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Posttranslational ruling of xanthine oxidase activity in bovine milk by its substrates

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Abstract

The aims of this study were to test the hypothesis that the substrates of xanthine oxidase (XO), xanthine and hypoxanthine, are consumed while the milk is stored in the gland between milkings, and to explore how XO activity responds to bacteria commonly associated with subclinical infections in the mammary gland. Freshly secreted milk was obtained following complete evacuation of the gland and induction of milk ejection with oxytocin. In bacteria-free fresh milk xanthine and hypoxanthine were converted to uric acid within 30 min ($T_{1/2} \sim 10$ min), which in turn provides electrons for formation of hydrogen peroxide and endows the alveolar lumen with passive protection against invading bacteria. On the other hand, the longer residence time of milk in the cistern compartment was not associated with oxidative stress as a result of XO idleness caused by exhaustion of its physiological fuels. The specific response of XO to bacteria species and the resulting bacteria-dependent nitrosative stress further demonstrates that it is part of the gland immune system.
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Xanthine oxidoreductase (XOR) is a complex molybdoflavoenzyme, present in milk and many other tissues [1]. The activity of XOR in bovine milk is intense compared with its activity in that of closely related species such as ovines and caprines, and also compared with that in human milk [2,3]. XOR is known as the terminal enzyme of purine catabolism; it oxidizes hypoxanthine (hXa) to xanthine (Xa), and Xa to uric acid. 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 [1,2]. In addition to its classical role in purine metabolism, XOR is considered as a component of the innate system in mammals [4], including the bovine mammary secretion system [5]. Biochemical, molecular, and pharmacological studies further implicated XOR as a source of reactive oxygen species involved in the pathophysiology of cardiovascular diseases [6].

In bovine milk XOR is distributed between two major pools: an inner-membrane mixture of XO and, predominantly, XD which probably has a non-enzymatic role in fat secretion; and a second pool of XO in milk serum or attached to the outside of phospholipid membranes where it is available for a role in the gland innate immune system [7]. These forms of XO may also affect the physicochemical oxidative properties of milk and mammary gland epithelial cells.

XO converts nitrate into nitrite, and so its presence may substantially increase the substrate for NO generation [8,9]. In parallel with this, lactoperoxidase (LPO), which is also abundantly expressed in bovine milk, may convert nitrite into the powerful bactericidal radical, nitric dioxide [10,11]. It has been shown recently that this property of XO is important for the formation of bactericidal activity against the major mammary gland pathogens, *Escherichia coli* and *Staphylococcus aureus*, in the mammary gland secretion of involuted cows [5].

One puzzle that needs to be solved in order to establish the role of XO in the innate immunity of the mammary

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gland relates to the fact that the classical substrates of XO, i.e., Xa and hXa, are not found in measurable concentrations in composite milk samples taken at milking (designated here as mature milk, MM) [12,13]. Mature milk contains uric acid at a concentration of ~40 μM [5,14], for which there are two possible explanations. The first is that uric acid is produced inside the alveolar epithelial cells and then secreted to the milk. In this situation, the radical as well as the antioxidant (uric acid) products of XOR are also produced within the alveolar cells. This possibility is incompatible with the hypothesis that XO plays an important role in the innate immunity of the mammary gland secretion system, with our previous finding of a dramatic increase in urate concentration when the activity of the epithelial cells was declining (during involution), and with the nitrogen dioxide-dependent creation of bactericidal environments. The second possibility is that, because secretion of milk into the gland lumen is continuous whereas its evacuation, by either suckling or milking, is intermittent, the change of Xa and hXa to urate may occur during the storage of milk in the gland.

The first aim of the present study was to test the hypothesis that uric acid and, therefore, also hydrogen peroxide, are actively produced in the mammary gland. To pursue this objective, we milked the cows after complete evacuation of their glands at the regular milking, and after two subsequent oxytocin treatments; this enabled us to obtain milk within a couple of minutes after its secretion to the gland lumen. The second aim was to study how XO activity changed in response to subclinical infections with common mammary gland bacterial pathogens.

Materials and methods

Experiment 1: experimental and analytical procedures. Milk (~50 ml per cow) was obtained from udders of six Israeli-Holstein cows. The sample from each cow was taken from a mixed yield of a single udder. Preliminary analysis [15] showed that the milk was taken from bacteria-free udders. Somatic cell counts (SCC) in these samples were ~70,000 ml^{-1}, which is typical of milk from bacteria-free udders; this milk was designated as mature milk (MM). After that milking, the sampled glands in these cows were completely emptied by hand milking. When no more milk could be obtained by hand milking, the cows were injected intramuscularly with a dose of 20 international units of oxytocin (Vetimex, Bladel, Holland). After 3–5 the mammary gland was hand milked again, to ensure that any residual milk left in the alveolus was evacuated. The cows were injected intramuscularly with a dose of 30 international units of oxytocin and after 3–5 min 30–50 ml of milk were sampled from each of the previously sampled glands; this milk was designated as fresh milk (FM). The MM and FM samples were stored in dry ice immediately after sampling, and were transported to a nearby laboratory, where they arrived at a temperature of 6–10 °C, and were analyzed within less than 20 min.

Samples of FM (5 ml in duplicate) were transferred to tubes held in a rotating water bath at a controlled temperature of 37 °C. An additional set of FM samples was analyzed as above, but with each tube containing 500 μM of alluporinol (Sigma, Rehovot, Israel). Subsamples (200 μl) were taken every 5 min for 30 min, for (Xa + hXa) and uric acid determination. Two sets of MM samples (5 ml in duplicate in each set) were held in a rotating water bath as described above. In this case one set was spiked with 30 μM of Xa, and the second set with 30 μM of Xa and 500 μM of alluporinol. Subsamples (200 μl) were taken every 5 min for 30 min, for Xa and uric acid determination.

The urate concentrations at the beginning of the incubations were determined with a commercial Kit 686-A (Sigma Co., Rehovot, Israel) according to the manufacturer's instructions. The subsamples were measured in the same solution of reagents that contained 10 U ml^{-1} of XO (Sigma, Rehovot, Israel). Within 1 min XO at this concentration would convert all the Xa and hXa in the samples to urate. Therefore, (Xa + hXa) concentrations in FM and the Xa concentrations in MM were determined, at each sampling point, as the difference between the total urate concentration (i.e., that determined when the uric acid reagent contained added XO) and that at the beginning of the incubation.

Experiment 2: experimental and analytical procedures. Thirty Israeli-Holstein dairy cows, in which one quarter or more of their udders were chronically infected with subclinical mastitis by one of the bacteria under study—*Staphylococcus aureus*, *E. coli*, *Streptococcus dysgalactiae*, and *Streptococcus chromogenes* (seven or eight cows with each type of infection) were included in the study. All infected quarters had been monitored for their bacterial condition for 2–3 months prior to the beginning of the study. Confirmation of bacterial identity was based on foremilk samples taken from an aseptic sampling and sent to the laboratory for analysis within 1 h, three times over 2 days [16]. The cows included in this study were multiparous, in their mid- to late-stage lactation. These cows were milked three times daily, at 05:00, 12:00, and 20:00, yielded between 28 and 52 l per day, and were fed a typical Israeli total mixed ration comprising 65% concentrate (17% protein) and 35% forage.

Milk was sampled from each quarter during the morning milking and was subjected to the following analyses, as described previously [15]. Briefly, SCC were quantified with a Z1 Coulter cell counter (Coulter Electronics, Luton, England), and cells were differentiated with an FACS Calibur flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) with anti-bovine monoclonal antibodies (VMRD Inc., Pullman, WA, USA). The monoclonal antibodies used were: anti-CD18/11a—BAT 75A (IgG-1), anti-CD4—GC 50A1 138A (IgM), anti-CD8—CACT 80 C (IgG-1), anti-CD21—BAQ 15A (IgM), anti-CD14—CAM 36A (IgG-1), and anti-polymorpho-nuclear (PMN) (G1) (IgM). All the monoclonal antibodies used were reactive with bovine cells. The secondary polyclonal antibodies (CALTAG Laboratories, Burlingame, CA, USA) used were: goat anti-mouse IgG-1 conjugated with TRI-COLOR (TC) and goat anti-mouse IgM conjugated with FITC. Uric acid and nitrate in the milk samples were determined calorimetrically [5].

XO activity in whole milk samples, reflecting the free pool of XO in milk (soluble XO + XO attached to the outer side of and of milk lipoprotein membranes) was determined as described by Silanikove and Shapiro [7].

The SCC, log SCC, PMN, CD4, CD8, CD14, uric acid, and nitrate data were analyzed with the aid of a two-way ANOVA linear statistical model that takes into account the cow and the quarter infection status:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + e_{ijk}$$

in which μ is the grand mean; α_i is the effect of the ith cow; β_j is the effect of the jth infection status; and e_{ij} represents the residual between-quarters error. Comparisons between pairs of infection groups were made by t-test using the Tukey-Kramer HSD.

Results

Experiment 1: purines are consumed in fresh milk within 30 min, which results in XO idling in mature milk because of lack of substrate

The (Xa + hXa) concentration in oxytocine-induced FM was ~40 μM, but this milk contained only ~3 μM of uric acid. On the other hand, no (Xa + hXa) could be detected in a composite sample of MM, which was sampled

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