 3 (group 1, n=5), 8 (group 2, n=6) or 15 (group 3, n=5) days after the milking on day 36 of lactation (day 0 of the trial). At the day 3, 8 or 15 milkings, the ewes were hand-milked as described previously, yields of secretion recorded and samples of secretion (pooled across the glands within ewes) placed on ice and transferred to the laboratory for analysis.

Samples of secretion were centrifuged at 3000 g for 20 min at 4°C, then to 9.5 ml of defatted secretion was added 0.1 ml of sodium azide (10% solution) and 0.4 ml of 75 mM e-amino-n-caproic acid. The mixture was left to stand for 2 hours at room temperature, followed by centrifugation at 100,000 g for one hour at 4°C. The supernatant fraction was transferred to autoanalyser cups and frozen at -20°C for subsequent analysis of plasmin and plasminogen concentrations. Plasminogen- derived and plasmin activities were determined in the supernatant samples using the assay described by Korycka-Dahl *et al.* (1983) as modified by Knutson *et al.* (1993). Yields of secretion, plasmin and plasminogen were corrected to a 24 h basis for the day 0 milking and to exactly 3, 8 or 15 days for the subsequent milkings (to account for small differences in actual versus nominal milking times for each ewe).

Data were subjected to analyses of variance in which parameters (concentrations of plasminogen and plasmin, yields of secretion, plasminogen and plasmin and plasminogen:plasmin ratios) on days 3 (group 1), 8 (group 2) and 15 (group 3) were compared with those on day 0 for groups (2 + 3), (1 + 3) and (1 + 2) respectively. In addition analysis of variance was used to test the significance of the differences for the various parameters between days 3, 8 and 15.

RESULTS AND DISCUSSION

The yields of secretion obtained from the mammary glands of ewes on days 3 (group 1), 8 (group 2) and 15 (group 3) are compared, in Table 1, with the production on day 0 of groups (2 + 3), (1 + 3) and (1 + 2) respectively. Secretion

TABLE 1: The means (± Standard Errors) for the yields of secretion, plasminogen, and plasmin together with the concentrations of plasminogen and plasmin in three groups of ewes milked on day 0 (groups 1, 2 and 3) and then next milked on day 3 (group 1), day 8 (group 2) and day 15 (group 3) after abrupt weaning.

	Day 0		Day 3	Day 8	Day 15
	Groups	Combined	Group (1)	Group (2)	Group (3)
Secretion Yield	2 + 3	$827^{a} \pm 63.9$	$598^{aA} \pm 94.8$	-	-
	1 + 3	$746^{a} \pm 67.2$	-	$478^{bA} \pm 86.8$	-
	1 + 2	$791^{a} \pm 44.9$	-	-	$51^{bB} \pm 66.6$
Plasminogen	2 + 3	$9.3^{a} \pm 4.3$	$149.6^{bA} \pm 27.3$	-	-
Concentration	1 + 3	$10.8^{a} \pm 3.5$	-	$45.5^{bB} \pm 4.5$	-
(ng/ml)	1 + 2	$9.0^{a} \pm 4.9$	-	-	$60.9^{bC} \pm 6.7$
Plasminogen	2 + 3	$8.15^{a} \pm 7.46$	$78.66^{bA} \pm 11.1$	-	-
Yield (µg)	1 + 3	$8.11^{a} \pm 3.53$	-	$22.37^{bB} \pm 4.56$	
	1 + 2	$6.96^{a} \pm 1.43$	-	-	$3.85^{aC} \pm 2.12$
Plasmin	2 + 3	$13.2^{a} \pm 7.46$	$274.7^{bA} \pm 26.8$	-	-
Concentration	1 + 3	$15.5^{a} \pm 8.5$	-	$118.2^{bB} \pm 11.0$	-
(ng/ml)	1 + 2	$17.0^{a} \pm 5.9$	-	-	$82.5^{bc} \pm 8.7$
Plasmin Yield	2 + 3	$10.6^{a} \pm 5.39$	$148.5^{bA} \pm 8.$	-	-
(µg)	1 + 3	$11.2^{a} \pm 6.13$	-	$51.6^{bB} \pm 7.92$	-
	1 + 2	$12.7^{a} \pm 1.56$	-	-	$4.9^{bC} \pm 2.32$
Plasmin:	2 + 3	$1.26^{a} \pm 0.23$	$2.59^{bA} \pm 0.37$		
Plasminogen	1 + 3	$1.59^{a} \pm 0.41$		$2.78^{aA} \pm 0.55$	
Ratio	1 + 2	$1.64^{a} \pm 0.29$			$1.53^{aA} \pm 0.39$

[a, b] Means within rows with differing superscripts are significantly different ($P < 0.05$)
[A, B, C] Means between days 3, 8, and 15 with differing superscripts are significantly different ($P < 0.05$)

yields declined linearly with increased periods between milkings. The declines were significant ($P<0.05$) from day 0 to days 8 and 15, and from days 3 and 8 to day 15.

The concentration of plasminogen in the secretion was approximately 15-fold higher in ewes in group 1 which had not been milked for 3 days, compared with that of groups (2 + 3) on day 0 (Table 1). Thereafter, the concentration of plasminogen in the secretion declined markedly. Although the concentration on day 15 was significantly lower than that on day 8 which in turn was significantly lower than that on day 3 ($P<0.05$), concentrations on all three days were significantly ($P<0.05$) greater than those on day 0 for the appropriate groups. As a result, yields of plasminogen were significantly ($P<0.05$) greater in ewes milked on day 3 than in those milked on days 0, 8 or 15 (Table 1) but the yield on day 15 was not significantly ($P>0.05$) different from that on day 0.

Concentrations of plasmin in the secretion followed a similar pattern to those of plasminogen (Table 1), although the increase in plasmin concentration at day 3 relative to day 0 (a 20-fold increase) was greater than that in plasminogen (a 15-fold increase). Both the concentration and yield of plasmin were significantly ($P<0.05$) greater in the ewes milked on day 3 than in those milked on days 0, 8 or 15. Similarly the concentrations and yields of plasmin on day 8 were significantly ($P<0.05$) greater than those on days 0 and 15. The nett effect of these changes was that the plasmin:plasminogen yield ratio (Table 1) was significantly greater ($P<0.05$) in ewes milked on day 3 after weaning than in the ewes at day 0.

The origin of plasminogen and plasmin in mammary secretions is still uncertain but blood is the most likely source in view of the large concentration of plasminogen in the blood relative to that of plasminogen plus plasmin in the milk. Moreover the results from a previous study with dairy cows in late lactation suggest that plasminogen moves together with blood serum albumin and the immunoglobulins from blood into milk, possibly through gaps in the glandular epithelium (Knutson *et al.*, 1993).

The concentrations of plasmin and plasminogen in ovine milk (Table 1) are much lower than those reported in the literature for bovine milk (Politis *et al.* 1989a, 1989b; Knutson *et al.*, 1993). The glandular epithelium may be less permeable to plasminogen in the ewe or plasminogen may be destroyed in ovine milk. The much higher plasmin:plasminogen ratio in ewes milk is perhaps suggestive of the latter possibility. Clearly the reason for the difference requires further investigation.

During the involution of the mammary gland of ewes there is a period of 2-3 days when the permeability of the glandular epithelium is markedly increased (Lascelles, 1962). Moreover, in ewes early in lactation this increase in permeability commences within 2-3 days after last milking. A similar increase in permeability would be anticipated in the present experiments and the 15-fold increase in the concentration of plasminogen and plasmin on the third day of involution is consistent with such a change.

The substantial fall in yield of plasmin and plasminogen by day 8 of involution implies that much of the plasmin and plasminogen in the gland on day 3 was subsequently removed. It is probable that the enzyme was broken down as it is unlikely that there would have been nett reabsorption against a concentration gradient. It is unclear whether plasminogen continued to enter the secretion after day 3 and was destroyed or whether the glandular epithelium became relatively impermeable again sometime between days 3 and 8. Reabsorption of proteins from the glandular lumen of mammary glands of ewes is greatly diminished after a period of rapid reabsorption early in involution (Lascelles, 1962; Mackenzie, 1968). Furthermore, in involuted glands the epithelium is sufficiently impermeable that blood serum

albumin is absorbed 2-3 times more rapidly than the larger IgG (Mackenzie, 1968). In this context the apparently linear rate of fluid removal from the involuting gland is perhaps unexpected in that it may have been anticipated that fluid loss would be more rapid at the time of greatest epithelial permeability around day 3 of involution.

The increase in the plasmin:plasminogen ratio on day 3 probably reflects an increase in the concentration of plasminogen activators in the secretion at this time. In rodents, abrupt weaning of the young at parturition or during established lactation is accompanied by marked changes in the mammary gland content of plasminogen activator. Thus plasminogen activator content of the gland increases sharply (by approximately 3-fold) in the first 2-3 days after weaning and then declines to preweaning levels after about 8 days (Ossowski *et al.*, 1979). Conversely, if the animals are permitted to lactate, plasminogen activator concentrations remain at basal (late pregnancy) levels. These changes in plasminogen activator concentrations in the mammary glands of mice at weaning are remarkably similar in their temporal pattern to the changes in the plasmin content of secretions observed in the present study. Since the infusion of the plasminogen activator urokinase into the mammary gland tissue with plasminogen leads to disintegration of alveolar epithelial cells (Hedkvist *et al.*, 1989), these results are consistent with the hypothesis that plasmin has a role in the initial stages of mammary gland involution.

The 15-20 fold increases in the concentrations of plasminogen and plasmin following the cessation of milking are much greater than the 1.15- to1.25- fold changes that occur when cows are switched from twice daily to once daily milking towards the end of lactation (Knutson *et al.*, 1993; K. Stelwagen, personal communication). The changes in concentration on once daily milking are consistent with relatively minor changes in epithelial permeability. The smaller changes on once daily milking may reflect, in part, a difference in the hormonal milieu to which the animals are exposed.

Ossowski *et al.* (1979) have shown that systemic administration of prolactin or oxytocin to weaned mice substantially reduces the elevation of mammary gland plasminogen activator content which typically accompanies abrupt weaning. Since both prolactin and oxytocin concentrations in circulation are elevated by suckling and milking (Cowie *et al.*, 1980), it may be that the daily stimulation of the mammary gland associated with once daily milking is sufficient to prevent a rise in plasminogen activator concentrations and so prevent an increased rate of conversion of plasminogen to plasmin. Although the routine administration of oxytocin to cows pre-milking has no effect on milk plasmin content, but causes a small increase in milk yield (Ballou *et al.*, 1993), this does not exclude the possibility that oxytocin prevents a weaning-induced rise in milk plasmin content. The effect of prolactin, which is more effective than oxytocin in suppressing the weaning-induced rise in mammary gland plasminogen activator content in mice, does not appear to have been investigated in ruminants.

REFERENCES

Ballou, L.U.; Bleck, J.L.; Bleck, G.T.; Bremel, R.D. 1993. The effect of daily oxytocin injections before and after milking on milk production, milk plasmin, and milk composition. *Journal of Dairy Science 76*: 1544-1549.

Bastian, E.D.; Brown, R.J.; Ernstrom, C.A. 1991. Plasmin activity and milk coagulation. *Journal of Dairy Science 74*: 3677-3685.

Benslimane, S.; Dognin-Bergeret, M.J.; Berdague, J.-L.; Gaudemer, Y. 1990. Variation with season and lactation of plasmin and plasminogen concentrations in Montbeliard cows' milk. *Journal of Dairy Research 57*: 423-435.

Busso, N.; Huarte, J.; Vassalli, J.-D.; Sappino, A.-P.; Belin, D. 1989. Plasminogen activators in the mouse mammary gland. *Journal of Biological Chemistry 264*: 7455-7457.

Cowie, A.T.; Forsyth, I.A.; Hart, I.C. 1980. Hormonal Control of Lactation, Springer-Verlag, Berlin and New York.

Hedkvist, L.; Höglund, S.; Persson, K.; Holmberg, O. 1989. Plasminogen activation in the bovine udder. *Acta Veterinaria Scandinavica 30*: 103-105.

Knutson, R.J.; Mackenzie, D.D.S.; Davis, S.R.; McCutcheon, S.N. 1993. The effect of once daily milking on concentrations and yields of plasminogen, plasmin and other whey proteins. *Proceedings of the New Zealand Society of Animal Production 53*: 55-158.

Korycka-Dahl, M.; Ribadeau Dumas, B.; Chene, N.; Martal, J. 1983. Plasmin activity in milk. *Journal of Dairy Science 66*: 704-711.

Lascelles, A.K. 1962. The absorption of serum albumin and casein from the mammary gland of the merino ewe. *Quarterly Journal of Experimental Physiology 47*: 48-56.

Mackenzie, D.D.S. 1968. Studies on the transfer of protein across the glandular epithelium of the mammary gland during involution. *Australian Journal of Experimental Biology and Medical Science 46*: 273-283.

Ossowski, L.; Biegel, D.; Reich, E. 1979. Mammary plasminogen activator: correlation with involution, hormonal modulation and comparison between normal and neoplastic tissue. *Cell 16*: 929-940.

Politis, I.; Block, E.; Turner, J.D. 1990. Effect of somatotropin on the plasminogen and plasmin system in the mammary gland: proposed mechanism of action for somatotropin on the mammary gland. *Journal of Dairy Science 73*: 1494-1499.

Politis, I.; Block, E.; Turner, J.D. 1989a. Plasmin and plasminogen in bovine milk: a relationship with involution? *Journal of Dairy Science 72*: 900-906.

Politis, I.; Ng Kwai Hang, K.F.; Giroux, R.N. 1989b. Environmental factors affecting plasmin activity in milk. *Journal of Dairy Science 72*: 1713-1718.

Politis, I.; Zavizion, B.; Barbano, D.M.; Gorewit, R.C. 1993. Enzymatic assay for the combined determination of plasmin and plasminogen in milk: revisited. *Journal of Dairy Science 76*: 1260-1267.