Casein-derived phosphopeptides disrupt tight junction integrity, and precipitously dry up milk secretion in goats

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Abstract

Mammary involution is triggered by local stimuli, but the precise mechanism has not been defined. Milk stasis accumulate local signals, which makes the tight junctions (TJ) leaky. The aim of the study was to check the hypothesis that casein hydrolyzates (CNH) compromise TJ integrity and dry up milk secretion. A single dose of CNH transiently (12 to 24 h) compromised TJ integrity in the treated udder. This was associated by a transient (12 to 96 h) decline in milk secretion. No such changes were recorded in the contralateral gland that served as a control. Four repeated doses of CNH after each milking caused drastic changes in mammary secretion and composition, which were associated with irreversible cessation of milk secretion within 96 h. No such changes were recorded in goats treated with de-phosphorylated casein (control). We conclude that CNH are the milk-borne factors that cause the disruption of TJ integrity and induction of involution, and that the serine-Ps in the CNHs are essential for the excretion of biological activity. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Secretion; Involution; Lactation; Mammary gland; Casein; Serine-P

Introduction

Mammary gland involution proceeds through several distinct stages that involve cessation of milking, apoptosis of epithelial cells and tissue remodeling. Unilateral cessation of
milking in goat’s [1], and teat sealing in mice [2–4] induced involution in the treated gland only. This specificity suggests that mammary involution is triggered by local stimuli, but the precise mechanism has not been defined [5,6]. Reinitiating milk removal can reverse the first stage of involution, but the second stage of involution is irreversible and is characterized by activation of protease that destroys the lobular-alveolar structure of the gland by degrading the extracellular matrix and basement membrane, and causes massive loss of alveolar cells [5,6].

Tight junction (TJ) in the epithelial cells of the mammary gland forms a barrier between the systemic (basolateral) and the milk (apical’ sides) and prevents paracellular transport [7,8]. Milk stasis causes the accumulation of local signals, which makes the TJ leaky [7]. The serine protease plasmin is the predominant protease in milk and is known to produce boiling-resistant peptides (proteose-peptones) from β-casein (CN) and αs1- and αs2-CN [9]. Plasmin is found in milk mainly in its inactive form plasminogen [9], and the conversion of plasminogen to plasmin is modulated by plasminogen activators (PA) [9]. The PA-plasminogen-plasmin (PPS) system is involved in control of milk secretion and tissue remodeling during involution [9,10]. Our group recently showed that stress activates the PPS system, leading to the release from β-CN (fraction 1–28) of multiphosphorilated peptide, that is a potent blocker of potassium channels in mammary epithelia apical membranes [11]. Reduction in milk production was correlated with the activities of plasmin and with channel-blocking activity in the milk of the tested cows. Furthermore, injecting a pure β-CN fraction 1–28 into the udder lumen of goats also leads to a transient reduction in milk production [11]. The O-phospho-L-serine residue in this peptide is essential for the excretion of its activity [11].

Caseinophosphopeptides, through their phospho-serine residues, may bind 20 to 40 mole of Ca2+ [12]. Maintenance of extracellular Ca2+ levels is essential for maintaining the TJ integrity of the mammary secretory epithelium [13,14]. Neville and Peaker [13] and Stelwagen et al. [15] found that introducing the Ca-chelator EGTA into the mammary gland induced transient loss of TJ integrity and transient reduction of milk yield. During the onset of involution, the PPS activity increase by as much as 500% [16], compared with an increase of 30% measured when cows were exposed to stress [11], which is consistent with the extensive degradation of casein during involution [17].

The aim of the present study was to test the following hypotheses: (i) that CN hydrolyzates (CNHs) are the milk-borne factors that disrupt epithelial cell TJ integrity, and induced dry-up of milk secretion in goats; (ii) that the function of the CNHs relates to the presence of O-phospho-L-serine residues; and (iii) that CNHs function as Ca-chelators, which induce a low concentration of Ca2+ in the milk, thus disrupting TJ integrity for a critical period, and inducing the second irreversible stage of involution. In order to test the hypotheses a dose of CNH was introduced into a single udder of goats, either as a single treatment, or repeatedly after each milking for several days. The contralateral gland served as a control. The results of the present experiment confirmed hypotheses (i) and (ii), but only partially confirmed hypothesis (iii). CNH indeed lowered the Ca2+ concentration in mammary secretion, and induced irreversible involution by preventing the restoration of TJ integrity for a critical period. However, reduction of Ca2+
concentration in the lumen of the gland was not essential for disrupting TJ integrity and for drying up milk secretion.

**Materials and methods**

**Preparation of CNH**

Commercial bovine CN (Sigma) was dissolved (100 g/l) in 25 mM Tris-buffer pH 8 and digested with trypsin (500 U/l) for 4 h at 37 °C. The solution was then acidified to pH 4.7 with HCl and the non-digested CN was pelleted by centrifugation. The supernatant was boiled for 15 min, cooled to room temperature and adjusted to pH 7 with NaOH solution. Material that had not dissolved under these conditions was discarded by centrifugation, and the CNH solution was sterilized by passage through a 22-μm sterile filter and kept frozen until use. The typical protein concentration (as measured by the Bradford method) in the CNH solution was 20 mg/ml and the osmotic pressure was 400 mOsmol/kg. Phosphate-free CNH was similarly prepared from de-phosphorylated bovine CN (Sigma), and served as a control.

**Animals and their maintenance**

The goats used in these experiments were lactating-pregnant Israeli Saanen goats (British Saanen crossed with local goats and back crossed with British Saanen bucks for three to six generations) in their second, third or fourth lactation; they weighed approximately 70 kg. The goats were in last trimester (late-lactation) of pregnancy, which is the typical period for drying their mammary secretion. Goats were machine milked twice daily at 0700, and 1600, and their milk yield were routinely recorded. Goats were fed the concentrate portion of their diet during milking. The diet comprised cubes of alfalfa hay (40% NDF and 14% protein) ad libitum, and 1.5 kg concentrates (18% protein). The basic milk yield and composition were recorded for three days before treatments from each gland of every goat.

**Experiment 1**

CNH (300 mg per 15 ml) was injected with a thin rounded plastic needle as a single dose into the cistern of single gland through the teat canal of four goats after the afternoon milking. The contralateral gland in each goat was treated with the same volume of the control solution. Mammary secretions from each gland were collected and sampled at each milking for a week after the treatment.

**Experiment 2**

CNH (300 mg per 15 ml) was injected as a single doses four successive times after each milking (i.e., four doses over two days) to the cistern of single gland through the teat canal of
four goats. In parallel, the contralateral gland in each goat was treated with the same volume of the control solution. Mammary secretions from each gland were collected and sampled at each milking for a week after the first treatment.

Experiment 3

The experimental procedures in experiment 3 were similar to that described in experiment 2, except that the CNH and control solutions contained 1.5 M CaCl₂.

Analytical methods

The concentrations of lactose, protein (total, whey, serum albumin and casein), and minerals (Na, K, Ca) in milk were determined as described by Shamay et al. [18]. The activity of plasminogen activator (PA) and plasmin in milk was determined as described by Silanikove et al. [11]. A calcium electrode (MeterLab pH/4201, a Portable pH Meter equipped with selective electrode 813D-12 and reference electrode REF251; Radiometer Analytical, Denmark) was used to determine the Ca²⁺ concentration in the milk, using the

Fig. 1. Effect of single dose of CNH on milk yield and milk lactose concentration. Values marked by asterisks are significantly different from the control (P < 0.05 or lower). ◆ — experimental, ■ — control.
repeated addition and ultrafiltration procedures to correct for the interference effect of casein [19].

**Statistical analysis**

The variation was analysed according to the General Linear Model of SAS [20] for repeated measurements. First, we checked if the post-treatment results differed significantly from the pre-treatment ones, by using the pre-treatment results of each goat at each sampling as co-variates. When the post-treatment results differed from the pre-treatment values only in the experimental group, the statistical significance ($P < 0.05$) was assessed by means of the PROC GLM software of SAS [20] to compare repeated measurements between treatments.

![Graphs showing effect of single dose of CNH on milk concentration of ionised calcium, Na$^+$, and K$^+$. Values marked by asterisks are significantly different from the control ($P < 0.01$ or lower).](image)

Fig. 2. Effect of single dose of CNH on milk concentration of ionised calcium, Na$^+$, and K$^+$. Values marked by asterisks are significantly different from the control ($P < 0.01$ or lower). ◆ — experimental, ■ — control.
Fig. 3. Effect of single dose of CNH on milk activities of PA and plasmin. Values marked by asterisks are significantly different from the control ($P < 0.01$ or lower). One unit of PA activity was defined as the amount of enzyme producing a change in absorbance of 0.1 in 60 min at 37 °C and 405 nM. One unit of plasmin was defined as the amount of enzyme producing a change in absorbance of 0.001 in 1 min at 37 °C and 405 nM. ■ — experimental, □ — control.

Fig. 4. Effect of 4-repeated dose of CNH on milk yield and milk lactose concentration. Experimental values are significantly different from the control ($P < 10^{-5}$ or lower). ■ — experimental, □ — control.
Results

Experiment 1 — Single-dose treatment

Milk secretion declined transiently in the CNH-treated glands; the lowest value (decline of 35%) was recorded at 48 h post-treatment, and it took 96–120 h for complete recovery (Fig. 1a). Lactose concentration declined transiently in the first milking post-treatment; the lowest value (decline of 30%, $P < 0.01$) was recorded at 36 h post-treatment, and it took 48–60 h for complete recovery (Fig. 1b). Sharp changes in $\text{Ca}^{2+}$ (decline of 35%, $P < 0.01$), $\text{Na}^+$ (threefold increase, $P < 0.001$), and $\text{K}^+$ (decline of 50%, $P < 0.01$) concentrations were recorded at the first milking post-treatment (12 h); they returned to pretreatment values in the

Fig. 5. Effect of 4-repeated dose of CNH on milk concentrations of $\text{Na}^+$, $\text{K}^+$ and total calcium. Experimental values are significantly different from the control ($P < 10^{-5}$ or lower). ◆ — experimental; ■ — control.
next milking (24 h post-treatment) in the CNH-treated glands (Fig. 2). PA and plasmin activities increased transiently in the CNH-treated glands (Fig. 3); PA activity was twice as high \((P < 0.01)\) at 24 h post-treatment, and plasmin activity at 24 and 46 h were 15-fold higher \((P < 0.001)\) than in the control glands. It took 48 h for the PA activity and 60–72 h for the plasmin activity to return to the pretreatment levels (Fig. 3).

Experiment 2 — Repeated dose treatments

Repeated doses of CNH induced drastic changes in the rate, appearance and composition of mammary gland secretion (Figs. 4, 5). The first sign of changes in appearance and

Fig. 6. Effect of 4-repeated doses of CNH on milk concentrations of proteose–peptone, serum albumin and whey protein. Experimental values are significantly different from the control \((P < 10^{-5}\) or lower). \(\bullet\) — experimental, ■ — control.
composition was excessive foaming of the milk at 24 h post-treatment. This was most likely due to the accumulation of proteose-peptones (natural CNH, Fig. 6), which are known to be foaming materials [21]. Between 36 and 96 h post-treatment the secretion contained numerous cells that were easily visible as sedimentation (~1/3 of the volume) in the collected secretion. Staining this fraction with trypan blue and its examination in light microscope showed that the sedimentation was composed mostly of lymphocytes and many phagocytes and dispersed epithelial cells. Occasionally, fat cells were observed. Most of the cells appeared viable, since the dye did not penetrate into them. By 96 h post-treatment, the scant mammary secretion resembled a turbid serum (beer-like fluid) having a pinky-yellowish color. This type of secretion resembles the secretion collected from fully involuted gland (i.e., two to three weeks after cessation of milking). In addition of being lactose-free (Fig. 4), this serum-like fluid was fat-free. The concentrations of Na⁺, K⁺, Ca²⁺, and serum albumin at 96 h post-treatment were similar to the expected blood plasma concentrations (Figs. 5, 6), indicating that this fluid was composed mostly of interstitial fluid. Concentrations of protein, whey and proteose-peptones in this serum-like fluid were much higher than in normal milk (Fig. 6). PA activity in the CNH-treated glands rose to six times that in the control at 12–24 h post-treatment and it stabilized at double the control level at 36 h post-treatment (Fig. 7a). Plasmin activity rose sharply stabilizing at 24 h post-treatment at a level 40 times greater than the control level at 24 h post-treatment (Fig. 7b).

\[ \text{Fig. 7. Effect of 4-repeated doses of CNH on milk activities of PA and plasmin. Experimental values are significantly different from the control (} P < 10^{-5} \text{ or lower).} \]

\[ \text{\bullet} \quad \text{experimental}; \quad \text{•} \quad \text{control.} \]
Experiment 3: repeated dose with CaCl₂

Treating goats with CNH and control solutions that contained 1.5 M CaCl₂ delayed the reduction in ionized calcium concentration in the CNH-treated glands during the first 24 h (data not shown). However, apart from this, changes in mammary secretion and composition in the glands treated with CNH were essentially similar to those described for Experiment 2.

Discussion

Casein hydrolyzates exhibits myriad bioactivities such as immunoregulation, and anti-microbial and antineoplastic actions, which support aspects of infant development, growth, and survival beyond that, provided by nutrition alone [12]. We have recently shown that casein enzymatic hydrolyzate may also be involved in local regulation of milk secretion [11]. A distinct plasmin-induced β-casein breakdown product (fraction 1–28) down-regulates milk secretion in goats and cows, an activity most likely related to its function as a potent blocker of potassium channels in the apical membranes of mammary epithelia [11]. The results of the present study suggest that CNH are among the milk-borne factors that cause the disruption of TJ integrity and induce dry-up of milk secretion upon milk stasis. It is also suggested that the O-phospho-L-serine residues in CNH are essential for excreting its biological activity. However, prevention of the fall in Ca²⁺ concentration by CNH did not prevent disruption of TJ integrity and its induction of dry-up of milk secretion. Presumably, the drop in Ca²⁺ concentration from ~3 mM to ~2 mM is not sufficient to trigger these events.

During lactation, when the ducts and alveoli are filled with milk, the secretory epithelium is positioned between two very different environments: the milk, containing high concentration of lactose and low concentrations of sodium and chloride, and interstitial fluid containing low concentration of lactose and high concentrations of sodium and chloride. Thus, when TJs are disrupted the Na⁺ concentration in the milk rises, whereas that of K⁺ declines [8]. The changes in Na⁺ and K⁺ concentrations in milk indicate that the TJs in the treated glands were compromised. The rapid restoration of Na⁺, K⁺ in milk in the single treated glands indicates TJ re-closure, and suggests that pre-existing junctional complexes are maintained during the first stage of involution.

The mechanism by which disruption of TJ affects milk secretion has not been established [8]. The stretching associated with milk stasis has been implicated in a mechanotransduction-signaling pathway, which in turn can alter both milk synthesis and TJ permeability [8]. However, the continuation of milking in the present experiments obviously prevented mechanical stress, indicating that decreased milk yield, in conjunction with disruption of TJ can occur in the absence of such stress. A change in the Na⁺/K⁺ ratio in milk could alter the intracellular Na⁺/K⁺ ratio in the mammary epithelial cells and thus affect their functioning [8]. However, ionic equilibration phenomena are fast, whereas in the present study, milk secretion remained depressed long after Na⁺/K⁺ ratio in milk had returned to its pretreatment level. Thus, the present results invalidated some of the prevailing explanations. We recently showed that β-CN fraction 1–28, which is part of the injected CNH may cause
similar transient reduction in milk production by a mechanism that does not relate to

The first visible stage of prostate and mammary involution is the disruption of interepi-
thelial adhesion before the onset of apoptosis [22]. This is consistent with disruption of the TJ
before onset of involution, and with the observation that the sloughed epithelial cells were
separated from each other. An increase in serum albumin in milk suggests paracellular
leakage of interstitial fluid into the milk [23]. However, as serum albumin is not a regular
constituent of interstitial fluid, it also suggests TJ disruption in the blood vessels surrounding
the alveolus. The disruptions of blood vessels and of alveolus integrity are typical events in
either inflammation or involution and account for the influx of lymphocytes and phagocytes
into the alveolar lumen. Thus, the precipitous dry-up of milk secretion may be related either
to necrosis caused by the inflammation or to induction of involution. The fact that all the
-treated goats resumed normal lactation after parturition suggests that we imitated natural
phenomena rather than inducing a necrotic response that would irreversibly damage the
secretory function of the udder.

Further support for the notion that CNH treatment induced involution is found in the
patterns of PA-plasmin-proteose-peptone concentration following treatment. The parallelism
between proteose-peptone concentration and plasmin activity is consistent with the findings
that proteose-peptones are composed mainly of natural plasmin-induced CNH products in
milk [11]. Thus, their accumulation because of progressively increasing exposure to plasmin
following milk stasis may explain the induction of involution associated with milk stasis.
The fact that most plasmin in goats and bovine milk is in the non-activated form, plasmi-
nogen explains the time lag of about 14 days between cessation of milking and induction
of involution.

In mouse, in the first stage of involution, alveolar cells undergo apoptosis, but there is no
remodeling of the lobular-alveolar structure [2]. During the second stage of involution the
lobular-alveolar structure of the gland is obliterated as proteinases degrade the basement
membrane and extra-cellular matrix. The two stages exhibit characteristic changes in gene
expression or activity. The present results do not enable us to identify precisely the transition
from the first stage to the second stage of involution; however, it should have occurred
between 36 and 72 h after the TJs were compromised. Stage 2 could be easily identified in the
present experiment because mammary cells were easily visible in the mammary secretion.
This also coincided with the marked increases in PA and plasmin activities, which appear to
be part of the enzymatic system that is involved with the obliteration of the lobular-alveolar
structure [2]. Thus, the present results suggest that the PA-plasmin system being involved in
stages 1 and 2 of involution.

In summary, CNH injections to the udder induce transient inflammatory response, lost of
TJ integrity followed by rapid dry up of mammary secretion. These events characterized
bovine and caprine involution [5,24], thus, the rapid rise of CNH in the udder seems to
accelerate and synchronized the natural phenomena. The effects of CNH on the cellular level
are not clear at this stage, and we do not know if CNH affect directly the three response, or if
for instance the lost of TJ integrity is consequence of the inflammatory response. Results of
studies in which the mammary gland was challenged with endotoxin and colchicine produced
similar response in terms of inducing inflammation and changes in milk composition [24]. Fluid volume at 7 days involution was 40% less in bovine udder halves infused with colchicine and cholchicine and endotoxin as compared to uninfused udder halves [24]. In comparison, CNH treatment induces complete dry up within 72 h in the treated glands. Thus, CNH appear to be much more effective then colchicine and cholchicine and endotoxin in inducing dry up of mammary secretion.

References


