

Original Contribution

Role of xanthine oxidase, lactoperoxidase, and NO in the innate immune system of mammary secretion during active involution in dairy cows: manipulation with casein hydrolyzates

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Abstract

The aims of this study were to test whether xanthine oxidase, lactoperoxidase, and NO are components of the innate immune system of mammary secretion during active involution in dairy cows, and whether the innate immune system is activated by casein hydrolysates. Our laboratory has shown recently that infusion of CNH into mammary glands induced involution and was associated with earlier increases in the concentrations of components of the innate immune system. Intact casein is inactive and served as control. Half of the glands of 8 Holstein cows scheduled for dry off (~60 days before parturition) were injected for 3 days with a single dose of casein hydrolyzates and the contralateral glands with a single dose of intact casein with the same concentration. Involution elicited marked increases in xanthine oxidase and lactoperoxidase activities, and accumulation of urate and nitrate. NO and H₂O₂ were constantly produced in the mammary gland secretion. Nitrite formed either by autooxidation of NO or by conversion of nitrate to nitrite by xanthine oxidase was converted into the powerful nitric dioxide radical by lactoperoxidase and H₂O₂ that is derived from the metabolism of xanthine oxidase. Nitric dioxide is most likely responsible for the formation of nitrosothiols on thiol-bearing groups, which allows an extended NO presence in mammary secretion. Nitrite is effectively converted to nitrate, which accumulated in the range of ~25 μM – 1 mM from the start of the experiment to the complete involution of glands. The mammary secretion in all glands was bactericidal and bacteriostatic during established involution, and this appeared sooner and more acutely in glands treated with casein hydrolyzates, within 8 to 24 h. It is concluded that xanthine oxidase, lactoperoxidase, and NO are components of the mammary innate immune system that form bactericidal and bacteriostatic activities in mammary secretions. The innate immune system play a major role in preventing intramammary infection during milk stasis and its activation may increase its effectiveness.

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Keywords: Mammary gland; Involution; XOR; LPO; H₂O₂; NO; NO₂[•]; Free radicals

Introduction

The selection of dairy cows for high milk yield during the last four decades has increased milk yields three- to fourfold in countries such as Israel and the United States, and the average annual yield now exceeds 10,000 L, in 300 days of lactation [1]. In the modern dairy industry, mastitis is the most debilitating disease, costing in the US dairy industry alone ~\$2 billion annually [2]. Mastitis in its subclinical and clinical forms is caused by intramammary infection (IMI)¹ with pathogens, mostly bacteria [2]. The proportion of IMI-infected animals in dairy cows ranges

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; CN, casein; CNH, casein hydrolyzate; DAN, 2,3-diaminonaphthalene; DMF, *N,N*-dimethylformamide; DPTA, diethylenetriaminepentaacetic dianhydride; DTNB, 5,5'-dithiobis-2-nitrobenzoate; IMI, intramammary infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LPO, lactoperoxidase; PBS, phosphate-buffered saline; TNB, 5-thio-2-nitrobenzoic acid; SDS, sodium dodecyl sulfate; XD, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxidoreductase.

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from 50 to 70% of the cows in most herds [2]. Sixty days before their expected parturition, dairy cows are dried by abrupt cessation of milking. The early stage of this dry period is associated with increased risk of acquiring IMIs [2]. Modern dairy cows are usually dried while still producing 20 to 40 L of milk per day, which exceeds 2 to 4 times the maximal yield of indigenous breeds of cows in the tropics. The first week of the dry period is associated with swelling of the udder to an extent that may cause conspicuous agony (the cows scream); it is frequently associated with leaking of mammary secretion, which substantially increases the risk of acquiring IMI [3].

Cessation of milk removal leads to rapid changes in the mammary secretion and initiation of the process of active mammary involution. This process comprises an extensive and highly ordered sequence of tissue and compositional changes that occur during the transition between lactating and nonlactating states [4,5]. In cows, involution is complete by 21 to 30 days after dry off, and during this period the mammary secretion becomes scant, watery, turbid (serum-like) and rich in leukocytes ($>1 \times 10^6/\text{ml}$) [4,6]. The compositional changes include a dramatic decrease in lactose and fat concentrations, and parallel increases in the concentrations of lactoferrin and immunoglobulins, which are part of the innate immune system [4,6]. It takes dairy cows considerably more time than rodents to reach involution [4].

Recently, we have shown that the process of active involution in goats and cows is triggered by the milk plasmin system that liberates active peptide from the N-terminal part of β -casein [6,7]. Plasmin in milk is found mostly in its inactive form, plasminogen, and the conversion of plasminogen to plasmin is modulated by plasminogen activators [8,9]. Following cessation of milking, plasmin activity in mammary secretion in dairy cows increased substantially within 13 days [6]. In mice, plasmin activity rose sharply immediately following the induction of dry off [10], which may explain the differences between the species in their rates of involution. In support of this hypothesis, we have shown that the infusion of casein hydrolyzates (CNH), which contains the active β -casein-derived peptide, dramatically accelerated the rate of involution in goats and cows, to the extent that it was completed within 3 days [6,7]. The infusion of CNH was associated with earlier increases in the concentrations of components of the innate immune system: lactoferrin (an antimicrobial protein) and immunoglobulin type G [6]. Therefore, it is possible that the increased risk of acquiring IMIs in modern dairy cows is associated with slow activation of the innate immune system.

Xanthine oxidoreductase (XOR) is a complex molybdo-flavoenzyme, which uses xanthine, hypoxanthine, and reduced nicotinamide adenine nucleotide as electron-donating substrates and catalyzes the last two steps in the formation of urate. The enzyme is synthesized as xanthine dehydrogenase (XD), but can be readily converted to xanthine oxidase (XO) by oxidation of sulfhydryl residues

or by proteolysis [11]. The milk of various mammals contains membrane-bound and free XO [12], which can generate weakly microbicidal superoxide and hydrogen peroxide [13]. Recent studies have shown that the microbicidal activity of XO in milk may also be related to the formation of NO from nitrite [14,15]; XO converts nitrate into nitrite, and so may increase substantially the substrate source for NO generation [16].

Lactoperoxidase (LPO), a heme-containing peroxidase, is commonly present in ruminant [17] and human [18] milk throughout lactation, and is likely to contribute to oxidative mechanisms in milk [19–21]. Nitrite reacts with mammalian peroxidase such as LPO to produce the potent radical, nitric dioxide ($\text{NO}_2^\cdot$) [22,23]. Thus, interaction between XO and LPO via NO and NO-derived species may serve as a potential mechanism for modulating their catalytic activities, influencing the regulation of local inflammatory responses and control infectious events *in vivo*.

The aims of the present study were to test the following hypotheses: (i) XO and LPO are part of the mammary gland innate immune system; (ii) reactive nitrogen species produced by XO and cycling between XO and LPO contribute to the innate defense against bacterial pathogens; and (iii) infusion of CNH into the mammary gland activates the innate system sooner and provides earlier protection against bacterial invasion.

Materials and methods

Preparation of experimental and control solutions

Casein hydrolyzate (experimental solution) and control solution (intact dissolved CN) were prepared from commercial CN, sterilized by passage through a 22- μm sterile filter, and kept frozen pending use [6].

Animals and their maintenance

Eight multiparous lactating pregnant Holstein cows that were scheduled for dry-off treatment (i.e., they were ~60 days before parturition) were used. The cows were fed throughout the trial with a typical Israeli total mixed ration that comprised 65% concentrates and 35% forage, and contained 17% protein. The cows were milked three times daily, at 0500, 1300, and 2000 and produced ~30 L of milk per day. The composition of the mammary secretion from each quarter was recorded for 3 days before the treatment.

Experimental procedures

A single dose of the CNH preparation containing 67.5 mg in 15 ml [6] 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 of each cow were treated

with the same volume and concentration of the control solution. This procedure was repeated after the afternoon milking, and similarly on the following two days (i.e., six postmilking doses over 3 days). At the time of the evening milking on those days, the cows were not taken to the milking parlor and were not milked or treated. The switch from thrice daily milking to twice daily milking may have reduced milk yield by 10%, but over such a short period, it is most unlikely to have affected milk composition [6]. After the last treatment, the cows were not milked throughout the dry period (~60 days), 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 days before the treatment, and on Days 4, 10, and 19 after the cessation of treatments.

Analytical methods

The pH in the mammary secretion samples was measured shortly after sampling (MeterLab pH/4201, Toledo, Spain), and then the samples were defatted and the skim milk samples were stored at -20°C for further analysis. Cl^{-} was measured with a Cl^{-} titrator (Chloride Titrator CMT 10, Radiometer, Copenhagen, Denmark).

The reagents used were purchased from Sigma Chemical Co. (Rehovot, Israel), unless otherwise mentioned. XO and XD activities were analyzed with pterin used as the substrate in a spectrofluorometer (Model 650–40; Hitachi, Tokyo, Japan), with excitation at 345 nm and emission at 390 nm [24]. The volume of the assay mixture was 1.0 ml in 50 mM sodium phosphate buffer, pH 7.6, which comprised 10 μM pterin and 10 μl of mammary secretion samples. Reactions proceeded for 10 to 60 min at 37°C . To distinguish between the XO and XD activities, the above reaction was carried out in the presence of 0.4 M NAD^{+} . To confirm the specificity of the activity, 50 μM allopurinol was added to the mixture and the reaction was carried out. The concentration of isoxanthopterin formed was calculated from the linear relationship between fluorescence intensity and standard concentration in the range of 0 to 10 nM.

LPO activity was assayed by the oxidation of ABTS [25]. The samples were diluted 1:10 and 10- μl aliquots were mixed with 200 μl of substrate mixture and read after 10 min in a Microplate reader (EL_x 800UV, Bio-Tek Instruments, VT). LPO diluted to produce activities in the range of 0.1 to 1 U/ml was run in parallel in the same volume to serve as standards.

Urate was analyzed with a commercial kit (Kit 686-A, Sigma Co., Rehovot, Israel) according to the manufacturer's instructions. Hydrogen peroxide was measured as described by Mottola et al. [26]. Peroxynitrite formation was measured by observing the conversion of dihydrorhodamine 123 to rhodamine 123 [27]. The reaction was carried out in a 3-ml glass cuvette, which contained 100 μM dihydrorhodamine 123 and 100 μM of the chelator DTPA in a total

volume of 1.4 ml of 100 mM potassium phosphate, pH 7.4. The reaction was initiated by adding various amounts of samples (10 to 400 μl) and monitoring the absorbance at 500 nm with a spectrophotometer (Ultraspec 2100 Pro, Amersham Pharmacia Biotech, UK). XO plus pterin was added to the reaction solution as a reference, according to Crow [27].

Superoxide ion formation was evaluated by measuring cytochrome *c* oxidation, essentially as used by Buescher et al. [28] with human colostrum.

Thiocyanate was determined according to Banks and Board [29], except that trichloroacetic acid was used at 20% instead of 60%, to precipitate protein.

Nitrate + nitrite concentration was measured by the Griess reagent as described by Bouchard et al. [30], with the following modification: instead of using 0.1 M sodium acetate to precipitate casein from skim milk, the samples were run in an ultracentrifuge (at 100,000g for 30 min), thus avoiding the need to neutralize the samples. Nitrite and nitrosothiols were measured by the fluorometric assay with DAN reagent as described by Sonoda et al. [31] after precipitating the casein with an ultracentrifuge. Nitrate concentration was calculated by subtraction of the nitrite concentration from the nitrate + nitrite concentration.

The discoloration of β -carotene was used to determine the oxidation reaction that results from the formation of free radicals in milk. The reaction took place in a cuvette that contained 14 μM β -carotene and 0.05% Tween 20, in 1.7 ml of 0.1 M sodium acetate buffer [32]. The reaction was started by adding a 0.3-ml sample. The decrease in absorbance at 460 nm as measured during the first 30 s was used to calculate the rate of β -carotene bleaching.

The conversion of DTNB into TNB was used as a measure of the formation of potent radicals, such as nitric dioxide [22]. TNB was prepared by reduction of 1 mM DTNB in 100 ml of 50 mM sodium phosphate buffer (pH 7.4) with 4 μl of 2-mercaptoethanol [22]. The test sample was placed in a cuvette that contained 40 μM TNB in 1.8 ml of 50 mM sodium phosphate buffer, pH 7.4. The reaction was started by adding a 0.2-ml sample. The decrease in absorbance at 412 nm as measured during the first 10 s was used to calculate the rate of oxidation of TNB to DTNB [22].

Antimicrobial assays

Bacterial inhibition (antibiotic-like activity) test

Bacillus termophilus and *Bacillus subtilis* inhibition were determined as described by Bennet et al. [33]. In brief, 8-mm-diameter wells were cut in agar that contained one of these bacteria, and were charged with 0.1 ml of mammary secretion samples and incubated overnight at 37°C . The diameter of the inhibition zones, corrected for well diameter, was used as indices for bacterial inhibition.

Bacterial killing test for deriving bactericidal and bacteriostatic activities

Bacterial killing was determined by using a colorimetric MTT method, according to Stevens et al. [34]. Briefly, bovine peripheral blood was collected by venipuncture into tubes (Becton Dickinson Vacutainer System, Plymouth, UK) and was submitted to hematological analysis. Plasma was removed and washed twice with PBS; the erythrocytes were lysed with 0.83% NH_4Cl in 0.17 M Tris buffer, washed twice with PBS, and resuspended in Eagle + FCS; the leukocytes were counted and resuspended at 2×10^6 neutrophils/ml. A 50- μl aliquot of the cell suspension was mixed with 50 μl of a suspension of one of the opsonized strains of *S. aureus* (AU3133) or of *E. coli* (P4) containing 2×10^7 cells/ml and 50- μl of skin milk, and poured into flat-bottomed 96-well plates (Nunc, Roskilde, Denmark) in triplicate or quadruplicate. The plates were incubated at 37°C for 30 min or 180 min. Then, the leukocytes were lysed with 50 μl /well of saponin (20 mg/ml; Sigma, St. Louis, MO) for 10 min at room temperature, and 50 μl of MTT-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) at a concentration of 2 mg/ml was added to each well. The formazan was dissolved with the addition of 50 μl of 50% *N,N*-dimethylformamide (DMF) (Riedel-deHaen, Sigma-Aldrich Laborchemikalien, Seelze, Germany) in 20% sodium dodecyl sulfate (SDS) (Sigma). The ability of live *S. aureus* or *E. coli* to reduce MTT to purple formazan was quantified by measuring the optical density (OD) at 570 nm with an ELISA reader (Sunrise, TECAN, Austria). The killing percentage was determined by comparison with a standard formazan curve, derived by adding MTT to known concentrations of bacteria-10, 40, 70, and 100% -derived from previously prepared bacterial suspensions (after opsonization). The standard killing curve was established by linear regression analysis. The percentage of bacteria killed was quantified according to the formula,

$$\{1 - [(OD \text{ sample}) - (OD \text{ leukocytes only}) - a]/b\} \times 100,$$

where *a* is the intercept of the standard curve and *b* is the slope. The bactericidal effect was defined as the number of bacteria that remained after incubation in mammary secretion samples for 30 min, and the bacteriostatic effect as the number of bacteria that remained after incubation for 180 min.

Statistical analysis

The datasets of this study were analyzed by using repeated-measures analysis to model correlated residuals within cow as described previously [6]. The analysis concentrated on the effects of treatment, day, and treatment $\times$ day interactions. The effects of parity and of days in milk were not significant ($P > 0.25$) and therefore were not included in the analyses presented here.

Results

pH and Cl^- concentration

In a previous study we showed that the disruption of mammary gland tight junction in cows by CNH treatment was followed immediately by dramatic changes in the concentrations of Na^+ and K^+ , whereas in the control glands such changes occurred 3 days after cessation of milk removal from the gland [6]. Continuing that investigation in the present study, we found that CNH treatment caused a sharp increase of the milk pH (from 6.65 to 7.12) within 8 h, with values of ~ 7.15 persisting for the rest of the experiment (Fig. 1a). In the control glands, the mammary secretion pH increased to ~ 6.9 , 3 days after cessation of milk removal (Day 7), and at Days 13 and 22 it reached the same levels as those in the experimental glands (Fig. 1a). CNH treatment also caused a rapid increase in Cl^- concentration (from 38 to 53 mM) within 8 h, after which the concentration continued to increase until it stabilized at ~ 90 mM (Fig. 1b). In the control glands, the Cl^- concentration in mammary secretion rose to the levels recorded in the treated gland at Day 7 (Fig. 1b). The changes in mammary secretion pH and Cl^- concentration most likely reflected an influx of bicarbonate and Cl^- from systemic fluids, since their concentrations in blood and interstitial fluid are much higher than those in milk (see [6] for detailed discussion).

XO activity

The XO activity in both the treated and the control glands was ~ 20 mU/ml (1 mU = 1 nM urate or isoxanthopterin/min) at the start of the experiment (Fig. 2a), which is within the range of XO activity of 49 ± 22 mU/ml found in Swedish breeds [35]. The lower activity in the Israeli cows is probably a dilution effect associated with their higher milk yield. XO activity rose to ~ 35 units/ml after the first treatment (8 h), though the response was significant only in the treated glands because of higher variability in the control glands (Fig. 2a). By Day 1, XO activity in the treated glands had increased to the maximal level (between 70 and 85 units/ml), and this was maintained throughout the experiment. In the control glands, a significant increase in XO activity (to ~ 60 units/ml) was recorded on Day 13 (i.e., 10 day after the cessation of milking), and at Day 22 the activity was similar to that in the treated glands (Fig. 2a).

Uric acid concentration was ~ 35 μM in all glands at the start of the experiment (Fig. 2b), which is consistent with typical values in cow's milk reported previously [36]. Uric acid concentration in the treated glands rose to ~ 65 μM on Days 2 and 3, reached a peak of ~ 100 μM on Days 3 and 7, and then stabilized at ~ 70 μM on Days 13 and 22. In the control glands, significant increases in uric acid concentration were recorded on Days 13 and 22, and

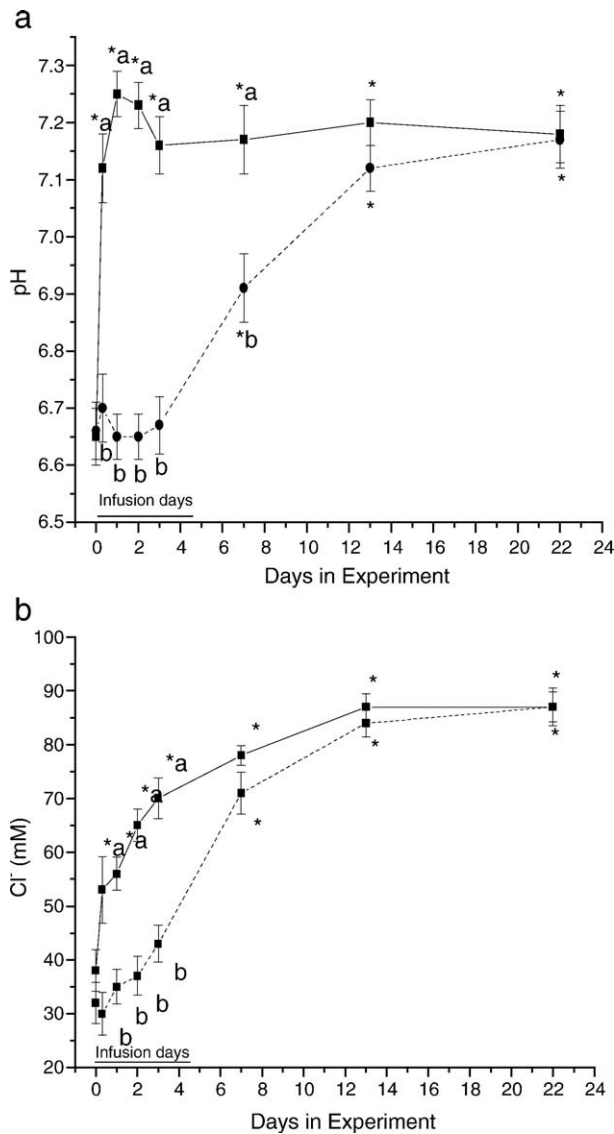


Fig. 1. Effects of repeated dose of CNH and drying off on pH (a) and Cl^- concentration (b) in mammary secretion. *Values (means $\pm$ SD) are significantly different from the pretreatment values ($P < 10^{-2}$ to 10^{-4}). ^{a,b}CNH values (solid line) are significantly different from the controls (dashed line; $P < 0.01$).

the concentration was similar to that in the treated glands (Fig. 2b).

XO also reduces oxygen to superoxide and hydrogen peroxide [11], but no formation of superoxide or peroxynitrite could be detected, throughout the experiment, in samples taken from the treated and control glands.

On the other hand, hydrogen peroxide was apparently generated and this was reflected in the records of H_2O_2 in milk. The hydrogen peroxide concentration was ~ 0.28 mM in all glands at the start of the experiment (Fig. 2c). It declined to 0.22 mM on Day 1, declined further to ~ 0.1 mM (experimental) and ~ 0.15 mM (control) on Day 2, reached a nadir of ~ 0.07 mM (treated) and ~ 0.13 mM (control) on Day 3, and then stabilized at ~ 0.15 mM in both treated and controls glands (Fig. 2c).

LPO activity

At the start of the experiment LPO activity in all glands was ~ 4 units/ml (Fig. 3a), which is within the range reported previously for dairy cows [37]. In the treated glands, LPO activity started to rise at the first sampling (8 h), became significant (~ 20 units/ml) on Day 1, reached a peak (~ 60 units/ml) on Day 2, stabilized at ~ 50 units/ml on Days 3 and 7, and then declined to ~ 30 units/ml on Day 13 and ~ 20 units/ml on Day 22. In the control glands, the first significant

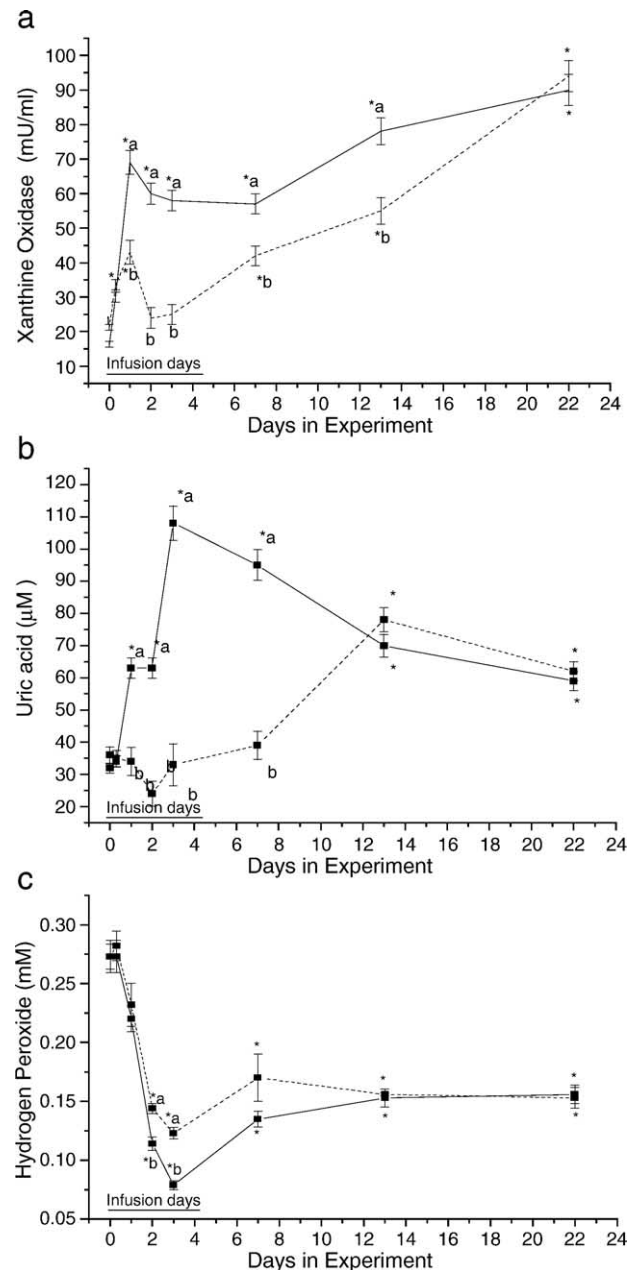


Fig. 2. Effects of repeated dose of CNH and drying off on XO activity (a) and on urate (b) and hydrogen peroxide (c) concentrations in mammary secretion. *Values (means $\pm$ SD) are significantly different from the pretreatment values ($P < 10^{-2}$ to 10^{-4}). ^{a,b}CNH values (solid line) are significantly different from the controls (dashed line; $P < 0.01$).

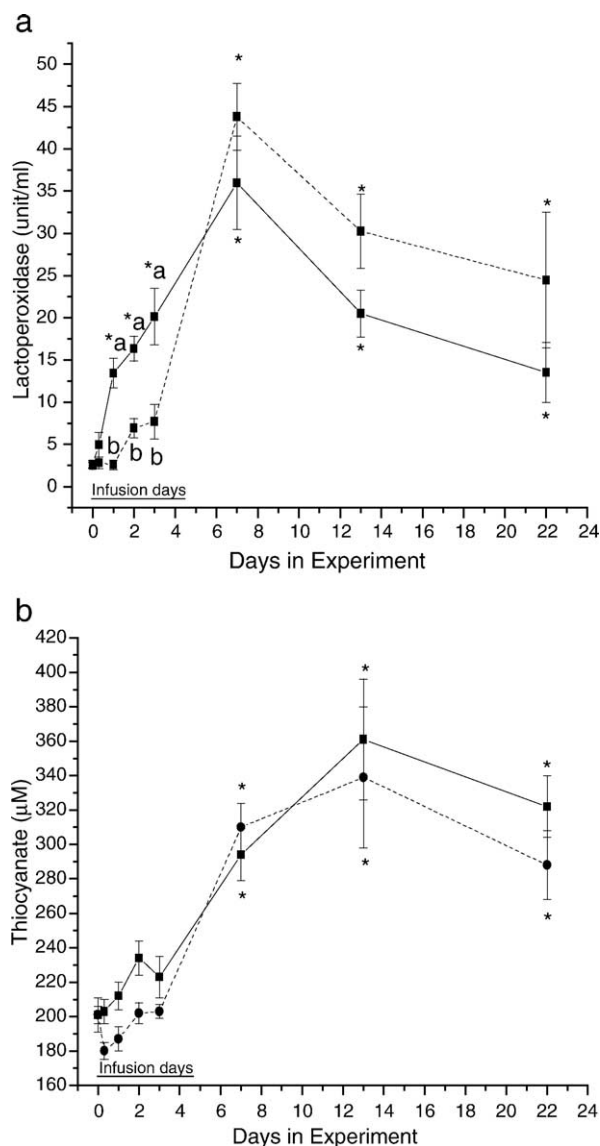


Fig. 3. Effects of repeated dose of CNH and drying off on LPO activity (a) and thiocyanate concentration (b) in mammary secretion. *Values (means $\pm$ SD) are significantly different from the pretreatment values ($P < 10^{-2}$ to 10^{-4}). ^{a,b}CNH values (solid line) are significantly different from the controls (dashed line; $P < 0.01$).

increase of LPO activity (~10 units/ml) was observed on Day 2; it then increased further to ~30 units/ml on Day 3 and stabilized at ~40 units/ml on Days 7, 13 and 22 (Fig. 3a).

Thiocyanate is considered to be the physiological substrate of LPO [38]. The concentration of thiocyanate of ~60 μmol at the start of the experiment (Fig. 3b) was consistent with previous reports [37]. However, as the LPO activity increased in CNH-treated cows or as the involution progressed, there was no associated reduction in thiocyanate concentration, indicating that thiocyanate was not consumed by the LPO activity. In fact, the thiocyanate concentration increased as involution progressed (Fig. 3b), and the increase was proportional to the increase in casein concentration (see Fig. 5 in [6]).

Nitrites nitrosothiols and nitrates

The initial nitrite concentration was ~400 nM (Fig. 4a). After CNH treatment, the nitrite concentration increased to ~2 μM within 8 h, and its concentration remained within the range 8 to 10 μM during the infusion days. After the infusion days the nitrite concentration stabilized at ~2 μM for the rest of the experiment. In the control glands, the nitrite concentration did not change during the infusion days, and after cessation of milking, its concentration was similar to that recorded in the treated glands (Fig. 4a).

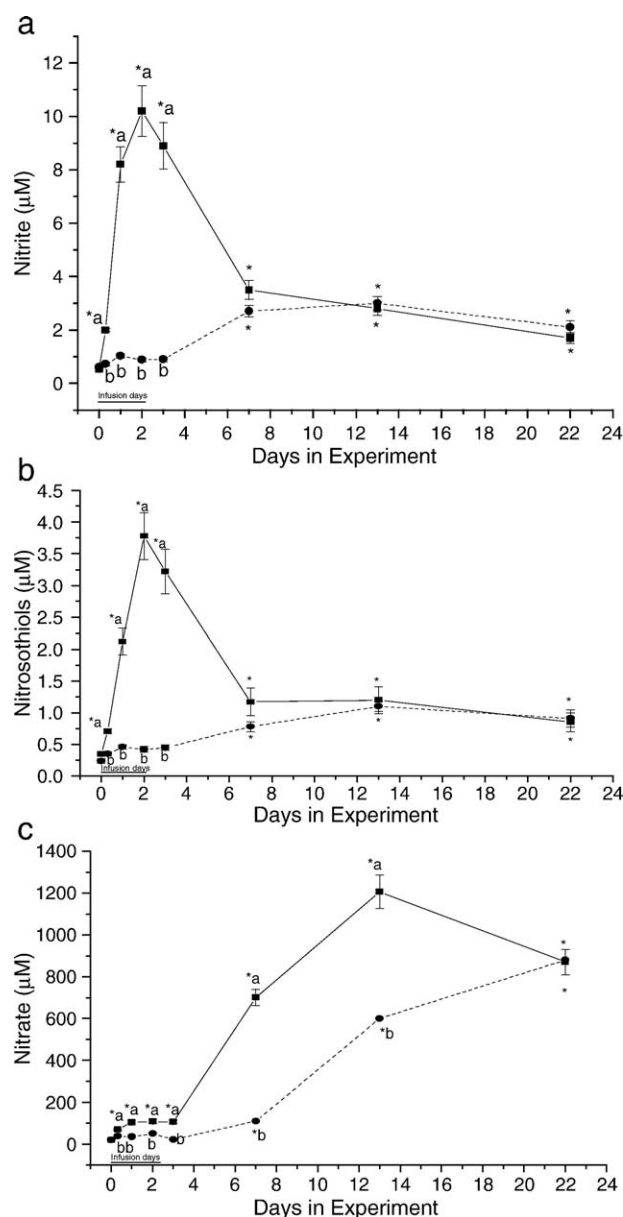


Fig. 4. Effects of repeated dose of CNH and drying off on nitrite (a), nitrosothiols (b), and nitrate (c) concentrations in mammary secretion. *Values (means $\pm$ SD) are significantly different from the pretreatment values ($P < 10^{-2}$ to 10^{-4}). ^{a,b}CNH values (solid line) are significantly different from the controls (dashed line; $P < 0.01$).

The initial nitrosothiols concentration was ~ 300 nM (Fig. 4b); it increased to ~ 700 nM within 8 h after the CNH treatment, and remained within the range of 2.2 to $3.8 \mu\text{M}$ during the infusion days. After the infusion days, the nitrosothiols concentration stabilized at $\sim 1 \mu\text{M}$ for the rest of the experiment. In the control glands, the nitrosothiols concentration did not change during the infusion days, and after cessation of milking it was similar to that recorded in the treated glands (Fig. 4b).

The nitrate concentration at the start of the experiment was $\sim 20 \mu\text{M}$ (Fig. 4c); it increased to $\sim 70 \mu\text{M}$ within 8 h after CNH treatment and remained at $\sim 100 \mu\text{M}$ during the infusion days. After the infusions days, the nitrate concentration continued to rise to $\sim 700 \mu\text{M}$ on Day 7, reached a peak of $1200 \mu\text{M}$ on Day 13, and fell back to $\sim 900 \mu\text{M}$ on Day 22 (Fig. 4c). In the control glands, the nitrate concentration did not change during the infusion days, and after cessation of milking it rose to $\sim 100 \mu\text{M}$ on Day 7, $\sim 600 \mu\text{M}$ on Day 13, and $\sim 900 \mu\text{M}$ on Day 22 (Fig. 4c).

β -Carotene bleaching and TNB oxidation

β -Carotene that was added to pretreated milk was oxidized (bleached) at a rate of $\sim 15 \mu\text{M/s}$ (Fig. 5a), whereas TNB was oxidized at rate of $\sim 5 \mu\text{M/s}$ (Fig. 5b). After CNH treatment, the β -carotene and TNB oxidation rates increased significantly within 8 h. During the infusion days, β -carotene bleaching increased threefold to sevenfold and TNB oxidation twofold to fivefold. β -Carotene and TNB oxidation rates increased in the control glands also, but the increase was much lower (Figs. 5a and b). From Day 7 onward β -carotene bleaching resumed at the pretreatment level, whereas TNB oxidation was even lower than the initial levels.

Antibiotic-like activity

During the infusion days, antibiotic-like activity against *Bacillus subtilis* and *Bacillus termophilus* was found in the CNH-treated glands, whereas no activity was recorded in the control glands. From Day 7 onward, antibiotic-like activity against these bacteria was recorded in all glands (Fig. 6).

Bactericidal and bacteriostatic activity

During the infusion days, bactericidal activity against *S. aureus* and *E. coli* was found in the CNH-treated glands treated with CNH, whereas no such activity was recorded in the control glands. From Day 7 onward, bactericidal activity against these bacteria was recorded in all glands (Fig. 7).

During the infusion days, bacteriostatic activity against *S. aureus* was found in the CNH-treated glands, whereas no such activity was recorded in the control glands. From Day 7 onward, bacteriostatic activity against these bacteria was reflected in the killing of all or almost all the bacteria in all

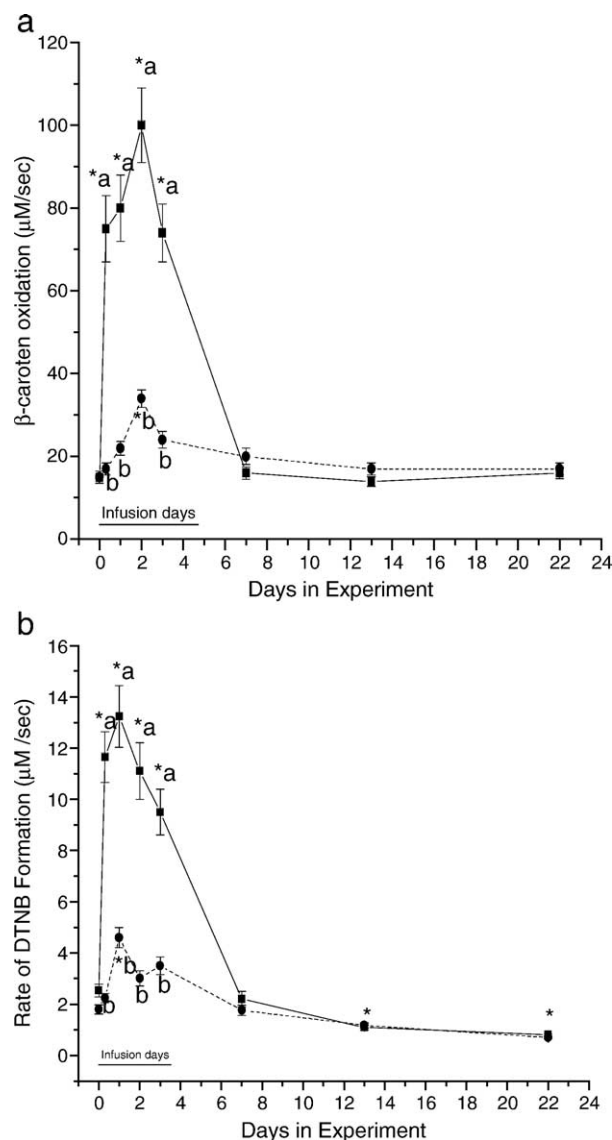


Fig. 5. Effects of repeated dose of CNH and drying off on β -carotene oxidation (a) and DTNB formation (b) in mammary secretion. *Values (means $\pm$ SD) are significantly different from the pretreatment values ($P < 10^{-2}$ to 10^{-4}). ^{a,b}CNH values (solid line) are significantly different from the controls (dashed line; $P < 0.01$).

glands (Fig. 8). The milk of late-lactating cows was naturally bacteriostatic against *E. coli*. This bacteriostatic effect of mammary secretions was augmented in the CNH-treated glands during the infusion days. From Day 7 onward, very few bacteria could survive the bacteriostatic effect of mammary secretion in all glands (Fig. 8).

Discussion

Role of XO

Irrespective of the treatment and time of sampling the pterin-derived isoxanthopterin production rate was allopurinol dependent, indicating that it reflected XOR activity

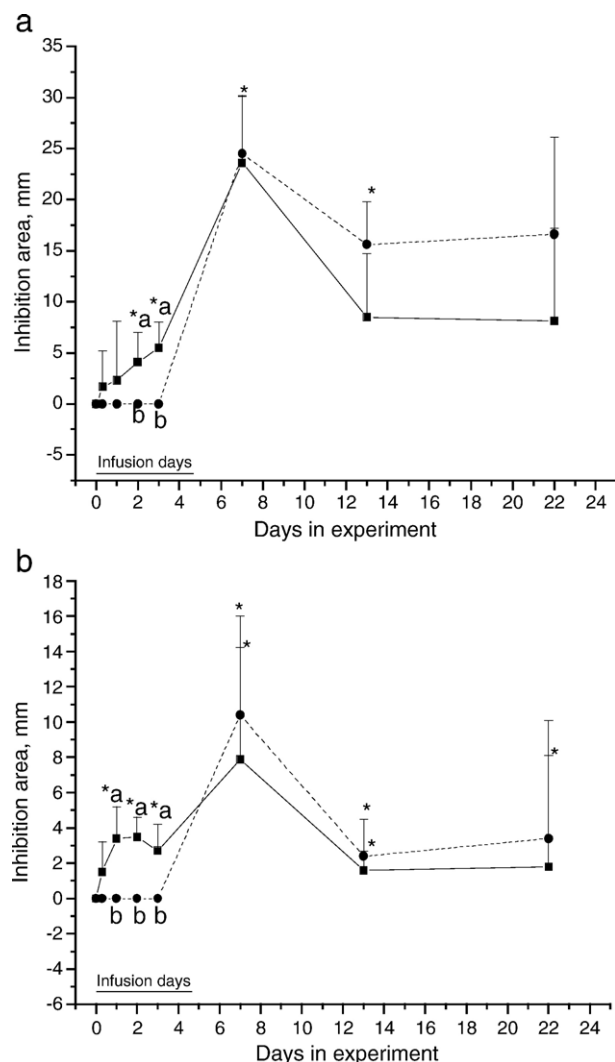


Fig. 6. Effects of repeated dose of CNH and drying off on antibiotic-like activity against *Bacillus subtilis* (a) and *Bacillus termophilus* (b) in mammary secretion. *Values (means $\pm$ SD) are significantly different from the pretreatment values ($P < 10^{-2}$ to 10^{-4}). ^{a,b}Experimental values (solid line) are significantly different from the controls (dashed line; $P < 0.01$).

[11]. The lack of response to the addition of NAD^+ in all the samples, irrespective of treatment and time of sampling, indicated that XOR was present in the mammary secretion as XO [35]. In cells, most XOR is in the XD forms, whereas in the systemic circulation most of it is in the oxidase form, as a result of XD to XO conversion by serum proteases [11]. As milk is rich in proteases, it can be considered that the dominance of the XO in milk is related to protease-induced conversion of XD to XO.

XO is known as the terminal enzyme of purine catabolism; it oxidizes hypoxanthine to xanthine, and xanthine to uric acid [11]. An increase in XO activity is expected to be associated with an increase in uric acid concentration, especially in involuting animals, because involution is associated with an increase in the rate of apoptosis [4], and therefore in the degradation of purine and pyrimidines. Thus, the increase in XO activity explains the

up to threefold increase in urate concentration. The basal level of urate in milk is similar to the typical level of urate in plasma in various mammals. As the tight junction of the mammary alveolus, which prevents intercellular free movement of solutes, is disrupted during involution [6], the maintenance of a positive gradient between gland lumen and blood plasma is indicative of continuous production of urate in the gland.

At the FAD active site, XO also reduces oxygen to superoxide and hydrogen peroxide [11]. The apparent lack of superoxide formation in the milk of late-lactating cows may relate to the rapid conversion of superoxide to hydrogen peroxide by SOD, which is an abundant enzyme in bovine milk [35]. In order to dismutate 100% of the superoxide anions formed by basal XO activity (20 mU/ml), SOD activity in the mammary secretion should be 2.9 U/ml [35], a value within the normal range of SOD activity (0 to 5 U/ml) in bovine milk. From this consideration it is also

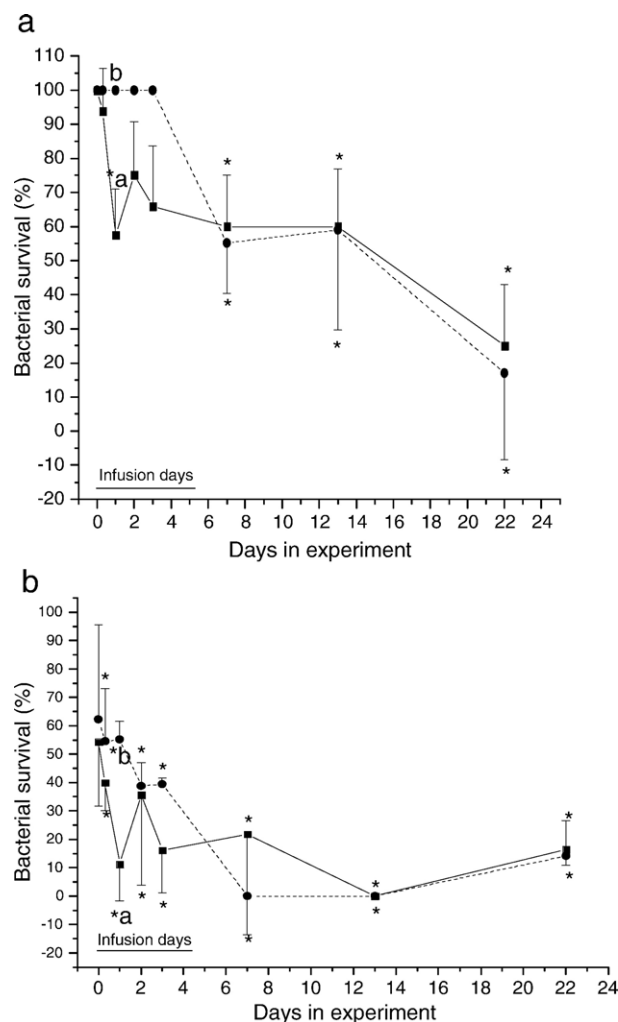


Fig. 7. Effects of repeated dose of CNH and drying off on bactericidal activity against *S. aureus* (a) and *E. coli* (b) in mammary secretion. *Values (means $\pm$ SD) are significantly different from the pretreatment values ($P < 0.05$). ^{a,b}Experimental values (solid line) are significantly different from the controls (dashed line; $P < 0.05$).

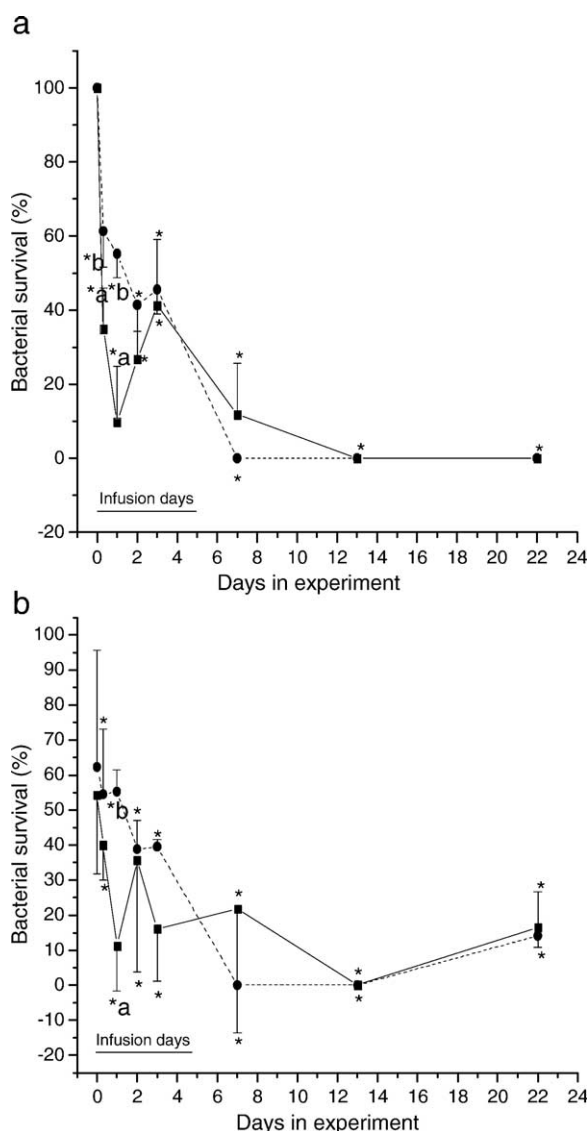


Fig. 8. Effects of repeated dose of CNH and drying off on bacteriostatic activity against *S. aureus* (a) and *E. coli* (b) in mammary secretion. *Values (means $\pm$ SD) are significantly different from the pretreatment values ($P < 0.05$). ^{a,b}Experimental values (solid line) are significantly different from the controls (dashed line; $P < 0.05$).

expected that as the involution progresses, the SOD activity in the mammary secretion could rise as high as 12 U/ml, to account for the lack of superoxide ion formation when XO activity reached 85 mU/ml. The apparent lack of superoxide formation also explains the apparent lack of peroxynitrite formation because the latter would require interaction between nitric oxide and superoxide in close proximity [39].

Under given conditions, urate and superoxide plus hydrogen peroxide formation by XO are stoichiometrically related to each other [40]. Assuming that superoxide was converted into hydrogen peroxide with a ratio of 2:1 between hydrogen peroxide and urate formation, ~ 4 mM/h of hydrogen peroxide would be formed in the milk of late-lactating cows, and ~ 17 mM/h in the mammary secretion of involuted glands. Nevertheless, the concentrations of these

two products of XO were inversely related. The explanation of this paradox is that hydrogen peroxide was consumed by other enzymes (see discussion below).

Our results show that XO is activated during active involution in cows, and they are consistent with overwhelming information that the plasma XOR activity and urate concentration increased under various inflammatory responses [11,41], and in particular with the observation that XOR in human mammary epithelial cells is activated in response to inflammatory cytokines [42].

XO also catalyzes the formation of NO from nitrite and the conversion of nitrate to nitrite; both reactions occur at the molybdenum site [11,16]. These reactions appear to be important in explaining the fate of NO-derived substances in the mammary gland during active involution (see Fig. 9 and related discussion).

XOR has been shown to be an essential protein in milk fat droplet secretion from the lactating mammary gland [43]. Our results have shown that XO plays an important role as part of the innate immune system in the mammary gland, and that this is consistent with the general view regarding the biological role of XOR in innate immunity [41]. The important functions of XO in mammary secretion are the formation of hydrogen peroxide, nitrite, and NO, which contribute to oxidative-antimicrobial activity [41], and of urate which is very effective in preventing oxidative damage by reactive nitrogen species in milk [19].

Role of LPO

A significant inverse linear interrelationship was found between LPO activity and H_2O_2 concentrations ($n = 288$; $r^2 = 0.62$; $P < 10^{-3}$). Thus, LPO is probably a major consumer of the H_2O_2 that is constantly produced in mammary secretions. LPO may function similarly in other biological systems; for instance, in normal human tracheal secretions LPO scavenges 80% of the H_2O_2 produced [44]. Catalase is an additional ubiquitous H_2O_2 -consuming enzyme in milk [45]; therefore, catalase activity may account for the remaining variability in H_2O_2 concentration.

Thiocyanate, considered to be the physiological substrate of LPO, is oxidized by the enzyme to hypothiocyanite, with H_2O_2 serving as the electron acceptor [46]. However, despite the increase in LPO activity and H_2O_2 formation in the present study no reduction in thiocyanate concentration was noted, indicating that it did not serve as the substrate for LPO. This is consistent with other workers' findings that the LPO/SCN/ H_2O_2 system in fresh raw milk is inactive [47], and that its activation requires the addition of exogenous thiocyanate and H_2O_2 to levels exceeding their physiological concentration in milk [46]. Several reports have shown that LPO cannot oxidize Cl^- to hypochlorite as myeloperoxidase does [22,48]. Thus, the large increase in Cl^- concentration in mammary secretion after the induction of involution probably does not contribute much to the formation of oxidized species.

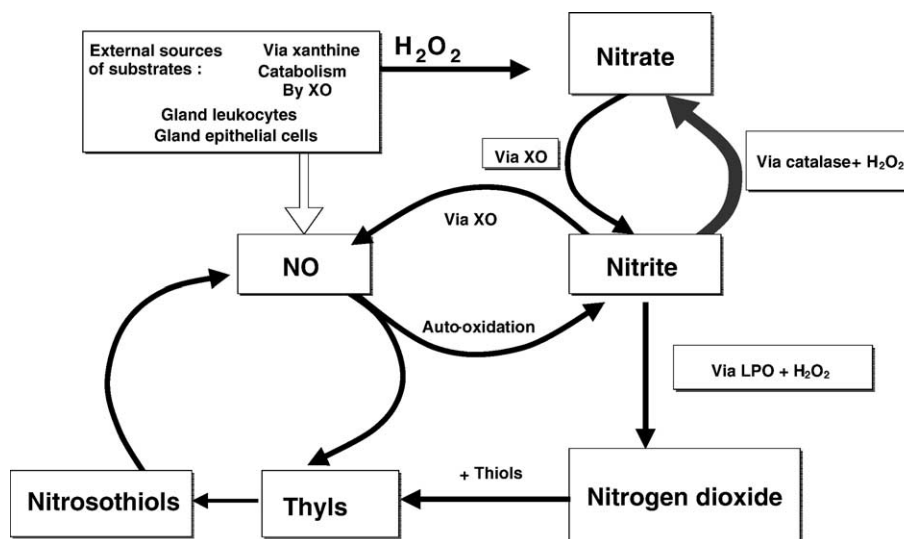


Fig. 9. Scenario of NO metabolism and cycling in mammary secretion: The major enzymes that are involved are XO, LPO, and catalase. The main substrates for this system are xanthine, a product of purine catabolism, and NO. Purines are derived from epithelial cells that are constantly sloughed into mammary secretion, and NO is constantly produced by epithelial cells and milk leukocytes. The input of these substrates as well as the activities of XO and LPO increased substantially under the inflammatory conditions characteristic of active involution. The conversion of xanthine to urate provides electrons to convert water and oxygen into superoxide ion and H_2O_2 by XO. Superoxide dismutase most likely plays an important role in converting superoxide into H_2O_2 , which is essential for the activities of LPO and catalase. The toxicity of radicals is harnessed for host defense at a cost to the host. The cost to the host in this case is minimized by formation of urate, which can effectively control NO_2 and by the effective conversion of nitrite into the more stable metabolite, nitrate. See text for more detailed explanations.

Recently, it was shown that nitrite at the physiological or pathological level catalyzes myeloperoxidase and LPO to form NO_2^+ , which may provide an additional pathway that contributes to cytotoxicity or host defense, associated with increased NO production [22,23]. At physiological concentrations, NO_2^+ oxidizes TNB chromophore into DTNB [22]. The concentration of H_2O_2 that is required to oxidize TNB-0.4M-far exceeds its physiological concentration in mammary secretions. Additional agents that could oxidize DTNB in physiological concentrations are peroxynitrite, hypothiocyanate, and hypochlorite [22], but, as discussed above, these are not likely to be formed in mammary secretion. Thus, the formation of DTNB after addition of TNB to milk may be regarded as evidence for the continuous formation of NO_2^+ . Also supporting this notion was the observation that the addition of 50 mM curcumin prevented TNB oxidation, since it has been shown that curcumin sequesters NO_2^+ but not NO, which indicates that curcumin could be useful for distinguishing between the actions of NO_2^+ and those of NO in various biological systems [49].

NO metabolism

Nitrite, nitrate, and nitrosothiols are well known to be indicators of NO metabolism. Thus, their accumulation in the mammary secretion of cows induced into involution indicates that NO production was accelerated. As the peak concentration of nitrite and nitrate exceeded the typical values expected to be found in the systemic fluid, and since nitrosothiols must be formed in situ, because of the limitation to NO diffusion, it is quite clear that the source

of NO and NO-derived species is the mammary gland. Mammary epithelial cells and various types of milk leukocytes can produce NO upon stimulation with lipopolysaccharide and proinflammatory cytokines such as IL-1 and IFN- γ [50,51]. Thus, the increase in the concentration of NO-derived species that followed the induction of involution is consistent with the inflammatory response, which is part of the involution process [4,6].

The concentrations of nitrite, nitrosothiols, and nitrate found in mammary secretion during active involution in the present study resemble the concentrations found in the plasma and synovial fluid of humans with pathological conditions such as rheumatoid arthritis, osteoarthritis, and chronic need for hemodialysis (see [52] for review). Nitrate concentration in blood or tissues is highly variable with 20 to 100 μM levels observed under normal physiological conditions, 400–500 μM under pathological conditions, and low millimolar concentrations in induced sputum of patients with asthma, or in patients treated with NO-donating vasodilator drugs [52]. Thus, the low millimolar concentration of nitrate found in the mammary secretion of involuted cows appears to be the highest concentration of this metabolite found in biological fluid under normal physiological conditions.

The concentrations of nitrite (mean 44 μM ; range 19 to 80 μM) and nitrate (mean 180 μM ; range 87 to 278 μM) in human milk during the first 3 to 5 days of lactation are quite high, which is apparently related to the provision of innate immune defense to breastfed neonates, by liberating NO in their stomach [53]. Nitrate concentrations during early lactation are much higher than that in mature human milk

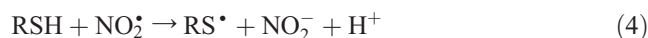
(45 $\mu\text{M} \pm 40$) [54], which suggests that during colostrum secretion in humans NO formation is activated or the conversion of nitrite to nitrate is suppressed (Fig. 9).

The detection of low levels of volatile *N*-nitroso compounds in human ($\sim 1.1 \mu\text{g/L}$) [54] and bovine ($\sim 0.1 \mu\text{g/kg}$) [55] milk suggests that endogenous *N*-nitrosamines are constantly formed by NO-derived species. We are not aware of any previous report on nitrosothiols concentration in mammalian milk; therefore, the present results are fundamental in this respect. The much higher concentration of nitrosothiols than of nitrosamines in cows' mammary secretion suggests that thiol groups serve as NO sinks, and so prevent the accumulation of more dangerous volatile *N*-nitroso compounds.

In vitro, the following reactions of $\text{O}_2^\bullet$ with NO nitrosate (RSNO) thiols (RSH) on proteins or peptides, via the formation of dinitrogen trioxide (N_2O_3) as the nitrosating agent [56,57], are



However, it is unlikely that nitrosothiols are formed in this way in vivo because of the low concentration and short life of NO, and because the rate of reaction (2) is too low [56]. In addition, typical O_2 concentrations, which are in the range of 1 to 50 μM in most tissues, will greatly limit the efficiency of this reaction [57]. Two explanations of the in vivo formation of nitrosothiols have been proposed: (1) N_2O_3 formation is significantly accelerated in a hydrophobic pocket of albumin, which enables the transfer of NO to thiols [56]; and (2) oxidation of thiol by $\text{NO}_2^\bullet$ forms thiyl radicals ($\text{RS}^\bullet$), which subsequently react with NO to form nitrosothiols [57].



As the rate of $\text{NO}_2^\bullet$ formation is second order with regard to NO, $\text{NO}^\bullet/\text{O}_2^-$ -mediated oxidation and nitrosation reactions are hampered by the availability of NO. In the present experiment, we found significant correlation between $\text{NO}_2^\bullet$ formation (TNB oxidation) and nitrosothiols concentration ($n = 288$; $r^2 = 0.66$; $P < 10^{-3}$). This positive interrelationship favors the mechanism described by reactions (4) and (5) as the most probable formation route of nitrosothiols in milk.

The association between thiols and NO is relatively weak; therefore, the slow dissociation constant of NO from nitrosothiols can exert a long-acting effect on NO [58,59], which is probably the main reason for the accumulation of NO-derived species in the mammary gland.

As in blood also in milk, NO-derived species which did not form adducts with protein or lipids were accumulated

mainly in the form of nitrate, which is much more stable than nitrite [52]. In blood, residual amounts of NO react with water to form nitrite which, in the presence of heme groups in proteins such as myoglobin or hemoglobin, or enzymes, rapidly oxidizes to nitrate and the corresponding met-heme protein [52]. For example, when NO diffuses into the red cell, it reacts rapidly with oxyhemoglobin to form methemoglobin and nitrite. In the presence of oxygen and methemoglobin reductase, these are rapidly converted back to hemoglobin and to nitrate. The equivalent mechanism in milk remains to be elucidated, but it is clear that it should be several orders or magnitude more active than the conversion of nitrate to nitrite by XO. The most promising candidate is catalase, which, in the presence of H_2O_2 , oxidizes rapidly nitrite to nitrate [60,61,62]. Indeed, in liver, kidney, and other organs catalase plays a key role in the conversion of nitrite to nitrate [61,63].

Based on the above discussion, the major outlines of NO metabolism in the mammary gland are presented in Fig. 9.

Radical formation and antimicrobial activity of mammary secretion

Our results are consistent with previous reports that demonstrated that the mammary secretion of involuted glands is bactericidal and bacteriostatic against a wide range of pathogens [64–66]. According to previous reports, this bactericidal and bacteriostatic environment is most likely to arise from the accumulation of antimicrobial factors such as immunoglobulins and lactoferrin. The present study highlighted the potential contributions of H_2O_2 and NO to this activity. The bactericidal activity of mammary secretion of the experimental glands, which started as soon as 8 h after the first treatment and persisted as long as the CNH treatments continued, is a novel finding. This effect can be related to the accelerated $\text{NO}_2^\bullet$ formation during the CNH treatment, as this radical is a powerful bactericide [67]. The accelerated $\text{NO}_2^\bullet$ formation and the rapid recruitment of leukocytes to the glands of cows treated with CNH account for the effectiveness of this mechanism in eradicating different types of pathogens from the gland [6]. The fact that accelerated $\text{NO}_2^\bullet$ formation persisted only during the days during which the cows were being treated suggests that CNH induced the secretion of short-lived cytokines, which in turn activated an NO burst that exceeded the antioxidant capacity of mammary secretion.

In previous reports we concluded that CNH treatments accelerated and synchronized the natural involution process [6,7]. Therefore, an important question that emerges is: Was the early bactericidal response induced by CNH artificial, or conversely, did it reactivate a redundant innate immune mechanism? Unpublished data from our laboratory support the second explanation: when cows approach the stage of dry off naturally and more gradually, producing only 10 L of milk per day, the plasmin activity is already close to its peak level. Furthermore, although the secretion looked like

normal milk, it was not possible to curdle it with rennin, because of excessive degradation and modification of the CN micelle. Thus, we hypothesize that involution process in cows that were not selected for high milk yield, or that are approaching involution and producing relatively small amounts of milk, is shorter and is associated with a spontaneous burst of NO₂ formation. In any case, CNH treatment appears to be a viable tool with which to activate the innate immune system, and thus to prevent bacterial infection at dry off.

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