Regulation of lactose production by ovine mammary acini in culture

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ABSTRACT

Our objectives were to examine the effects of milk stasis, *in vivo*, and cell density, *in vitro*, on lactose production by cultured mammary acini from lactating ewes. In each of six lactating ewes, lambs were prevented from suckling one mammary gland to cause milk stasis in that gland. The opposite gland served as the control. Mammary acini were isolated and their lactose production during 24 h of incubation was measured.

Milk stasis markedly reduced lactose output (4.8 vs 2.5 fmol/cell/h; $P<0.001$). This effect was not altered by varying cell density or adding lactogenic hormones and foetal calf serum to culture medium. However, acini seeded at 2.5×10^5 cells/well produced nearly 5 times more lactose than those seeded at 2.5×10^6 cells/well (6.0 vs 1.3 fmol/cell/h; $P<0.001$). In further experiments, doubling the media volume from 1 to 2ml/well did not affect lactose output or relieve the density effect ($P>0.10$). In contrast, doubling the plating area significantly increased lactose output ($P<0.05$).

These data suggest that the milk stasis effect is exerted upon the mammary secretory cells because the effect was carried over into culture. The density effect appears to be mediated by cell to cell contact, rather than by nutrient limitation or diffusible inhibitors.

Keywords: Lactose; ovine; mammary; milk stasis; cell density; cell culture.

INTRODUCTION

We previously reported the development and initial characterisation of an *in vitro* model system for study of lactose synthesis and secretion by ruminant mammary tissue (Davis *et al.*, 1993). As part of that study, ewes were starved prior to collection and culture of their mammary tissue. In contrast to the well-established effect of short-term starvation on milk production of ruminants (50% reduction in milk yield; Linzell, 1967), we observed no effect of this treatment on lactose production *in vitro* (Davis *et al.*, 1993). This result raised the question of whether the model system accurately reflected functionality *in vivo*. Reduced milking frequency or absence of milking also suppress milk yield *in vivo*. Bauman *et al.* (1974) reported that cessation of milking markedly down-regulated lactose synthesis by mammary tissue from lactating ewes. Accordingly, our first objective was to examine if the inhibitory effect of milk stasis on milk secretion *in vivo* would be carried over into the *in vitro* culture system.

Our previous results also revealed a marked effect of cell density on lactose output *in vitro* by mammary acini from lactating sheep (Davis *et al.*, 1993). This confirmed earlier studies using dispersed mammary cells from lactating cows (Rao *et al.*, 1975; Larson, 1976), but conflicted with findings of experiments on rat mammary cells (Park and Chandler, 1976). Neither our previous studies nor those reported by Larson (1976) provided an adequate explanation of this density effect. Hence, our second objective was to further evaluate the effects of cell density on lactose output *in vitro*.

MATERIALS AND METHODS

Animals

Lactating Coopworth ewes, 5-6 weeks in lactation, were used in this study. Initially, 8 ewes were allocated for use in the trial, but one was eliminated due to an unbalanced udder and data from a second ewe were omitted due to extremely low lactose production. Hence, data represent mammary tissue from six ewes.

Experiment 1

The first experiment examined the effect of milk stasis on production of lactose by acini, *in vitro*. Forty hours prior to slaughter, one side of the mammary gland was fitted with a cover to prevent suckling (stasis gland) and secured with a harness of nylon mesh netting. The other gland was left uncovered (control gland) and the lamb was allowed to suckle *ad libitum*. After 40 h, ewes were injected with 5IU oxytocin, *i.v.*, immediately prior to milking out both glands by hand. The ewes were slaughtered humanely and the entire udder was removed immediately and transported within 10 minutes to the laboratory. Preparations of mammary acini were made from stasis and control glands of each ewe by collagenase digestion of secretory tissue samples essentially as described by Davis *et al.* (1993).

Treatments, *in vitro*, were arranged in a 2x2 factorial design and included two levels of culture medium: basal or basal supplemented with 10% foetal calf serum and hormones (insulin 5ug/ml; hydrocortisone 1ug/ml; prolactin 1ug/ml; prostaglandin E_2 0.5ug/ml); and two levels of plating density: 2.5×10^5 or 2.5×10^6 cells/culture well.

Experiment 2

The second experiment utilised only acini isolated from the control glands, i.e., glands that were not subjected to milk stasis. All cultures were conducted in basal medium. To further examine the negative effect of cell density on lactose output, a 2^3 factorial arrangement of treatments was applied to cultures. Factors included plating area (2 or 4cm^2), medium volume (1 or 2ml/well), and cell density (0.25 or 2.5×10^6 cells/

well). The difference in plating area was achieved by culturing acini in 24-well (2cm^2) or 12-well (4cm^2) culture plates. Volume and cell density were varied by altering additions of medium and cells, respectively, to wells.

Culture

In all cultures, acini were plated onto wells previously coated with Matrigel (Integrated Sciences Pty. Ltd., Crows Nest, NSW, Australia) at 15ul/cm^2. Treatments were run in triplicate and cultures were incubated for 24 hrs. Medium in the plates was sampled initially after adding acini and allowing them to settle (approximately 30 min) and again after 24hrs of incubation. The lactose content of the culture medium was determined by a bioluminescence assay (Arthur *et al.*, 1989), after deproteinisation of samples with $ZnSO_4$ and NaOH as previously described (Davis *et al.*, 1993). Bioluminescence was measured on a Perkin-Elmer LS-50 Spectrophotometer.

Statistical Analysis

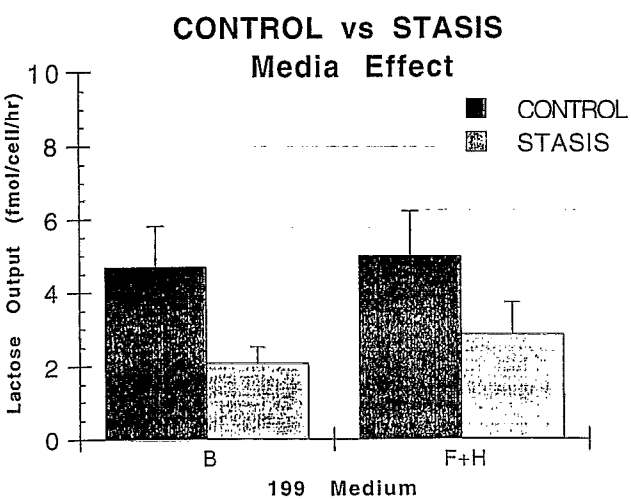
Data were analysed using the general linear models procedure of SAS. Where main effects or interactions were significant (P<0.05) means were separated by least significant difference. Lactose output is presented as fmol/cell/h to normalise for cell number, medium volume and plating area.

RESULTS

Experiment 1

Lactose production of acini from glands subjected to 40h of milk stasis was reduced to approximately one-half that of acini from control glands (4.8 vs 2.5 fmol/cell/h; P<0.001). Adding hormones and foetal calf serum to the medium did not significantly alter lactose output (P=0.40), nor was there any interaction between the effects of stasis and hormone addition (P=0.70; Figure 1).

FIGURE 1: Effects of milk stasis and medium additives on production of lactose by cultured ovine mammary acini from lactating ewes. Acini were prepared from suckled (control; solid bars) or unsuckled (40 h of milk stasis; open bars) mammary glands from ewes (n=6) at 5-6 weeks of lactation. Cultures were in basal medium alone or supplemented with 10% FCS, Prl and HC at 1 ug/ml, PGE2 at 0.5ug/ml and INS, 5ug/ml. Data represent least squares means and standard errors of the means. There was no significant interaction between stasis and medium (P=0.70), nor did additives affect lactose production (P=0.40). The main effect of stasis was highly significant (P<0.001). Pooled SEM = 0.62.



Increasing the plating density (cells/cm^2) by 10-fold reduced lactose output per cell nearly 5-fold (6.0 vs 1.3 fmol/cell/h; P<0.001). At both high and low cell density, lactose output by acini from stasis glands was approximately half that of control cultures. But, the reduction in lactose output due to high cell density obscured the stasis effect such that the difference was significant only at the low cell density, leading to a density by stasis interaction (P<0.01; Figure 2).

Addition of hormones and FCS to medium did not significantly affect lactose output at either cell density (P>0.10; data not shown).

FIGURE 2: Effects of milk stasis and plating density (cells/well) on production of lactose by lactating ovine mammary acini, *in vitro*. Acini were from suckled (control; solid bars) or nonsuckled (stasis; open bars) glands as described in Figure 1. Acini were plated at 0.25 or 2.5 x 10^6 cells/culture well in 24-well plates. Data are least squares means and standard errors of the means. Main effects of stasis and density were highly significant (P<0.001). In addition, there was a significant interaction between effects of stasis and density (P<0.01). Stasis significantly reduced lactose output at low density (P<0.001) but the reduction was not significant at high density (P>0.10). Pooled SEM=0.62.

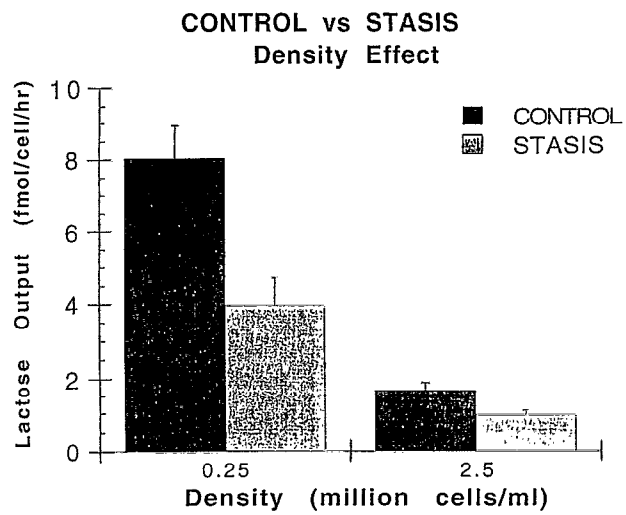
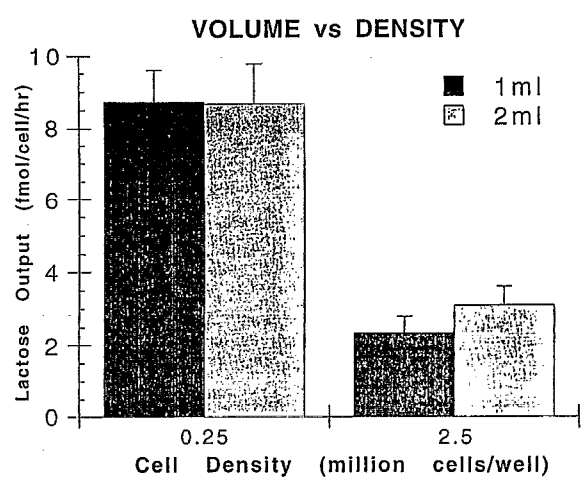


FIGURE 3: Effects of plating density and medium volume on lactose production by lactating ovine mammary acini, *in vitro*. Acini were from suckled glands and were cultured in basal medium. Acini were plated at 0.25 or 2.5 x 10^6 cells/well in wells containing 1 or 2ml medium. Data are least squares means and standard errors of the means. The main effect of plating density was significant (P<0.001), but volume had no effect (P=0.66), nor was the interaction significant (P=0.60). Pooled SEM = 0.76.

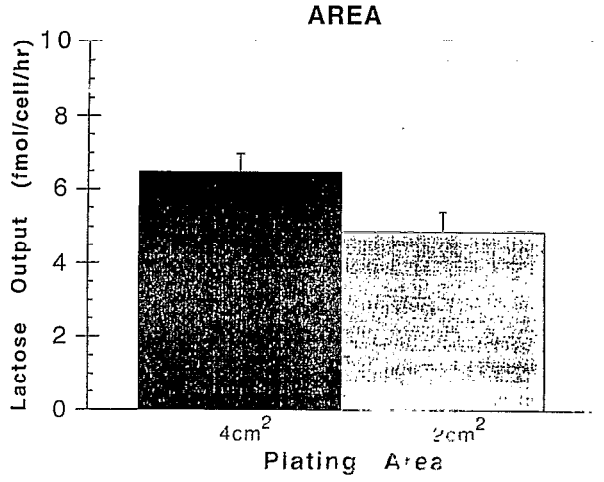


Experiment 2

The second experiment used only acini from lactating glands ("control" glands of experiment 1) and basal medium. As depicted in Figure 3, doubling the volume of medium from 1 to 2ml/well had no effect on lactose output (main effect of volume: P=0.66). Nor did it relieve the marked inhibition of lactose production due to high cell density (main effect of density: P<0.001) as indicated by the lack of significant interaction between volume and density (P=0.60).

In contrast to varying media volume, doubling the plating area increased lactose output by 33% (main effect of area: P<0.05; Figure 4). The average increase was 16% at the lower cell density ($2.5x10^5$ cells/well) but more than 200% at high cell density ($2.5x10^6$ cells/well). Consistent with Figure 3, doubling media volume had no effect on lactose output. Moreover, effects of plating area and media volume were independent (area by volume interaction: P=0.96).

FIGURE 4: Main effect of plating area on lactose production by lactating ovine mammary acini, *in vitro*. Acini were from suckled glands and were cultured in basal medium in 12-well ($4cm^2$) or 24-well ($2cm^2$) culture plates. Data are least squares means and standard errors of the means. The main effect of plating area was significant (P<0.05). Media volume had no effect (P=0.65), hence the data shown are the average of wells containing 1 or 2ml medium. There was no interaction between plating area and media volume (P=0.96). Pooled SEM = 0.76.



DISCUSSION

We previously reported that short-term starvation of lactating ewes had no effect on subsequent lactose output of their mammary tissue *in vitro* (Davis *et al.*, 1993). A similar result was reported by Wilde and Kuhn (1979) using acini from starved, lactating rats. In contrast, the results of experiment 1 clearly demonstrated that acini from glands subjected to milk stasis *in vivo*, produced substantially less lactose *in vitro*. These results show that, for milk stasis, the behaviour of the *in vitro* model did reflect the functionality of the gland, *in vivo*. The discrepancy in results obtained after starvation or milk stasis implies that the loss of milk production associated with these states in the lactating animal (Bauman *et al.*, 1974; Linzell, 1967) arises from different mechanisms. Indeed, our data suggest that the effect of starvation is at the nutritional, rather than cellular level. Hence, upon restoration of adequate substrate supply, *in vitro*, acini from starved glands were able to resume normal lactose output. Alternatively, starvation may evoke production of an inhibitor of lactation which is active *in vivo*, but inactivated or diluted out *in vitro*.

In contrast to starvation, the milk stasis effect appears to be at the cellular level because the reduction of milk output *in vivo* is still apparent, in terms of lactose output, *in vitro*. The lack of response to serum and lactogenic hormones in both control and stasis cultures suggests that the stasis defect is not due to a reduction in hormonal stimulation. Others found that the cell density effect in bovine mammary cell cultures was independent of incubation time, nutrient supply, and feedback inhibition by lactose itself (Rao *et al.*, 1975; Larson, 1976).

To elucidate the nature of the cell density effect, we varied volume of medium per cell and plating surface area per cell. The results demonstrated that doubling media volume did not relieve the density effect but doubling plating area increased lactose output significantly. The lack of improvement with increased media volume suggests that the density effect is not mediated by nutrient limitation or accumulation of soluble inhibitors in medium. This is consistent with our previous finding that frequent media changes (every 3h) had no effect on lactose output (unpublished data). Larson (1976) was also unable to attribute the cell density effect to nutrient limitation. Instead, our data suggest that physical contact between adjacent cells or acini may cause the density effect.

In conclusion, these findings suggest that effects of milk stasis, starvation and cell density on lactose output *in vitro* are mediated by independent mechanisms. Such mechanisms may also determine the synthetic and/or secretory activity of mammary acini *in vivo*. The relationship between cell density and lactose output in this culture system suggests that experiments requiring many cells, such as those of Wheeler *et al.* (1993, 1995), should be accommodated by increasing the plating area.

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