Lipopolysaccharide challenge of the mammary gland in cows induces nitrosative stress that impaired milk oxidative stability

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Abstract

The aim of this work was to study the effects of mastitis induced by intramammary lipopolysaccharide challenge on milk oxidative stability, as well as to understand the underlying biochemical processes that cause such changes. Lipopolysaccharide challenge was associated with nitric oxide burst from surrounding mammary epithelial cells and consequently provoked nitrosative stress that was induced by formation of NO₂⁻ from nitrite by lactoperoxidase. This response was associated with ~3 fold increased formation of hazardous compounds: nitrotyrosines, carbonyls and lipid peroxides. We sustained the involvement of xanthine oxidase as a major source of hydrogen peroxide. In consistent with previous findings, catalase has been shown to have a major role in modulating the nitrosative stress by oxidizing nitrite, to nitrate. The current hygienic quality criteria cannot detect mixing of low-quality milk, such as milk with high somatic cells and nitrite with high-quality milk. Thus, developing of improved quality control methodology may be found important for the production of high quality milk.
Keywords: milk; cow; mastitis; NO, NO₂⁺; plasmin system

Running title: LPS challenge of the mammary gland

Abbreviations: bulk milk tank – BMT; curd firmness - CF; lactoperoxidase – LPO; lipopolysaccharide – LPS; milk yield – MY; nitric oxide - NO; nitrotyrosine – Ntyr; plasmine – PL; plasminogen – PG; plasminogen activator – PA; rennet clotting time – RCT; somatic cell counts – SCC
Implication

In this study we show that intramammary treatment with LPS induced NO burst that was associated with nitrosative stress that reflected formation of hazardous compounds in milk: nitrotyrosines, carbonyls and lipid peroxides. These changes were associated with impairment of milk quality. Currently, very little is known about the presence of oxidative substances in commercial milk and about the industrial and health implications of their present there. As mastitis presents a prevalent problem in the dairy industry, this study stresses the importance to gain further information on the effect of different forms of mastitis on milk composition.

Introduction

Milk plays a fundamental role in modern western diets. It is an easy accessible matrix rich in a wide variety of essential nutrients such as minerals, vitamins and easily digested proteins with balanced amino acid profiles, which are important in supporting
most body functions (Drewnowski and Fulgoni, 2008; Silanikove et al., 2010). High-quality raw milk constituents are required to obtain high yields and good quality dairy products such as yogurt and cheese (Leitner et al., 2008; Oliviera et al., 2002). Nowadays, dairy plants grade milk upon reception according to its hygienic quality based on acceptable standards. In most countries, these standards comprise limits on maximal transporting temperature, maximal bacterial count, antibiotics residues and somatic cell count (SCC) (PMO, 2007).

Activation of indigenous enzymatic systems in milk takes an important role in promoting these changes. The simultaneous reduction in milk quality and milk secretion in infected glands was related to activation of plasminogen activator (PA), plasminogen (PG), and plasmine (PL) derived active peptides from milk β-casein (Shamay et al., 2002 and 2003; Silanikove et al., 2000 and 2005 and 2006 and 2009a). Enzymes linked to the metabolism of nitric oxide (NO) have also shown recently to affect considerably the milk composition of the inflamed mammary glands Silanikove et al., 2005 and 2006 and 2009a and
Xanthine oxidase, lactoperoxidase and their respective substrates, xanthine/hypoxanthine and NO are components of the mammary innate immune system. These components interactively function to form an effective bactericidal environment towards major mammary gland pathogens. Accordingly, H$_2$O$_2$ and NO are being constantly surged from the surrounding epithelial cells and milk leukocytes. Nitric oxide cycles in milk through its auto-oxidation to nitrite and the conversion of nitrite to NO$_2$$^\cdot$ by H$_2$O$_2$-dependent lactoperoxidase (LPO) activity (Silanikove et al., 2005). In turn, NO$_2$$^\cdot$ contributes to formation of bacteriocidic environment in milk and interacts with thiol-bearing groups on proteins to form thiyl radicals (Silanikove et al., 2005) and with tyrosine on proteins to form nitrotyrosine (Ntyr) (Silanikove et al., 2009b). The interaction of NO with thiyl radicals forms nitrosothiols, which serve as a pool that constantly delivers NO into the system (Silanikove et al., 2005 and 2009b). The above described NO-cycle includes two important regulatory modes which modulate and restrain NO$_2$$^\cdot$ activity. The first is catalase, which converts nitrite, the substrate of LPO, to the more stable...
metabolite nitrate, and the second is the formation of nitrosothiols, as alternative to formation of much more reactive Ntyr (Silanikove et al., 2005 and 2009b). Few recent studies have shown that under certain conditions, such as extended storage and mastitis, milk nitrite could be in the low µM range, which is above the permitted level in foods (Silanikove et al., 2005; Titov et al., 2010).

Mastitis is one of the most common diseases in dairy cows, inflicting 25 to 50% of the udders in modern dairy herds (Rainard and Riollet, 2006). The above information points out that mastitis has great effect on milk quality and potentially may also affect its safety for human consumption; therefore, it is imperative that its effect on milk composition be methodically understood.

Lipopolysaccharides (LPS) are glycolipids from the outer membrane of Gram-negative bacteria. LPS stimulates immune system cells, resulting in acute increased production of pro-inflammatory cytokines, over expression of cell adhesion molecules and matrix-degrading enzymes (Heumann and Roger, 2002). Exposure of a tissue to LPS induces rapid inflammation,
which resemble to a large extend the inflammatory response induced by *Escherichia coli* infection. Therefore, LPS challenge is a frequent model to study the inflammatory response induced by *E. coli* and its endotoxins in the mammary gland (e.g., Bruckmaier, 2005; Vangroenweghe *et al.*, 2004).

The general aim of this study was to explore the effects of intramammary LPS challenge on milk composition and the reflection of these changes on milk quality and safety. The specific aims were: (i) how LPS affects the NO-cycle in milk and milk composition, (ii) how LPS affects the PA-PG-PL-casein derived peptides (proteose peptones) and how these changes affect milk yield (MY) and composition (iii) to show that catalase plays a role in restraining the nitrosative stress induced by LPS challenge.

### 2. Materials and Methods

All protocols were approved by the Institutional Animal Care Committee of the Agricultural Research Organization, which is the legitimate body for such authorizations in Israel.
2.1. Study layout

The layout details of the study are described in Silanikove et al. (2011). Briefly, twelve Israeli Holstein heifers with low SCC, (27,000 ± 5000 cells mL⁻¹) and no bacterial detection that produced 33.2 ± 3.1 milk d⁻¹, were divided into two groups of six cows each. In the control cows, two mammary quarters, one front and one rear were infused with 10 mL sterile non-pyrogenic saline solution (Teva Pharmaceutical Industries Ltd., Israel) (r-Cont). The other two counter quarters (c-Cont) served as control to the procedure. In the experimental cows, two mammary quarters, one front and one rear were infused with LPS; 10 μg of LPS (E. coli, O55B5; Sigma Chemical Co., St. Louis, MO, USA) dissolved in 10 mL of sterile non-pyrogenic saline (e-LPS), while the two counter quarters served as control to the treatment (c-LPS). Intramammary infusion was injected with a special applicator following careful sterile cleaning of the teat. During the 4 days of the study (-24 h, 0 h, +24 h, +48 h and +72 h, where 0 h refer to day of infusion) every quarter of each cow was separately milked
into individual containers and milk samples were taken after
recording MY. Milk was discarded for 7 days following the
infusion.

2.2. Analytical procedures

One sub-set of samples was sent to a central laboratory for the
determination of fat, protein, lactose, urea and SCC. The amount
of milk components secreted and the concentration of particular
component at each point of time were calculated from the MY.
The quality of milk for cheese production by measuring curd
firmness (CF) and rennet clotting time (RCT) was tested with an
Optigraph (Ysebaret, Frepillon, France) (Merin et al., 2008).

Additional sub-sets of milk samples were defatted under cold
conditions (Silanikove et al., 2007) and analyzed according to
previously described procedures: concentration of casein, whey
proteins, proteose peptones, lactoferrin (ELISA), albumin
(ELISA), Na$^+$ and K$^+$ and the activity of PA, PLG and PL
(Silanikove et al., 2000 and 2009b); the activity of XO, LPO,
catalase and the concentration of nitrite (fluorometrically by the
DAN reagent), nitrate (colorimetrically by the Griess reaction).

The formation of potential harmful products in milk was assessed by analyzing the concentration of nitrotyrosine in whey proteins by ELISA, the concentration of carbonyls in whey protein and formation of lipid peroxides in milk lipids by colorimetric reactions (Silanikove et al., 2005).

2.3. Statistical analysis

The datasets of this study were analyzed using repeated measures analysis modeling correlated residuals within cow (SAS, 1990) as described before (Shamay et al., 2003). Briefly, the analysis considered the effects of treatment, day, and treatment × day interactions. The effect of days in milk was not significant (P > 0.25) and, therefore, was not included in the analyses presented here.

The model used was:

\[ Y_{ijkm} = \mu + C_i + T_j + T_jD_k + Qm(T_jCI) + e_{ijkm} \]

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, \( TjDk \) is the treatment day interaction effect, 
\( Qm(TjCI) \) is the quarter within cow treatment error term for 
treatment effect; and \( eijkm \) is the residual error.

3. Results

All the measured metabolites were not affected in the 
control cows, whether the glands were treated with saline or not, 
which indicates that the responses were specific to the LPS 
treatment.

3.1. Milk yield, SCC and lactose

This data was reported elsewhere (Silanikove et al., 2011).

Briefly, the dose of LPS applied in this experiment induced acute 
inflammation that was reflected by increase of SCC (up to \( 5-6 \times 
10^6 \) cells mL\(^{-1}\)), rectal temperature, plasma cortisol concentration 
and NAGase activity. These changes were associated with a 
maximum drop of \(~30\%\) in MY at 24 h post-treatment in the 
experimental glands. MY recovery occurred after 4 to 5 days. MY 
in e-LPS glands dropped by \(~20\%\), indicating that LPS induced a
negative effect on MY in the non-treated glands, though the effect
was significantly milder in comparison to the effect on treated
glands and was apparent only 24 h post-treatment. LPS challenge
induced a significant transient drop in lactose concentration
(~20% at 24 h post-treatment) and lactose secretion (~40% at 24 h
post-treatment) in the e-LPS glands. A drop of ~9 % and ~6 % in
lactose concentration at 24 h and 48 h post-treatment and of ~14%
and ~11% in lactose secretion was recorded in treatment c-LPS,
respectively.

3.2. Casein, whey proteins and proteose peptones

LPS challenge in the e-LPS glands induced a significant
transient increase in casein, whey proteins, and proteose peptones
concentrations. Casein concentration increased ~1.2 fold at 24 h
post-treatment, (Table 1), however, despite the increase in it
concentration, casein secretion dropped by ~21% at 24 h post-
treatment. Furthermore, the proportion of casein / total protein in
the LPS challenged glands was lower than in control treatments
(0.78 vs. 0.84; Table 1). Whey proteins concentration increased
~1.6 fold at 24 h post-treatment, (Table 1). Thus, despite the decrease in MY, the secretion of whey proteins actually increased by 1.21 fold at 24 h post-treatment in the LPS challenged glands ($P < 0.05$). No changes in whey proteins concentration was recorded in the control treatments (Table 1). Thus, the ~20% drop in whey protein secretion in treatment c-LPS was proportional to the drop in MY.

LPS challenge in the e-LPS glands induced a dramatic transient increase in proteose peptones concentration of 4.4 fold at 24 h post-treatment (Table 1). Thus, despite the decrease in MY, the yield of proteose peptones actually increased by ~ 2.7 fold at 24 h post-treatment in the LPS challenged glands ($P < 0.01$). No changes in proteose peptones concentration was recorded in the control treatments (Table 1). Thus, the ~20% drop in proteose-peptones content in treatment c-LPS was proportional to the drop in MY.

3.3. Plasminogen activator-plasminogen-plasmin system and milk clotting
LPS challenge in the e-LPS glands induced a transient increase in PA activity of ~2.1 fold at 24 h post-treatment and a corresponding decrease of ~1.7 fold in PG activity, an increase of ~11 fold in PL activity (Table 1), which was reflected by a decrease in PG/PL ratio from ~27 to ~1.5 at 24 h post-treatment. Whereas milk coagulating parameters were in the normal range in the control samples (curd firmness (CF) 11.60 ± 1.01 V; rennet clotting time (RCT) 950 ± 210 s), it was not possible to curdle the milk in samples collected 24 and 48 h after LPS challenge (Table 1). At 72 h post-treatment, milk clotting parameters were significantly different than in the control (CF 6.81 ± 0.92 V; RCT 2750 ± 195 s), indicating impairment in milk quality (Table 1).

3.4. *Soluble components of the innate immune system: lactoferrin and albumin*

LPS challenge induced a dramatic transient increase of lactoferrin concentration (2.7 fold) and albumin (4.7 fold) at 24 h post-treatment in the e-LPS glands (Table 2). These increases in lactoferrin and albumin concentrations were greater than the
decline in MY, indicating that a net increase in their secretion occurred. The response was specific to LPS treatment as no changes in lactoferrin and albumin concentrations were detected in c-LPS.

3.5. Enzymes of the innate immune system along with components of their precursors and products

LPS challenge in the e-LPS glands induced a transient increase in XO activity of 2.3 fold at 24 h post-treatment and 2.5 fold at 48 h post-treatment. The corresponding figures for c-LPS were increase by 1.4 at 24 h post-treatment and by 2.0 fold at 48 h post-treatment (Fig. 1A). These increases in XO activity were greater than the decline in MY, indicating that a net increase in its secretion occurred.

Uric acid in milk is a product of milk XO activity (Silanikove et al., 2007). LPS challenge in the e-LPS glands induced a transient increase in uric acid concentration, which was parallel to the changes in XO activity: an increase of 3.1 fold at 24 h post-treatment and 6.7 fold at 48 h post-treatment. The corresponding
figures for c-LPS were increase of 2.3 fold at 24 h post-treatment and 4.8 fold increase at 48 h post-treatment (Fig. 1B). These increases in uric acid concentrations were greater than the decline in MY, indicating that a net increase in the secretion of its precursors, xanthine and hypoxanthine, occurred (Silanikove et al., 2007).

Lactoperoxidase (LPO) is a major whey protein (Silanikove et al., 2005), explaining its high initial activity (~5 U mL\(^{-1}\)). LPS challenge in the e-LPS glands induced a transient increase in LPO activity of 1.4 fold at 24 h and 48 h post-treatment (Fig. 1C). The corresponding figure for c-LPS was increase of 1.1 LPO activity.

The increase in LPO in e-LPS glands and c-LPS glands was roughly equal in the case of treated glands, or smaller than the decline in MY in c-LPS, indicating that unlike with XO, no net increase in the secretion of LPO occurred.

LPS challenge in the e-LPS glands induced a dramatic transient increase in nitrite and nitrate concentration of 9 and 4.4 folds, respectively, at 24 h post-treatment, and 16 and 3.1 fold, respectively, at 48 h post-treatment. These responses were sharp
and at 72 h post-treatment, nitrite and nitrate concentrations fell to the pre-treated values (Table 2).

Fresh raw milk catalase activity was ~2 U mL$^{-1}$ (Fig. 1D). LPS challenge in the e-LPS glands induced a transient 3 to 3.5 fold increase in catalase activity to ~7 U mL$^{-1}$ at 48 h post-treatment and ~6.2 U mL$^{-1}$ at 72 h post-treatment (Fig. 1D). Thus, the increase in catalase activity lagged behind the increase in XO and LPO activities and remained high for a longer period. The increase in catalase activity was significantly higher than the reduction in MY, indicating a net increase in catalase secretion. No increase in catalase activity was recorded in c-LPS glands.

3.6. Oxidized substances

Fresh raw milk contained ~100 nmole g$^{-1}$ of Ntyr in whey proteins (Table 2). LPS challenge in the e-LPS glands induced a transient increase in Ntyr concentration, which was parallel to the changes in LPO activity: an increase of 5.3 fold at 24 h post-treatment and 5.7 fold at 48 h post-treatment. The response was
specific to LPS treatment as no changes in Ntyr concentration were detected in c-LPS glands.

The concentration of carbonyls and lipid peroxide followed similar kinetics to that of Ntyr. A summary of control and values of Ntyr, carbonyls and lipid peroxides at 24, 48, and 72 h are presented in Table 2. The data show a peak in their concentration at 48 h post-treatment, which was ~3 fold higher than in the controls. Although the concentration of Ntyr, carbonyls and lipid peroxides started to drop at 72 h post-treatment, it was still significantly higher (~2 fold) than in the controls (Table 2).

3.7. Changes in Na\(^+\) and K\(^+\) concentrations

LPS challenge in the e-LPS glands induced a transient increase in Na\(^+\) concentration of ~4 fold (from ~22 mM to ~113 mM). LPS challenge in the e-LPS glands induced a transient decrease in potassium concentration of ~4 fold (from ~33 mM to ~8 mM) at 24 h post-treatment and the kinetics was inversely proportional to that of sodium concentration (Table 2). These responses were sharp and at 48 h post-treatment Na\(^+\) and K\(^+\)
concentrations resumed to ~ half-way of the pre-treatment level and returned to the pre-treatment values at 72 h post-treatment. No significant changes in Na\(^+\) and K\(^+\) concentrations were recorded in c-LPS glands.

4. Discussion

4.1 Changes in milk composition in respect to milk quality and safety

The present study provides significant evidence that supports the generally accepted rule in the dairy industry that milk from clinical mastitic udders should not be used for human consumption. Considerable amounts of milk are typically used in many western countries for the production of cheese. Typically, nowadays SCC content in the bulk milk tank (BMT) in most EU countries is around 250,000 mL\(^{-1}\), whereas the upper permitted level is 600,000 mL\(^{-1}\) (EEC, 1994). Mastitis usually inflects a single gland and typically has SCC 1×10\(^6\) mL\(^{-1}\) and above (Rainard and Riollet, 2002). Thus, according to the current SCC-based hygienic criterion, such milk in small quantities but with
high SCC may perhaps enter the BMT, and the milk will still
meet the above-described criteria. The amount of low-quality
milk that can enter the BMT without violating these criteria is
inversely related to the SCC level. Analyzing the quality of bulk
milk in 11 tanks of Israeli dairy farms has shown great variability
in milk quality for curdling that was not related to SCC (Leitner et
al., 2008). It was suggested that this variability was related to
mixing milk from post clinical infection when the milk appear
normal and from sub-clinically infected udders with the general
milk. However, mixing milk from infected udders with milk from
non-infected ones could not be detected by measures, such as
SCC, proteose peptones content and % of casein, which worked
well on the individual cow level as predictors of udder
inflammation (Leitner et al., 2008). Thus, the results of this and
former studies (Forsback et al., 2009, 2010. Leitner et al., 2008)
implicate that it is important for the dairy industry to develop
analytical tools that will allow to prevent combining low-quality
milk, such as milk rich in somatic cells and nitrite with high-
quality milk in order to ensure optimal yield and quality of curd from milk designated for cheese production.

The concentration of nitrite in milk infected by bacteria may exceed the permitted safety levels of ~1 μM in milk (present study; Silanikove et al., 2009b; Titov et al., 2010). Whether nitrites and nitrates in foods and drinking water affect adversely human health is a controversial issue (Milkowski et al., 2010). However, as discussed below, the high level of Ntyr, Lpx, and carbonyls in mastitic milk is associated with the high level of nitrite and nitrate in that milk. Ntyr, Lpx, and carbonyls are considered as causative agents and hallmarks of cancer, atherosclerosis and other inflammatory diseases (Ohshima et al., 2003). Thus, obviously their presence in food is undesirable, though, as recently mentioned, there is lack of information regarding the significance of their occurrence in foods (Silanikove et al., 2009b).

A critical question that arises from this study is: does the current in use milk quality hygienic criteria are sufficient to ensure the highest quality that the present stage of knowledge
permits? A single report from Brazil, carried out at 1998, has shown that all samples of bulk milk collected from 36 farms exceeded the Brazilian legislation level for nitrite, and that 14.3% of commercial pasteurized milk samples were above that level (Seraphim et al., 1988). Because the present experimental set up induced a very strong inflammatory response it is still premature to draw conclusions pertaining to more common situations that prevailed in dairy farms. We also could not find more information regarding the effect of mastitis on milk safety in the scientific literature. Thus, more research on the basic cow/gland level and on the BMT level in different sites, both within and between countries is required to clarify this issue.

4.2. LPS challenge is reflected by accumulation of inflammatory indices in milk

The present result are consistent with previous finding that show that the inflammatory response induced by LPS disrupt tight junction integrity as reflected by changes in milk Na and K+ concentrations (Shamay et al., 2002 and 2003), increased the
secretion of soluble constituents of the innate immune system (Ibeagha-Awemu et al., 2010; Silnikove et al., 2006), including the secretion of albumin from mammary epithelial cells (Shamay et al., 2005).

Our results are also consistent with previous studies showing that LPS challenge induces significant casein degradation and identifies milk plasmin as a major cause for that (Moussaoui et al., 2002; Rossi et al., 2009; Silnikove et al., 2006). The biological implications of these findings were discussed before (Leitner et al., 2006 and 2011; Silnikove et al., 2006) and, therefore, these aspect are not discussed here in detail.

4.3. LPS induced acute nitrosative response and accumulation of oxidative substances in milk

It is known that LPS challenge and inflammatory mediators (cytokines) induce the formation of NO burst from various cells, including mammary gland cells (Ara et al., 2008; Boulanger et al., 2001; Ito et al., 2010; Silnikove et al., 2005; Zheng et al., 2006). Here we show for the first time that such NO burst is
reflected by accumulation of NO-derived metabolites that in turn impaired the oxidative stability of proteins and lipids in bovine milk. In previous study carried out during mammary gland involution, it was suggested that the main bactericidal effect of NO in milk may be related to conversion of nitrite to NO$_2^\cdot$ in a hydrogen peroxide depended manner by LPO according to reactions 1-3 (Silanikove et al., 2005 and 2009b).

LPO + H$_2$O$_2$ $\rightarrow$ LPO-compound I + H$_2$O (1)

LPO-compound I + NO$_2^-$ $\rightarrow$ LPO-compound II + NO$_2^\cdot$ (2)

LPO-compound II + NO$_2^-$ $\rightarrow$ LPO + NO$_2^\cdot$ (3)

This proposition is further supported in the present study by the increased LPO activity and large increase in Ntyr content in whey proteins. Ntyr cannot be produced directly by NO, but it can be formed by interaction with NO$_2^\cdot$ (Johnston and DeMaster, 2003; Sala et al., 2004).

The present results are also consistent with the previous proposition that increased NO$_2^\cdot$ production is also responsible for the accumulation of carbonyls and oxidized fat in milk.
(Silanikove et al., 2005 and 2009b), as also found in other tissues and cells (Jung et al., 2007).

Nitrosative stress can be defined as a condition that occurs when the production of highly reactive nitrogen-containing chemicals, such as NO$_2^\bullet$, exceed the ability of biological regulated system such as milk to neutralize and eliminate it and consequently can lead to reactions that alter normal protein and other organic components structure in that system. The rather dramatic increase in the content of Nitryl, carbonyls and oxidized fat in milk indicate that acute inflammation, such as that induced by LPS challenge induce nitrosative stress. This suggests that formation of NO$_2^\bullet$ at peak inflammatory response exceeds the capacity of the two regulatory systems that function to modulate NO$_2^\bullet$ to eliminate it. These are formation of nitrosothiols instead of more reactive NO$_2^\bullet$ (reactions 5 and 6) and conversion of nitrite to nitrate (discussed below).

\[
\text{RS}^- + \text{NO}_2^- \rightarrow \text{RSNO} \quad (5)
\]

\[
\text{RS}^- + \text{NO}^- \rightarrow \text{RSNO} \quad (6)
\]
The association between thiols and NO (RSNO) is relatively weak, resulting in slow dissociation of NO from nitrosothiols (Silanikove et al., 2005). Thus, RSNO are probably the main reason for the constant cycling and accumulation of NO-derived species in milk (Silanikove et al., 2005). As the rate of NO$_2$• formation is second order with regard to NO-nitrite mediated oxidation (reactions 1-3), nitrosation reactions are limited by the availability nitrite, which is a product of NO autooxidation. Thus, by maintaining constant NO-cycle, an ability to respond rapidly to bacterial infection is preserved as rather small increase in NO surge will exceed the capacity of RSNO formation and nitrite oxidation by catalase to restrain the formation of NO$_2$•. All and all, the continuous accumulation of nitrate following LPS treatment support this model as summarized in Fig. 2.

4.3. NO products accumulate in milk, mainly as nitrate: the role of catalase

Catalases are enzymes ubiquitously found in all kinds of living organisms and are best known for catalyzing the
decomposition of hydrogen peroxide to water and oxygen.

However, catalase contains porphyrin heme (iron) groups in its center and thus can oxidize various acceptors, including nitrite by functioning as peroxidase according to classical 3 steps reactions of peroxidases (reactions 7-9):

\[
\text{Catalase} + \text{H}_2\text{O}_2 \rightarrow \text{Catalase-compound I} + \text{H}_2\text{O} \quad (7)
\]

\[
\text{Catalase-compound I} + \text{NO}_2^- \rightarrow \text{Catalase-compound II} + \text{NO}_3^- \quad (8)
\]

\[
\text{Catalase-compound II} + \text{NO}_2^- \rightarrow \text{Catalase} + \text{NO}_3^- \quad (9)
\]

We have previously shown that conversion of nitrite to nitrate by catalase is the main function of milk catalase that serves as a basic mechanism for prevention of excessive nitrosative stress in milk ((Silanikove et al., 2005 and 2009b). The increase of nitrate to levels of ~ 80 µM in the treated glands, suggest that the need to convert nitrite to nitrate under LPS challenge increased substantially (Silanikove et al., 2009b). The increase in uric acid (the product of xanthine or hypoxanthine oxidation in milk)
concentration to ~ 300 µM imply similar increase in the secretion of XO substrates, xanthine and/or hypoxanthine that led to proportional increase in hydrogen peroxide production (Silanikove et al., 2007). The levels of uric acid measured in the present experiment suggest that Vmax of catalase exceeds the normal catalase activity in milk (Silanikove et al., 2009b). Thus, the large increase in catalase activity in the present results suggests that it plays an important role in protecting the gland from nitrosative stress and is consistent with recent finding that the presence of intact catalase in milk is essential for preventing accumulation of basal Ntyr, Lpx, and carbonyls (Silanikove et al., 2009b). Thus, the present results reemphasize our previous conclusion that in light of its essential contribution to milk-quality maintenance and because of the importance of cow’s milk in human nutrition, it might be important to ensure that this enzyme is fully functional in each cow acquired by a commercial dairy farm.

4.4. LPS induced an increase in XO activity in milk
Hydrogen peroxide formation is essential for conversion of nitrite to NO₂⁻ by LPO and oxidation of nitrite to nitrate by catalase. XO was identified as the major source of hydrogen peroxide in bovine milk (Silanikove et al., 2007 and 2009b). Under subclinical infection with *E. coli*, which induced much milder nitrosative stress than in this study, XO activity was up-regulated by increasing the secretion of xanthine + hypoxanthine, which are the main physiological substrates of XO in milk. However, under acute inflammation, as in the present work and under involution, the activity of XO was up-regulated both by increasing its net secretion into the milk and by rising the secretion of its substrates, xanthine + hypoxanthine. This most likely reflects the large increase in the need for H₂O₂ and is reflected by the large increase in uric acid concentration, consistent with previous reports (Silanikove et al., 2007 and 2009b).

The conversion of xanthine + hypoxanthine to uric acid is stociometrically linked to superoxide and H₂O₂ formation (reactions 10-13) (Silanikove et al., 2007):
Hypoxanthine + H₂O + O₂ \rightarrow xanthine + 

2H⁺ + 2O₂⁻ \rightarrow \text{(10)}

Hypoxanthine + H₂O + O₂ \rightarrow xanthine + 

2H⁺ + H₂O₂ \rightarrow \text{(11)}

Xanthine + H₂O + O₂ \rightarrow Uric acid + 

2H⁺ + 2O₂⁻ \rightarrow \text{(12)}

Xanthine + H₂O + O₂ \rightarrow Uric acid + 

2H⁺ + H₂O₂ \rightarrow \text{(13)}

Whether superoxide or hydrogen peroxide would be coupled to xanthine + hypoxanthine oxidation at the molybdenum active site of XO depends on the number of electrons produced during the reaction, which depends on reaction conditions. Fridovich (1970) has originally shown that under physiological conditions ~80% H₂O₂ and ~20% O₂⁻ are produced, whereas production of 100% O₂⁻ requires an environment of 100% O₂ at pH 10, which is quite un-physiological. In accordance, it was shown that superoxide is not produced by milk XO (Silanikove et al., 2005) and scarcely produced by XO attached to the apical surface of bovine aortic endothelial cells (Kelley et al., 2010), whereas
hydrogen peroxide is the main product of XO oxidation under both conditions. The lack of evidence for O$_2^\cdot$ production in milk is consistent with the fact that milk is much more hypoxic in comparison to blood. In systemic fluid, XO is considered frequently as a source for formation of harmful radicals, such as peroxynitrite and superoxide. The situation in milk is quite complicated. On the one hand, it provides H$_2$O$_2$ for the conversion of NO to NO$_2^\cdot$, which is essential for the glandular defense, but impairs milk composition. On the other hand, it provides H$_2$O$_2$ for the conversion of nitrite to nitrate by catalase, which is essential to the resolution of inflammatory response and in maintaining milk quality during its storage in the udder and under commercial farming conditions (Silanikove et al., 2009b)

**Conclusions**

This study supports the concept that LPS induced NO burst from surrounding mammary epithelial cells. The consequential nitrosative stress was most likely induced by formation of NO$_2^\cdot$ from nitrite by LPO. This response was associated with ~3 fold
increased formation of hazardous compounds: nitrotyrosines, carbonyls and lipid peroxides and is associated with impairment of milk quality. Currently, very little is known on the presence of oxidative substances in commercial milk and about the health implications of their presence there. Thus, our study stresses the importance to gain further information in this direction.

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Figure Legends

Figure 1. Effect of lipopolysaccharide on xantine oxidase activity (mU mL⁻¹; panel A), uric acid concentration (µM; panel B), lactoperoxidase activity (U mL⁻¹; panel C) concentrations and catalase activity (U mL⁻¹; panel D) in treated (circles) control-LPS (triangles) and control-control (squares) glands. The results are presented as mean ± SD. Results at 24, 48 and 72 h in the experimental treatment are significantly higher ($P < 0.001$) than in the controls.

Figure 2. Schematic model that describes the simultaneous activation of the PA-PLG-PL system and the NO-derived cycle following challenge of the mammary gland with lipopolysaccharide (LPS), and the interaction between them. LPS challenge activate the PL system, and the increased PL activity liberates peptides from the casein micelle, which in turn down-regulate milk secretion and casein micelle clotting; see Leitner et al. (2011) for further details. In parallel, LPS up regulate NO-cycle metabolism, which results in increased formation of nitrotyrosine, carbonyls and lipid peroxide; see Silanikove et al (2005 and 2009b) for further details. The increased content of oxidized component in milk most likely increased their susceptibility to proteolysis Silanikove et al. (2006). Casein derived active peptides: ; casein micelle: .
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