Infusions of Casein Hydrolyzates into the Mammary Gland Disrupt Tight Junction Integrity and Induce Involution in Cows

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ABSTRACT

Milk stasis triggers local stimuli, which make the tight junctions leak and trigger involution. The aim of the study was to test the hypothesis that casein hydrolyzates compromise tight junction integrity and dry-off milk secretion in dairy cows. Six repeated doses of casein hydrolyzates after each milking during 3 d caused drastic changes in mammary secretion and composition, which were associated with irreversible cessation of milk secretion. No such changes were recorded in the control glands that had been treated with nonhydrolyzed casein. Treatment with casein hydrolyzates disturbed tight junction integrity within 8 h (as indicated by changes in Na^+ and K^+ concentrations), reduced the concentrations of lactose precipitously, activated the plasmin activator-plasminogen-plasmin system, and induced the secretion of immunoglobulin type G and lactoferrin. At the end of the 3-d treatments, we stopped milking the experimental and control glands. Milk composition 19 d later was similar in the experimental and control glands and was consistent with the composition expected in fully involuted glands. We conclude that casein hydrolyzates are among the milk-borne factors that cause the disruption of tight junction integrity and induce involution in cows. The process induced by casein hydrolyzate was more rapid and synchronized than the involution induced at drying-off.
(**Key words:** involution, mammary gland, secretion, tight junction)

Abbreviation key: CNH = casein hydrolyzate, **Lf** = lactoferrin, **PA** = plasmin activator, **TJ** = tight junction.

INTRODUCTION

Enzymatic hydrolysis of casein (**CN**) liberates peptides that may contribute to the health and proper development of young (FitzGerald, 1998) and serve as local regulators of mammary gland function (Silanikove et al., 2000; Shamay et al., 2002). A peptide derived from the activity plasmin on β-CN (fraction 1-28) downregulates milk secretion in cows and goats; its activity was correlated with its ability to block potassium channels in the apical membranes of mammary epithelia (Silanikove et al., 2000). In goats, injection of casein hydrolyzates (**CNH**) into the udder induced a local inflammatory response and loss of tight junction (**TJ**) integrity, followed by rapid drying-off mammary secretion (Shamay et al., 2002). As such events also characterize the involution induced by cessation of milking in cows and goats (Olivier and Smith, 1983; Hurley, 1989; Capuco and Akers, 1999), it was suggested that an artificial increase of the CNH concentration in the udder mimics the natural phenomenon. The process induced by CNH was more rapid and synchronized than that induced at drying-off (Shamay et al., 2002).

The serine protease, plasmin, is the predominant protease in milk and is known to produce boiling-resistant peptides (proteose-peptones) from β-CN and αs1- and αs2-CN (Politis, 1996). Plasmin in milk is found mainly in its inactive form, plasminogen, of which the conversion to plasmin is modulated by plasminogen activators (**PA**; Politis, 1996). The PA-plasminogen-plasmin system is involved in control of milk secretion and tissue remodeling during involution (Ossowski et al., 1979; Politis, 1996). We have shown that both the antisecretory properties of β-CN fraction 1-28 and the involuting properties of CNH are related to the activity of the PA-plasminogen-plasmin system in milk (Silanikove et al., 2000; Shamay et al., 2002). However, only a modest increase in plasmin activity is required for activation of the antisecretory properties of β-CN fraction 1-28 (Silanikove et al., 2000), whereas the involution induced either by CNH (Shamay et al., 2002) or at the drying-off of mammary secretion was associated with a dramatic increase in plasmin activity (Aslam and Hurley, 1997; Athie et al., 1997). Consequently, the concentration of natural CNH (i.e., proteose peptones) increased dramatically in the mammary secretion of involuting goats (Shamay et al., 2002).

There is no information on the effects of CNH on mammary secretion in cows. The objectives of the present study were to test the hypotheses: 1) that introducing CNH to the mammary gland disrupts epithelial cell TJ integrity, and induces dry-off of milk secretion in the treated glands, and 2) that this treatment imitates and accelerates the involution induced at drying-off.

MATERIALS AND METHODS

Preparation of Casein Hydrolyzate

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 nondigested 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 removed by centrifugation and discarded, and the CNH solution was sterilized by passage through a 22-μm sterile filter and kept frozen pending use. The typical protein concentration in the CNH solution (as measured by the Bradford method) was 20 mg/ml. No measurable activity of PA, plasminogen, or plasmin was found in the CHN preparation administered to the udder.

Control solution was prepared as follows: Trypsin was deactivated by boiling at 100°C for 30 min. The commercial CN was then treated with the inactivated trypsin solution as described above. The pelleted CN was dissolved (20 mg/ml) in 25 mM Tris-buffer, pH 8, adjusted to pH 7 with NaOH, and sterilized and stored as described above. The osmotic pressure of the experimental and control solutions was 400 mOsmol/kg.

Animals and Their Maintenance

The eight cows used in these experiments were multiparous (second, third, or fourth lactation) lactating and pregnant (last trimester) Holsteins that were scheduled for dry-off treatment (i.e., they were ~300 DIM). The cows selected for the experiment had SSC of <300,000 cells/ml. The cows were fed a typical Israeli TMR that comprised 65% concentrates and 35% forage and contained 17% protein throughout the trial. The cows were milked three times daily, at 0500, 1300, and 2000 h, and produced ~30 L milk per day. The composition of the mammary secretion from each quarter was recorded for 3 d before the treatment.

Experimental Procedures

A single dose of the CNH preparation (67.5 mg per 15 ml as determined both by the spectrometric and the macro-Kjeldhal assays and with absorbance of 20,000 at 280 nm) was injected with a thin, rounded plastic needle, through the teat canal, into the cistern of two single glands (quarters) of each cow (i.e., 16 glands) after the morning milking. The contralateral quarters in each cow were treated with the same volume of the control solution. This procedure was repeated after the afternoon milking and similarly on the next 2 d (i.e., six postmilking doses over 3 d). At the time of the evening milking on those days, the cows were not taken to the milking parlor and were not milked nor treated. The switch from thrice daily milking to twice daily milking may have reduced milk yield by 10%, but for such a short period, milk composition was most likely not affected (Sorensen et al., 2001). After the last treatment, the cows were not milked throughout the dry period (~60 d), and milking was resumed in the next lactation cycle. Mammary secretions (~100 ml) from each gland were collected and sampled at each dosing. In addition, a sample was taken from each gland daily at 0600, for 3 d before the treatment, and on d 4, 10, and 19 after the cessation of treatments.

Analytical methods. The concentration of protein in the CNH preparation was determined by a spectrometric assay (Vernon and Bernlohr, 1977), by the macroKjeldhal method (N × 6.35), and with absorbance at 280 nm. The concentrations of total protein, whey proteins, total casein, and proteose peptone in mammary secretions were determined as described by Shamay et al. (2000). Enzyme-linked immunoassays were used for the determination of albumin (Stelwagen et al., 1994), lactoferrin (**Lf**) (Shuster and Harmon, 1996), and IgG (Kummer et al., 1992) concentrations. The activities of PA, plasminogen, and plasmin in milk were determined as described by Silanikove et al. (2000). Lactose concentration was determined enzymatically according to Shapiro et al. (2002). Mammary secretions were prepared for mineral (Na, K, Ca, Mg, P, and S) analysis as described by Shamay et al. (2000), and the concentrations of these minerals were measured with an Inductively Coupled plasma-atomic emission spectrometer (Spectro, Germany).

Statistical analysis. The datasets of this study were analyzed using repeated measures analysis (SAS PROC MIXED) modeling correlated residuals within cow (SAS, 1988). The analysis concentrated on the effects of treatment, day, and treatment × day interactions. The effect of parity and DIM were not significant ($P > 0.25$) and, therefore, were not included in the analyses presented here.

The model used was

$$Y_{ijkm} = \mu + C_i + T_j + T_jD_k + Q_m(T_jC_I) + e_{ijkm},$$

where Y_{ijkm} is the variable within cow, treatment, quarter, and day, C_i is the cow class effect, T_j is the treatment class effect, D_k is the day class effect, T_jD_k is the treatment × day interaction effect, $Q_m(T_jC_I)$ is the quarter within cow treatment error term for treatment effect; and e_{ijkm} is the residual error.

Covariance between residuals within cow was modeled either as compound symmetry or as heterogeneous compound symmetry (SAS, 1988). Analyses relative to first treatment infusion used observations from the first 8-h (0.3-d) sampling and onward with pooled observations made before infusion as a covariate. Running this model using general linear model procedures and type III mean squares (SAS, 1988) yielded similar results to those obtained by the repeated measures analysis using either the compound symmetry or the heterogeneous compound symmetry analysis of residuals covariance.

RESULTS

Volume, Appearance, and Osmotic Components

Repeated doses of CNH induced precipitous dry-off of mammary gland secretion in the treated gland, whereas the control gland maintained its milk secretion. At the third day posttreatment, the scanty mammary secretion collected from the treated gland (100 to 300 ml compared with a typical initial volume of ~2.5 L) was watery, fat-free, turbid (serum-like), and contained numerous amount of leukocytes (> then 5×10^6/ml), mostly (90%) in the form of polymorphonuclear neutrophils. This type of secretion resembled the secretion collected from the control glands at 10 and 19 d postmilking.

The concentration of lactose dropped precipitously in the treated glands, whereas in the control glands, it dropped only after cessation of milking (Figure 1). The drop in lactose was significant after the first milking (0.3 d), by d 1 it had dropped to ~50% of its initial concentration (~120 mmol), and by d 2 and 3, it was down to one-sixth of the initial concentration. In the control glands, after 4 d of dry-off (i.e., at d 7 of the experiment), lactose had dropped to ~50% of the initial concentration. At d 13 and 22, the concentration of lactose was ~20 mmol in both the control and experimental glands (Figure 1).

In the experimental glands, the concentration of Na^+ rose precipitously following the first treatment, whereas that of K^+ dropped precipitously (Figure 1). In the control glands, equivalent changes were observed only following cessation of milking. At d 13 and 22 of the experiment, the Na^+ (~120 mmol) and K^+ (~12 mmol) concentrations in the control and experimental glands were similar (Figure 1).
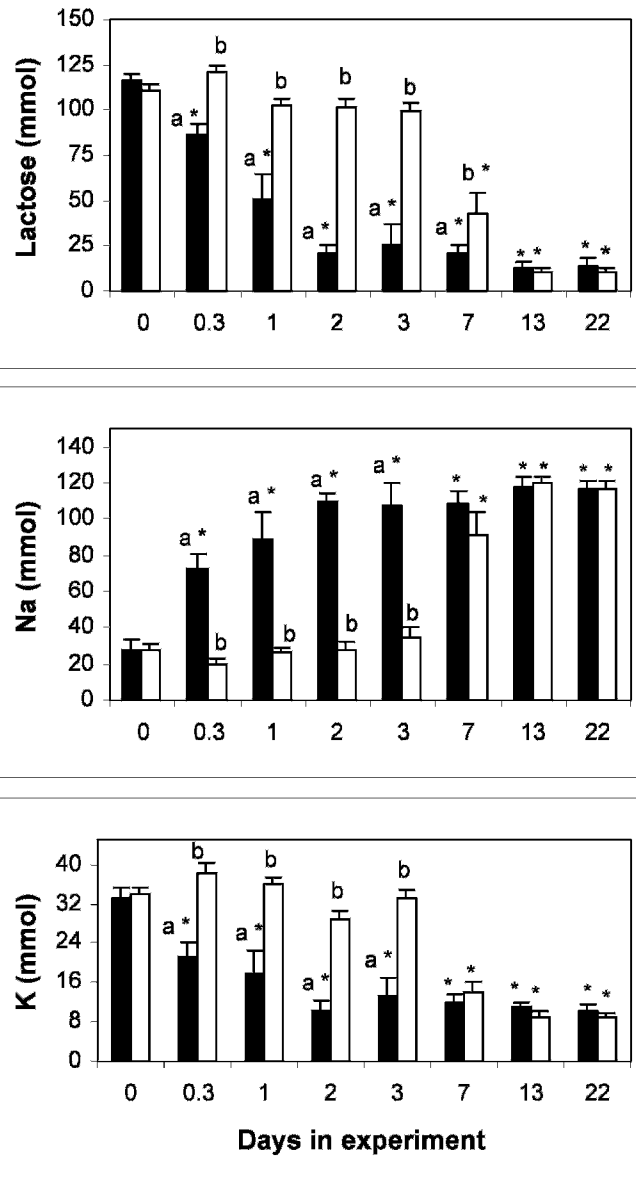
Figure 1. Effect of repeated dose of CNH and drying-off on lactose, Na^+, and K^+ concentrations in mammary secretion. *Values are significantly different from the pretreatment values ($P < 10^{-2}$ to 10^{-4}). [a,b]Experimental values (filled bars) are significantly different from the control (open bars; $P < 10^{-2}$).

PA-Plasminogen-Plasmin System

PA activity at the start of the experiment was ~1100 U/ml (Figure 2). In the experimental glands, the PA activity rose significantly after the first treatment, and activities at the highest range (3200 to 3500 U/ml) were measured at d 1, 2, 3, and 7 to the experiment; on d 13, the PA activity dropped significantly and fell further, to ~1200 U/ml on d 22. In the control glands, a nadir in
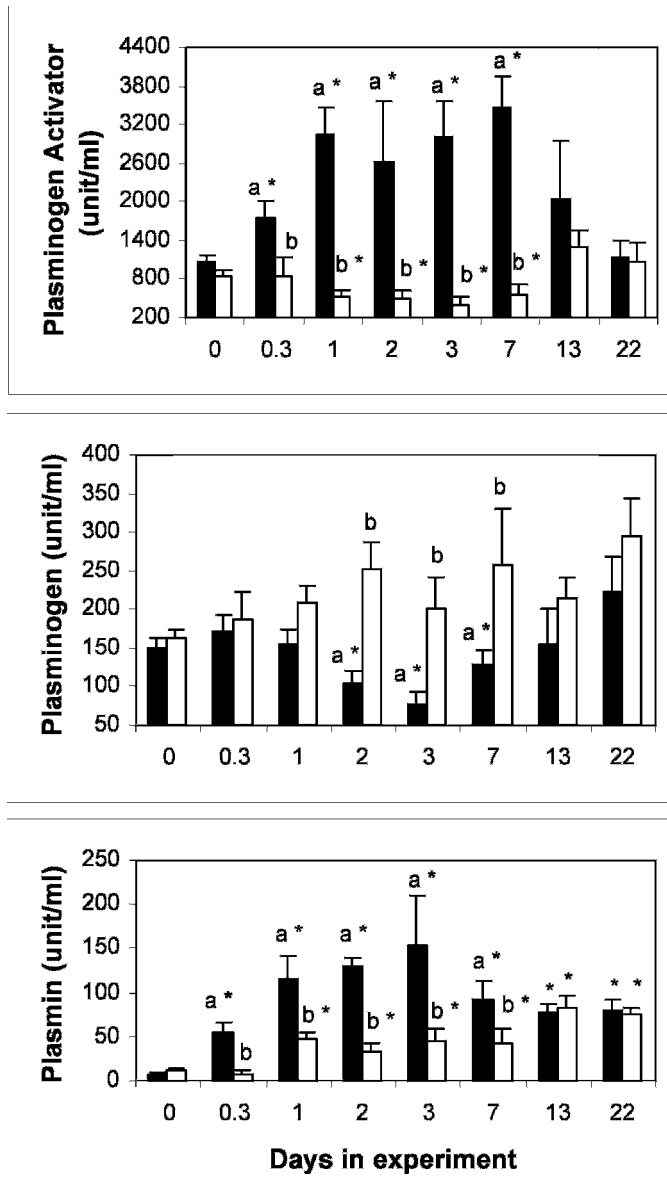
Figure 2. Effect of repeated dose of CNH and drying-off on plasminogen activator (PA), plasminogen, and plasmin activities in mammary secretion. One unit of PA, plasminogen, or plasmin activity was defined as the amount of enzyme producing a change in absorbance of 0.001 in 1 min at 37°C and 405 nm. *Values are significantly different from the pretreatment values ($P < 0.01$). [a,b]Experimental values (filled bars) are significantly different from the controls (open bars; $P < 0.01$).

PA activity (400 to 600 U/ml) was observed on d 1, 2, 3, and 7 of the experiment; it was followed by a rise to ~1200 U/ml on d 13 and 22 (Figure 2).

Plasmin activity at the start of the experiment was ~5.5 U/ml (Figure 2). In the experimental glands, the plasmin activity rose significantly after the first treatment, and activities in the highest range (120 to 150

U/ml) were measured on d 1, 2, and 3 of the experiment, after which it fell to ~100 U/ml on d 7, 13, and 22. In the control glands, plasmin activity rose to ~50 U/ml on d 1, 2, 3 and 7, and to ~100 U/ml on d 13 and 22 (Figure 2).

Plasminogen activity at the start of the experiment was ~150 U/ml (Figure 2). In the experimental glands, a nadir in plasminogen activity was observed on d 2, 3, and 7 of the experiment, and was followed by a return to 160 and 220 U/ml on d 13 and 22, respectively. In the control glands, plasminogen activity tended to increase with time; however, because of high variability, these changes were not statistically significant (Figure 2).

Proteins

In the experimental glands, the total protein concentration on d 2 and 3 (~55 mg/ml) was 1.8 times higher than the initial concentration of ~30 mg/ml (Figure 3). In the control glands, there was a tendency for protein concentration to increase during this period. On d 7, 13, and 22 of the experiment, the protein concentration was ~70 mg/ml in both the control and the experimental glands (Figure 3).

Initially, in both the control and experimental glands, CN (at ~24 mg/ml) constituted 80% of the total protein, whereas by d 22 (at ~28 mg/ml), it constituted only 40% of the total protein (Figure 3). A significant increase in CN concentration (to ~40 mg/ml) was observed on d 2, 3, and 7 of the experiment in the experimental glands and on d 7 in the control glands (Figure 3).

Initially, whey protein (at ~6 mg/ml) constituted 20% of the total protein, but by d 22 (at ~40 mg/ml), it constituted 60% of the total protein in both the experimental and control glands (Figure 3). In the experimental glands, the concentration of whey protein increased steadily from the first treatment, and by d 7 of the experiment, it had reached 88% of the maximal values attained on d 13 and 22. In the control glands, the concentration of whey protein increased significantly only after the cessation of milking, and on d 7, it was significantly lower than that in the experimental glands (Figure 3).

In both the experimental and control glands, the proteose-peptone concentration increased more than fourfold, from ~2 mg/ml at the start of the experiment to ~9 mg/ml at the end (Figure 3). The pattern of increase in proteose-peptone concentration resembled that described for total whey protein, namely, a steady increase from the beginning in the experimental glands and a rise that started only after the cessation of milking in the control glands.

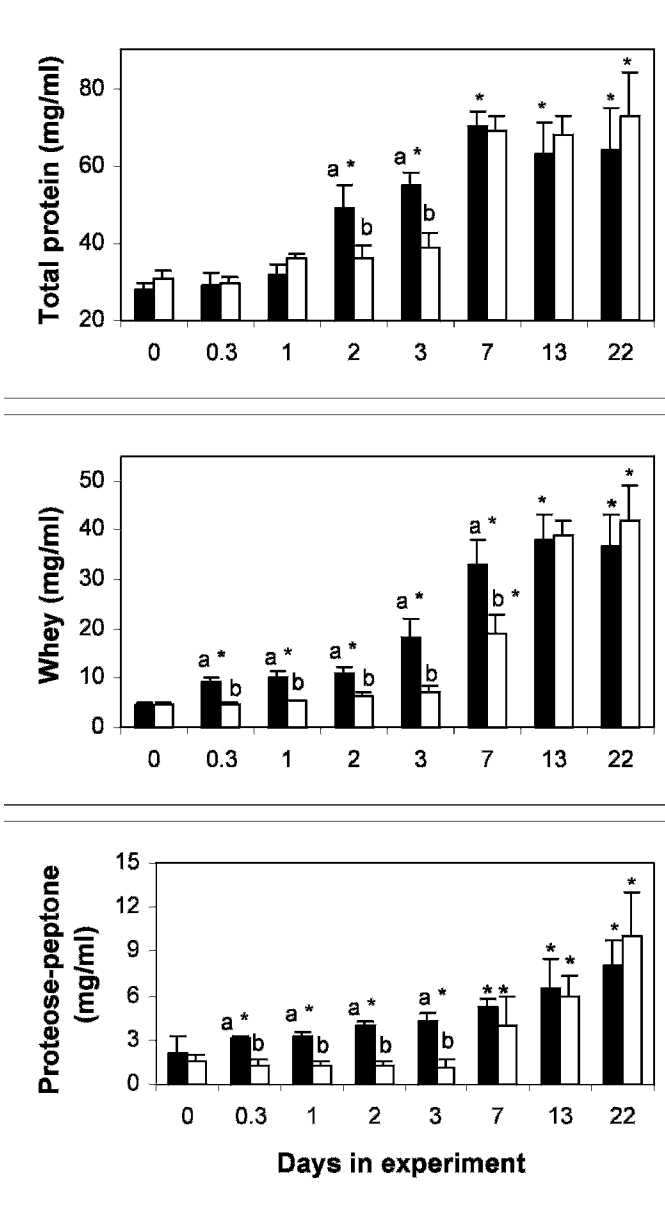Albumin concentration in both the experimental and control glands increased almost ninefold, from ~120 μg/

Figure 3. Effect of repeated dose of CNH and drying-off on total protein, whey protein, and proteose peptones concentrations in mammary secretion. *Values are significantly different from the pretreatment values ($P < 10^{-2}$). [a,b]Experimental values (filled bars) are significantly different from the controls (open bars; $P < 10^{-2}$).
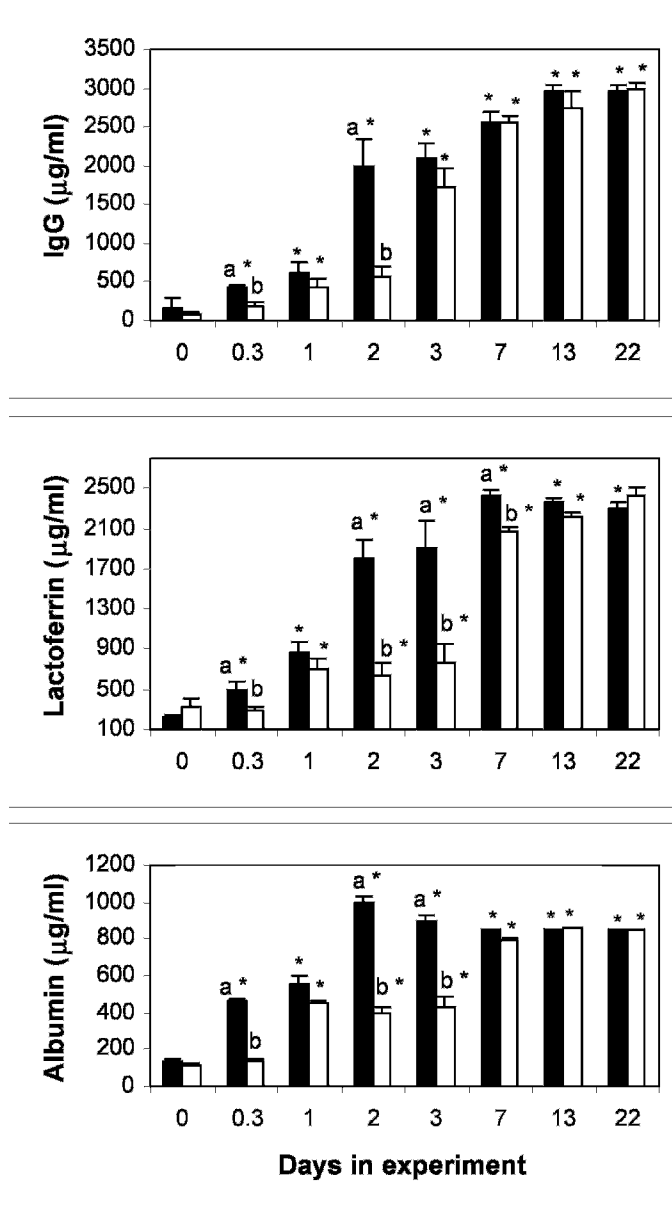
Figure 4. Effect of repeated dose of CNH and drying-off on immunoglobulin type G (IgG), lactoferrin (Lf), and albumin concentrations in mammary secretion. *Values are significantly different from the pretreatment values ($P < 10^{-2}$ to 10^{-4}). [a,b]Experimental values (filled bars) are significantly different from the controls (open bars; $P < 0.01$).

ml at the start of the experiment to ~900 μg/ml at the end (Figure 4). In the experimental glands, a significant increase in albumin concentration was observed after 0.3 d, and values in the highest range were already measured from d 2. In the control glands, a significant increase in albumin concentration (to ~480 μg/ml) was measured at d 2, 3, and 4 of the experiment and concentrations in the highest range from d 7 (Figure 4).

The concentration of Lf in both the experimental and control glands increased more than 12-fold, from ~180 μg/ml at the start of the experiment to ~2200 μg/ml at the end (Figure 4). In the experimental glands, a significant increase in Lf concentration was observed after 0.3 d; on d 2 and 3, the concentration had risen to ~1900 μg/ml, and concentrations in the highest range were measured from d 7. In the control glands, a significant increase in Lf concentration (to ~700 μg/ml)

was measured on d 1, 2, and 3, ~1900 μg/ml on d 7, and concentrations in the highest range from d 13 (Figure 4).

The concentration of IgG in both the experimental and control glands increased 30-fold, from ~120 μg/ml at the start of the experiment to ~3000 μg/ml at the end (Figure 4). In the experimental glands, a significant increase in IgG concentration was observed after 0.3 d; the IgG concentration had risen to ~2100 μg/ml on d 2 and 3, and to ~2500 μg/ml on d 7, and concentrations in the highest range were measured from d 13. In the control glands, a significant increase (to ~500 μg/ml) was measured on d 1 and 2, and from d 7, the concentrations were similar to those recorded in the experimental glands (Figure 4).

Macrominerals

The fluctuations in the concentrations of total phosphorus, total calcium, and total magnesium during the first 7 d of the experiment resembled those of casein (Figure 5). The r^2 values of the linear correlations between CN and calcium (0.724), CN and magnesium (0.688), and CN and phosphorus (0.633) were highly significant (n = 288; $P < 10^{-3}$ to 10^{-4}). As with CN, by d 7 of the experiment, the concentrations of these ions were significantly higher than those on d 0. However, by d 22, the phosphorus concentration had dropped markedly, and there was a clear falling trend in calcium and magnesium concentrations but without a change in CN concentration (Figure 5).

The total sulfur concentration in both the experimental and control glands increased almost threefold, from ~9 mmol at the start of the experiment to ~26 mmol at the end (Figure 5). The patterns of changes in sulfur concentration in the experimental and control glands closely resembled that described for the changes in whey proteins.

DISCUSSION

The difference between the responses of the experimental and control glands during the first 3 d of the experiment highlights two important points: First, it eliminates the possibility that the experimental glands responded nonspecifically to proinflammatory endotoxin. Endotoxins such as lipopolysaccharide may be present as contamination in the materials used and could induce some of the responses observed in the experimental glands (Olivier and Smith, 1983; Shuster and Harmon, 1991; Persson et al., 1992). Secondly, it shows that the antisecretory and inflammation-inducing properties of CN take effect only after hydrolysis of the mother molecules to peptides.
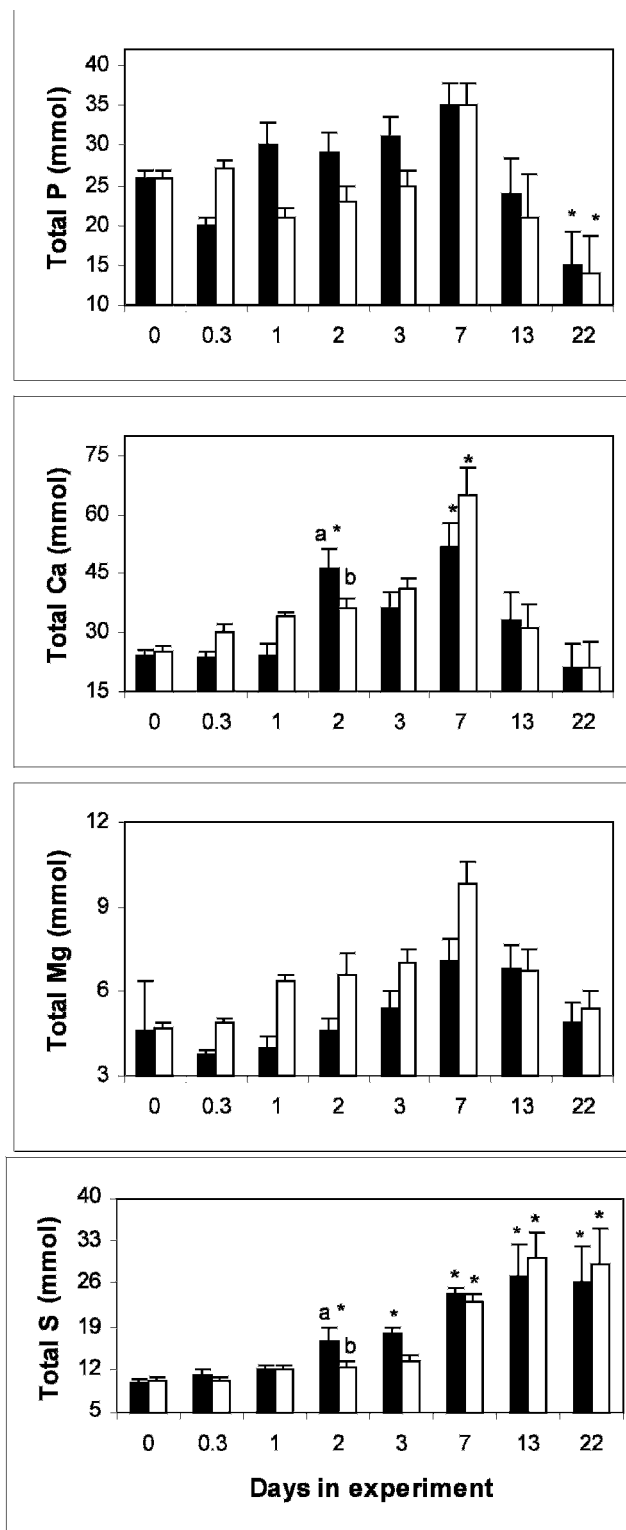

Figure 5. Effect of repeated dose of CNH and drying-off on total phosphorus, total calcium, and total magnesium and total sulfur concentrations in mammary secretion. *Values are significantly different from the pretreatment values ($P < 10^{-2}$). [a,b]Experimental values (filled bars) are significantly different from the controls (open bars; $P < 10^{-2}$).

The process of involution of the mammary gland comprises the extensive and highly ordered sequence of ultrastructural and compositional changes that occur during the transition period between lactating and non-lactating states (Olivier and Smith, 1983; Hurley, 1989; Capuco and Akers, 1999). One of the most basic features of mammary secretion is that the total osmotic pressure of the secretion remains approximately constant and equal to that of the blood (Mepham, 1987; Holt, 1993). As lactose is the main osmotic component in milk, the secretion volume follows the changes in the secretion of lactose very closely (Shamay et al., 2000). Accordingly, the secreted fluid volume declined precipitously between 3 and 7 d after dry-off, in keeping with previous findings (Hurley, 1989; Noble and Hurley, 1999). In the glands treated with CNH, the secretion rate was reduced dramatically after 1 d of treatment, and only a minimal amount of mammary secretion could be milked by d 3. These results are consistent with findings on the effect of CNH on goats (Shamay et al., 2002).

The increase in total protein concentration in the first weeks of involution most likely reflects the reduction in the volume of mammary secretion. However, the increases in the concentrations of IgG, Lf, and proteose peptones during the first weeks of involution reflect remodulation of mammary function, in keeping with previous results in cows (Olivier and Smith, 1983; Aslam and Hurley, 1997, 1998). Treatment with CNH induced similar changes in the pattern of protein secretion, but these changes were induced earlier, and are also found in goats (Shamay et al., 2002).

Unilateral cessation of milking in goats (Li et al., 1999) and teat sealing in mice (Li et al., 1997) induced involution in the treated gland only. This specificity suggests that mammary involution is triggered by local stimuli (Capuco and Ackers, 1999; Wilde et al., 1999). Thus, the findings that CNH induced involution only in the treated gland, both in cows (present experiment) and in goats (Shamay et al., 2002), suggest that it serves as a local stimulus.

Tight junction (**TJ**) in the epithelial cells of the mammary gland forms a barrier between the systemic (baso-lateral) and the milk (apical sides) and prevents para-cellular transport (Nguyen and Neville, 1998), but milk stasis causes the accumulation of local signals, which makes the TJ leaky (Nguyen and Neville, 1998). Because of the difference in the populations of ions in interstitial fluid and milk, when TJ are disrupted, the Na^+ concentration in the milk rises, whereas that of K^+ declines (Nguyen and Neville, 1998). The changes in Na^+ and K^+ concentrations in the mammary secretion are consistent with disruption of the TJ at or before the onset of involution. In the CNH-treated glands, the disruption of the TJ occurred within 8 h after the first treatment, in keeping with the previously reported response of goats to the same treatment (Shamay et al., 2002). It is not possible, on the basis of the sampling protocol, to determine the exact time when the TJ were compromised in the cows that were induced into involution at drying-off. However, as leaky TJ reduce milk yield (Allen, 1990; Stelwagen et al., 1994; Silanikove et al., 2000), it can be assumed that TJ leakiness had been established in those glands by d 3 after the cessation of milking, though the TJ had probably started to become leaky earlier (Stelwagen et al., 1994).

The mechanism by which disruption of TJ affects milk secretion has not been established (Nguyen and Neville, 1998). The stretching associated with milk stasis has been implicated in a mechanotransduction-signaling pathway, which, in turn, could alter both milk synthesis and TJ permeability (Nguyen and Neville, 1998). However, the continuation of udder emptying during the first 3 d of the experiment in cows (present experiment) and goats (Shamay et al., 2002) obviously prevented mechanical stress, indicating that decreased milk yield, in conjunction with disruption of TJ, can occur in the absence of such stress.

Silanikove et al. (2000) showed that injection of β-CN fraction 1-28 into the mammary gland in cows and goats down-regulated milk secretion in the treated gland through down-regulation of lactose secretion (Shamay et al., 2000). This finding is consistent with the hypothesis that leaky TJ reduces milk secretion in response to a reduction in lactose synthesis, which may be mediated by the cytoskeleton (Olivier and Smith, 1983; Noble and Hurley, 1999). Interestingly, the injected CNH contains β-CN fractions that are similar to fraction 1-28 (i.e., fractions 1-25 and 2-25; Adamson and Reynolds, 1977), and accelerates the formation of natural fractions of CN (present results, Shamay et al., 2002). It has been found in cows that β-CN fractions accumulate in the mammary gland following cessation of milking (Aslam and Hurley, 1998). Thus, we postulate that active components of β-CN may be responsible for the precipitous dry-off of mammary secretion following milk stasis.

Proteose-peptones (boiling-resistant peptides) in milk are composed mainly of plasmin-induced CN products (Andrews, 1983). Thus, the gradual increase in the concentration of proteose-peptones following milk stasis is consistent with the progressively increasing exposure of casein to plasmin and with the increase in plasmin activity. The notable increase in the activity of the PA-plasminogen-plasmin system following CNH treatment, as also found in goats (Shamay et al., 2002), suggests that casein hydrolysis is self-accelerated once a critical level of proteose-peptones accumulates in the gland. The changes in proteose-peptone concentration,

however, were not synchronized with the changes in plasmin activity in the CNH-treated gland, as indicated by the finding that the highest concentration of proteose-peptones was recorded when plasmin activity had already dropped. Unlike the situation in the experimental glands, we did not find the expected reduction in plasminogen activity in the control glands along with the increase in plasmin activity. Plasmin activity in mammary secretions is affected by many factors, such as tissue type and urokinase type of PA (Politis, 1996), PA inhibitors (Politis, 1996), plasmin inhibitors (Precetti et al., 1997), and insulin-like binding proteins, which may increase the availability of the casein substrate (Flint et al., 2001). Thus, the steep increase in proteose-peptones concentration that was observed in both treatments from d 3 after cessation of milking may be related to a decrease in plasmin inhibition and an increase in CN availability.

Roughly, two-thirds of the calcium and magnesium secreted in the milk of cows are bound to O-phospho-L-serine residues in the CN micelles (Mepham, 1987; Neville et. al., 1994), which explain the parallel fluctuations in the concentration of CN, phosphorus, calcium, and magnesium (present experiment; Shamay et al., 2000). The increase in the total sulfur concentration with advancing involution most likely reflects the increased proportion of whey proteins, such as albumin, which are richer in sulfated amino acids than CN.

CONCLUSIONS

Injections of CNH into the udder induce loss of TJ integrity, which is followed by rapid dry-off of mammary secretion and local inflammatory response. The fact that all the treated glands resumed normal lactation after parturition supports the conclusion that we imitated natural phenomena rather than inducing a necrotic response that would irreversibly damage the secretory function of the udder. In several aspects (induction of inflammation and changes in milk composition), CNH treatment resembles studies in which the mammary gland was challenged with endotoxin and colchicine (Olivier and Smith, 1983; Persson et al., 1992). However, these treatments cannot induce complete dry-off of mammary secretion. Furthermore, Shuster and Harmon (1991) had shown that the mammary gland is partially refractory to frequent intramammary endotoxin infusions. Thus, the antisecretory effects of CNH are probably independent of its proinflammatory effects.

We had already treated more than 100 cows with CNH, with 98% of the treated quarters being dried-off irrespective of cow's parity and stage of lactation (Shamay and Silanikove, 2002). This treatment ap-

pears to be a viable tool for the following indications: 1) to reduce the agony associated with drying-off cows yielding 30 l/d or more milk, 2) to dry the secretion in quarter with SCC so high that it negatively affects the whole tank milk quality, which allows to continue milking the three other quarters for the rest of the lactation cycle, and 3) to eradicate from infected glands several types of bacteria that cause subclinical and clinical mastitis.

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