

SELENIUM ASSOCIATED OXIDATIVE STRESS IN THE MAMMARY GLAND OF
PERIPARTURIENT DAIRY COWS

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Comparative Medicine and Integrative Biology

2012

ABSTRACT

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Dairy cows experience tremendous stressors during the periparturient period leaving them susceptible to numerous metabolic and infectious diseases. Mastitis continues to be the number one disease afflicting dairy cows during this time period. An inability to compensate for fetal demands and the onset of lactation alters a dairy cow's nutritional status significantly around the time of calving. Antioxidant deficiencies as a consequence of low selenium levels during the periparturient period are associated with increased susceptibility to mastitis. The benefit of selenium to mammary gland health may be attributable to its incorporation into selenoproteins, which participate in immune regulation, partly due to their ability to control oxidative stress. Oxidative stress is the result of decreased antioxidant potential and increased reactive oxygen species production. Increased levels of reactive oxygen species contribute to inflammation by causing either direct cellular damage or altering signaling pathways in immune cells. The ability of selenoproteins to control oxidative stress, such as during the periparturient period, may reduce the incidence and severity of mastitis lessening economic burdens to the dairy industry. The goal of this thesis is to examine selenoprotein activity within the mammary gland of periparturient dairy cattle and determine if it may contribute to oxidative stress and pro-inflammatory gene expression during this time period.

ACKNOWLEDGEMENTS

A special thanks to members of the Meadow Brook lab, in particular, Dr. Lorraine Sordillo, Jeff Gandy, and Chris Corl, for their technical and moral support and incredible amount of patience. I would also like to thank my committee members, Dr. Lorraine Sordillo, Dr. Ron Erskine, Dr. Jane Maddox, and Dr. Vilma Yuzbasiyan-Gurkan for their unconditional support, expertise, and guidance through the program.

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CHAPTER 1

IMMUNOPATHOLOGY OF MASTITIS: INSIGHTS INTO DISEASE RECOGNITION AND RESOLUTION

By

Stacey L. Aitken, Christine M. Corl, and Lorraine M. Sordillo

Published in JOURNAL OF MAMMARY GLAND BIOLOGY AND NEOPLASIA, September 2011, 16:291-304, *Immunopathology of Mastitis: Insights into Disease Recognition and Resolution*, by Stacey L. Aitken, Christine M. Corl, and Lorraine M. Sordillo

ABSTRACT

Mastitis is an inflammation of the mammary gland commonly caused by bacterial infection. The inflammatory process is a normal and necessary immunological response to invading pathogens. The purpose of host inflammatory responses is to eliminate the source of tissue injury, restore immune homeostasis, and return tissues to normal function. The inflammatory cascade results not only in the escalation of local antimicrobial factors, but also in the increased movement of leukocytes and plasma components from the blood that may cause damage to host tissues. A precarious balance between pro-inflammatory and pro-resolving mechanisms is needed to ensure optimal bacterial clearance and the prompt return to immune homeostasis. Therefore, inflammatory responses must be tightly regulated to avoid bystander damage to the milk synthesizing tissues of the mammary gland. The defense mechanisms of the mammary gland function optimally when invading bacteria are recognized promptly, the initial inflammatory response is adequate to rapidly eliminate the infection, and the mammary gland is returned to normal function quickly without any noticeable clinical symptoms. Suboptimal or dysfunctional mammary gland defenses, however, may contribute to the development of severe acute inflammation or chronic mastitis that adversely affects the quantity and quality of milk. This review will summarize critical mammary gland defense mechanisms that are necessary for immune surveillance and the rapid elimination of mastitis-causing organisms. Situations in which diminished efficiency of innate or adaptive mammary gland immune responses may contribute to disease pathogenesis will also be discussed. A better understanding of the complex interactions between mammary gland defenses and mastitis-causing pathogens should prove useful for the future control of intramammary infections.

Key Words: mastitis, mammary gland, immunology, immunity, inflammation, immunopathology

Abbreviations

| | |
|----------------|---|
| Ig | immunoglobulin |
| LPS | lipopolysaccharide |
| IL | interleukin |
| IFN | interferon |
| TNF | tumor necrosis factor |
| TGF | transcription growth factor |
| PAMPs | pathogen-associated molecular patterns |
| TLR | toll-like receptor |
| TIR | toll-interleukin 1 receptor |
| MyD88 | myeloid differentiation primary response gene 88 |
| TIRAP | toll-interleukin 1 receptor domain containing adaptor protein |
| TRIF | toll-receptor-associated activator of interferon |
| TRAM | toll-receptor-associated molecule |
| NF- κ B | nuclear factor kappa B |
| CD | cluster of determination |
| LBP | lipopolysaccharide binding protein |
| COX | cyclooxygenase |
| LOX | lipoxygenase |
| PG | prostaglandin |
| TX | thromboxane |
| PUFA | polyunsaturated fatty acid |
| HETE | hydroxyeicosatetraenoic acid |
| HPETE | hydroperoxyeicosatetraenoic acid |
| ICAM1 | intercellular adhesion molecule 1 |
| LX | lipoxin |
| LT | leukotriene |
| MDP | muramyl dipeptide |
| LTA | lipoteichoic acid |
| NET | neutrophil extracellular trap |
| NOD | nucleotide-binding oligomerization domain |
| PMN | polymorphonuclear leukocyte |

INTRODUCTION

Mastitis is an inflammatory condition of the mammary gland that is most often associated with lactating mammals and is mainly caused by bacterial infection. Epidemiological studies in humans found that up to a third of all lactating women will suffer from mastitis, where the clinical form of the disease is a primary reason why mothers will stop breast feeding [1, 2]. Adequate diagnosis and treatment of the disease in women are necessary to avoid lactation failure, recurrent mastitis, breast abscess, and even death in some situations [2]. Intramammary infections are an important health concern in food-producing animals such as dairy cattle, goats, and sheep [3, 4]. Mastitis is the most costly disease to the dairy industry as a consequence of decreased milk production and quality, treatment costs, replacement animal costs, and reduced ability to market dairy products. Despite ongoing research efforts to develop more effective preventative or curative control measures, mastitis remains a significant health problem in both human and veterinary medicine.

The development of mastitis is partially related to the degree that mammary glands are exposed to bacterial pathogens. A broad range of gram-positive and gram-negative pathogens cause mastitis. The establishment and severity of infection can be influenced by the expression of bacterial virulence factors. Increased susceptibility to mastitis and the extent of disease severity also is influenced by several host-related factors including nutrition, genetics, oxidative stress, environmental stressors, and physiological transition of the mammary gland from involution to lactation [5-8]. Indeed, the incidence and severity of mastitis in most mammals is more pronounced around the time of parturition. In humans, mastitis is most common within the first 3 weeks of lactation, and approximately 74-95% of all new mastitis cases occur within 12 weeks postpartum [2]. Dairy cattle also are especially susceptible to new intramammary infections

beginning 3 weeks prior to parturition through early lactation [9]. The ability to control mastitis, especially during the periparturient period, is ultimately reliant upon an efficient immune system that rapidly clears bacterial pathogens and restores mammary gland function and milk production.

Innate and Adaptive Immune Responses

The mammary gland immune system consists of a diverse array of physical, cellular, and molecular factors that function within innate or adaptive (acquired) immune responses. The innate immune system constitutes the primary line of defense during the initial stages of infection and is a key determinant of mastitis outcome. Innate defense mechanisms can be pre-existent within the mammary gland, but are activated quickly upon exposure to bacteria. Depending on the efficiency of the innate defense mechanisms, pathogens may be eliminated within minutes to hours following invasion. Rapid elimination of bacteria often will not result in any noticeable changes in mammary gland function or milk quality. Components of the innate defense system include nonspecific physical barriers of the teat end, pattern recognition receptors, phagocytes (i.e., neutrophils and macrophages), and various soluble factors (i.e., cytokines, complement, and lactoferrin). The efficiency of the innate arm of the immune system not only determines if new intramammary infections occur, but also influences the severity and duration of mastitis by influencing the nature of the adaptive immune response.

The adaptive immune response is triggered when innate immune mechanisms fail to eliminate a pathogen. The adaptive immune response is characterized by the generation of antigen-specific lymphocytes and memory cells with the ability to recognize specific antigenic determinants of a pathogen. When the mammary gland is re-exposed to the same antigen, a heightened state of immune reactivity occurs as a consequence of immunological memory and

clonal expansion of antigen-specific effector cells. A memory immune response would be much faster, considerably stronger, longer lasting, and often more effective in clearing an invading pathogen when compared to a primary immune response. In contrast to the innate immune response, adaptive immunity can take days to develop because of the clonal expansion of B and T lymphocytes specific to the invading pathogen. Antigen-specific B lymphocytes synthesize and secrete antibodies that recognize and counteract specific bacterial virulence factors. Effector functions of T lymphocytes include the production of cytokines that facilitate cell-mediated immunity by regulating the magnitude and duration of the immune response. The unique features of the adaptive immune response form the basis of mastitis vaccine protocols. Both innate and adaptive immune defenses of the mammary gland must be highly interactive and coordinated in order to provide optimal protection from mastitis.

Pathogen-Dependent Immune Responses

The outcome of host-pathogen interactions within the mammary gland is variable and can result in acute or chronic symptoms that may present as a range of severity from subclinical to clinical mastitis. Gram-positive and gram-negative pathogens are known to elicit different mammary gland immune responses during intramammary infections [10]. Differences in the magnitude and duration of host responses are determined, in part, from specific bacterial virulence factors. For example, *Staphylococcus aureus* adheres to and internalizes within host cells enabling evasion of the initial innate immune response which often results in subclinical, chronic infections. Alternately, coliform infections are associated with rapid bacterial multiplication, toxin release, eicosanoid biosynthesis, and cytokine production that may result in acute, clinical mastitis. The host response to each bacterial pathogen is the result of

bacterial recognition and communication between various cell types within the mammary gland. The ultimate goal of the immune response is to eliminate invading pathogens and restore the mammary gland to normal function. However, an overly robust immune response may cause tissue damage, so it is important that the mastitis-causing pathogen is neutralized and eliminated rapidly before extensive bystander tissue damage can occur. Therefore, a delicate balance of pro-inflammatory and inflammatory-resolving activities is critical to prevent inadvertent damage to the mammary gland and restore homeostasis to the immune system. Understanding critical host-pathogen interactions during mastitis pathogenesis will enable the development of novel interventions aimed at optimizing natural immune defenses of the mammary gland and avoiding immune-related pathology.

Mammary Immunobiology

Physical and Chemical Barriers of the Teat

The port of entry for bacterial pathogens is the teat canal. The teat sphincter and keratin lining provide a physical and chemical barrier to invading pathogens. Sphincter muscles surrounding the teat end opening can hinder bacterial penetration by maintaining tight closure between periods of milk removal. Patency of these muscles is directly related to increased susceptibility to new intramammary infections [11]. The stratified squamous epithelium lining the teat duct produces a keratin layer that also is crucial to maintaining the barrier function of the teat end between milking periods. The teat canal can become completely occluded during the nonlactating period when there is creation of a keratin plug. Bacteria are physically trapped within the keratin lining preventing subsequent migration into the gland cistern. Removal of keratin from the teat end was correlated to increased bacterial invasion and

colonization in dairy cattle [11, 12]. The lipid components of keratin, including esterified and nonesterified fatty acids, were shown to have bacteriostatic properties [13]. Whereas the precise mechanisms of antibacterial activity are unknown, some evidence suggests that the long chain fatty acids found in keratin may disrupt the lipid integrity within bacterial cell walls and result in perforation and death of invading pathogens. Despite the ability of the teat to trap pathogens, intramammary infections still occur and the mammary gland must rely on additional antimicrobial defense mechanisms to inhibit bacterial growth.

Endogenous Soluble Defenses

Bacteria that are able to traverse the teat canal and enter the gland cistern are confronted with a number of soluble antibacterial factors (i.e., peptides, proteins, enzymes) that can target the invading organism. In the healthy mammary gland microenvironment, some of these pre-existing factors include lactoferrin, complement, lysozyme, cytokines, immunoglobulins (Ig), and other soluble molecules with known bactericidal and bacteriostatic properties. The presence of these factors changes during different stages of lactation and have variable efficacy against different mastitis-causing pathogens.

Lactoferrin is among the well-characterized antimicrobial proteins of the mammary gland. Produced by epithelial cells and leukocytes, lactoferrin is an iron-binding protein with known bacteriostatic capabilities. In the presence of bicarbonate, lactoferrin can sequester free ferric ions present in milk and therefore hinder the growth of bacteria which have iron requirements, such as staphylococci and coliforms. In ruminants, lactoferrin and Ig were shown to act synergistically to inhibit the growth of certain gram-negative bacteria. [14]. A recent in vitro study using a bovine mammary epithelial cell line found that lactoferrin may marginally neutralize the

cytotoxic effects of endotoxin by binding the lipid A portion of lipopolysaccharide (LPS), thus potentially altering the course of gram-negative intramammary infections [15]. Certain bacteria, however, are resistant to the antibacterial effects of lactoferrin. For example, *Streptococcus agalactiae* can utilize lactoferrin as an iron source following binding with citrate or cell surface receptors. More recent studies showed that *Strep. uberis* also may utilize lactoferrin as a molecular bridge between adhesion molecules on the bacterial surface and lactoferrin receptors on mammary epithelial cell surfaces [16]. The lactoferrin bridge leads to internalization by mammary epithelial cells and protection from the action of local immune defense mechanisms. Lactation stage greatly influences the amount and effectiveness of lactoferrin's antibacterial properties. For example, the concentration of lactoferrin is low in the milk of healthy lactating mammary glands, but increases dramatically during involution and inflammation [17]. Lactoferrin, in the presence of other milk proteins such as β -lactoglobulin, appears to have synergistic antibacterial effects against mastitis-causing pathogens such as *S. aureus* and *Strep. uberis* [18].

Complement is another component of the innate defense system and consists of a collection of proteins present in serum and milk. The proteins that comprise the complement system are synthesized mainly by hepatocytes, but other cellular sources include monocytes and tissue macrophages. Concentrations of complement are highest in colostrum, inflamed mammary glands, and during involution [19]. Activation of the complement system results in the generation of several pro-inflammatory fragments, of which the C5a fragment is especially associated with mastitis [20]. Direct bactericidal activities of complement result from the deposition of pore-forming complexes onto the surface of bacteria. Gram-negative mastitis-causing pathogens, such as *Escherichia coli*, are especially sensitive to complement-mediated

lysis [19]. Other important biological functions of complement that contribute to early microbial killing include opsonizing bacteria and priming or activating host immune cells for phagocytosis and intracellular killing [20]. Complement also is a potent chemoattractant for neutrophils and monocytes during the early stages of infection [20]. Activation of the complement cascade in the milk of healthy mammary glands, however, is thought to play only a minor bactericidal role due to its relatively low concentrations.

The role of cytokines in the physiology and immunology of the mammary gland has been studied extensively over the last several decades. Cytokines not only are critical for normal physiological functions, but also are known to play a central role in essentially all aspects of inflammation and immunity [10, 21-24]. The cytokine network consists of a diverse group of proteins produced by both immune and non-immune cells throughout the entire body and under diverse circumstances. The physiological and immunomodulatory capacity of the cytokine network is complex. Individual cytokines can interact with other cytokines synergistically, additively, or antagonistically on multiple cell targets. Several different cytokines can affect biological processes in the same way, as there is considerable functional redundancy within the cytokine network. Most cytokines have very short half-lives, so their synthesis and function usually occurs in bursts of activity. Cytokines are able to influence cellular functions through high affinity receptors for each cytokine located on mammary gland cells. Therefore, the activity of any responder cell is a function of not only the quantity and type of cytokine in the mammary gland microenvironment, but also the relative expression of cytokine receptors.

The concentration and composition of cytokines expressed within tissues and secretions changes dramatically during different physiological transitions of the mammary gland. For example, expression of IL2 and IFN was lower during the periparturient period compared to the

fully involuted bovine mammary glands [25]. In contrast, expression of IL4, IL10, and TNF α were reported to be higher in milk and mammary tissues [26, 27]. The increased expression of TNF α and TNF receptors during pregnancy and early lactation was suggested to play a role in the growth and development of rat mammary glands [28]. Higher expression of TGF α and TGF β 1 in bovine mammary glands during mammatogenesis and involution were correlated to periods of active proliferation and reorganization of mammary tissues [29]. In addition, TGF β 1 was shown to play a critical role in regulating mammary gland regenerative capacity during successive cycles of lactation and involution in mice [30]. More recent studies showed that TNF α , IL6, IL10 and TGF β 1 also play an important role in remodeling of mammary tissues [31-33].

In contrast to other soluble defenses within the mammary gland, there is no evidence that cytokines have direct antibacterial functions. Instead, cytokines play an essential role in host defense by orchestrating the antimicrobial functions of mammary gland effector cell populations following exposure to invading pathogens. Therefore, pre-existing concentrations of cytokines in the healthy mammary gland likely exert their biological effects by influencing normal physiological functions and maintaining immunological homeostasis [21, 24]. As such, cytokines exert their diverse effects on mammary gland defense mechanisms by escalating innate and adaptive immune responses, activating inflammation, and initiating the migration of leukocytes from blood into infected tissues following bacterial recognition by local cell populations. The pattern of cytokine expression by cells in the mammary gland will differ depending on the mastitis-causing pathogen that elicits their response [10]. In general, however, gram-negative bacteria initiate a greater magnitude of pro-inflammatory cytokine responses (i.e., IL1, IL6, IL8, and TNF α) when compared to gram-positive bacteria that tend to express a weaker and slower cytokine response during the early stage of infection.

In addition to several pre-existing innate defense mechanisms, Ig also can function in the surveillance and early elimination of mastitis-causing pathogens from the mammary gland. Immunoglobulins are produced by antigen-activated B lymphocytes that subsequently proliferate and differentiate into antibody-secreting plasma cells. Concentrations of Ig in lacteal secretions are synthesized locally, are selectively transported, or present in transudate from serum [34]. Recent findings also reported the expression of Ig heavy and light chain transcripts by mammary gland epithelial cells of lactating mice [35]. In addition to plasma cells, these data suggest that at least some Ig found in the colostrum and milk may be produced by mammary gland epithelial cells. There are 4 classes of Ig that are known to influence mammary gland defense against mastitis-causing pathogens, namely IgG₁, IgG₂, IgA, and IgM, which all differ in their physiochemical and biological properties. In general, Ig concentrations are maximal during colostrogenesis and during intramammary infections. In bovines, IgG₁ is the predominant isotype in healthy mammary glands, while IgG₂ increases substantially during mastitis. Indeed, there is some evidence to suggest that low concentrations of the IgG₂ isotype correlated to an increased incidence of bovine mastitis [7]. In the milk of women, however, IgA is found in highest concentrations especially during the immediate postpartum period. Increased susceptibility to mastitis in women is correlated to concentrations of IgA in milk. Indeed, the concentrations of IgA in the normal milk of women that ultimately developed mastitis was significantly lower when compared to women that remained mastitis-free, suggesting that IgA deficiency is a risk factor for human mastitis [36]. The presence of all Ig isotypes in colostrum and milk can facilitate antimicrobial defenses of the mammary gland. For example, several Ig isotypes (IgG₁, IgG₂, and IgM) can act as opsonins to enhance phagocytosis by neutrophils and macrophages. In addition to its role in opsonization, IgM is efficient at complement fixation. Whereas IgA does

not aid in bacterial opsonization, it does function in bacterial agglutination that can impede the ability of certain pathogens to spread throughout the mammary gland. Another important role of IgA in the defense of the mammary gland is its ability to neutralize some bacterial toxins. Clearly, both the concentration and isotype composition of Ig found in lacteal secretions can have a profound influence on the establishment of new intramammary infections.

Pattern Recognition Receptors

The ability to sense the presence of bacteria within the mammary gland is essential for early host defense. Both immune and non-immune cell populations within the healthy mammary gland play a significant role in surveillance and activation of the innate immune response to invading pathogens via pattern recognition receptors (Table 1) (see below). Pattern recognition receptors can be expressed on the cell surface, secreted, or expressed intracellularly and function to recognize a diverse array of conserved motifs unique to specific microbes that are referred to as pathogen associated molecular patterns (PAMPs) [37, 38]. These PAMPs can differentiate a range of bacterial factors associated with mastitis-causing bacteria including lipopeptides of gram-positive bacteria and LPS of gram-negative bacteria. After binding to their ligand, pattern recognition receptors can initiate intracellular signaling cascades that result in initiation of immune responses or can facilitate antimicrobial activity directly.

The Toll-like receptor (TLR) family of pattern recognition receptors was among the first to be discovered and are pertinent to bacterial intramammary infections. To date, 10 TLRs were identified in humans and 12 in mice, with agonist specificity varying between species [37, 38]. The TLRs may work independently, antagonistically, or synergistically upon stimulation to modulate the immune response [39]. The TLR consists of a leucine-rich repeat area that recognizes the PAMP, a

Table 1. Pathogen Recognition Receptors

| Factor | Role | Reference |
|--------------------------|--|-----------|
| Innate Immunity | | |
| CD14 | Binds LPS. Membrane version is expressed on several cell including monocytes, macrophages, neutrophils, dendritic cells, and B cells. The soluble version may compete with mCD14 for LPS and is essential in the activation of non-mCD14 expressing cells, including epithelial and endothelial cells, by LPS. | [44, 120] |
| PGRP | Expressed in differentiated, lactating epithelium where it binds and hydrolyzes peptidoglycans. | [121] |
| TLR2 | Recognizes peptidoglycan and LTA from gram + bacteria and lipoarabinomannan from mycobacteria. May form a heterodimer with TLR1 to recognize triacylated lipopeptides from gram – bacteria and mycoplasma or with TLR6 to recognize diacylated lipopeptides from gram + bacteria and mycoplasma. | [84, 88] |
| TLR3 | Detects double-stranded RNA. | [84, 88] |
| TLR4 | Recognizes LPS of gram – bacteria, heat-shock proteins, fibrinogen, and polypeptides. | [84, 88] |
| TLR5 | Recognizes bacterial flagellin. | [84, 88] |
| TLR9 | Intracellular recognition of CpG-containing oligodeoxynucleotides (ODNs). | [84, 88] |
| Acquired Immunity | | |
| Memory B Cells | Produced during B cell division along with plasma cells following pathogen recognition. Their presence allows for a rapid response to a previously encountered antigen. | [7, 21] |
| Memory T Cells | Rapidly divide upon recognition of a previously encountered antigen. Longer life-span than memory B cells. | [7, 21] |
| Fc Receptors | Expressed on macrophages, neutrophils, and natural killer cells and recognize antibodies of infected cells or pathogens. | [7, 21] |

transmembrane domain, and an intracellular toll-interleukin 1 receptor (TIR) domain for downstream signaling. Toll-like receptors recruit TIR domain-containing adaptor molecules, including MyD88, TIRAP, TRIF, and TRAM, to activate downstream signaling pathways. The adaptor molecule, MyD88, is used by all TLRs except TLR3 and activates NF κ B to initiate the production of inflammatory cytokines. Multiple intracellular signaling pathways may be upregulated in response to the activation of TLR in addition to pro-inflammatory mediator production, including

apoptotic pathways. Both TLR2 and TLR4 are the most significant during bacterial mastitis infections and are primarily activated in response to gram-positive and gram-negative infections, respectively.

The recognition of LPS from gram-negative pathogens by TLR4 is facilitated by additional proteins, CD14, LPS-binding protein (LBP), and myeloid differentiation protein 2. Myeloid differentiation protein-2 is a secreted protein that is associated with the extracellular portion of TLR4 and is critical for LPS signaling. LPS-binding protein is an acute phase protein in serum that facilitates the subsequent binding of LPS to membrane bound CD14 (mCD14). In addition, LBP may facilitate the transfer of LPS to soluble CD14 (sCD14) aiding the complex recognition and activation in endothelial cells [40]. CD14 is a glycosylated phosphatidylinositol-anchored protein located on the membranes of monocytes, macrophages, and neutrophils. Cells lacking mCD14, such as endothelial and epithelial cells, utilize sCD14 present in serum and milk to aid in LPS recognition by TLR4. Soluble CD14 may compete with mCD14 for the binding of LPS to prevent activation of monocytes and macrophages [41]. Studies of murine mastitis revealed that treatment with recombinant bovine sCD14 not only resulted in the recruitment of neutrophils to the mammary gland, but also increased survival of mice following *E. coli* infusion [42, 43] [44]. Transgenic mice expressing sCD14 in milk also were shown to be less susceptible to edema induced by *E. coli* mastitis [45]. Infusion of a plant-derived recombinant bovine sCD14 following experimental bovine *E. coli* mastitis resulted in decreased viable bacterial numbers and decreased clinical severity [46]. Collectively, these studies support the contention that CD14 plays a central role in the pathogenesis of gram-negative pathogens. Strategies to increase sCD14 in milk may reduce the severity of coliform mastitis.

Inflammation

Inflammation is a critical component of the innate defense system that involves complex biological responses of vascular tissues to harmful stimuli such as bacterial pathogens. The inflammatory process is initiated by cells already present within the mammary tissues. Resident cells that express pattern recognition receptors are activated by bacterial PAMPs and release various inflammatory mediators including cytokines and eicosanoids that initiate the inflammatory cascade. These mediator molecules initially increase vasodilation that enhances blood flow. The permeability of blood vessels also changes, causing the leakage of plasma components (i.e., serum albumin, complement, and acute phase proteins) into localized areas of affected tissues resulting in edema. Cytokines and other mediator molecules act directly on vascular endothelial cells to enhance the adhesion and migration of leukocytes from the blood to the site of injury. Neutrophils are the predominant cell type to undergo extravasation during the early stages of inflammation. Neutrophils first marginate and then adhere to the local endothelium near the site of infection. Cytokines and eicosanoids stimulate adherent neutrophils to move between endothelial cells and pass across the basement membrane into the damaged tissue. The movement of neutrophils is facilitated by chemotaxis gradients created by inflammatory mediator molecules at the localized site of infection.

Eicosanoids

Eicosanoids can regulate several inflammatory processes such as vascular permeability, leukocyte infiltration, localized edema, and fever [47] and eicosanoid concentrations are increased in the milk and plasma of mastitic cows [48, 49]. Eicosanoid biosynthesis occurs from the oxygenation of fatty acids through the cyclooxygenase (COX), lipoxygenase (LOX), or P450

enzymatic pathways. Depending on the timing and magnitude of expression, certain eicosanoids can either enhance or resolve the inflammatory response. The COX pathway is composed of 2 major isoforms. COX1 is constitutively expressed in most tissues and synthesizes low levels of prostaglandins (PG), such as prostacyclin (PGI₂), that functions in the maintenance of normal physiological functions and vascular homeostasis. Conversely, COX2 is highly inducible in response to pro-inflammatory stimuli and it is primarily associated with the biosynthesis of pro-inflammatory mediators such as PGE₂, PGF₂ α and thromboxane B₂ (TXB₂). Increased PGE₂, PGF₂ α and TXB₂ concentrations in milk were detected in both experimental and natural cases of mastitis caused by *Strep. uberis* as well as other mastitis-causing pathogens [50-53]. Increased COX2 expression during the resolution of inflammation, however, is associated with the presence of metabolites, such as PGD₂ and 15 d-PGJ₂, which can inhibit leukocyte adhesion to endothelial cells and decrease cytokine expression by blocking NF κ B activation [54]. Non-steroidal anti-inflammatory drugs can inhibit PG biosynthesis by targeting COX activity and are used widely to treat a variety of inflammatory-based diseases including mastitis in dairy cows, but with variable results [55-58].

LOX is a heterogeneous family of non-heme enzyme dioxygenases with the ability to oxidize polyunsaturated fatty acids (PUFA). There are several different LOX isoforms including 5LOX and 15LOX where the nomenclature is defined by the capability of each enzyme to introduce molecular oxygen on a specific carbon of the fatty acid structure [59]. Metabolism of arachidonic acid by the 5LOX pathway gives rise to hydroxyl and hydroperoxy derivatives (5-hydroxyeicosatetraenoic acid (HETE) and 5-hydroperoxyeicosatetraenoic acid (HPETE), respectively), that are often elevated during inflammation. The 15LOX1 isoform is characterized as an inducible enzyme expressed in endothelial cells, epithelial cells, reticulocytes,

and macrophages with the ability to oxygenate PUFA during inflammation. The initial oxygenated product formed during arachidonic acid metabolism by 15LOX1 is 15HPETE, which is the biosynthetic precursor of 15HETE and other leukotrienes [60]. Increased 15LOX1 activity is recognized as an important factor in the development of certain inflammatory-based diseases such as atherosclerosis [61]. Metabolites of the 15LOX1 pathway enhanced intercellular adhesion molecule 1 (ICAM1) expression and monocyte adhesion in vessel walls during disease progression in humans [62, 63]. These data suggest that 15LOX1 may facilitate pathogenesis by enhancing the pro-inflammatory phenotype of endothelial cells. Increased expression of 15HPETE is found in bovine mammary endothelial cells as a result of selenium deficiency [64] and this metabolic product upregulates the expression of ICAM1 in other bovine endothelial cell types [65]. Conversely, evidence suggests that the LOX pathways also play a significant role in the biosynthesis of lipoxins (LX) that are a unique class of eicosanoids with dual anti-inflammatory and pro-resolving functions [66]. Relative to mastitis, an imbalance of LXA4:LTB4 occurs during chronic mastitis and was reportedly due to the dramatic reduction in LXA4 biosynthesis within infected mammary glands [52]. The gene expression of 15LOX1 is increased in mammary tissues in early lactation dairy cows [67], however, its contribution to mammary gland health or disease during this time period is unknown and should be a focus a future research.

Mammary Vascular Endothelium

The mammary vascular endothelium has received relatively little attention with regards to bovine mastitis despite the significant role endothelial cells play in the pathogenesis of inflammatory-based diseases. The vascular endothelium is composed of a single layer of cells lining blood vessels that provides a barrier between blood components and extravascular tissues.

Anatomical features of the mammary microvasculature were described extensively in rodents and to a lesser extent in the bovine [68]. The mammary capillary network forms a basket-like structure around alveoli and the endothelium lining these vessels is the primary site of exchange of metabolites from blood to tissue. The mammary gland depends upon an adequate supply of blood-derived nutrients and hormones to both initiate and sustain milk synthesis. Microscopic examination of human [69, 70] and rodent [71, 72] mammary glands suggest that the capillary endothelium is largely continuous, with some minor areas of fenestration. Rodent and bovine mammary gland capillaries exhibit numerous marginal folds and microvilli beginning from late pregnancy to peak lactation, thus increasing the surface area for the exchange of molecules from blood to tissue [71-74]. Despite the critical role of the vasculature to mammary physiology, its complex functions in milk-producing mammals, such as dairy cows, are not fully understood.

In addition to its role in supplying nutrients to the mammary gland, the mammary vascular system actively participates in the inflammatory response to infectious pathogens. Mammary vascular endothelial cells are activated in response to numerous stimuli released from cell populations in the mammary gland, bacterial toxins, reactive oxygen and nitrogen species, and potent lipid mediators [64, 75-78]. The endothelium responds in various ways including changes in vascular tone and blood flow to accommodate leukocyte slowing and migration, production of pro-inflammatory cytokines and adhesion molecules, and production of reactive oxygen and nitrogen species important in intracellular signaling [5]. Under normal physiological conditions the vascular endothelium is able to maintain its integrity. During prolonged or excess inflammation, however, a disturbance of endothelial homeostasis may occur, resulting in the loss of vascular barrier functions and the influx of serum components into lacteal secretions [17, 40, 79, 80]. Consequently, the loss of vascular integrity may contribute to the development of

severe or chronic mastitis. The outcome of new intramammary infections may be dependent upon the functional integrity of mammary endothelial cells.

Localized Cellular Components of Inflammation

Epithelial cells lining the teat canal, gland cistern and alveoli are among the first cells to recognize pathogens and participate in triggering an inflammatory response. Recently it was shown that the teat canal provides an early, active immune response to pathogens aside from the physical and chemical barrier [81, 82]. Rinaldi et al. (2010), reported rapid and intense immune gene changes within teat tissue following experimental *E. coli* infection. Genes shown to change within 12 h following infection were involved with pathways participating in the inflammatory response and leukocyte recruitment, antimicrobial peptide production, apoptosis, acute phase response, and bacterial recognition receptors [81]. Although the extent of participation of the teat canal in host defense is not completely known at this time, epithelial cells and leukocytes present within the teat end tissues have the capacity to respond to invading pathogens and may be important to the initiation and resolution of infection. Future studies that investigate how the teat tissues respond to pathogen invasion earlier than 12 h seem prudent in light of these recent findings.

Alveolar epithelial cells also are involved in bacterial recognition and initiation of the innate immune system. A bovine mammary epithelial cell line, MAC-T, was found to express both TLR2 and TLR4 following exposure to LPS [83]. Others showed that primary bovine mammary epithelial cells responded robustly to *E. coli*-induced activation of TLR4-dependent signaling pathways with the enhanced expression of pro-inflammatory cytokines such as TNF α and IL8 [84, 85]. Whereas *S. aureus* also could properly induce TLR2 signaling in mammary secretory

epithelial cells, the expression of TNF α and IL8 was muted due to inadequate NF κ B activation [84]. Bougam et. al. (2010) also found that some staphylococcal PAMPs (i.e. muramyl dipeptide (MDP) and lipoteichoic acid (LTA)) failed to increase the protein expression of pro-inflammatory cytokines in the culture supernatant of bovine mammary epithelial cells. However, they did find that MDP and LTA synergized to induce the production of several neutrophil chemoattractants by mammary epithelial cells that was dependent on NF κ B activation [86]. Bacterial pathogens can upregulate TLR on mammary epithelial cells as well as produce non-specific bactericidal factors, such as cytokines, chemokines, and β -defensins [87, 88]. Collectively, these studies suggest that mammary epithelial cells could differentially affect the overall inflammatory response depending on how they recognize and respond to different bacterial PAMPs. Therefore, the severity and duration of mastitis may be related not only to TLR expression, but also how TLR-induced signaling pathways become activated in mammary alveolar epithelial cells.

Milk somatic cells from healthy glands are primarily composed of macrophages, but also include lymphocytes, neutrophils, and mammary epithelial cells. These cells function to survey the mammary gland for invading pathogens. The composition of milk somatic cells changes upon bacterial recognition and infection development. A shift to a predominately neutrophil population in the mammary gland occurs following bacterial recognition and release of chemoattractants (i.e., complement components, cytokines and eicosanoids) by macrophages and epithelial cells. Neutrophils function to eliminate pathogens primarily by phagocytosis and intracellular killing. Neutrophils can kill pathogens by several antibacterial mechanisms: neutrophil extracellular trap (NET) formation, respiratory burst, antibacterial peptides and defensins. Following binding of complement components and Ig on neutrophil receptors, neutrophils are activated and initiate a

respiratory burst releasing high concentrations of oxidizing agents to kill ingested bacteria. Neutrophil granules also contain antibacterial peptides, such as cathelicidins, hydrolases, proteases, and lysozyme. The phagocytic and oxidative burst functions of neutrophils are drastically reduced in the presence of milk due to the ingestion of fat and casein [89]. Alternate neutrophil functions, such as the release of NETs, do not appear to be affected in the presence of milk [90]. Activated neutrophils release nuclear and granular material in a web-like fashion, called NETs, which physically trap bacteria. Bacterial entrapment by NETs contains the pathogen and places them in an environment with a high local concentration of antimicrobial agents released by neutrophils to enhance bacterial kill [91]. NETs may be important factors in neutrophil bacterial death during intramammary infection [90].

Macrophages, in addition to neutrophils, are responsible for bacterial phagocytosis. Upon bacterial recognition, macrophages activate the immune system by release of cytokines and other pro-inflammatory mediators and facilitate the innate immune response, including neutrophil migration and bactericidal functions. Macrophages appear to play a role in *E. coli* mammary epithelial invasion and colonization in a murine model of mastitis [92]. Addition of TLR4 expressing macrophages into milk spaces of TLR4 depleted mice was able to significantly decrease bacterial invasion of epithelial cells. Additionally, macrophages present bacterial antigens to lymphocytes for initiation of a specific immune response. Macrophages have a role in resolution of infection as well by phagocytizing aged neutrophils to minimize cellular and tissue damage by toxic antibacterial components released by neutrophils [93]. Macrophages are involved at multiple levels during mastitis and are indispensable to bacterial recognition and elimination.

Cellular communication between somatic and epithelial cells propagates the innate

immune response. Maintaining the health of each cell type is necessary to rapidly resolve infection without causing irreversible mammary gland damage that will affect subsequent milk production.

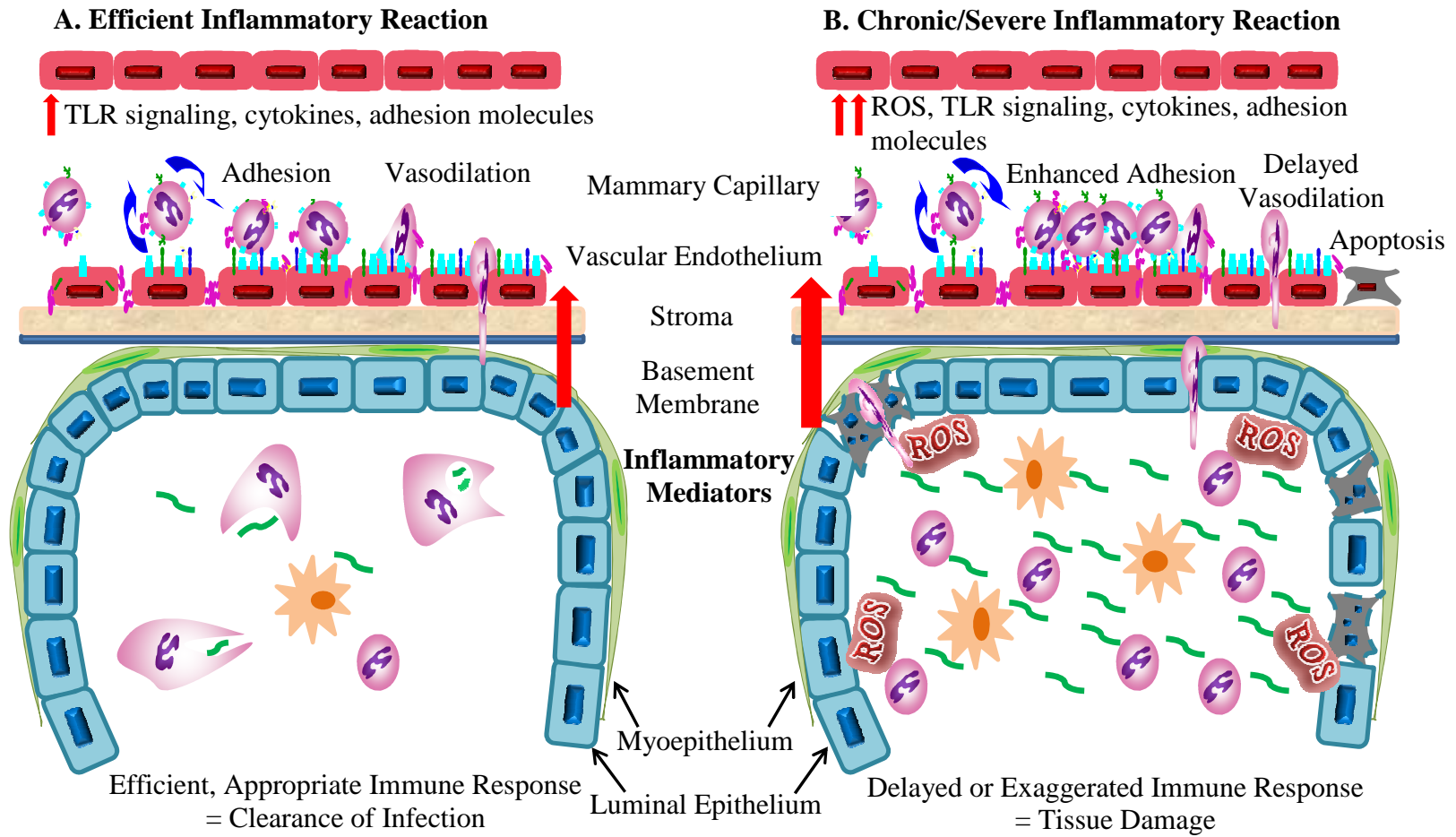
Immunopathogenesis

Mammary gland innate and adaptive immune responses are complex, interconnected, and crucial for defense against mastitis-causing pathogens. There are some situations, however, when some mammary immune responses may facilitate disease pathogenesis and lead to deleterious consequences. There are several ways that host defense mechanisms can fail and subsequently lead to immunopathogenesis. The inability of local mammary gland defenses to adequately detect and eliminate pathogens can facilitate wide-spread immune evasion and the development of chronic inflammation. Conversely, the uncontrolled recruitment and activation of inflammatory cell types, especially neutrophils and macrophages, can result in the accumulation of toxic levels of cytokines, lipid mediators, and reactive oxygen species that can severely damage host tissues with possible systemic complications leading to death. The delicate balance between a robust immune response needed to eliminate mastitis-causing pathogens during the early stages of infection and the generation of anti-inflammatory mechanisms needed to restore mammary gland immune homeostasis influence the extent of immunopathology and the outcome of disease (Figure 1) (see below).

An effective immune response occurs when pathogens are promptly detected and eliminated without excessive or prolonged inflammation. Various bacterial species, however, are capable of epithelial invasion and colonization and contribute to the severity and chronicity of mastitis. Subclinical, chronic *S. aureus* mastitis, for example, is associated with a suboptimal

Figure 1 (see p. 25). The mammary gland immune response to bacterial invasion results in prompt elimination and resolution of infection or delayed detection, rapid bacterial replication/growth, overproduction of inflammatory mediators and subsequent tissue damage. **a** Resident milk macrophages and neutrophils phagocytize invading pathogens. The immune system is activated following bacterial recognition by mammary macrophages and epithelial cells. A regulated release of cytokines and inflammatory mediators recruit leukocytes from the vasculature to the infection site and facilitate prompt bacterial removal with minimal damage to surrounding tissue. **b** Delayed or exaggerated immune responses, as occurs during the periparturient period, results in chronic or severe inflammation with subsequent tissue damage. Altered leukocyte migration or function promotes bacterial propagation. Excess release of bacterial toxins, inflammatory mediators, and leukocyte proteases, lysozymes and reactive oxygen species results in the breakdown of the blood-milk barrier and leads to irreversible epithelial and endothelial damage and ultimately lost milk production or death. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

Figure 1 (cont'd)



innate immune response and the ability to evade adaptive mammary gland defenses. Experimental intramammary infection with *S. aureus* decreased expression of intracellular receptors, NOD1 and NOD2, in bovine teat canal tissue that may be important to detection of this pathogen because of its ability to invade epithelial cells [82]. Cytokine gene expression for IL1 β and TNF α did not significantly change across tissues within the mammary gland, consistent with other reports of subclinical *S. aureus* infection [82]. Gunther et al. (2011) demonstrated an inability of *S. aureus* to upregulate inflammatory genes through TLR MyD88-dependent activation in primary bovine mammary epithelial cells. Both *S. aureus* and *E. coli* were able to activate TLR2/4 signaling in mammary epithelial cells; however, neither *S. aureus* nor its active component LTA were able to activate NF κ B. Conversely, a strong induction by *E. coli* and LPS was able to induce gene expression of IL8 and TNF α by an NF κ B-mediated pathway (Yang et al., 2008). Some suggest that the lack of NF κ B signaling by staphylococcal PAMPs may be due to the increased production of TGF β in the mammary gland by *S. aureus*, thereby blocking the MyD88-dependent signaling as found by Naiki et al. (2005). Additionally, exotoxin production is an *S. aureus* virulence factor that facilitates immune evasion. Leukotoxins form pores in leukocytes inhibiting bacterial phagocytosis and permitting bacterial persistence within the mammary gland [94]. Causing a suboptimal immune response may be a bacterial adaptation to evade host bactericidal responses and promote bacterial survival within the mammary gland. Decreased ability to recognize pathogens alone or in combination with a reduced immune response to bacterial invasion may contribute to the ability of some mastitis-causing pathogens to evade the innate immune response and therefore result in subclinical, chronic mastitis.

Alternate to bacterial immune evasion with weakened host responses is an excess inflammatory response that causes local tissue damage or systemic consequences. Severe coliform

infections are associated with an exacerbated inflammatory response following rapid bacterial growth. Mammary tissue damage may occur as a consequence of the release of bacterial toxins or bactericidal components from infiltrating inflammatory cells. Lipopolysaccharide triggers cellular signaling and upregulation of pro-inflammatory factors that, when produced in excess, may ultimately result in cellular damage or death [95]. For example, activation of NF κ B in lactating murine mammary epithelium decreased milk production and was associated with apoptosis in involuting mammary epithelial cells [96]. A study by Long et al. (2001) reported increased expression of apoptotic factors in mammary tissue following experimental *E. coli* infection and is consistent with the findings of others that mammary tissue damage is induced by apoptosis or necrosis [97, 98]. Excess LPS release and NF κ B activation during coliform mastitis may have an impact on decreased milk production through mammary epithelial apoptosis. Additionally, massive leukocyte influx during acute mastitis results in the release of reactive oxygen species, proteases and lysozymes causing indiscriminate damage to surrounding mammary tissue [97]. Mammary epithelial cell damage occurs secondary to activated leukocytes and release of reactive oxygen species such as myeloperoxidase and free radicals. Antioxidant supplementation reduced the severity of PMN cytotoxicity in mammary epithelial cells as well as MAC-T cells [99]. Preventing excessive leukocyte influx into the mammary gland will minimize mammary tissue damage and milk loss.

The vascular endothelium is a target of LPS released from replicating or dying gram-negative bacteria and is implicated in the pathogenesis of sepsis. Endothelial cells can respond to LPS ligation with the TLR4 signaling complex by either production of pro-inflammatory factors or initiation of endothelial apoptosis. Excess LPS can result in enhanced production of IL1, IL6 and TNF α . Many of the severe clinical symptoms of coliform mastitis are attributed to the actions of TNF α [100, 101]. Vascular dysfunction may be a result of excess exposure of the endothelium to TNF α resulting from

enhanced leukocyte adhesion and transmigration, increased reactive oxygen and nitrogen species production, and ultimately apoptosis [102]. Although some cell types are susceptible to the apoptotic effect of TNF α , bovine mammary endothelial cells appear to have some resistance [75]. Recent studies suggest that upregulation of anti-apoptotic proteins may be a protective mechanism [103]. Prolonged exposure to high doses of TNF- α or in combination with other cytokines may leave bovine mammary endothelial cells vulnerable to apoptosis.

Vascular oxidative stress is associated with many human inflammatory-based diseases and may be another factor contributing to the severity of mastitis. An imbalance between reactive oxygen species and antioxidant production or availability results in oxidative stress. Excess reactive oxygen species production results in direct cellular and tissue damage from unstable molecules interacting with cellular nucleic acids, lipids, and proteins or may indirectly trigger intracellular signaling and promote a pro-inflammatory or pro-apoptotic cellular phenotype. Oxidative stress occurs in transition dairy cattle when several inflammatory-based diseases, such as mastitis, are most prevalent [104, 105]. Vitamin E and selenium, micronutrients with antioxidant functions, were related to decreased mastitis duration and severity in the periparturient period [106, 107]. Antioxidant supplementation decreases mammary epithelial cell cytotoxicity as previously discussed and also may protect the vascular endothelium against direct or indirect oxidant-induced damage.

Impacts of reactive oxygen species on the endothelium include increased permeability, increased adhesion of leukocytes, as well as altered cellular signaling and redox-regulation of transcription factors [108]. Neutrophil migration is a multi-step process that requires the upregulation of adhesion molecules on both the immune cell and the endothelium. Adhesion molecule gene expression changes in bovine mammary tissue during the transition period and is

positively correlated with expression of several antioxidant enzymes, including selenoproteins [67]. Moreover, delayed neutrophil migration is associated with the severity of coliform mastitis [109]. Adhesion molecules in neutrophils have been studied extensively in the context of mastitis, however, little is known regarding their role in endothelial cells [110]. Selenium supplementation increased the speed of neutrophil migration into the bovine mammary gland during *E. coli* infection compared to selenium-deficient animals [106]. In bovine mammary and aortic endothelial cells, selenium deficiency causes oxidative stress and results in greater ICAM1 expression and neutrophil adherence [65, 78]. An immediate oxygenation product of 15LOX1 metabolism of arachidonic acid, 15-HPETE, upregulates ICAM1 expression in selenium- deficient bovine aortic endothelial cells [65]. Interestingly, 15LOX1 mRNA expression is significantly upregulated in early lactation cows compared to pre-partum cows [67]. Neutrophil-endothelial adhesion initiates intracellular signaling through adhesion molecules, such as ICAM-1, and enhanced expression of this adhesion molecule is associated with pathologic pro-inflammatory conditions [111]. Increased expression of adhesion molecules and tight neutrophil-endothelial binding may indicate an inability to rapidly migrate to the infection site, thereby allowing bacterial growth and endotoxin release contributing to the severity of coliform mastitis.

Transmigration of leukocytes across the endothelium is necessary for mounting a proper immune response to infection. However, activation of immune cells in the process may have negative consequences on endothelial integrity. For example, activated neutrophils are able to kill pathogens by NET formation [112]. It is possible that release of these substances during the transmigration process may inadvertently cause endothelial damage. Activated human umbilical vein endothelial cells stimulated NET formation that consequently resulted in endothelial injury [113]. Excess neutrophil migration, NET formation and endothelial activation may contribute to

endothelial damage during mastitis.

The speed of leukocyte influx into the mammary gland affects the outcome of mastitis. Downregulation of neutrophil adhesion molecules is known to contribute to periparturient immunosuppression and increased mastitis susceptibility [114]. Other mechanisms may be involved in the relative rapidity of leukocyte migratory responses, such as activation of the uroplasminogen cascade that is involved with breakdown of the basement membrane and extracellular matrix required for diapedesis and migration to the infection site. Ovine blood monocytes and neutrophils taken from healthy and mastitic ewes infected with *Strep. agalactiae* were analyzed for changes in genes associated with the plasminogen activation cascade [115]. Consistent with this theory, cells from mastitic ewes showed increased uroplasminogen and uroplasminogen receptor expression. Further research in this area is required to fully understand the mechanisms for impaired leukocyte migratory responses and increased susceptibility to mastitis.

Other factors contribute to the severity of mastitis and may involve complement components. High concentrations of complement component C5a are produced during sepsis [116]. In a recent study, C5a induced TLR4 signaling in bovine neutrophils and resulted in the production of IL8 in the absence of other stimulatory factors [117]. The ability of complement components to initiate TLR4 signaling may contribute to the severity of mastitis during prolonged inflammation. In contrast, C5a was not detected in milk following intramammary *S. aureus* infection [118]. This likely is secondary to a suboptimal immune response and decreased serum proteins in the mammary gland following infection. Human-specific *S. aureus* strains possess additional virulence factors, such as staphylococcal complement inhibitor that blocks C3b formation protecting it from neutrophil phagocytosis [119]. Future research in this area may reveal the significance of complement to mastitis pathology.

Conclusions

Mammary gland innate and adaptive immune responses are complex and highly interconnected. Optimal host defenses against mastitis-causing pathogens occur when mammary immune mechanisms are tightly regulated to effectively eliminate the injurious insults and return the immune system to homeostasis. Rapid resolution of intramammary infections will eliminate bystander tissue damage and prevent any noticeable changes to milk quantity or quality. Some mastitis-causing pathogens, however, have intrinsic properties that make the efficient elimination by the immune system difficult, and attempts by local mammary gland defenses to achieve control often results in significant tissue damage and reduced milk production. Whereas antibiotic therapy remains the mainstay for the treatment of mastitis in both human and veterinary medicine, there is a need for alternative and adjunct therapeutic options that target host immune responses. The challenge is to selectively down-modulate harmful host responses without diminishing beneficial responses that facilitate elimination of invading pathogens. In contrast to antimicrobial drugs used to treat mastitis, strategies that target host responses will minimize the risk that drug resistant bacteria will emerge.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number #2007-35204-18463, the Agriculture and Food Research Initiative Competitive Grants Program, grant number #2011-67015-30179, and by an endowment from the Matilda R. Wilson Fund (Detroit, MI).

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CHAPTER 2

EVALUATION OF ANTIOXIDANT AND PROINFLAMMATORY GENE EXPRESSION IN BOVINE MAMMARY TISSUE DURING THE PERIPARTURIENT PERIOD

By

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Published in JOURNAL OF DAIRY SCIENCE, February 2009, 92:589-98, *Evaluation of Antioxidant and Proinflammatory Gene Expression in Bovine Mammary Tissue During the Periparturient Period*, by S. L. Aitken, E. L. Karcher, P. Rezamand, J. C. Gandy, M. J. VandeHaar, A. V. Capuco, and L. M. Sordillo

ABSTRACT

The incidence and severity of mastitis can be high during the period of transition from pregnancy to lactation when dairy cattle are susceptible to oxidative stress. Oxidative stress may contribute to the pathogenesis of mastitis by modifying the expression of proinflammatory genes. The overall goal of this study was to determine the relationship between critical antioxidant defense mechanisms and proinflammatory markers in normal bovine mammary tissue during the periparturient period. Mammary tissue samples were obtained from 12 cows at 35, 20, and 7 d before expected calving and during early lactation (EL, 15 to 28 d in milk). Enzyme activities for cytosolic glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase were relatively low during the dry period, but increased during EL, whereas activity of thioredoxin reductase 1 did not change significantly as a function of time. In contrast, gene expression for these antioxidant selenoproteins and for heme oxygenase-1 gradually decreased as parturition approached and then increased during EL. The expression of intercellular vascular adhesion molecule-1 and vascular cell adhesion molecule-1 followed a similar trend where mRNA abundance gradually declined as parturition approached with a slight rebound in EL. Gene expression of the pro-oxidant, 15-lipoxygenase 1, which is known to increase during times of oxidative stress, also increased dramatically in mammary tissue from EL cows. Expression of the proinflammatory cytokines, IL-1 β , IL-6, and IL-8 did not change significantly during the periparturient period. Strong positive correlations were found between several antioxidant enzymes (cytosolic glutathione peroxidase, thioredoxin reductase 1, and heme oxygenase-1) and vascular adhesion molecules (intercellular vascular adhesion molecule-1, vascular cell adhesion molecule-1) suggesting a protective response of these antioxidants to an enhanced proinflammatory state. Ability to control oxidative stress through manipulation

of key antioxidant enzymes in the future may modify the proinflammatory state of periparturient cows and reduce incidence and severity of some diseases such as mastitis.

Key words: mammary gland, periparturient period, oxidative stress, inflammation

INTRODUCTION

Dairy cattle experience increased incidence of disease, such as mastitis, during the periparturient period when host defense mechanisms are compromised. Several physiological changes occur in periparturient dairy cows that may contribute to altered immune and inflammatory responses including overall nutritional status, energy metabolism, and changes in hormone profiles (Sordillo, 2005). Another factor contributing to compromised immunity and increased incidence of disease may be the progressive development of oxidative stress (Miller et al., 1993; Sordillo, 2005; Sordillo and Aitken, 2009). Oxidative stress occurs when there is an imbalance between production of reactive oxygen species (**ROS**) and reduced host antioxidant capabilities (Valko et al., 2007). During the periparturient period, dairy cows experience extreme shifts in cellular metabolism as the mammary gland prepares for the ensuing lactation (Sordillo and Aitken, 2009). The onset of copious milk synthesis and secretion requires large amounts of molecular oxygen for aerobic metabolism. Free radicals are formed as a normal end product of cellular metabolism arising from either the mitochondrial electron transport chain or from stimulation of NADPH2 (Valko et al., 2007). Therefore, the considerable increase in oxygen requirements during heightened metabolic demands results in augmented rates of ROS production. Indeed, several recent studies showed that production of excess ROS in the peripheral blood of dairy cattle during the periparturient period can overwhelm certain antioxidant defenses, resulting in increased oxidative stress (Bernabucci et al., 2005; Castillo et al., 2005; Sordillo et al., 2007).

Dairy cattle have several known endogenous antioxidant defense mechanisms that can counteract the harmful effects of ROS accumulation, but it is the selenium-dependent selenoproteins that have been studied extensively with respect to mammary gland health

(Sordillo and Aitken, 2009). Selenium supplementation reduces the incidence and severity of mastitis (Smith et al., 1984). The beneficial effects of selenium supplementation are thought to be due to the actions of certain antioxidant selenium-dependent enzymes, which have a selenocysteine residue incorporated into their active site. These selenoenzymes function in part by reducing harmful ROS and other fatty acid hydroperoxides to less-reactive waters and alcohols, respectively. Approximately 25 selenoproteins have been identified in humans (Papp et al., 2007), but the selenoprotein most often associated with antioxidant functions in cattle is cytosolic glutathione peroxidase (**GPX1**; Smith et al., 1997). Indeed, GPX1 activity is often used as a diagnostic tool when assessing the selenium status of dairy cows or as an indicator of increased ROS accumulation. Several recent studies, however, now document the presence of other selenoprotein enzymes in the blood and tissues of dairy cattle that may play an important role in controlling oxidative stress, including thioredoxin reductase 1 (**TrxR1**) and phospholipid hydroperoxide glutathione peroxidase (**GPX4**; Bruzelius et al., 2007; Sordillo et al., 2007). In addition to their ROS scavenging functions, many selenoproteins are essential for regulating cellular redox status and influencing the expression of redox-regulated genes. For example, TrxR1 regulates expression of other important antioxidant defenses such as heme oxygenase 1 (**HO-1**), which is upregulated in response to ROS and converts the pro-oxidant heme into bilirubin, carbon monoxide, and iron (Balla et al., 2007). Although HO-1 has not been studied in dairy cattle in vivo, HO-1 is upregulated in cultured bovine aortic endothelial cells under oxidative stress conditions following reduced TrxR1 activity (Trigona et al., 2006). Despite significant evidence supporting a role for antioxidants in enhancing resistance to mastitis (Sordillo and Aitken, 2009), there is no information as to how the expression of specific antioxidant defenses change in mammary tissues during the periparturient period when dairy

cattle experience increased oxidative stress.

There are several human inflammatory-based diseases that occur as a consequence of oxidative stress, including cardiovascular disorders, diabetes, and cancer (Valko et al., 2007; Bonomini et al., 2008). The pathologies of these diseases may result from the enhanced expression of redox regulated proinflammatory factors such as eicosanoids and cytokines. For example, the metabolism of arachidonic acid through the 15-lipoxygenase 1 (**15-LOX1**) pathway causes the formation of 15-hydroperoxyeicosatetraenoic acid (**15-HPETE**), which is enhanced during oxidative stress (Cao et al., 2000). Increased 15-HPETE concentration in tissue and cells is associated with enhanced expression of certain proinflammatory genes such as intercellular adhesion molecule-1 (**ICAM-1**) and vascular cell adhesion molecule-1 (**VCAM-1**) (Bonomini et al., 2008). Vascular adhesion molecules are essential for transendothelial leukocyte migration to the site of infection. Enhanced expression of either ICAM-1 or VCAM-1, however, can lead to pathologic proinflammatory conditions (Radi et al., 2001; Sordillo et al., 2008). Relative to dairy cattle health, oxidative stress enhanced 15-LOX1 activity and accumulation of 15-HPETE in bovine endothelial cells and caused a significant increase in ICAM-1 expression (Sordillo et al., 2008). The expression of VCAM-1 protein in bovine mammary tissues also was reported to increase significantly during colostrogenesis when dairy cattle are known to experience oxidative stress (Hodgkinson et al., 2007).

Oxidative stress increases the expression of acute phase cytokines that also can exacerbate tissue damage during severe inflammatory responses (Cuschieri and Maier, 2007). proinflammatory cytokines are thought to play an important role in the mammary gland's response to a variety of mastitis-causing organisms including *Staphylococcus aureus*, *Streptococcus uberis*, and *Escherichia coli* (Oviedo-Boyso et al., 2007). Indeed, numerous

studies showed that tumor necrosis factor- α (TNF- α), IL-1 β , IL-6, and IL-8 were linked with the severity of coliform mastitis during the periparturient period when dairy cattle experience oxidative stress (Oviedo-Boyso et al., 2007). Expression of TNF- α from isolated mononuclear cells in either peripheral blood or supramammary lymph nodes was greater in the periparturient period compared with mid to late lactation (Sordillo et al., 1995). An inverse relationship between TrxR1 activity and TNF- α production by peripheral blood mononuclear cells obtained from cows experiencing oxidative stress also was recently reported (O'Boyle et al., 2006).

Collectively, these previous data support the contention that reduced antioxidant capacity and enhanced proinflammatory status may be related and that this relationship may play a role in dairy cattle disease susceptibility during the periparturient period. However, there is no information linking the antioxidant and inflammatory status in normal bovine mammary tissue during the periparturient period when dairy cattle are susceptible to increased incidence of disease. Such information may be useful in finding ways to decrease the incidence or severity of periparturient cow diseases. The goal of this study was to determine the relationship between expression of critical antioxidant defense mechanisms and proinflammatory markers in normal mammary tissue during the periparturient period when dairy cattle experience oxidative stress.

Materials and Methods

Animals

Use of animals for these investigations was approved by the Beltsville Agricultural Research Center's Animal Care and Use Committee. Mammary tissue samples were obtained from Holstein dairy cows in the dry period (35 to 7 d before expected parturition date) and early

lactation (**EL**). Multiparous (second or greater lactation) cows were dried off 60 d before expected calving date at which time average milk yield (mean \pm SEM) was 10.2 ± 3.00 kg/d (data not shown). One week before initiation of the study, SCC and bacteriological analyses were performed on foremilk samples aseptically collected. All cows were free of mastitis. Dry cows were killed at the USDA abattoir (Beltsville, MD) at -35 d ($n = 3$), -20 d ($n = 3$), and -7 d ($n = 3$) before expected calving date and mammary tissue samples were obtained after removing extra-parenchymal tissues and processed as described previously (Capuco et al., 1997). Mammary tissue samples were also obtained from lactating cows that were between 15 and 28 DIM (EL; $n = 3$); these samples were parenchymal biopsies as described previously (Sharma et al., 1994). All tissue samples were immediately frozen and stored at -70°C .

Quantitative real-time PCR

Total RNA was isolated from mammary tissue using the RNeasy Lipid Tissue Mini Kit from Qiagen (Valencia, CA). The RNA was DNase digested using the RNase-Free DNase Set (Qiagen) and cDNA was then synthesized using the High Capacity cDNA reverse transcriptase kit with RNA inhibitor (Applied Biosystems, Foster City, CA). All of the primers used in the present study were derived from the *Bos taurus* genome (GenBank) and are shown in Table 2. Real-time quantitative PCR (**qPCR**) was carried out in a 7500 Fast Real-Time PCR system (Applied Biosystems) using custom-designed TaqMan minor groove binding probes from Applied Biosystems. The PCR was performed in triplicate using a 20- μL reaction mixture per well, containing 10 μL of TaqMan Fast Universal PCR Master Mix (2 \times , Applied Biosystems), 1 μL of (20 \times) Custom TaqMan Gene Expression Assay Mix (Applied Biosystems), 100 ng of cDNA, and the balance was nuclease-free water. Targeted genes were amplified with the

reaction mixture described above. A (20×) Custom Taqman Gene Expression Assay Mix for bovine ribosomal protein 9 (**RPS9**) was generated by Applied Biosystems as an endogenous control (Bionaz and Loor, 2007). The thermal cycling conditions for fast 2-step PCR were used: stage 1 enzyme activation, 95°C for 20 s; stage 2, 95°C for 3 s; stage 3, 60°C for 30 s; with 40 replications through stages 2 and 3. Quantification was carried out with the relative quantification method (Livak and Schmittgen, 2001). The abundance of target genes, normalized to RPS9 (as the internal control) and relative to a calibrator, are illustrated by $2^{-\Delta\Delta C_t}$, where C_t is the cycle number at which the fluorescence signal of the product crosses an arbitrary threshold set with exponential phase of the PCR and $\Delta\Delta C_t = (C_{t_{\text{target gene unknown sample}}} - C_{t_{\text{RPS9 unknown sample}}}) - (C_{t_{\text{target gene calibrator sample}}} - C_{t_{\text{RPS9 calibrator sample}}})$. Averaged abundance of target genes at 35 d before expected calving date was considered as the calibrator.

GPX1 and GPX4 Activities

Mammary tissue samples (−35 d, n = 3; −20 d, n = 3; −7 d, n = 3; and EL, n = 3) were examined for both GPX1 and GPX4 activities. Mammary tissue samples were weighed, suspended in a collecting buffer (pH 7.4) containing 263 mM sucrose, 21 mM Trizma hydrochloride, and 0.1% Triton X-100 (Sigma, St. Louis, MO), and homogenized on ice for 1 min using a Polytron homogenizer (Kinematica Inc., Bohemia, NY). Samples were then centrifuged at $13,000 \times g$ for 20 min at 4°C, after which supernatants were collected. The activities of GPX1 and GPX4 in supernatants obtained from mammary tissue samples were determined as described previously (Sordillo et al., 1998). In the coupled enzymatic assays, while oxidation of NADPH2 was spectrophotometrically monitored, enzyme activities were

determined using either hydrogen peroxide or phosphatidylcholine hydroperoxide as the substrates (for GPX1 and GPX4 activities, respectively). One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 micromole of NADPH₂ per minute.

Data were normalized per milligram of total protein content of tissue supernatants.

TrxR1 Activity

Mammary tissue samples (n = 3 per time point) were weighed, suspended in a collecting buffer (pH 7.25) containing 1× PBS with 0.02 μM EDTA (Sigma), and homogenized on ice for 1 min using a Polytron homogenizer. Samples were then centrifuged at 13,000 × g for 20 min at 4°C, after which supernatants (1 mL) were concentrated using a centrifugal filter device (Millipore, Billerica, MA) with membrane nominal molecular weight limit of 30,000 Da at 3,273 × g at 4°C for approximately 10 min reaching a final volume of 500 μL. Enzyme activity was determined using the standard insulin-based method previously developed (Holmgren and Bjornstedt, 1995). Each well of a 96-well plate contained 33 μL of a reaction mixture consisting of 0.08 M HEPES, 3.03 mM EDTA, 0.6048 mM of insulin, and 0.61 mM of NADPH₂. Samples were run in the presence and absence of 16 μM *E. coli* thioredoxin (Sigma). The reaction was started by addition of 9 μL of tissue supernatants. Following a 60-min incubation at 37°C, the reaction was stopped by addition of 150 μL of stopping buffer consisting of 1.0 mM 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB)/6 M guanidine hydrochloride in 0.2 M Tris-HCl, pH 8.0. The absorbance was read at 415 nm. The reaction without thioredoxin was subtracted from the thioredoxin-dependent reaction and TrxR activity was expressed as A₄₁₅ units × 1,000/(min × mg protein) (Hill et al., 1997).

Statistical Analyses

All statistical analyses were conducted using SAS, version 9.1.2 for Windows (SAS Institute Inc., Cary, NC). Pearson correlation coefficients (r) were computed to determine relationships between antioxidants and proinflammatory markers and adhesion molecules. The effect of lactation stage (35, 20, 7 d before expected calving date and EL) on the relative abundance of mRNA and antioxidant enzyme activities of the mammary tissue was tested by the MIXED procedure of SAS. Cow was considered as a random factor. Protected least significant difference was used to compare least squares means and data are reported as least squares means \pm standard error of the means. Significant differences were declared at $P \leq 0.05$.

Results and Discussion

Antioxidant Defense

The incidence and severity of mastitis can be high during the periparturient period when dairy cattle are known to experience oxidative stress (Bernabucci et al., 2005; Castillo et al., 2005; Sordillo et al., 2007). Selenium supplementation to periparturient cows reduces the incidence and severity of mastitis probably through the actions of selenoproteins (Smith et al., 1984). Cytosolic glutathione peroxidase is the predominant intracellular form of glutathione peroxidase and is the most extensively studied selenoprotein in dairy cattle (Smith et al., 1997). It converts ROS to less reactive metabolites and thus protects tissues against oxidative damage (Papp et al., 2007). In the current study, GPX1 mRNA abundance was highest at 35 d before expected calving, gradually declined as parturition approached, but rebounded slightly during EL ($P < 0.03$; Figure 2A). Enzyme activity for GPX1 in mammary tissue remained relatively low during the dry period and increased during EL ($P < 0.02$; Figure 2B). An increase in GPX1

activity during EL may be due to a cytoprotective response against oxidative damage that occurs during the onset of copious milk synthesis and secretion. Changes in GPX1 enzyme activity observed in this study are consistent with previous reports in plasma and whole blood where GPX activity increased at calving and during EL (Bernabucci et al., 2005; Sordillo Sordillo et al., 2007). The disparate shifts in GPX1 mRNA and enzyme expression may be attributable to several factors. The expression of GPX1 mRNA is particularly sensitive to any changes in ROS accumulation, such that increases in GPX1 mRNA are an excellent indicator of oxidative stress. Therefore, real-time qPCR, as used in this study, may permit enhanced detection of alterations in GPX1 mRNA abundance during periods when ROS concentrations may fluctuate but overall changes in GPX1 enzymatic activity may be minor. Furthermore, enzyme activity of GPX1 can be influenced by other factors within the tissue microenvironment such as the availability and/or oxidized status of key substrates such as glutathione. Therefore, disparate trends in GPX1 mRNA expression and GPX1 enzymatic activity also may be explained by changes in the redox status of tissues at the time of sample collection.

Phospholipid hydroperoxide glutathione peroxidase (GPX4) is present in cytosolic, nuclear, and mitochondrial membranes of the cell. This enzyme has the unique ability to reduce lipid peroxides as well as soluble hydroperoxides (Papp et al., 2007). This study defines, for the first time, changes in GPX4 mRNA and enzyme activity across the periparturient period. Expression of GPX4 mRNA gradually declined as parturition approached, and increased to maximal abundance during EL ($P < 0.05$; Figure 3A). The GPX4 enzyme activity, however, followed a similar pattern as GPX1 activity and was greatest in EL compared with all other time points ($P < 0.01$; Figure 3B). Although a similar trend for GPX1 and GPX4 enzyme activities was demonstrated in the current study, the mRNA expression of GPX4 declined 37% between 35

and 7 d prepartum, whereas the mRNA expression of GPX1 declined by 56% during the same time period. In contrast to GPX1, the enzyme activity of GPX4 is more slowly depleted and tends to be conserved during periods of selenium deficiency (Bruzelius et al., 2007).

Furthermore, knocking out the GPX4 gene in mice results in embryonic lethality, whereas GPX1 knockout mice develop normally (Brigelius-Flohe, 2006). Based on these data, we suggest that GPX4 plays a vital role for countering oxidative stress in the periparturient dairy cow.

Similar to GPX1, TrxR1 is present in the cytosol and is capable of the direct reduction of lipid hydroperoxides and hydrogen peroxide (Papp et al., 2007). As observed for GPX1 and GPX4, TrxR1 mRNA expression in mammary tissue declined as parturition approached and was lowest at d -7 ($P < 0.01$; Figure 4A). Thus, the mRNA expression values for all 3 selenoproteins were lowest in mammary tissue at 7 d before expected calving. In contrast to the enzymatic activities for the other selenoproteins examined, TrxR1 activity did not change relative to time of calving (Figure 4B). Perhaps this is partly because the TrxR1 enzymatic activity in mammary tissues was extremely low regardless of lactation stage. Indeed, the relative mammary tissue TrxR1 activity was lower when compared with even the lowest standard that was within the reliable detection limits of the enzymatic assay used in this study. Sordillo et al. (2007) demonstrated TrxR1 activity to be decreased in peripheral blood mononuclear cells (PBMC) isolated from dairy cows at 3 wk postpartum compared with prepartum values: however, this systemic decrease may not reflect changes occurring locally within mammary tissue. Results from Bruzelius et al. (2007) suggest that TrxR1, although not as sensitive as GPX1, is sensitive to changes in selenium status as TrxR1 activity was positively correlated to selenium concentrations in their study. However, TrxR mRNA expression and enzyme activity in rat aortas have demonstrated that other regulatory factors may be more important for TrxR activity

other than selenium status such as relative ROS accumulation (Wu et al., 2003).

Although the antioxidant potential of various selenoproteins were defined, we also were interested in evaluating the expression of the non-selenium-dependent antioxidant, HO-1, because of its importance in counteracting oxidative stress in human disease. Heme oxygenase 1 is the rate-limiting enzyme in heme degradation and is responsible for catalyzing the reaction that produces biliverdin, ferritin, and carbon monoxide from free heme (Balla et al., 2007). This is important because free heme is cytotoxic to cells and promotes inflammation. Biliverdin and ferritin are both substances with antioxidant properties. This is the first study to report the expression of HO-1 transcripts in mammary tissue of periparturient dairy cows; however, HO-1 has been shown to be regulated by selenoproteins in bovine endothelial cell cultures (Trigona et al., 2006) and can also participate in the inflammatory process (Wagener et al., 1999). Expression of HO-1 was highest at d -35 relative to all other time points ($P < 0.01$) (Figure 5). Unlike our findings for GPX4 and TrxR1, the rebound in HO-1 mRNA in EL tissues was not statistically significant. The lack of an increase in HO-1 expression at parturition may indicate an inadequate response of additional protective antioxidants such as bilirubin. Although not measured in this study, bilirubin levels decline at calving and continue to decline during the first month of lactation (Bionaz et al., 2007). The results of HO-1 mRNA expression are supportive of previous reports demonstrating the inability of periparturient dairy cows to adequately respond to oxidative stress (Bernabucci et al., 2005; Castillo et al., 2005; Sordillo et al., 2007).

Proinflammatory Gene Expression

Several previous studies have documented changes in proinflammatory cytokine expression around the time of calving in infected mammary glands. For example, increased

expression of proinflammatory cytokines, including IL-1 β , IL-6, IL-8, and TNF- α have been linked to the pathology of acute mastitis during the periparturient period (Oviedo-Boyso et al., 2007). Macrophages are the main immune cell type present in milk and tissues of healthy, lactating mammary glands and can facilitate neutrophil recruitment via release of various cytokines such as interleukins and TNF- α , at the initiation of inflammation. In the current study, gene expression was detected for IL-1 β , IL-6, and IL-8, but mRNA abundance did not change significantly during the periparturient period (Table 3). The trends for IL-6 and IL-8 mRNA abundance were to decline through involution and increase slightly during EL. A declining trend in gene expression of TNF- α , however, was detected from 20 to 7 d before expected parturition and mRNA abundance remained low through EL ($P < 0.05$). Sordillo et al. (1995) found that mononuclear cells isolated from peripheral blood and supramammary lymph nodes from periparturient dairy cows produced a greater amount of TNF- α than did mid to late lactating dairy cows following LPS stimulation. If changes in mammary expression of TNF- α are primarily due to the presence of macrophages in the tissue, the differential expression of this cytokine may arise from differences in inherent production from monocytes in the circulation versus those in the mammary gland, as well as activation of these cells following stimulation. Perhaps most importantly is that TNF- α expression in this study was determined in mammary tissues that comprised several different cell populations with different capacities to produce TNF- α or any other cytokine. There is a limited amount of data evaluating interleukins during the periparturient period in the absence of infection. One study did show that concentrations of IL-6 in serum collected from the whole blood of periparturient dairy cows declined dramatically as parturition approached and remained low until the last sampling point at 8 wk postpartum (Ishikawa et al., 2004). Plasma IL-8 concentrations remained low in periparturient cows (–14

d to 14 d relative to calving) compared with concentrations at calving (Kimura et al., 2002). Low cytokine mRNA expression in this study may be attributable to lack of expression in the absence of agonist within the local mammary tissue microenvironment.

Local endothelial cell activation is triggered by cytokines, including IL-1, IL-8, and TNF- α , as well as other proinflammatory mediators (i.e., LPS) and results in enhanced expression of adhesion molecules. Adhesion molecules, including ICAM-1 and VCAM-1, are essential for the recruitment of inflammatory cells to the site of infection (Radi et al., 2001). In the current study, ICAM-1 mRNA expression was highest at d -35, declined as parturition approached, but increased in EL ($P < 0.004$; Table 3). Expression of VCAM-1 followed a similar pattern of mRNA abundance ($P < 0.02$; Table 3). Hodgkinson et al. (2007) demonstrated varying abundance of VCAM-1 protein expression in mammary tissue and supramammary lymph nodes from late gestation to EL. The authors proposed that VCAM-1 protein expression is not constitutive, but is activated by certain physiological events such as inflammation or colostrogenesis (Hodgkinson et al., 2007). Elevated expression of ICAM-1 and VCAM-1 mRNA at d -35 relative to d -20 and -7 in this study is consistent with the physiology of mammary involution. The significance of elevated vascular adhesion molecules in mammary tissues during EL is not known, but may be related to the increase in oxidative stress during this time. In humans, there is significant supporting evidence linking several inflammatory-based diseases to enhanced vascular adhesion molecule expression and oxidative stress (Valko et al., 2007; Bonomini et al., 2008). In this study, significant correlations were demonstrated between several antioxidants (GPX1, TrxR1, and HO-1) and proinflammatory factors (ICAM-1 and VCAM-1; Table 4). Increases in proinflammatory markers during a period when oxidative stress is known to occur, and their correlation with antioxidant markers, may indicate a role for oxidative stress

in the pathogenesis of dairy cattle diseases occurring during the periparturient period. Additional research is needed in this area to further elucidate the potential protective roles of antioxidants during the periparturient period.

Increased enzymatic activity of 15-LOX1 is associated with several inflammatory-based diseases including atherosclerosis (Bonomini et al., 2008). Metabolism of arachidonic acid by the 15-LOX1 pathway leads to the production of proinflammatory eicosanoids capable of generating ROS and exacerbating oxidative stress. Although 15-LOX1 has not previously been investigated in mammary tissue, this enzyme also may contribute to the pro-oxidant conditions associated with the periparturient period. For example, a recent study demonstrated that 15-LOX1 and its immediate metabolites were responsible for enhanced ICAM-1 expression in cultured bovine endothelial cells subjected to oxidative stress (Sordillo et al., 2008). In the current study, mammary tissue samples from EL had a greater abundance of 15-LOX1 mRNA compared with those obtained at other time points ($P < 0.01$; Figure 6). An increase in 15-LOX1 activity during the early postpartum period may contribute to the oxidative stress experienced by the cow by increasing ROS within the mammary tissue. Several selenoproteins can regulate the activity of 15-LOX1. For example, TrxR1 regulates the cellular abundance of fatty acid hydroperoxides generated following oxidation of arachidonic acid via the 15-LOX1 pathway (Cao et al., 2000; Weaver et al., 2001; Yu et al., 2004). The increase in both selenoprotein and 15-LOX1 mRNA during EL is suggestive of an association among these factors in mammary tissue as well. The findings from this descriptive study support a possible role of 15-LOX1 in the development of oxidative stress during the periparturient period and warrants further investigation.

Conclusions

Dairy cattle are exposed to oxidative stress during the periparturient period. Oxidative stress may be an underlying cause of increased susceptibility to diseases such as mastitis. Increased gene expression of proinflammatory factors may be a consequence of increased oxidative stress within the mammary gland during the onset of copious milk synthesis and secretion. This study demonstrates that the mRNA expression of several antioxidant enzymes decreases as parturition approaches and then increases after parturition. The expression of 2 key proinflammatory adhesion molecules, however, also decreases as parturition approaches and then increases afterwards. Results from this descriptive study provide no evidence for cause and effect mechanisms, but we suggest that further studies are warranted. It may be possible to target some of these antioxidant defense mechanisms for intervention during oxidative stress in an attempt to control inflammation and decrease susceptibility to acute mastitis in transition dairy cattle.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the National Research Initiative of the USDA Cooperative State Research, Education, and Extension Service, grant number # 2007–35204–18463 and by an endowment from the Matilda R. Wilson Fund (Detroit, MI).

Table 2. Bovine primers used for reverse transcription-PCR

| Gene | Accession Number | | Sequence (5' to 3') |
|---------------|------------------|-----------------------------|---|
| 15 LOX-1 | NM_174501 | Forward Reverse Probe | GTGCCTTCCGTCTATACATCCTATG CCCGGATGTTAATTTCCATGGTGTA CCCGGATGTTAATTTCCATGGTGTA |
| IL-1 β | NM_174093 | Forward Reverse Probe | GCTCTCCACCTCCTCTCACA CTCTCCTTGCACAAAGCTCATG CAGAACACCACTTCTCG |
| IL-8 | NM_173925 | Forward Reverse Probe | GCTCTCTTGGCAGCTTTCCT GGCATCGAAGTTCTGTACTCATTCT CAGAACTGCAGCTTCAC |
| TNF- α | NM_173966 | Forward Reverse Probe | GCCCCAGAGGGAAGAG CCAGAGGGCTGTTGATGGA CCCCAGGTGGCCCC |
| ICAM-1 | NM_174348 | Forward Reverse Probe | GCAGGTGGTCCACAAACAC GCAATCCCGCTGGTCTAGTC ATGTCCTGTACGGCCCC |
| VCAM-1 | NM_174484 | Forward Reverse Probe | ACAAAGGCAGAGTACACAAACACTT GAGGAGGGACTGACCAAGATG CCTGGGAGCAACATTA |
| TrxR-1 | NM_174625 | Forward Reverse Probe | GACCAAACCATCGAAGGAGAGTAT CTCGTGCAAGCATCTCTTCCT ATTGCCAGCAATACCG |
| GPX-4 | NM_174770 | Forward Reverse Probe | CTCAAGCCAGCGCTACTCT CGGGACGCGCACATG CCGGCGCAGCCAGA |
| GPX-1 | NM_174076 | Forward Reverse Probe | CTTCCCCTGCAACCAGTTTG GGCAATTCAGGATCTCCTCGTT TTGGCGTTTTCTTGATGCC |
| HO-1 | NM_001014912 | Forward Reverse Probe | GTGAGCTGACCCAAGAAGGTTT CCTCCAGGGCCACATAGATG ACGCCATCACCAGCTTA |
| RPS9 | XM_864261 | Forward Reverse Probe | GGCGGCTCGTCCGTATC AATCTTCAGGCCCAGGATGTAATC CCCTCATCCAGCACCC |

¹ 15 LOX-1 = bovine arachidonate 15-lipoxygenase 1; IL-1 β = bovine interleukin 1, β ; IL-8 = bovine interleukin 8; TNF- α = bovine tumor necrosis factor (TNF superfamily, member 2); TrxR-1 = bovine thioredoxin reductase-1; GPx-4 = bovine glutathione peroxidase-4; GPx-1 = bovine glutathione peroxidase-1; HO-1 = bovine heme oxygenase (decycling)-1; ICAM-1 = bovine intercellular adhesion molecule-1; VCAM-1 = bovine vascular cell adhesion molecule-1.

² Predicted: bovine similar to ribosomal protein S9, transcript variant 3 (LOC533892), mRNA.

Table 3. Relative mRNA abundance of proinflammatory cytokines and adhesion molecules in bovine mammary gland tissues obtained at –35 d, –20 d, and –7 d before calving and during early lactation (EL: 2–4 wk postpartum)¹

| Gene ² | -35 d | -20 d | -7 d | EL | SEM | <i>P</i> -value |
|-------------------|-------------------|--------------------|-------------------|-------------------|------|-----------------|
| IL-1 β | 1.16 | 0.62 | 0.79 | 0.57 | 0.26 | 0.45 |
| IL-6 | 1.59 | 0.17 | 0.20 | 0.99 | 0.49 | 0.55 |
| IL-8 | 1.19 | 0.22 | 0.35 | 3.66 | 1.53 | 0.49 |
| TNF- α | 1.02 ^a | 0.95 ^a | 0.52 ^a | 0.41 ^b | 0.12 | 0.05 |
| ICAM-1 | 1.04 ^a | 0.66 ^b | 0.38 ^c | 0.62 ^b | 0.09 | 0.004 |
| VCAM-1 | 1.00 ^a | 0.71 ^{ab} | 0.36 ^b | 0.55 ^b | 0.12 | 0.02 |

^{a-c} Means with different superscripts are significantly different ($P < 0.05$).

¹ Data were analyzed by the $2^{-\Delta\Delta C_t}$ method with –35 d as the reference expression point. Data reported as least squares means \pm SEM.

² TNF- α = tumor necrosis factor- α ; ICAM-1 = intercellular adhesion molecule-1; VCAM-1 = vascular cell adhesion molecule-1.

Table 4. Pearson correlation coefficients

| Gene ¹ | | GPX1 | GPX4 | TrxR1 | HO-1 |
|-------------------|----------|--------|------|---------|---------|
| 15LOX | <i>r</i> | -0.24 | 0.61 | 0.07 | 0.00001 |
| | <i>P</i> | 0.40 | 0.02 | 0.79 | 1.0 |
| ICAM-1 | <i>r</i> | 0.81 | 0.36 | 0.70 | 0.85 |
| | <i>P</i> | 0.0004 | 0.20 | 0.01 | 0.0001 |
| VCAM-1 | <i>r</i> | 0.71 | 0.57 | 0.94 | 0.65 |
| | <i>P</i> | 0.004 | 0.03 | <0.0001 | 0.01 |

¹ GPX1 = glutathione peroxidase; GPX4 = phospholipid hydroperoxide; TrxR1 = thioredoxin reductase; HO-1 = heme-oxygenase; 15-LOX = 15-lipoxygenase 1; ICAM-1 = intercellular adhesion molecule-1; VCAM-1 = vascular cell adhesion molecule-1.

Figure 2. Data reported as least squares means \pm SEM. Significant differences ($P < 0.05$) between days are represented by different letters. (A) Alterations in mRNA abundance of glutathione peroxidase 1 (GPX1) in the bovine mammary tissue obtained at -35, -20, and -7 d relative to calving and at early lactation (EL). Data were analyzed by the $2^{-\Delta\Delta C_t}$ method with -35 d as the reference expression point. (B) Enzyme activity of GPX1 in the bovine mammary tissue obtained at -35, -20, and -7 d relative to calving and at EL

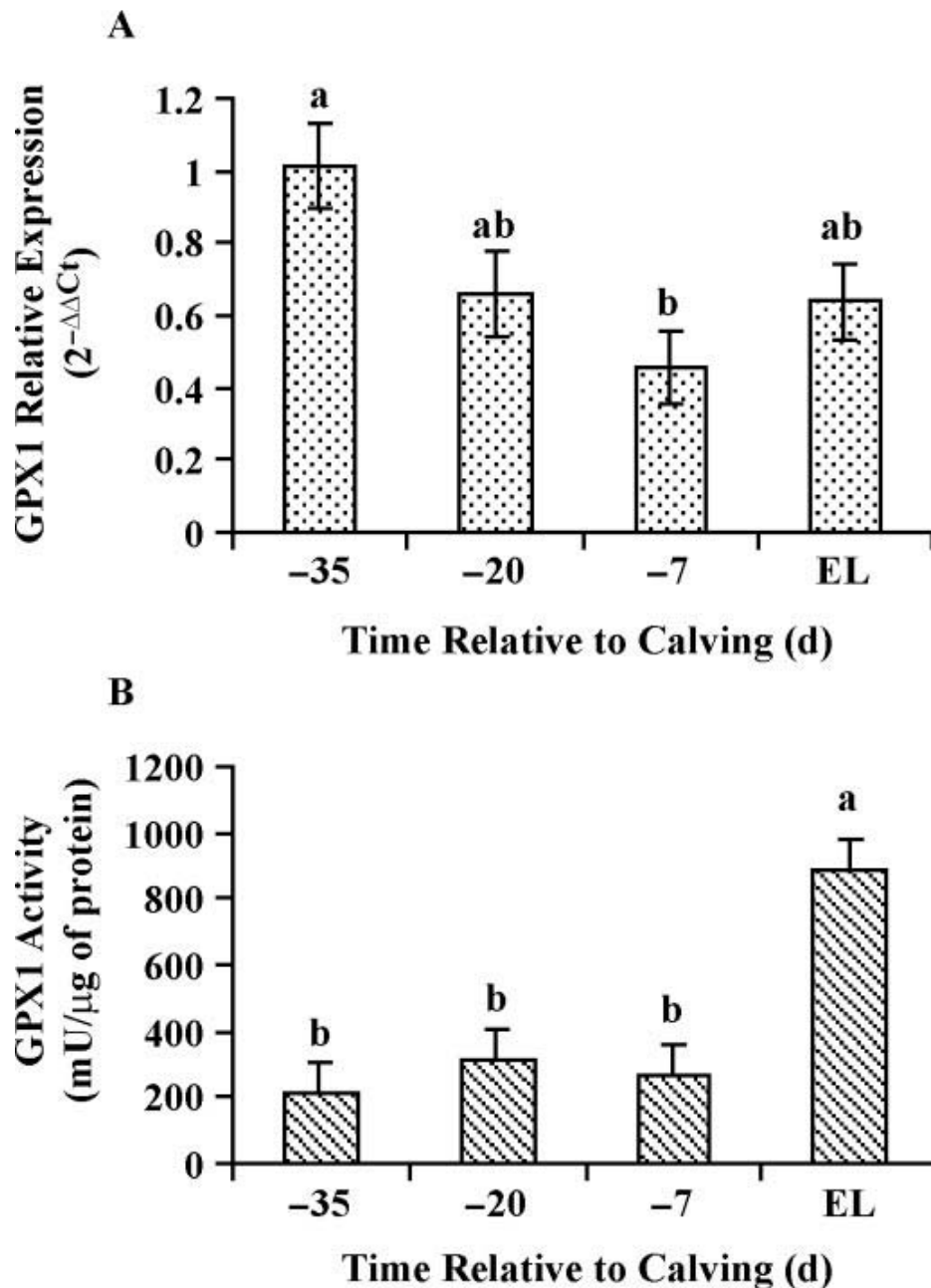


Figure 3. Data reported as least squares means \pm SEM. (A) Alterations in mRNA abundance of phospholipid hydroperoxide (GPX4) in the bovine mammary tissue obtained at -35, -20, and -7 d relative to calving and at early lactation (EL). Data were analyzed by the $2^{-\Delta\Delta C_t}$ method with -35 d as the reference expression point. Significant differences ($P < 0.05$) between days are represented by different letters. (B) Enzyme activity of phospholipid hydroperoxide (GPX4) in the bovine mammary tissue obtained at -35, -20, and -7 d relative to calving and at early lactation (EL)

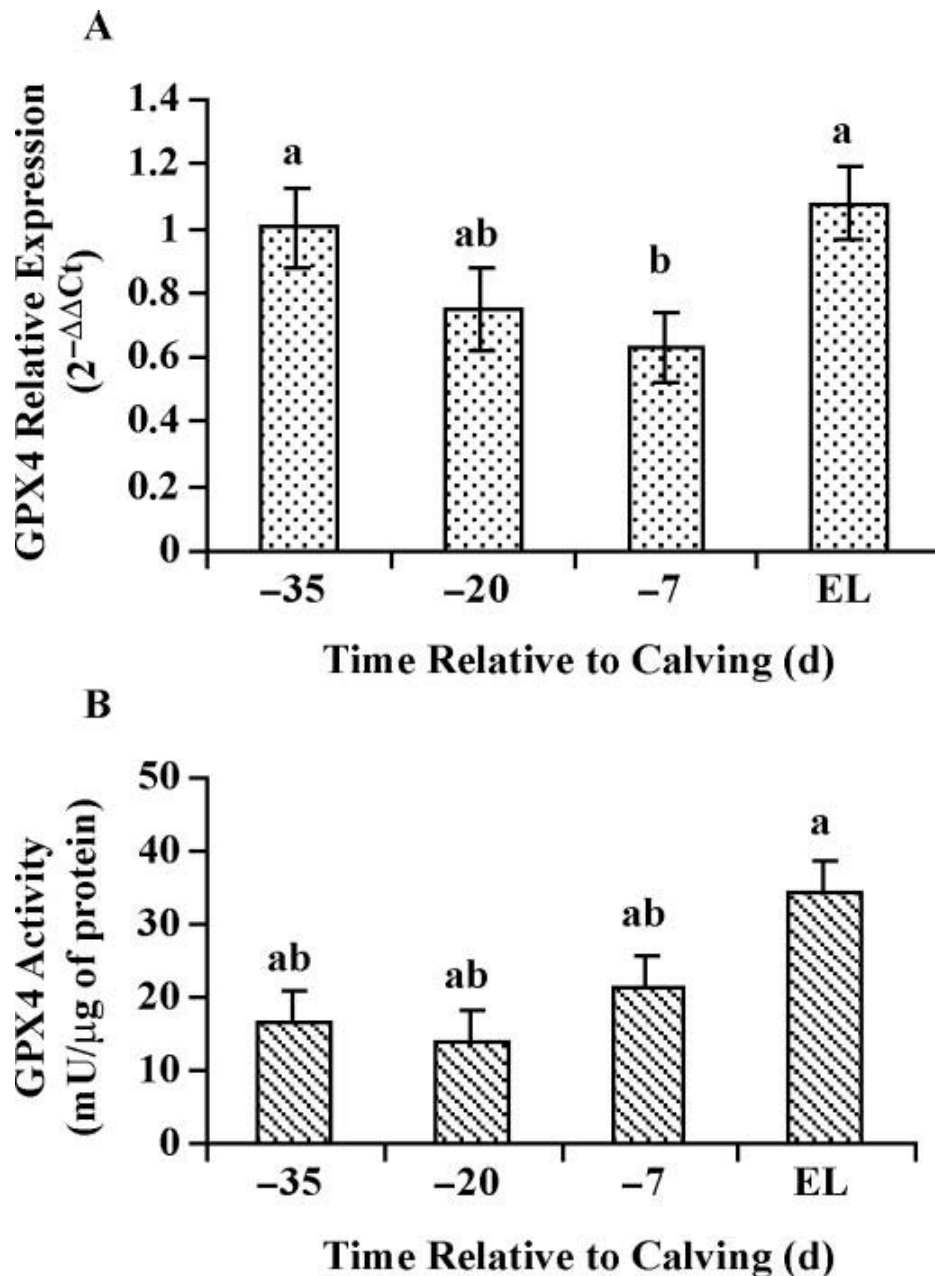


Figure 4. Data reported as least squares means \pm SEM. (A) Alterations in mRNA abundance of thioredoxin reductase (TrxR1) in the bovine mammary tissue obtained at -35 , -20 , and -7 d relative to calving and at early lactation (EL). Data were analyzed by the $2^{-\Delta\Delta C_t}$ method with -35 d as the reference expression point. Significant differences ($P < 0.05$) between days are represented by different letters. (B) Enzyme activity of TrxR1 in the bovine mammary tissue obtained at -35 , -20 , and -7 d relative to calving and at EL. The enzyme activity is expressed as A412 units \times 1000/(min \times mg protein)

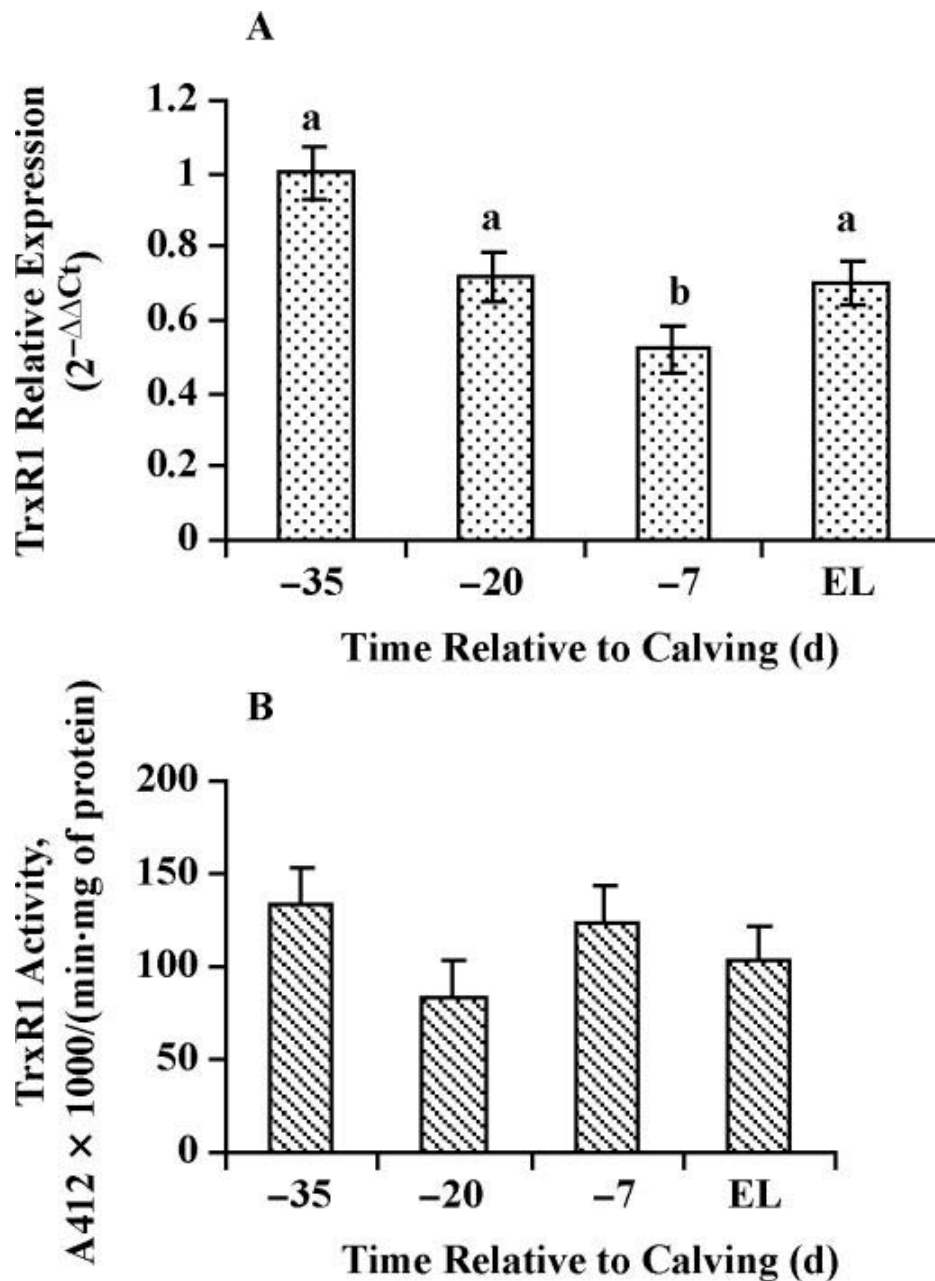


Figure 5. Alterations in mRNA abundance of heme oxygenase-1 (HO-1) in the bovine mammary tissue obtained at -35, -20, and -7 d relative to calving and at early lactation (EL). Data were analyzed by the $2^{-\Delta\Delta Ct}$ method with -35 d as the reference expression point. Data reported as least squares means \pm SEM. Significant differences ($P < 0.05$) between days are represented by different letters

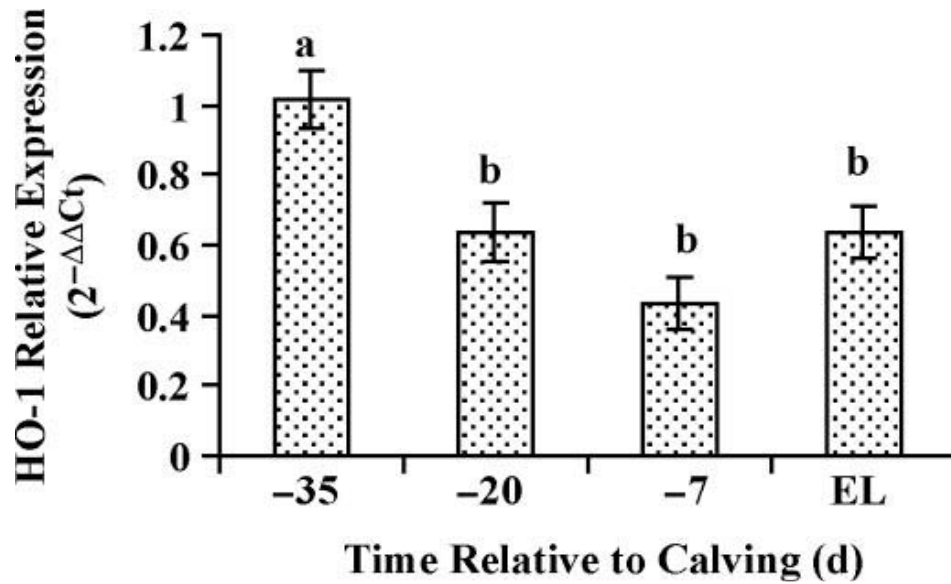
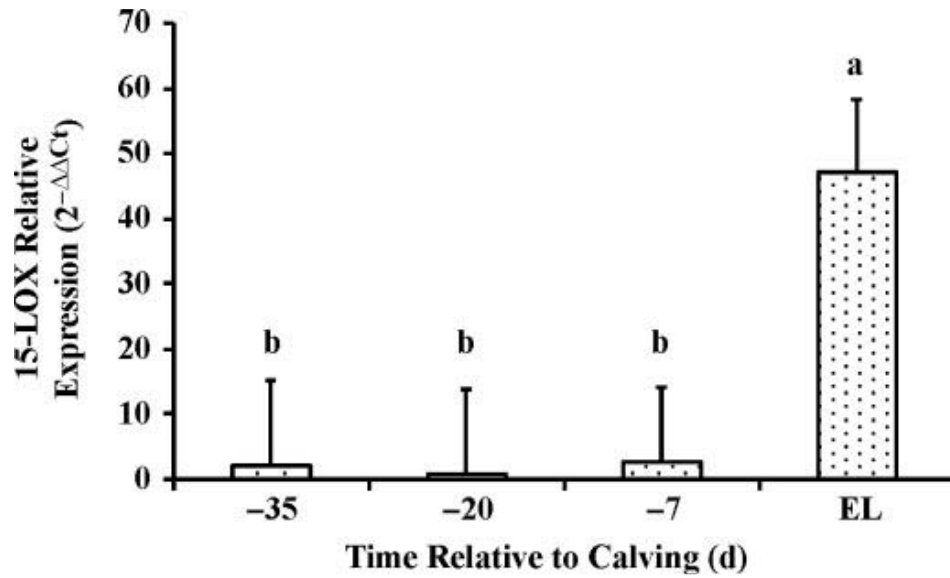


Figure 6. Alterations in mRNA abundance of 15-lipoxygenase 1 (15-LOX1) in the bovine mammary tissue obtained at -35, -20, and -7 d relative to calving and at early lactation (EL). Data were analyzed by the $2^{-\Delta\Delta C_t}$ method with -35 d as the reference expression point. Data reported as least squares means \pm SEM. Significant differences ($P < 0.05$) between days are represented by different letters



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