Elevated plasma cortisol reduces permeability of mammary tight junctions in the lactating bovine mammary epithelium

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Abstract

Induction of tight junction permeability in the mammary epithelium decreases milk secretion, and in cows tight junctions become leaky after 17 h of milk accumulation. *In vitro* studies demonstrate the importance of glucocorticoids for the formation and maintenance of tight junctions. In this study we examined whether cortisol can prevent mammary tight junction permeability in the lactating gland *in vivo*, and inhibit the associated milk loss, using our milk-accumulation model to challenge tight junction patency. Following a 4-day control period Jersey cows were subjected to a 24-h period in which they were milked twice at 0700 and 1500 h (TM; *n*=6), once at 0700 h (OM; *n*=7), or once and treated with ACTH (40 IU per 2 h, starting after 14 h of milk accumulation) to increase endogenous cortisol levels (OM+ACTH; *n*=7). Frequent blood samples for cortisol, lactose and glucose analyses were taken via indwelling jugular catheters.

ACTH treatment resulted in a sustained elevation of systemic cortisol concentrations. Plasma lactose, an indicator of tight junction leakiness, was not changed in TM cows, but began to increase rapidly at 17 h of milk accumulation in OM cows. Treatment with ACTH prevented the increase in plasma lactose, although levels were slightly, but not significantly, higher than in TM cows, indicating that elevated plasma cortisol reduced mammary tight junction leakiness. Milk yield was reduced by 12% in both once-milked groups, despite cortisol preventing tight junction leakiness. However, the milk loss in the latter group may not be related to leaky tight junctions, but be due to a reduction in milk precursor uptake by the mammary gland. Consistent with this notion was a 34% increase in plasma glucose levels in OM+ACTH cows only.

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Introduction

The tight junction or zonula occludens, a 'gasket-like' structure, is part of the junctional complex and surrounds endothelial or epithelial cells in close proximity to the cell's apical domain. When intact, it prevents the vectorial movement of small molecules and ions between the basolateral and apical sides of cells. This 'barrier' function of the tight junction permits the existence of pools of ions and/or small molecules differing in concentration on either side of the cell. In addition, tight junctions are instrumental in maintaining the polarised state of secretory cells, by preventing redistribution of plasma membrane components (e.g. ion channels and ion pumps) and maintaining a difference in lipid and protein composition between the basal and apical side of the plasma membrane. The latter function of the tight junction is often referred to as its 'fence' function (Schneeberger & Lynch 1992, Mandel *et al.* 1993).

In the mammary gland, epithelial tight junctions, when 'tight', form a barrier between the blood, or more precisely

the interstitial fluid (basolateral side), and milk in the alveolar lumen (apical side), thus preventing serum components from entering into milk and vice versa (Stelwagen *et al.* 1995). In the periparturient period (Linzell & Peaker 1974) and again as the mammary gland involutes, towards the end of lactation (Fleet & Peaker 1978), tight junctions are 'leaky'. However, it was shown recently that the permeability of tight junctions can increase during established lactation if milk is allowed to accumulate. In cows, tight junctions switch to a leaky state after approximately 17 h of milk accumulation (Stelwagen *et al.* 1997a), and in goats after 21 h (Stelwagen *et al.* 1994b). Furthermore, this increase in permeability is associated with a reduction in milk secretion (Neville & Peaker 1981, Stelwagen *et al.* 1994b, 1997a), and an increase in protease activity in milk (Stelwagen *et al.* 1994c).

Although there is evidence from *in vitro* experiments on colonic and intestinal epithelia that insulin-like growth factors-I and -II and insulin are involved in modulating the permeability of tight junctions (McRoberts *et al.* 1990, McRoberts & Riley 1992), little is known about

endocrine factors that regulate mammary epithelial tight junctions. Growth hormone, although important for maintaining milk secretion, does not appear to be important for the maintenance of mammary tight junctions in rats (Flint & Gardner 1994) and cows (Stelwagen *et al.* 1994a). Lactogenesis, the period during which mammary tight junctions are formed, requires the presence of prolactin, insulin and glucocorticoids (Forsyth 1982), but neither prolactin nor insulin was found to affect tight junctions in a murine mammary epithelial cell line (Zettl *et al.* 1992). In contrast, addition of dexamethasone, a synthetic glucocorticoid, to the medium of cultured mammary cells significantly reduces tight junction permeability (Zettl *et al.* 1992). Those authors concluded that glucocorticoids play an important role in the maintenance and formation of mammary tight junctions. The objectives of the present study were to examine whether cortisol reduces the permeability of mammary tight junctions *in vivo*, in the lactating mammary epithelium, and prevents the associated reduction in milk secretion. Our previously described milk-accumulation model (Stelwagen *et al.* 1997a) was used to challenge mammary tight junctions.

Materials and Methods

Animal care and use

Twenty multiparous Jersey cows during the early stage of lactation (68 ± 10 days postpartum) were used. During the study the cows were kept in large, partly covered, individual pens. The diet of freshly cut clover-ryegrass pasture was fed at 0800 and 1700 h. Fresh water was available throughout the experiment. Cows were machine milked at 0700 and 1600 h, or, in the case of once-daily milking, at 0700 h. Animal experimentation was conducted in accordance with the guidelines of the Ruakura Animal Ethics Committee.

Experimental protocol

Following a control period of 4 days, during which all cows were treated in the same manner, they were randomly assigned to one of three groups at the start of the subsequent 24-h experimental period. Groups were a control group milked twice (TM, $n=6$), a group milked only once (OM, $n=7$), and a group that was milked once and treated with adrenocorticotrophin (ACTH) (OM+ACTH, $n=7$). A dose of 0.05 mg (40 IU) of ACTH (ACTH₍₁₋₂₄₎, Synacthen, CIBA-Geigy, Switzerland) was administered five times in a total volume of 2 ml saline per dose into a jugular vein. Treatments were administered every 2 h, starting at 14 h of milk accumulation. The dose level was chosen based on previous data (Verkerk *et al.* 1994), to ensure that an elevation in plasma cortisol concentration was sustained with a low dose of ACTH and as few injections as possible. The time for the initial dose

(i.e. at 14 h) was chosen so that cortisol levels were elevated before mammary tight junctions became leaky, shown recently to occur consistently at 17 h of milk accumulation (Stelwagen *et al.* 1997a).

Three days before the experimental period, indwelling jugular catheters (Cavafix Certo, B Braun Melsungen, Melsungen, Germany) were inserted in each cow to facilitate repeated blood sampling and administration of ACTH. Following each blood sampling, catheters were flushed with heparinised (50 IU/ml; Leo Pharmaceutical Products, Ballerup, Denmark) saline. Blood samples were collected every 2 h during the first 10 h and hourly for the remainder of the 24-h experimental period. To more accurately describe the systemic cortisol profiles, samples were also taken from OM+ACTH cows 30 min following each ACTH treatment.

Milk yields were recorded at each milking throughout the control and experimental periods. Composite whole milk samples (25 ml) were also collected from each cow at every milking.

A mammary biopsy was taken after 23 h of milk accumulation from two randomly chosen cows from each group. No data of tissue analyses are reported, but all subsequent milk yield and composition data from these six cows are not included in the statistical analyses of the present study.

Chemical analyses

Blood samples were collected in heparinised vacutainers and stored on ice until centrifugation at 1500 *g* for 15 min. Plasma was aspirated and stored at -20°C until analysed for cortisol, lactose or glucose. Total cortisol was measured by a fluorescence polarisation assay, as previously reported (Verkerk *et al.* 1994). Inter- and intra-assay coefficients of variation were less than 10% and assay sensitivity was 0.5 ng/ml. Recovery of unlabelled antigen was 93%. Lactose in plasma was analysed using an enzymatic method as described previously (Stelwagen *et al.* 1994a). Glucose in plasma was analysed using a Hitachi 717 random access autoanalyser (Boehringer Mannheim NZ Ltd, Auckland, New Zealand).

The somatic cell count and the concentration of fat, protein and lactose were measured in fresh whole milk samples using a cell counter (somatic cell count) and standard infra red analyses (milk composition) as outlined previously (Stelwagen *et al.* 1994a).

Statistical analyses

All statistical analyses were carried out using SAS (SAS System for Windows, Release 6.11 1996, SAS Institute Inc., Cary, NC, USA). Differences between treatments were evaluated by ANOVA, including a covariate as appropriate ($P<0.05$). The covariate used for the ANOVA of milk yield, somatic cell count, fat, protein or lactose

percentage was the average for each respective variable during the 2 days preceding the experimental period. Milk yield was also included as a covariate for the ANOVA of plasma lactose. Because it was important to compare treatments at different time points and the likelihood that data were correlated, plasma lactose data were first subjected to multivariate analyses of variance (MANOVA). The MANOVA indicated a significant treatment effect, hence univariate ANOVAs were carried out subsequently for each time point.

The treatment profiles for cortisol appeared to increase over time. To determine whether these increases were significant, regression lines were fitted through the data of each animal, starting from 14.5 h. Regression coefficients were then tested per treatment to determine whether they were significantly greater than zero, using paired *t*-test analysis. To determine whether the slopes differed among treatment groups, regression coefficients were analysed by ANOVA.

The calculations to determine when the level of lactose in the plasma began to increase (i.e. when tight junction permeability changed) have been described previously (Stelwagen *et al.* 1997a). Briefly, the average concentration of the first four 2-hourly measurements is compared with the concentration at each subsequent time point. Then the time point at which the difference becomes significant ($P < 0.05$) is taken to indicate the onset of tight junction leakiness.

Means presented are least square means. In the case of multiple means (ANOVA) standard errors (S.E.M.) are reported as the average S.E.M. for the three treatment groups.

Results

Concentration of cortisol in plasma

The first dose of ACTH caused a rapid (<0.5 h) increase in the concentration of plasma cortisol in cows in the OM+ACTH group (Fig. 1A). Subsequent treatments sustained these significantly elevated cortisol levels throughout the desired time frame, whereas no differences were observed between the TM and OM groups. Mean plasma cortisol concentrations between 14.5 and 23 h of milk accumulation were: TM vs OM vs OM+ACTH, 4.6^a vs 7.3^a vs $54.7^b \pm 2.4$ ng/ml ($^{ab}P < 0.0001$). Even at its lowest point, at 18 h, the concentration was still significantly higher than in the TM and OM groups (TM vs OM vs OM+ACTH, 1.6^a vs 2.6^a vs $32.7^b \pm 4.3$ ng/ml, $^{ab}P < 0.0001$).

There appeared to be a slight, albeit significant ($P < 0.05$), increase in the cortisol profiles as the time of milk accumulation progressed. The increase was greater for OM+ACTH cows than for cows in the other two groups, for which the regression coefficients did not differ (TM vs OM vs OM+ACTH, 0.63^a vs 1.20^a vs $3.18^b \pm 0.28$, $^{ab}P < 0.0001$).

Figure 1 Concentrations of cortisol (A) and lactose (B) in plasma of cows milked twice (TM) or once (OM) during the 24-h experimental period. One OM group received 40 IU of ACTH every 2 h starting at 14 h of milk accumulation (OM+ACTH). Arrows indicate when ACTH was administered. Values are the mean $\pm$ S.E.M. of six (TM) or seven (OM, OM+ACTH) cows. Where error bars are not visible the S.E.M. was smaller than the plotting symbol.

Concentration of lactose in plasma

The concentrations of lactose in plasma, an indicator of mammary tight junction permeability, are shown in Fig. 1B. In cows in the TM group the concentration did not increase, and, in fact, remained at basal levels throughout the experimental period. The profile for OM cows, on the other hand, showed a typical rapid increase, becoming significant after 17 h of milk accumulation. The concentration of plasma lactose in cows with elevated plasma cortisol levels (ACTH treatment) was significantly lower compared with that in OM cows. Although lactose concentrations tended ($P > 0.10$) to be higher from 19 h onwards in ACTH-treated cows than in TM cows, at no time point were they statistically different. Mean concentrations of plasma lactose at the final measurement at 23 h of milk accumulation were: TM vs OM vs OM+ACTH, 29.4^a vs 444.2^b vs $132.9^a \pm 66.3$ μ M ($^{ab}P < 0.01$).

Figure 2 Changes in the concentrations of fat, protein and lactose in the milk of cows milked twice (TM) or once (OM) during the 24-h experimental period. One OM group received 40 IU of ACTH every 2 h starting at 14 h of milk accumulation (OM+ACTH). ^{a,b}Means differ at $P < 0.05$. ^{x,y}Means differ at $P < 0.07$. Values are the mean $\pm$ S.E.M. of six (TM) or seven (OM, OM+ACTH) cows.

Milk composition and yield

Means for milk fat, protein and lactose content at the end of the experimental period are shown in Fig. 2. Fat content was significantly elevated by once-daily milking, but this increase appeared to be prevented by ACTH treatment (i.e. in OM+ACTH cows). Protein content of the milk, on the other hand, was greater for both once-milked groups, and was not affected by ACTH treatment. Compared with the TM group, milk lactose, the major osmoregulatory component in milk, tended to be lower in the OM group, but not in the OM+ACTH cows. The somatic cell count, an indicator of mammary health status, comprises predominantly white blood cells. Counts were low ($< 50\,000/\text{ml}$) throughout the control and experimental period, and did not differ among the treatment groups (log-transformed somatic cell counts: TM vs OM vs OM+ACTH, 4.63 vs 4.60 vs 4.62 ± 0.1 cells/ml). Compared with that of TM cows, milk yield was approximately 12% lower in the once-milked cows, regardless of ACTH treatment (TM vs OM vs OM+ACTH, 16.3^a vs 14.4^b vs $14.4^b \pm 0.4$ kg/day, ^{ab} $P < 0.05$).

Concentration of glucose in plasma

The concentrations of glucose in the plasma, measured at 23 h of milk accumulation, were: TM vs OM vs OM+ACTH, 4.20^a vs 4.21^a vs $5.64^b \pm 0.1$ mM (^{ab} $P < 0.001$). Concentrations were significantly greater in ACTH-treated cows, but did not differ between TM and OM groups. Because glucose is the major precursor for lactose, we estimated how much milk the increase in plasma glucose represents if it were to be used as a milk precursor, using the following assumptions: (1) the concentration of

glucose in the plasma rises to a constant level within 3 h of the first ACTH administration, based on cortisol infusion data (Stewart & Thompson 1984); (2) no other precursors are limiting, and plasma-derived glucose is the sole source of mammary glucose; (3) 2 mol glucose are required for 1 mol lactose; and (4) 85% of the glucose taken up by the mammary gland is used for lactose synthesis (Annison & Linzell 1964). The glucose excretion rate is a function of its clearance from the plasma and its concentration. Using the clearance rate of 17.2 ml/min per $\text{kg}^{0.75}$ body weight, obtained by Hartmann & Kronfeld (1973) for lactating cows of similar body weight and milk production as the cows in the present study, it follows that the decrease in mammary glucose uptake in ACTH-treated cows is 23.4 g/h or 139.3 g in 7 h with an 85% utilisation rate. At 2 mol glucose per 1 mol lactose this represents 70 g of lactose and 1.3 kg of milk (i.e. 52.4 g of lactose per kg of milk, Fig. 2), a figure that is quite close to the actual decrease in milk yield of 1.9 kg.

Discussion

Earlier studies have demonstrated the importance of mammary tight junctions in maintaining milk secretion (Stelwagen *et al.* 1994b, 1995, 1997a). Moreover, glucocorticoids play an important role in the formation and maintenance of mammary tight junctions *in vitro* (Zettl *et al.* 1992). In the present study we used an *in vivo* model to examine whether elevated levels of plasma cortisol decrease permeability of tight junctions in the lactating gland, and in consequence prevent milk loss associated with increased tight junction leakiness.

Cortisol levels were successfully elevated by ACTH during the desired time frame. However, in all groups the cortisol profiles increased towards the latter part of the treatment period. This may be due to stress from the frequent blood sampling regime and/or interference with rumination and sleep patterns, because stress elevates cortisol levels in cows (Verkerk *et al.* 1998). Furthermore, Lefcourt *et al.* (1993) reported that cows exhibit weak circadian rhythms, with cortisol levels reaching a maximum at 0530 h. Indeed, levels also peaked in cows in the TM and OM groups at that time (i.e. 22 h).

Lactose in plasma was the main indicator of mammary tight junction permeability in the present study. Lactose is only produced in the mammary gland, and because it is not secreted basolaterally (Stelwagen *et al.* 1998b) its presence in blood can only be explained by its movement from milk into blood via leaky tight junctions. We have extensively validated the use of plasma lactose as a reliable indicator of tight junction permeability (Stelwagen *et al.* 1994b, 1995, 1997a). The increase in plasma lactose at 17 h of milk accumulation in OM cows is in agreement with tight junctions becoming consistently leaky after this time (Stelwagen *et al.* 1997a, 1998a). Compared with that in OM cows, the concentration of lactose in plasma was significantly reduced in ACTH-treated cows. These data represent the first *in vivo* evidence that cortisol can reduce tight junction permeability in the lactating mammary gland, and support *in vitro* studies (Zettl *et al.* 1992). Although concentrations in ACTH-treated cows were not statistically different from those in TM cows, they tended to be higher after 19 h of milk accumulation. This suggests that although the onset of leakiness was delayed by 2 h, cortisol did not completely prevent the effect of milk accumulation on the tight junctions. The reason(s) for this remain(s) elusive. A higher level of cortisol may be required, or levels need to be elevated earlier. It is possible that the initiation of the change in permeability of tight junctions, which are closely linked to the cytoskeleton of the cell (Schneeberger & Lynch 1992), started before cortisol was elevated. Alternatively, as mentioned earlier, the frequent sampling protocol may have elicited a stress response towards the end of the 24-h period, and preliminary data suggest that stress may adversely affect mammary tight junctions (Stelwagen *et al.* 1997b, McFadden & Stelwagen 1998). The small changes in milk composition between TM and OM cows are consistent with previous data (Stelwagen *et al.* 1994a, 1997a). The fact that the lactose level in milk of ACTH-treated cows was not different from that in TM cows supports the data on plasma lactose, because when tight junctions are open lactose 'leaks' from milk into plasma (Stelwagen *et al.* 1997a).

The 12% decrease in milk yield in OM cows is well within the range previously observed with once-daily milking (Stelwagen *et al.* 1994a, 1996, 1997a). The reduction in milk secretion is due to local, intramammary, mechanisms (Wilde *et al.* 1987, Stelwagen & Knight

1997). Although the exact mechanisms underlying such control are not known, they are likely to involve a putative inhibitory protein which has been isolated from milk (Wilde *et al.* 1995), as well as modulation of mammary tight junction patency (Stelwagen *et al.* 1994b, 1995, 1997a). Therefore, given the fact that ACTH reduced tight junction permeability, we did not expect milk yield to be reduced to the extent as shown for the ACTH-treated cows. However, milk loss in these cows may be related to factors other than tight junctions. In ruminants exogenous glucocorticoids significantly lower glucose extraction by the mammary gland, resulting in higher systemic glucose levels (Hartmann & Kronfeld 1973, Kronfeld & Hartmann 1973, Stewart & Thompson 1984). Therefore, the fact that plasma glucose levels were not different between TM and OM groups, but were 34% higher in the ACTH-treated cows, suggests that mammary uptake of glucose was reduced in the latter group. With glucose being the major precursor for milk lactose, which is the predominant osmoregulatory component in milk, the lower milk yield in the ACTH-treated cows may thus be due to a decrease in precursor supply, rather than being related to tight junction patency. Indeed, our calculations (see Results) show that the increase in plasma glucose in the OM+ACTH group represents a precursor pool that might explain the milk loss in these cows. It may be possible to overcome the confounding effects of glucocorticoids on milk secretion, by minimising the cortisol increase necessary to maintain tight junction patency. After all, cortisol levels in the present study were approximately three times higher than levels occurring naturally during lactogenesis, when mammary tight junctions are formed (Paterson & Linzell 1971, Hudson *et al.* 1976).

Finally, the present study does not address the mechanism by which cortisol modulates tight junctions. However, disruption of phosphorylation/dephosphorylation events and transforming growth factor- α can override the effects of glucocorticoids on mammary tight junctions *in vitro*, by causing a redistribution of the tight junction-associated protein, ZO-1 (Singer *et al.* 1994, Buse *et al.* 1995a,b). The ZO-1 protein is a cytoplasmic plaque protein, linking the integral tight junction protein occludin to the actin-based cytoskeleton (Fanning *et al.* 1996). To ascertain that such a mechanism underlies the *in vivo* changes observed in the present experiment requires further study.

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