Role of endothelial cells in bovine mammary gland health and disease

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Abstract

The bovine mammary gland is a dynamic and complex organ composed of various cell types that work together for the purpose of milk synthesis and secretion. A layer of endothelial cells establishes the blood–milk barrier, which exists to facilitate the exchange of solutes and macromolecules necessary for optimal milk production. During bacterial challenge, however, endothelial cells divert some of their lactation function to protect the underlying tissue from damage by initiating inflammation. At the onset of inflammation, endothelial cells tightly regulate the movement of plasma components and leukocytes into affected tissue. Unfortunately, endothelial dysfunction as a result of exacerbated or sustained inflammation can negatively affect both barrier integrity and the health of surrounding extravascular tissue. The objective of this review is to highlight the role of endothelial cells in supporting milk production and regulating optimal inflammatory responses. The consequences of endothelial dysfunction and sustained inflammation on milk synthesis and secretion are discussed. Given the important role of endothelial cells in orchestrating the inflammatory response, a better understanding of endothelial function during mastitis may support development of targeted therapies to protect bovine mammary tissue and mammary endothelium.

Keywords: endothelial cells, mammary gland, inflammation, mastitis, lactation.

Introduction

The mammary gland is a specialized organ in female mammals that evolved for the purpose of milk synthesis and secretion to feed offspring. Due to advancements in genetic selection, however, the lactating dairy cow produces milk volumes that far exceed the neonate's nutritional requirement. As an example, the bovine neonate consumes approximately 11 kg of milk at 30 days of age if allowed *ad libitum* consumption, whereas the average commercial dairy cow produces on average four times more milk (Jasper and Weary, 2002). In addition to the remarkable milk volume synthesized by the mammary gland, milk is also rich in nutrients, including protein, carbohydrates, fats, minerals, and vitamins (Haug *et al.*, 2007). The ability of the mammary gland to synthesize and secrete milk requires a structure that is as unique as its function. In particular, the vascular endothelium is fundamental for the mammary gland to grow and develop and to initiate and sustain milk production. A thin, single layer of mammary capillary endothelial cells forms a semipermeable barrier that facilitates the exchange of serum components to provide oxygen, remove carbon dioxide, and transfer solutes and macromolecules for cellular energy and metabolism (Prosser *et al.*, 1996; Andres and Djono, 2010). Endothelial cells also support the robust synthesis and secretion of milk by facilitating a high rate of transfer of blood-derived components, including amino acids and glucose that are necessary for milk production (Aleman *et al.*, 2009; Mattmiller *et al.*, 2011). To maintain adequate delivery of milk components and substrates, endothelial cells directly orchestrate vascular tone, blood fluidity, and vascular permeability to support both the vasculature and underlying milk-producing tissue (Prosser *et al.*, 1996).

In addition to supporting lactation physiology, the endothelium also plays a direct role in orchestrating host defense to infectious bacterial pathogens. The mammary gland is exposed to outside environment through the teat canal. If bacteria are able to penetrate the teat end barrier, then the efficiency of mammary gland inflammatory responses will determine if mastitis manifests. Vascular endothelial cells play a central role in the

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facilitating movement of soluble and cellular host defense mechanisms into mammary tissues during the initial stages of bacterial invasion. An efficient inflammatory response will recognize and eliminate invading bacteria promptly without causing any discernable changes to mammary tissues (Aitken *et al.*, 2011a). When the initial inflammatory response fails to prevent the establishment of bacterial infection, however, clinical and/or chronic mastitis will cause impaired milk yield and quality. Indeed, mastitis costs the US dairy industry an estimated 2 billion dollars a year due to reduced milk production, discarded milk following antibiotic treatment, veterinary costs, and replacement animal costs (National Mastitis Council, 2004). Inadequate inflammatory responses can be attributed to deficiencies in normal endothelial cell functions. Endothelial dysfunction is characterized as facilitating the unregulated accumulation of leukocytes at the site of infection, enhanced leakage of plasma proteins into mammary tissues, and disruption in blood flow that contributes to mammary tissue damage and loss of function (Scalia and Lefer, 1998; Knepler *et al.*, 2001; Cassuto *et al.*, 2014). Thus, optimal endothelial cell functions are needed to tightly regulate inflammatory responses and prevent immunopathology during bacterial invasion.

Because endothelial cells play a central role in mammary gland health, knowledge of the delineating factors between a successful self-limiting inflammatory response and immunopathology is critical. This review will cover three major topics that discuss how and why: (1) endothelial cells are necessary for the growth and development of the unique mammary gland; (2) endothelial cells modify their phenotype to orchestrate the initiation and resolution of inflammation; and (3) endothelial dysfunction contributes to immunopathology and subsequent tissue damage. A better understanding of the biological mechanisms regulating endothelial cell function is essential for optimizing mammary gland health and increasing the efficiency of milk production in dairy cattle.

Endothelial cells contribute to the specialized structure of the mammary gland

Mammary gland growth and development

Mammogenesis, the first stage of mammary gland development, culminates in the formation of a complex epithelial and endothelial network. Previous studies show that a disruption in the normal progression of murine mammogenesis hinders the ability of the mammary gland to adequately secrete milk (Rossiter *et al.*, 2007). Mammogenesis begins during embryogenesis and continues following the birth of the succeeding neonate, at which time it overlaps with the onset of lactation. Studies on mammogenesis were conducted in rats and mice, and from those studies the development of the bovine mammary endothelium can be inferred (Yasugi *et al.*, 1989; Matsumoto *et al.*, 1992; Abdul Awal *et al.*, 1996; Rossiter *et al.*, 2007). The mammary endothelium is composed of a thin layer of simple squamous endothelial cells, and along with myoepithelial cells and connective tissue, forms a complex vascular network known

as the mammary vasculature. A unique feature of the mammary gland vasculature is the basket-like capillary beds surrounding bronchi-like alveoli clusters as demonstrated in murine models (Yasugi *et al.*, 1989). Capillary beds surrounding alveoli maximize the surface area for exchange of oxygen and nutrients. To further facilitate exchange, but also maintain a selective barrier, the endothelial surface is largely continuous with some minor areas of fenestration. A continuous endothelium is an uninterrupted layer of cells that restricts the free movement of large solutes and proteins, but allows for exchange of water and other very small molecules (Levick and Smaje, 1987; Clough, 1991; Michel and Curry, 1999). Fenestrations are small pores in endothelial cells that permit passage of small molecules and proteins. Development of the murine and human mammary endothelium establishes a semipermeable barrier that is primarily continuous with some areas of fenestration, which suggests that the bovine mammary gland maintains a similar structure (Stirling and Chandler, 1976; Matsumoto *et al.*, 1992). To further facilitate nutrient transfer for optimal mammary growth, mammogenesis is characterized by an increase in cell number and surface area to provide a maximal interface for nutrient transfer and milk secretion. In murine models, the increase in surface area of the luminal endothelium occurs early in pregnancy by the formation of microvilli and marginal folds on individual endothelial cells (Matsumoto *et al.*, 1992). Evidence of increased endothelial surface area by the formation of microvilli and marginal folds, minor areas of fenestrations, and basket-like capillary beds surrounding alveoli suggest that optimal mammary function is unequivocally dependent on mammary structure.

Onset of milk synthesis and secretion

Lactogenesis is the acquisition of functional properties enabling the mammary gland to synthesize and secrete large volumes of milk. While much of the research regarding lactogenesis focuses on the differentiation of epithelial cells, endothelial cells also experience structural and functional changes in an effort to support initial and sustained milk production. For example, the number of mitochondria increases in rat mammary endothelial cells, conceivably for the increased energy required for maintaining blood flow, vascular tone, and regulating transport of components needed for milk synthesis (Abdul Awal *et al.*, 1996). Pinocytotic vesicles also increase in rat endothelial cells to support efficient transportation of plasma solutes and molecules, such as glucose (Abdul Awal *et al.*, 1996). Recent research demonstrates that several glucose transporter molecules (GLUT) are expressed in the bovine mammary gland, including GLUT1, GLUT3, GLUT4, GLUT5, GLUT8, and GLUT12, named according to their order of discovery. In the bovine mammary gland, the mRNA expression of GLUT1, GLUT8, and GLUT12 increased substantially from late in gestation to early lactation (Zhao and Keating, 2007). Similarly, the mRNA and protein expression of GLUT1 was significantly increased in mammary tissue from early lactation cows compared to non-lactating cows (Komatsu *et al.*, 2005). Mammary tissue from early-lactation dairy cows exhibited increased GLUT1, which

was localized to endothelial cells and epithelial cells, compared to 15 days prior to the onset of lactation (Mattmiller et al., 2011). Though the mechanisms that stimulate increased expression of GLUT1 during late pregnancy and early lactation are unclear, previous research demonstrates that hypoxia in the mammary gland elicits a hypoxia inducible factor-α dependent mechanism that results in GLUT1 upregulation (Shao et al., 2014). In contrast, the regulation of GLUT4 expression is not clear. Some research suggests GLUT4 increases during late lactation in bovine mammary tissue, whereas others suggest that GLUT4 is not expressed in mammary tissue (Komatsu et al., 2005; Mattmiller et al., 2011). However, the expression of glucose transporters, GLUT1 and GLUT4, by primary bovine mammary endothelial cells (BMEC) was confirmed in an in vitro study (Mattmiller et al., 2011). Because glucose transport is a rate-limiting step in optimal lactation, more investigation into the role of GLUT in mammary endothelial cells are necessary. Nonetheless, the localization of glucose transporters to the endothelium and increased mRNA expression in BMEC from lactating cows suggests endothelial cells play an active role in metabolism and nutrient transfer during lactation.

In conjunction with increased transport molecules, murine models demonstrated increased capillary permeability during early lactation, which would support enhanced transfer of fluids and molecules for milk synthesis (Matsumoto et al., 1994). To further support nutrient transfer, capillaries with thinner walls were in closer contact with the alveoli during late pregnancy and early lactation compared to late lactation (Matsumoto et al., 1992). Perhaps most telling about the importance of the mammary vasculature in rats is that the development of the vasculature, measured as number of capillaries per individual lobular ductule, surpassed alveolar lobular ductule development during lactation (Ramirez et al., 2012). Though additional research is still required in dairy cattle, research in other species support the notion that changes in both structure and optimal function of the bovine vasculature during lactogenesis directly affects the ability of epithelial cells to synthesize milk components.

Mammary gland involution

To support maximal milk production in the next lactation, a period of involution is needed for regeneration of bovine mammary tissues (Holst et al., 1987). Dairy cows milked continuously until calving produced approximately 75% less milk compared to the twin given a 60-day non-milking period prior to calving (Swanson, 1965). Involution across species involves a regression in lobulo-alveoli complexity, decreased cell number, and a loss of milk-synthesizing function (Holst et al., 1987; Walker et al., 1989; Tatarczuch et al., 1997; Djonov et al., 2001). Involution in murine, goat, and dairy cow mammary glands suggests epithelial cells experience apoptosis at a very high rate following cessation of milking (Wilde et al., 1997). However, other bovine studies demonstrate very little epithelial cell loss during involution even though alveolar lumen area decreases (Holst et al., 1987). Nonetheless, previous research in lactating goats suggests that induction of epithelial cell apoptosis may be initiated by

accumulation of milk in the mammary gland (Quarrie et al., 1994). Whether regression of the bovine vasculature occurs during involution is not clear, although changes in the endothelium during murine and sheep involution may be used to infer changes in the bovine. In the involuting mouse mammary gland, capillary density decreases to a density similar to early pregnancy (Pepper et al., 2000). Studies in sheep suggest that a decrease in mammary capillary density may be a result of endothelial apoptosis. During involution of the ovine mammary gland, apoptosis of endothelial cells was observed in addition to the presence of apoptotic bodies found within the cytoplasm of healthy endothelial cells (Tatarczuch et al., 1997). Interestingly, murine models of involution featured endothelial cell apoptosis that was preceded by epithelial cell apoptosis (Djonov et al., 2001). The timing of endothelial cell apoptosis relative to epithelial apoptosis suggests the endothelial cells may be adapting to or dying from an altered microenvironment. However, the major temporal differences between epithelial and endothelial regression during sheep and rat mammary gland involution was not demonstrated, thus more research is required to understand these processes (Walker et al., 1989; Tatarczuch et al., 1997). Degradation of the proteins in the basement membrane also occurs during involution and matrix metalloproteinases (MMP) are largely responsible for this event. Data demonstrate that MMP1 and MMP14 are increased in epithelial cells during late involution, whereas MMP2 was only localized to mammary endothelial cells and only during involution (Rabot et al., 2007). In a human umbilical vein endothelial cell (HUVEC) model, withdrawal of growth factor to induce apoptosis resulted in increased MMP2 expression in apoptotic endothelial cells (Levkau et al., 2002). Activation of MMP2 in HUVEC enables association of MMP2 with receptors for fibronectin and vitronectin, both glycoproteins responsible for the formation of the extracellular matrix and cell adhesion, suggesting apoptosis of endothelial cells during mammary gland involution may be integrin-mediated (Levkau et al., 2002). Apoptosis may not be solely responsible for decreased vascular networks in the murine mammary gland where capillary regression was noted in the absence of apoptotic endothelial cells (Djonov et al., 2001). The conflicting research in rodent models and the limited amount of research in involuting bovine mammary glands suggests that more research is needed to better understand the role of endothelial cells in the remodeling process.

Endothelial cells prevent mastitis by orchestrating inflammation

The inflammatory response is a complex, tightly regulated series of events to protect the normal milk-synthesizing function of the bovine mammary gland from mastitis-causing organisms. Endothelial cells regulate a wide variety of homeostatic mechanisms, including maintenance of proper vascular tone, regulation of vascular permeability, modulation of blood fluidity, and regulation of immune responses for protection of the mammary gland during pathogen exposure (Prosser et al., 1996; Aitken et al., 2011a). Exposure of microbes and microbial components

Table 1. Summary of inflammatory mediator production during bovine mastitis

Models	Selected findings	References
Experimental intramammary challenge		
LPS	⬆ mammary tissue TNFα, COX-2, LF, LZ, and iNOS mRNA	Schmitz et al., 2004
LPS	⬆ plasma PGE_2, 15-keto-$PGF_{2\alpha}$, LTB_4, and LTC_4 and milk TXB_2	Anderson et al., 1986; Piotrowska-Tomala et al., 2012
E. coli	Severity correlated with ⬆ plasma TNF-α and milk PGE_2	Pezeshki et al., 2011
E. coli	⬆ milk IL-1, IL-6, IL-8, complement fragment C5a, and TNFα	Shuster et al., 1997
E. coli	⬆ milk IL-1β, IL-12, IFN-γ, IL-8, IL-10, and TNFα	Bannerman et al., 2004b
K. pneumoniae	⬆ plasma $PGF_{2\alpha}$ and milk histamine, 5-HT, TxB_2, PGE_2, and $PGF_{2\alpha}$	Zia et al., 1987
S. marcescens	⬆ milk IL-1β, IL-8, IL-10, IL-12, IFN-γ, TNF-α, and C5a	Bannerman et al., 2004a
S. aureus	⬆ milk IL-1β, IL-12, IFN-γ, IL-10	Bannerman et al., 2004b
S. uberis	⬆ milk IL-1β, IL-8, IL-10, IL-12, IFN-γ, TNF-α, and C5a	Rambeaud et al., 2003; Bannerman et al., 2004a
Natural infection		
Mixed bacteria	⬆ milk $PGF_{2\alpha}$, PGE_2, and TXB_2, LXA_4, and LTB_4 in milk	Boutet et al., 2003, Atroshi et al., 1987
E. coli	⬆ milk 6-keto $PGF_{1\alpha}$, PGE_2, HODE, 5-HETE, 15-HETE and plasma 6-keto $PGF_{1\alpha}$, PGE_2, and RvD_2	Mavangira et al., 2015
E. coli or S. aureus	⬆ plasma coagulation parameters including ATT and PT time	Ismail and Dickinson, 2010
S. aureus	⬆ mammary tissue TGF-β protein expression	Andreotti et al., 2014

⬆, Increased; LF, lactoferrin; LZ, lysozyme; LPS, lipopolysaccharide; IL, interleukin; TNF-α, tumor necrosis factor-α; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; PG, prostaglandin; LT, leukotriene; TX, thromboxane; IFN-γ, interferon-γ; 5-HT, serotonin; HODE, hydroxyoctadecadienoic acid; HETE, hydroxyeicosatetraenoic acid; RvD, resolvin; ATT, activated partial thromboplastin time; PT, prothrombin time; TGF, transforming growth factor-β.

initially triggers a response from epithelial cells and resident macrophages to produce and release a variety of inflammatory mediators including cytokines and oxylipids (Table 1). The inflammatory mediators initially recruit phagocytic immune cells and robustly activate endothelial cells. Human and murine models indicate vasodilation of capillaries and increased vascular permeability are necessary for the influx of neutrophils to contain pathogens and limit extravascular tissue damage (Van Nieuw Amerongen and Van Hinsbergh, 2002; Bazzoni and Dejana, 2004). Endothelial cells also promote active resolution of inflammation to protect the integrity of the vasculature and interstitial tissue (Kadl and Leitinger, 2005). During mastitis, however, the inflammatory response can either be inefficient, excessive, or prolonged, resulting in severe tissue and vascular damage. Thus, it is necessary to differentiate between a normal inflammatory response (self-limiting) and immunopathology (mastitis). The following sections will discuss the change in endothelial cell phenotype needed to orchestrate an efficient inflammatory response.

Endothelial cells modulate changes in vascular tone and blood flow during inflammation

A change in blood flow and vascular tone, characterized by the degree of constriction relative to dilation, is among the initial responses of the vasculature during pathogen exposure (Lacasse et al., 1996; Prosser et al., 1996; Kobayashi et al., 2013). Myoepithelial cells and endothelial cells work synergistically to either constrict or relax in response to signals primarily produced by endothelial cells (Prosser et al., 1996). Endothelial cells produce a variety of vasoactive mediators, such as nitric oxide (NO), prostacyclin (PGI_2), endothelin-1, and histamine that function in an autocrine, juxtacrine, and paracrine manner to regulate myoepithelial contraction or relaxation. The most well-studied endothelial-derived regulators of vascular tone during homeostasis are NO and PGI_2 in lactating goats, though less is known in the bovine (Nishida et al., 1992; Nielsen et al., 1995; Lacasse et al., 1996). At the onset of inflammation, endothelial nitric oxide synthase (eNOS) is activated as a result of increased intracellular calcium. Activation of eNOS metabolizes arginine to citrulline and NO. Subsequently, NO activates guanylyl cyclase to produce cGMP, which inhibits calcium influx into the endothelial cell allowing relaxation of the actin cytoskeleton (Busse and Mulsch, 1990). In lactating goats, infusion of the mammary external pudendal artery with an eNOS inhibitor significantly reduces mammary blood flow (Lacasse et al., 1996). Mice deficient in eNOS fail to exhibit an early change in the vasculature and the later phase is markedly reduced, suggesting without eNOS activation the inflammatory response cannot be efficiently mounted or proceed normally (Bucci et al., 2005).

Similar to eNOS activation and NO biosynthesis, constitutive cyclooxygenase-1 (COX-1) is activated by an increase in intracellular calcium to facilitate the synthesis of PGI_2, an oxylipid. An oxylipid is an oxidized fatty acid and may be synthesized

enzymatically or non-enzymatically from many different fatty acids, such as arachidonic acid and linoleic acid. Increased calcium also activates cytosolic phospholipase A_2, which cleaves fatty acids from membrane phosphatidylcholine to generate a free fatty acid and lysophosphatidylcholine moiety (Lombardo et al., 1986). During early inflammation, COX-1 oxidizes arachidonic acid to prostaglandin G_2 (PGG_2), which is quickly reduced to PGH_2 by intrinsic peroxidase activity of COX-1 (Fitzpatrick and Soberman, 2001). Next, PGH_2 is converted to PGI_2 by PGI_2 synthase. Activation of the PGI_2 receptor, a G-protein-coupled receptor (GPCR), causes an increase in adenylyl cyclase-dependent cAMP and subsequent vasodilation as a result of protein kinase A activation (Murata et al., 1997). Furthermore, sustained PGI_2 is dependent on COX-2 and also is activated by increased calcium, but its optimal expression and activity requires transcription and translation induced by pro-inflammatory mediators. Increased expression of COX-2 would aide in sustaining vasodilation during the progression of inflammation. In lactating goats, infusion of the external pudendal artery with PGI_2 increased blood flow in the mammary gland, whereas infusion with a PGI_2 synthase inhibitor significantly decreased mammary blood flow suggesting the endothelium is sensitive to changes in PGI_2 and can alter mammary blood flow (Nielsen et al., 1995; Lacasse et al., 1996). A change in mammary blood flow following PGI_2 and NO exposure suggests that the vasculature in the bovine mammary gland is sensitive to potent vasodilatory mediators. By modulating vascular tone, endothelial cells strive to provide an optimal endothelial surface to facilitate rolling, attachment, and migration of leukocytes to control infection and inflammation.

Though vasodilation is critical to the progression of an appropriate immune response, vasoconstriction during the very early stages of infection and inflammation is protective in the event of mechanical injury and bleeding. Because vasoconstriction limits blood flow, and therefore may compromise the health of the mammary tissue, the release of vasoconstrictors is short-lived and balanced by the sustained release of vasodilators (Prosser et al., 1996). Two vasoconstrictors that are rapidly synthesized at the onset of inflammation are platelet-activating factor (PAF) and thromboxane A_2 (TXA_2). Lysophosphatidylcholine, a lipid moiety generated following the cleavage of arachidonic acid from phosphatidylcholine, acts as a precursor for the production of PAF (Lombardo et al., 1986). Early production of PAF in BMEC and bovine aortic endothelial cells (BAEC) was confirmed following lipopolysaccharide (LPS) exposure (Corl et al., 2008; Corl et al., 2010). In murine pulmonary artery endothelial cells, PAF also induced increased production of NO suggesting the presence of a negative feedback loop to prevent sustained vasoconstriction (Predescu et al., 2013). Another well-known vasoconstrictor produced during inflammation is TXA_2, an unstable oxylipid that is degraded to TXB_2. The biosynthesis of TXA_2 is similar to PGI_2 except that thromboxane synthase converts PGH_2 to TXA_2. Interestingly, BAEC stimulated with a TXA_2 mimetic induced the synthesis of the PGI_2, further supporting a negative feedback loop established by the production of vasoconstrictors (Clesham et al., 1992). In fact, the balance of TXA_2 and PGI_2 may be more important than

absolute concentrations in modulating vascular tone. The production of vasoconstrictors, which also contributes to vasodilator release, suggests that modulation of vascular tone during the initial inflammatory response is tightly regulated to prevent unnecessary damage to blood vessels and interstitial tissue.

In conjunction with changes in vascular tone, reduced blood fluidity also is required during the inflammatory response. During early inflammation, endothelial cells initiate and propagate coagulation by increasing procoagulant properties and decreasing anticoagulants. In BAEC and HUVEC models, thrombus formation is initiated by upregulation of tissue factor expression in response to bacterial toxins and cytokines (Nawroth and Stern, 1986; Crossman et al., 1990; Fei et al., 1993). The coagulation cascade, initiated by the binding of tissue factor to factor VIIa, acts on factor X to form Factor Xa, which in turn complexes with prothrombin, Factor Va, and calcium to produce thrombin (Nawroth and Stern, 1986; Rao and Rapaport, 1987). Thrombin then converts fibrinogen to fibrin resulting in the formation of a fibrin clot, thus slowing blood flow at the site of coagulation. Much of the research on the modulation of coagulation in bovine endothelial cells is focused on the effect of cytokines. In BAEC, tumor necrosis factor-α (TNF-α) exposure decreases the expression of thrombomodulin, a major regulatory mechanism of coagulation that prevents the formation of thrombin by generating active protein C (Nawroth and Stern, 1986). Further supporting the effect of pro-inflammatory cytokines on BAEC, TNF-α dose-dependently decreased activated protein C, an antioxidant serine protease that inactivates Factor Va and VIIIa (Conway and Rosenberg, 1988). Antithrombin, another anticoagulant factor, binds to heparin-like glycosaminoglycans on the endothelial luminal surface to prevent serine protease activity (Olson et al., 2010). However, research suggests pro-inflammatory cytokines reduce the expression of heparin sulfate, the major target for antithrombin, by about 50% in porcine aortic endothelial cells (Kobayashi et al., 1990). Furthermore, thrombin increased cytokine-induced leukocyte migration across a HUVEC monolayer and in an in vivo rabbit dermal inflammation model, supporting the idea that the coagulation cascade is critical for inflammation (Drake et al., 1992). The purpose of coagulation is to reduce blood fluidity to prevent the systemic spread of bacteria and toxins as well as to enhance leukocyte contact with the endothelial cells to facilitate leukocyte migration.

Vascular permeability is altered during inflammation

Endothelial cells mediate the type and quantity of solutes and leukocytes that move back and forth between tissue and blood using selective cellular junctions (Bazzoni and Dejana, 2004). Perturbation of the endothelium alters endothelial junctions and permeability leading to leakage of plasma proteins and leukocytes. Several endothelial junction proteins can control vascular permeability by regulating cell-to-cell contact and thus forming a highly selective barrier (Blum et al., 1997; Jiang et al., 1998). The series of events modulating bovine mammary vascular permeability during inflammation is unknown, but

previous studies suggest inflammatory mediators and toxins produced during a normal inflammatory response directly alter endothelial junctions (Bannerman *et al.*, 1998; Zhang *et al.*, 2013). Studies using HUVEC models demonstrate that endothelial tight junctions are loosened by GPCR activation in response to various extracellular mediators, including fatty acids and cytokines (Goeckeler and Wysolmerski, 1995; Blum *et al.*, 1997; Jiang *et al.*, 1998; Michel and Curry, 1999). Tight junctions are composed of transmembrane proteins (e.g., claudins and occludins) and cytosolic proteins (zona occludins), which bridge to cytoskeleton actin filaments. Similarly, in a HUVEC model of inflammation LPS decreased endothelial tight junction proteins concomitant with increased monolayer permeability (Zhang *et al.*, 2013). Claudin-5 (a tight junction protein) deficient mice exhibited a leaky blood–brain barrier with larger molecules able to move across this extremely restrictive barrier (Nitta *et al.*, 2003). Unrestricted movement of macromolecules may contribute to destruction of extravascular tissue and allow systemic infection. Tight junctions are not the only cell connections altered during inflammation. Exposure of bovine pulmonary artery endothelial cells to LPS destabilized endothelial adherens junctions by cleaving β-catenin, thereby disrupting the anchor to the cell cytoskeleton (Bannerman *et al.*, 1998). Additionally, blocking VE-cadherin binding in HUVEC increased permeability to solutes and leukocytes suggesting any modulation in endothelial junction expression may severely alter the course of inflammation (Hordijk *et al.*, 1999). Thus, without proper organization and function of tight junctions and adherens junctions in the mammary endothelium, exchange of solutes and cells is dysregulated and may contribute to tissue damage due to accumulation of plasma components. However, some rearrangement or modulation of endothelial junctions must occur to facilitate effective clearance of mastitis-causing pathogens by phagocytic immune cells.

In dairy cows, most research on barrier permeability is focused on epithelial cells without considering the important contributions of endothelial cells. Early studies suggested that an increase in sodium and chloride in milk from cows with mastitis was indicative of epithelial barrier disruption (Linzell and Peaker, 1972). However, researchers also appreciated the presence of serum albumin in milk as indicative of increased permeability at the blood–milk barrier (Kobayashi *et al.*, 2013). Endothelial cells and associated endothelial junctions directly contribute to changes in milk components, such as albumin. Albumin is one of the most important proteins that regulate oncotic pressure and disruption of oncotic pressure typically causes edema in interstitial tissue (Rippe *et al.*, 1979). Additionally, albumin can bind many different molecules and act as a chaperone across the endothelium (Spector, 1975). Thus, increased albumin in secretory products, i.e., milk, would indicate radical changes at the endothelium. A similar occurrence is demonstrated during early involution, when albumin concentration in milk increases (Poutrel *et al.*, 1983; Breau and Oliver, 1985). The increase in serum albumin may be related to a minimal increase in vascular permeability to support passive immunoglobulin transfer during early involution. In contrast, the increased concentration of serum albumin in early involution

may simply be an artifact of decreased milk production with no appreciable change yet in plasma protein transfer (Poutrel *et al.*, 1983). Understanding how the endothelium regulates vascular permeability is necessary for discovering new and innovative therapies to prevent systemic infection, as well as add insight to the complex changes that occur during mammary regression.

Leukocyte movement increases across the mammary endothelium during inflammation

The movement of leukocytes into the infected tissue is critical to prevent the progression of mastitis. Leukocytes present in the blood stream are not innately adhesive, but do interact with the endothelium. Under basal conditions neutrophils roll along the vascular barrier and communicate through random and reversible interactions with neutrophil surface selectins. Neutrophil leukocyte–selectin (neutrophil associated; L-selectin) association with the murine endothelium allows contact, but not tethering, capture, or firm adhesion (Kaplanski *et al.*, 1993; Ley *et al.*, 1995). The purpose of neutrophil rolling is to survey the vasculature for cytokines, oxylipids, or reactive oxygen species (ROS) signaling microbial infection (Ley *et al.*, 1995). The production of ROS is increased during early inflammation due to increased adenosine triphosphate (ATP) production through the electron transport chain and as a result of respiratory burst by phagocytic cells (Parnham *et al.*, 1987; Aon *et al.*, 2012). At the initiation of inflammation, activation of GPCRs induces the release of pre-synthesized platelet-selectin (P-selectin) from specialized endothelial organelles called Weibel–Palade bodies as demonstrated in HUVEC (McEver *et al.*, 1989). Binding of P-selectin to leukocyte-associated P-selectin glycoprotein ligand-1 forms a tight, but reversible, attachment between endothelial cells and leukocytes. In BMEC, the mRNA expression of P-selectin is increased following exposure to TNF-α and in P-selectin deficient mice, rolling of neutrophils on the endothelial surface and neutrophil migration are reduced, illustrating the vital role of initial P-selectin binding in leukocyte migration (Mayadas *et al.*, 1993; Aitken *et al.*, 2011b). Capture, but not firm adhesion, of neutrophils is complete with endothelial-selectin (E-selectin), but its transcription and expression requires gene transcription and translation. In both BMEC and HUVEC, increased temporal synthesis and expression of P-selectin and E-selectin is the result of various inflammatory mediators, including cytokines and ROS (Scholz *et al.*, 1996; Maddox *et al.*, 1999; Aitken *et al.*, 2011b). Stimulation of BMEC with TNF-α induced an increase in E-selectin expression at 3 and 6 h, whereas P-selectin expression was only significantly increased at 3 h (Maddox *et al.*, 1999). The time-dependent expression of adhesion molecules involved in tethering and capture during inflammation further supports the temporal, cooperative effort between P- and E-selectin that must exist in the bovine mammary gland. The contribution of endothelial cells to tethering and capture of neutrophils during inflammation is unequivocal and necessary for an appropriate, timely response.

Firm adhesion follows capture of leukocytes and is necessary for diapedesis of leukocytes into the inflamed tissue. Firm adhesion is achieved by neutrophil ligand binding to intercellular adhesion molecule-1 (ICAM-1), ICAM-2, and vascular cell adhesion molecule-1 (VCAM-1) (Sans *et al.*, 1999). Macrophage-1 antigen binds to endothelial ICAM-1, whereas lymphocyte function associated antigen-1 preferentially binds ICAM-2. *In vivo* data during bovine mammary gland inflammation are limited regarding firm adhesion, but murine and human studies provide information about endothelial cells in orchestrating leukocyte adhesion and migration (Sans *et al.*, 1999; Hafezi-Moghadam *et al.*, 2007). Very late antigen-4 (VLA-4) is the ligand for VCAM-1, and when VLA-4 is blocked on rat leukocytes, adhesion to the endothelium is significantly reduced (Hafezi-Moghadam *et al.*, 2007). In a rat model of colitis, blockade of ICAM-1 slightly reduced leukocyte adhesion, but blockade of VCAM-1 prevented all adhesion of leukocytes to colonic venules suggesting VCAM-1 may be the predominant molecule in firm adhesion (Sans *et al.*, 1999). In bovine *in vitro* models, several studies demonstrated the ability to increase the expression of ICAM-1 and VCAM-1 following exposure to TNF-α and PAF (Sordillo *et al.*, 2005; Corl *et al.*, 2008). Additionally, a mixture of saturated and unsaturated fatty acids increased the expression of ICAM-1 in BAEC (Aitken *et al.*, 2011b; Contreras *et al.*, 2012a, b). In contrast, omega-3 fatty acids reduced ICAM-1 and VCAM-1 expression in cytokine-stimulated human endothelial cells (De Caterina *et al.*, 1994). Furthermore, arachidonic acid-derived 15-hydroperoxyeicosatetraenoic acid (15-HPETE) and linoleic acid-derived 13-hydroperoxyoctadecadienoic acid, both produced early in the inflammatory response, induced an increase in BAEC and HUVEC ICAM-1 expression at low doses (Friedrichs *et al.*, 1999; Sordillo *et al.*, 2008). Rapid biosynthesis of oxylipids supports robust and acute expression of endothelial adhesion molecules to expedite neutrophil migration into the infected tissue. The sensitivity of the endothelium to fatty acids and oxylipids is important because it suggests that the inflammatory response may be modulated with only small changes in autocrine, juxtacrine, and paracrine mediators.

Though research focusing on the process of leukocyte migration is limited, there is no doubt that endothelial–leukocyte cooperation is necessary to successfully bring neutrophils to the site of infection. Platelet–endothelial cell adhesion molecule-1 (PECAM-1) is thought to be the primary molecule that mediates migration of leukocytes between across the endothelium through the formation of homodimers between endothelial cells and neutrophils (Schenkel *et al.*, 2002). Though there was no change in BMEC PECAM-1 mRNA expression following TNF-α exposure, previous human studies showed that blocking neutrophil PECAM-1 or HUVEC prevents transmigration, suggesting without PECAM-1 binding an efficient inflammatory response would not occur (Muller *et al.*, 1993; Aitken *et al.*, 2011b). Moreover, expression of PECAM-1 was upregulated in an *in vivo* murine model following exposure to cytokines, such as IL-1β and TNF-α, (Thompson *et al.*, 2001). Additionally, PECAM-1 receptors may rearrange and move to junctional complexes to support leukocyte migration. For example, TNF-α and IFN-γ induced PECAM-1 redistribution on the HUVEC (Romer *et al.*, 1995). Expression of PECAM-1 during bovine mammary inflammation

is not well characterized and requires further exploration. However, based on other human and murine inflammatory models, it is appreciated that an optimal inflammatory response requires a coordinated effort between endothelial cells and surrounding cell types to facilitate neutrophil migration to the site of inflammation.

Resolution of inflammation is necessary to prevent vascular and mammary tissue damage

The process of active resolution is not completely understood, but current research suggests that there is a switch from a pro-inflammatory to an anti-inflammatory/pro-resolving cellular phenotype (Serhan *et al.*, 2008a). A proper inflammatory response is self-limiting with the final phase being resolution. The sequence of events that lead to resolution of inflammation and tissue repair is not well defined due to the involvement of many different cells and pathways. Thus, the topics and order of discussion for the following endothelial mechanisms is based on their hierarchal importance in reducing leukocyte migration and promoting tissue repair.

Endothelial cells are sensitive to ROS and free radicals, collectively called pro-oxidants, and during inflammation there is increased pro-oxidant production surrounding and within endothelial cells (Zweier *et al.*, 1994). Intracellular pro-oxidants are derived from the mitochondrial electron transport chain during ATP production and extracellular pro-oxidants are predominantly derived from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity during phagocytic respiratory burst (Karlsson *et al.*, 1995; Aon *et al.*, 2012). The continued presence of pro-oxidants can contribute to the development of oxidative stress. Oxidative stress is an imbalance of antioxidant mechanisms and pro-oxidant molecules resulting in damage to cellular lipids, proteins, and DNA (Sordillo *et al.*, 2009). To mediate resolution, antioxidants must be produced to counteract the damaging effects of pro-oxidants. The most well-studied enzymatic antioxidant defenses include glutathione peroxidase (GPx), superoxide dismutase (SOD), and thioredoxin reductase (TrxR) (Bowler *et al.*, 2004; Aon *et al.*, 2012). Superoxide dismutase either adds or removes an electron from O_2^- to form O_2 or H_2O_2, which will be catalyzed to oxidized glutathione and H_2O by GPx (Bowler *et al.*, 2004). The *in vivo* action of TrxR, GPx, and SOD is unknown during self-limiting bovine mammary gland inflammation, but *in vitro* models in bovine, murine, and human endothelial cells provide an opportunity to discuss how the lack of antioxidants defenses promotes inflammation. The overexpression of GPx-1 protected BAEC-derived NO production in an endothelial injury model (Weiss *et al.*, 2001). Conversely, deficiency of GPx-1 during TNF-α stimulation in human microvascular endothelial cells exacerbated the expression of VCAM-1 and ICAM-1 and subsequently enhanced ROS production (Lubos *et al.*, 2011). Mice deficient in extracellular endothelial-derived SOD demonstrated significantly exaggerated lung inflammation characterized by robust and sustained pro-inflammatory cytokine production (Bowler *et al.*, 2004). Importantly, neutrophil infiltration was persistent in SOD knockout mice, which was consistent with sustained

endothelial adhesion molecule expression, suggesting clearance of pro-oxidants by endothelial cells is critical for decreased leukocyte migration and resolution of inflammation (Bowler *et al.*, 2004).

While GPx and SOD directly reduce ROS and free oxygen radicals, TrxR reduces oxidized thioredoxin, so that it may reduce other pro-oxidants (Trigona *et al.*, 2006). Following low-dose H_2O_2 exposure in HUVEC, thioredoxin mRNA and protein expression were significantly increased indicating, its role in antioxidant defense (Haendeler *et al.*, 2004). Interestingly, TrxR can also reduce GPx in human plasma after pro-oxidant quenching suggesting redundancy in antioxidant defense system (Bjornstedt *et al.*, 1994). However, it is unknown whether this occurs in the bovine mammary gland or in the mammary endothelial cells. Overall these studies suggest that for a self-limiting inflammatory response to occur, adequate antioxidants and antioxidant enzymes must be available to counteract increased pro-oxidants during inflammation.

Another new and burgeoning area of research that involves active resolution of inflammation is the production of anti-inflammatory and pro-resolving lipid mediators. The major classes of anti-inflammatory and pro-resolving oxylipids include resolvins (RV), protectins (PD), and lipoxins (LX), which are synthesized from fatty acids. The RV are synthesized from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and PD are derived from DHA. Resolvins derived from EPA are enzymatically formed by the sequential oxidation of cytochrome P450 and 5-LOX, whereas DHA-derived RV (RvD) are generally derived by the sequential oxidation 15-LOX and 5-LOX, similar to AA-derived LX. On the other hand, PD is formed by 15-LOX oxidation and subsequent hydrolysis. There is not a great deal of *in vivo* bovine research available for review in this new area, however, research in *in vitro* bovine models demonstrate the anti-inflammatory effects of increased EPA and DHA on cellular phenotype and subsequent oxylipid production. For example, in BAEC increased omega-3 fatty acid content (DHA and EPA) reduced pro-inflammatory cytokine expression, adhesion molecule expression, ROS production, and pro-inflammatory oxylipid production (Contreras *et al.*, 2012a). Also, the production of DHA-derived oxylipids, including RvD and PD, and AA-derived LXA_4, were increased in BAEC exposed to increased omega-3 fatty acids (Contreras *et al.*, 2012a). In a HUVEC model of inflammation, RvD_1 prevented the reorganization of tight junction proteins and abrogated any changes in vascular permeability induced by LPS (Zhang *et al.*, 2013). Similar to RV function, PD also has the ability to promote endothelial barrier function. In human microvascular endothelial cells, PD1 effectively prevented the LTB_4-induced migration of neutrophils (Serhan *et al.*, 2006). In addition, PD1 administration also was able to abrogate the inflammatory response after induction of murine peritonitis (Serhan *et al.*, 2006). Thus, research suggests that administration of omega-3-derived oxylipids may be beneficial in preventing immunopathogenesis; however, the complexity of oxylipid biosynthesis necessitates further research in human and veterinary species.

Oxylipids derived from omega-6 fatty acids may also play a very important role in protecting the endothelial barrier. Arachidonic acid-derived LX are primarily synthesized with the cooperative effort of both 5-LOX and 15-LOX (Serhan *et al.*, 2008b). Lipoxins inhibit chemotaxis and migration of neutrophils, reduce ROS production, and prevent activation of nuclear factor-κB (NF-κB) (Serhan *et al.*, 2008a). In the context of endothelial barrier integrity, LXA_4 prevents LPS- or PAF-induced increased vascular permeability suggesting LXA_4 acts as an anti-inflammatory protector of the endothelium (Ereso *et al.*, 2009). In addition, LXA_4 induces IL-10 production in a HUVEC model while concomitantly inhibiting production of pro-inflammatory cytokines and vascular adhesion molecules (Baker *et al.*, 2009). Consequently, active resolution is dependent on the contribution of potent anti-inflammatory oxylipids and given their ability to modulate many aspects of inflammation warrants continued research in human and veterinary species.

As oxylipids may directly modulate cytokine production to promote resolution, other negative feedback loops and checkpoints also exist to prevent sustained inflammation. Cytokine expression, as a result of NF-κB signaling, is a major pro-inflammatory pathway activated following the detection of bacterial pathogens (Nadjar *et al.*, 2005). Thus, it makes sense that NF-κB signaling should be tightly regulated to prevent sustained activation. One such checkpoint is inhibition of NF-κB signaling pathway by inhibitor of κB (IκB). The inhibitor IκBα prevents NF-κB binding, and thus prevents activation of pro-inflammatory gene expression. Though IκBα is degraded at the onset of inflammation, sustained activation of NF-κB initiates the expression of IκB, which acts as a negative feedback loop (De Martin *et al.*, 1993). Additionally, IκBα may directly decrease the expression of IL-1, IL-6, IL-8, and VCAM in porcine aortic endothelial cells (Wrighton *et al.*, 1996). Aside from pro-inflammatory cytokines, endothelial cells also produce anti-inflammatory cytokines, including IL-10, IL-1 receptor agonist, and transforming growth factor-β (TGF-β), which decrease the expression of pro-inflammatory cytokines and also promote tissue repair (Cromack *et al.*, 1993). In HUVEC, exposure to TGF-β decreased both baseline E-selectin and TNF-α-stimulated E-selectin mRNA and protein expression by more than 50% (Gamble *et al.*, 1993). Though the mechanism by which tissue repair is initiated remains unclear, TGF-β contributes substantially through activation of angiogenesis and induction of matrix component synthesis (Ignotz and Massague, 1986; Ferrari *et al.*, 2009). Decreased expression of pro-inflammatory cytokines, in conjunction with increased anti-inflammatory cytokines and growth factors, is necessary for controlling chronic inflammation and immunopathology.

Endothelial dysfunction as an underlying cause for immunopathology

Bovine mastitis results when there is not a self-limiting inflammatory response and may be a result of endothelial cell dysfunction. Endothelial dysfunction can contribute to an aberrant inflammatory response as a result of breakdown of tight junction proteins, loss of barrier integrity, increased attachment of leukocytes and platelets, and in the most severe cases, leading to sepsis and tissue hypoperfusion (Levi *et al.*, 1997; Van
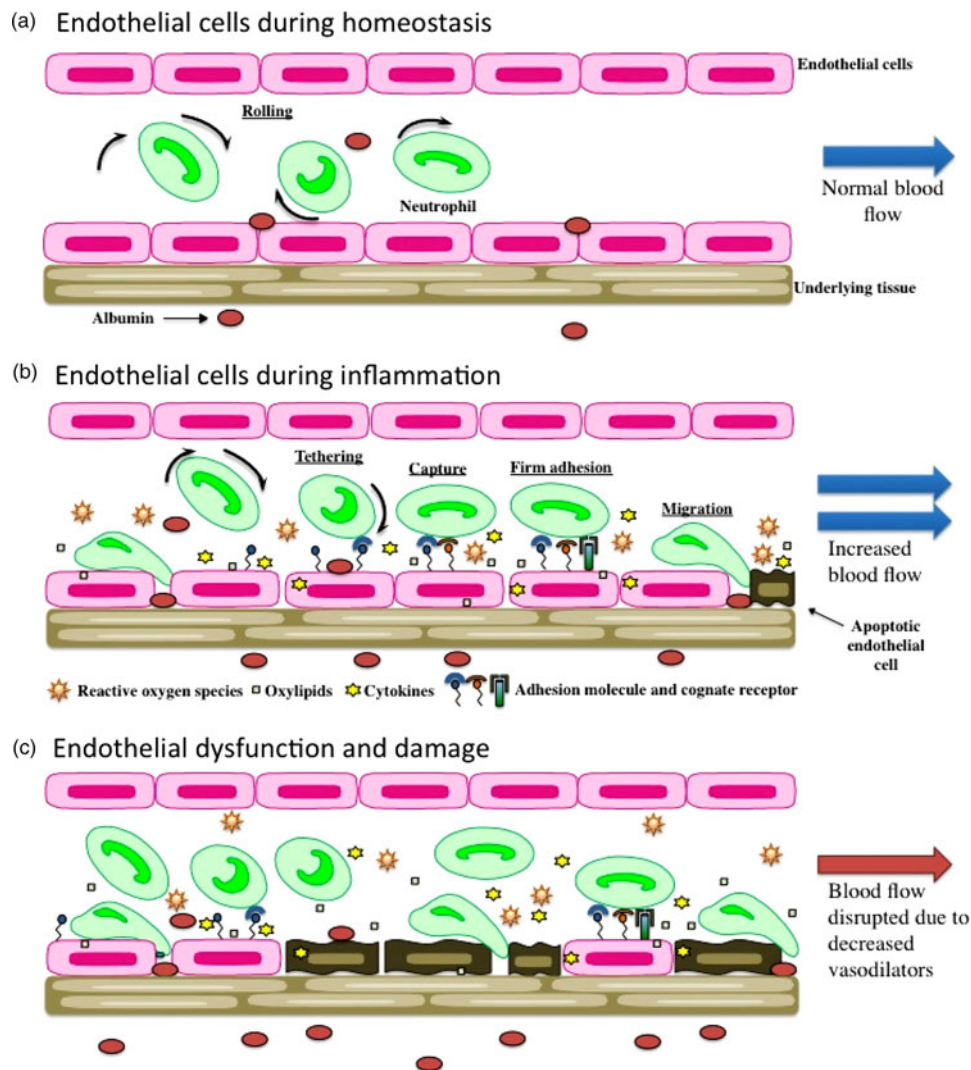
Fig. 1. (a) During homeostasis, endothelial cells maintain adequate vascular tone, permeability, and leukocyte migration. (b) At the onset of infection-induced inflammation, endothelial cells acquire new properties to facilitate clearance of the bacteria. Activated endothelial cells express adhesion molecules and produce increased concentrations of ROS, vasoactive lipid mediators, and cytokines. Increased vasodilation and chemokines elicit neutrophils to adhere to and cross the endothelium. Some endothelial cells may exhibit morphological changes during inflammation, those of which are eliminated during the resolution and wound healing phase of inflammation. (c) An unregulated immune response results in prolonged activation of endothelial cells and accumulation of activated neutrophils at the endothelial surface causing irreversible cell damage. Oxidative stress as a result of accumulated ROS, free radicals, and lipid hydroperoxides further contributes to cellular damage and apoptosis of endothelial cells.

Nieuw Amerongen and Van Hinsbergh, 2002; Winn and Harlan, 2005). Based on previous *in vitro* BMEC and BAEC studies, as well as research in other species and disease models as a reference, it is possible to speculate how endothelial cell dysfunction can contribute to immunopathology during mastitis (Fig. 1). The following sections discuss the impact of sustained inflammation and accumulation of pro-oxidants on endothelial function and survival.

Oxidative stress negatively affects antioxidant pathways and oxylipid biosynthesis

Oxidative stress contributes to immunopathology by modulating endothelial function. During inflammation, endothelial metabolism is increased to support a variety of mechanisms, including increased adhesion molecule expression, cytokine expression, and antioxidant pathways (Kadl and Leitinger, 2005). Functional roles of antioxidant systems may be decreased due to quenching by existing ROS and free radicals, down regulation of transcriptional expression, and inactivation of antioxidant enzymes (Blum and Fridovich, 1985). In BAEC, reduced TrxR activity during oxidative stress resulted in decreased heme oxygenase-1 (HO-1), an enzyme that catalyzes the degradation of ROS-inducing free heme (Trigona *et al.*, 2006; Fortes *et al.*, 2012). Decreased HO-1 would exacerbate oxidative stress and potentially result in endothelial cell death. In fact, pro-apoptotic markers were increased during oxidative stress in BAEC, a finding that was correlated to decreased HO-1

and TrxR (Trigona *et al.*, 2006). *In vitro* BMEC models shed further light on how oxidative stress affects cellular function eventually leading to endothelial dysfunction. Endothelial cells (BMEC and BAEC) increased production of TXB_2 and PAF, and decreased production of PGI_2 during oxidative stress potentially compromising maintenance of vascular tone (Cao *et al.*, 2000; Weaver *et al.*, 2001). Additionally, changes in endothelial cell metabolism during oxidative stress resulted in the accumulation of bioactive oxylipids, such as 15-HPETE (Weaver *et al.*, 2001). Arachidonic acid-derived 15-HPETE is a lipid hydroperoxide derived by 15-LOX oxidation and can have similar effects on endothelial cell health as ROS. Such changes in oxylipid biosynthesis during oxidative stress can cause apoptosis of bovine endothelial cells, which may lead to disruption of the endothelial barrier (Sordillo *et al.*, 2005). Our understanding of these mechanisms during bovine mastitis is less clear; however, *in vitro* reports support the contention that oxidative stress can compromise endothelial integrity.

Oxidative stress contributes to decreased NO

In human diseases, endothelial dysfunction is classically characterized as the reduced availability of NO resulting in altered vascular tone and blood flow. The availability of NO ensures that the mammary vasculature remains relaxed, as opposed to being in a state of vasoconstriction (Ohashi *et al.*, 1998). Chemical inhibition of eNOS in HUVEC induces intracellular oxidative stress contributing to increased adhesion of neutrophils (Niu *et al.*, 1994). During a dysfunctional inflammatory response, NO levels may be reduced dramatically as a result of several different mechanisms. First, reduced NO may be a function of eNOS uncoupling. Uncoupling of eNOS occurs when available O_2 is oxidized to form superoxides preventing its combination with L-arginine to generate NO (Vasquez-Vivar *et al.*, 1998). In addition to reduced NO synthesis, superoxide, generated during oxidative stress, is toxic to endothelial cells and other cells if not reduced by superoxide dismutase. Aside from superoxide production by eNOS uncoupling, activated neutrophils arrested at the endothelial barrier produce superoxide and other ROS/free radicals by the action of NADPH oxidase (Karlsson *et al.*, 1995; Aon *et al.*, 2012). The excess ROS further uncouples eNOS by oxidizing BH4, a cofactor for eNOS activity (Xia *et al.*, 1998). Though eNOS activation or uncoupling during bovine mastitis is unknown, accumulation of neutrophils at the endothelial interface was demonstrated during *Streptococcus uberis* mastitis along with destruction of the surrounding tissue (Thomas *et al.*, 1994). Levels of NO also may be reduced by ROS consumption. Accumulation of activated neutrophils would allow for increased ROS production, such as superoxide, at the endothelial surface, which would be able to act on NO directly rather than affecting eNOS activity. Peroxynitrite, formed by the reaction of NO and superoxide, induced barrier dysfunction, endothelial protein oxidation, and cytoskeleton rearrangement in porcine pulmonary artery endothelial cells (Knepler *et al.*, 2001). Thus, reduced NO through eNOS uncoupling or quenching by oxygen radicals prevents endothelial cells from maintaining proper vascular tone and perpetuates a dysfunctional endothelial phenotype.

Prolonged or excessive inflammation induces apoptosis of endothelial cells

The end result of persistent endothelial dysfunction is apoptosis, which may be activated through both intrinsic and extrinsic pathways. Apoptosis of endothelial cells would compromise the mammary endothelium thereby disrupting the semipermeable endothelial barrier. The extrinsic pathway is initiated through receptor activation by death ligands, including TNF-α and LPS. Exposure of BMEC to TNF-α induced a pro-apoptotic response, suggesting cytokines meant to activate endothelial cells to facilitate pathogen clearance may also damage the gatekeeper cells at the blood–milk barrier (Aitken *et al.*, 2011b). Furthermore, apoptosis was documented during oxidative stress in a BAEC model supporting the intrinsic pathway of apoptosis (Sordillo *et al.*, 2005; Trigona *et al.*, 2006). The intrinsic pathway is activated by cellular DNA damage and/or accumulation of intracellular ROS, which causes mitochondrial damage triggering apoptosis. During bovine mastitis, ROS and free radicals accumulate at the endothelial barrier due to the activated nature of endothelial cells and activated neutrophils that are migrating or immobilized at the barrier (Thomas *et al.*, 1994). Previous HUVEC models demonstrate that oxygen radicals activate protein kinase D and ASK-1/JNK, which activates the JNK/p38 MAPK apoptosis pathway (Zhang *et al.*, 2005). Additionally, a HUVEC model demonstrated that apoptotic endothelial cells possess procoagulant and prothrombotic properties, including increased platelet adhesion and decreased thrombomodulin, heparin sulfate, and tissue factor pathway inhibitor (Bombeli *et al.*, 1997, 1999). Furthermore, caspase cleavage during apoptosis directly disrupts adherens junctions, allowing the transfer of plasma proteins and fluid in bovine pulmonary artery endothelial cells (Bannerman *et al.*, 1998). During bovine mastitis, it seems likely that an inability to rectify the inflammatory response may be due to damaged and apoptotic endothelial cells. Unfortunately, it is unclear when the inflammatory response can be targeted to prevent destruction of the bovine mammary vascular endothelium and represents a substantial gap in our current knowledge.

Conclusions

Endothelial cells are no longer viewed as static barriers at the interface between blood and tissue. Endothelial cells regulate transfer of solutes and macromolecules, leukocyte migration, vascular tone, and blood flow during homeostasis and inflammation. The ability of a thin layer of endothelial cells to serve such a prominent role is directly related to their proximity to the circulation and to the underlying interstitial tissue. Specifically, capillaries encircling milk-producing alveoli in the bovine mammary gland offer increased surface area for the principle functions of lactation and immune surveillance. However,

research is still limited outlining the role of endothelial cells in initiation and resolution of mammary inflammation. Through modulation of vascular tone and blood fluidity, vascular permeability, and endothelial adhesiveness, the endothelium enables the progression of a self-limiting inflammatory response to support clearance of the pathogen and protect the milk-producing tissue. However, mediators that are important for progression of mastitis and clearance of pathogens may also be responsible for damage to alveoli in the mammary gland. In particular, oxidative stress is especially detrimental to endothelial function and survival. Thus, understanding how endothelial activation becomes endothelial dysfunction represents a point of potential therapeutic intervention, e.g, preventing or ameliorating oxidative stress. Future studies should focus on the mechanisms that initiate the switch from activation to resolution. Such research would be important to modify exacerbated and sustained inflammatory responses that contribute to inflammatory-based diseases such as bovine mastitis.

Acknowledgments

References

Abdul Awal M, Matsumoto M, Toyoshima Y and Nishinakagawa H (1996). Ultrastructural and morphometrical studies on the endothelial cells of arteries supplying the abdomino-inguinal mammary gland of rats during the reproductive cycle. *Journal of Veterinary Medical Science* **58**: 29–34.

Aitken SL, Corl CM and Sordillo LM (2011a). Immunopathology of mastitis: insights into disease recognition and resolution. *Journal of Mammary Gland Biology and Neoplasia* **16**: 291–304.

Aitken SL, Corl CM and Sordillo LM (2011b). Pro-inflammatory and pro-apoptotic responses of TNF-alpha stimulated bovine mammary endothelial cells. *Veterinary Immunology and Immunopathology* **140**: 282–290.

Aleman G, Lopez A, Ordaz G, Torres N and Tovar AR (2009). Changes in messenger RNA abundance of amino acid transporters in rat mammary gland during pregnancy, lactation, and weaning. *Metabolism* **58**: 594–601.

Anderson KL, Kindahl H, Smith AR, Davis LE and Gustafsson BK (1986). Endotoxin-induced bovine mastitis: arachidonic acid metabolites in milk and plasma and effect of flunixin meglumine. *American Journal of Veterinary Research* **47**: 1373–1377.

Andreotti CS, Pereyra EA, Baravalle C, Renna MS, Ortega HH, Calvinho LF and Dallard BE (2014). *Staphylococcus aureus* chronic intramammary infection modifies protein expression of transforming growth factor beta (TGF-beta) subfamily components during active involution. *Research in Veterinary Science* **96**: 5–14.

Andres AC and Djono V (2010). The mammary gland vasculature revisited. *Journal of Mammary Gland Biology and Neoplasia* **15**: 319–328.

Aon MA, Stanley BA, Sivakumaran V, Kembro JM, O'rourke B, Paolocci N and Cortassa S (2012). Glutathione/thioredoxin systems modulate mitochondrial H2O2 emission: an experimental-computational study. *Journal of General Physiology* **139**: 479–491.

Atroshi F, Parantainen J, Kangasniemi R and Osterman T (1987). Milk prostaglandins and electrical conductivity in bovine mastitis. *Veterinary Research Communications* **11**: 15–22.

Baker N, O'meara SJ, Scannell M, Maderna P and Godson C (2009). Lipoxin A4: anti-inflammatory and anti-angiogenic impact on endothelial cells. *Journal of Immunology* **182**: 3819–3826.

Bannerman DD, Sathyamoorthy M and Goldblum SE (1998). Bacterial lipopolysaccharide disrupts endothelial monolayer integrity and survival signaling events through caspase cleavage of adherens junction proteins. *Journal of Biological Chemistry* **273**: 35371–35380.

Bannerman DD, Paape MJ, Goff JP, Kimura K, Lippolis JD and Hope JC (2004a). Innate immune response to intramammary infection with Serratia marcescens and Streptococcus uberis. *Veterinary Research* **35**: 681–700.

Bannerman DD, Paape MJ, Lee JW, Zhao X, Hope JC and Rainard P (2004b). Escherichia coli and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. *Clinical and Diagnostic Laboratory Immunology* **11**: 463–472.

Bazzoni G and Dejana E (2004). Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiological Reviews* **84**: 869–901.

Bjornstedt M, Xue J, Huang W, Akesson B and Holmgren A (1994). The thioredoxin and glutaredoxin systems are efficient electron donors to human plasma glutathione peroxidase. *Journal of Biological Chemistry* **269**: 29382–29384.

Blum J and Fridovich I (1985). Inactivation of glutathione peroxidase by superoxide radical. *Archives of Biochemistry and Biophysics* **240**: 500–508.

Blum MS, Toninelli E, Anderson JM, Balda MS, Zhou J, O'donnell L, Pardi R and Bender JR (1997). Cytoskeletal rearrangement mediates human microvascular endothelial tight junction modulation by cytokines. *American Journal of Physiology* **273**: H286–H294.

Bombeli T, Karsan A, Tait JF and Harlan JM (1997). Apoptotic vascular endothelial cells become procoagulant. *Blood* **89**: 2429–2442.

Bombeli T, Schwartz BR and Harlan JM (1999). Endothelial cells undergoing apoptosis become proadhesive for nonactivated platelets. *Blood* **93**: 3831–3838.

Boutet P, Bureau F, Degand G and Lekeux P (2003). Imbalance between lipoxin A4 and leukotriene B4 in chronic mastitis-affected cows. *Journal of Dairy Science* **86**: 3430–3439.

Bowler RP, Nicks M, Tran K, Tanner G, Chang LY, Young SK and Worthen GS (2004). Extracellular superoxide dismutase attenuates lipopolysaccharide-induced neutrophilic inflammation. *American Journal of Respiratory Cell and Molecular Biology* **31**: 432–439.

Breau WC and Oliver SP (1985). Accelerated bovine mammary involution induced by infusion of concanavalin A or phytohemagglutinin. *American Journal of Veterinary Research* **46**: 816–820.

Bucci M, Roviezzo F, Posadas I, Yu J, Parente L, Sessa WC, Ignarro LJ and Cirino G (2005). Endothelial nitric oxide synthase activation is critical for vascular leakage during acute inflammation *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 904–908.

Busse R and Mulsch A (1990). Calcium-dependent nitric oxide synthesis in endothelial cytosol is mediated by calmodulin. *FEBS Letters* **265**: 133–136.

Cao YZ, Reddy CC and Sordillo LM (2000). Altered eicosanoid biosynthesis in selenium-deficient endothelial cells. *Free Radical Biology and Medicine* **28**: 381–389.

Cassuto J, Dou H, Czikora I, Szabo A, Patel VS, Kamath V, Belin De Chantemele E, Feher A, Romero MJ and Bagi Z (2014). Peroxynitrite disrupts endothelial caveolae leading to eNOS uncoupling and diminished flow-mediated dilation in coronary arterioles of diabetic patients. *Diabetes* **63**: 1381–1393.

Clesham G, Parsaee H, Joseph S, Mcewan JR and Macdermot J (1992). Activation of bovine endothelial thromboxane receptors triggers release of prostacyclin but not EDRF. *Cardiovascular Research* **26**: 513–517.

Clough G (1991). Relationship between microvascular permeability and ultrastructure. *Progress in Biophysics and Molecular Biology* **55**: 47–69.

Contreras GA, Mattmiller SA, Raphael W, Gandy JC and Sordillo LM (2012a). Enhanced n-3 phospholipid content reduces inflammatory responses in bovine endothelial cells. *Journal of Dairy Science* **95**: 7137–7150.

Contreras GA, Raphael W, Mattmiller SA, Gandy JC and Sordillo LM (2012b). Nonesterified fatty acids modify inflammatory response and eicosanoid biosynthesis in bovine endothelial cells. *Journal of Dairy Science* **95**: 5011–5023.

Conway EM and Rosenberg RD (1988). Tumor necrosis factor suppresses transcription of the thrombomodulin gene in endothelial cells. *Molecular Cell Biology* **8**: 5588–5592.

Corl CM, Gandy JC and Sordillo LM (2008). Platelet activating factor production and proinflammatory gene expression in endotoxin-challenged bovine mammary endothelial cells. *Journal of Dairy Science* **91**: 3067–3078.

Corl CM, Contreras GA and Sordillo LM (2010). Lipoxygenase metabolites modulate vascular-derived platelet activating factor production following endotoxin challenge. *Veterinary Immunology and Immunopathology* **136**: 98–107.

Cromack DT, Porras-Reyes B, Purdy JA, Pierce GF and Mustoe TA (1993). Acceleration of tissue repair by transforming growth factor beta 1: identification of *in vivo* mechanism of action with radiotherapy-induced specific healing deficits. *Surgery* **113**: 36–42.

Crossman DC, Carr DP, Tuddenham EG, Pearson JD and Mcvey JH (1990). The regulation of tissue factor mRNA in human endothelial cells in response to endotoxin or phorbol ester. *Journal of Biological Chemistry* **265**: 9782–9787.

De Caterina R, Cybulsky MI, Clinton SK, Gimbrone MA Jr and Libby P (1994). The omega-3 fatty acid docosahexaenoate reduces cytokine-induced expression of proatherogenic and proinflammatory proteins in human endothelial cells. *Arteriosclerosis Thrombosis and Vascular Biology* **14**: 1829–1836.

De Martin R, Vanhove B, Cheng Q, Hofer E, Csizmadia V, Winkler H and Bach FH (1993). Cytokine-inducible expression in endothelial cells of an I kappa B alpha-like gene is regulated by NF kappa B. *EMBO Journal* **12**: 2773–2779.

Djonov V, Andres AC and Ziemiecki A (2001). Vascular remodelling during the normal and malignant life cycle of the mammary gland. *Microscopy Research and Technique* **52**: 182–189.

Drake WT, Lopes NN, Fenton JW II and Issekutz AC (1992). Thrombin enhancement of interleukin-1 and tumor necrosis factor-alpha induced polymorphonuclear leukocyte migration. *Laboratory Investigation* **67**: 617–627.

Ereso AQ, Cureton EL, Cripps MW, Sadjadi J, Dua MM, Curran B and Victorino GP (2009). Lipoxin a(4) attenuates microvascular fluid leak during inflammation. *Journal of Surgical Research* **156**: 183–188.

Fei H, Berliner JA, Parhami F and Drake TA (1993). Regulation of endothelial cell tissue factor expression by minimally oxidized LDL and lipopolysaccharide. *Arteriosclerosis Thrombosis and Vascular Biology* **13**: 1711–1717.

Ferrari G, Cook BD, Terushkin V, Pintucci G and Mignatti P (2009). Transforming growth factor-beta 1 (TGF-beta1) induces angiogenesis through vascular endothelial growth factor (VEGF)-mediated apoptosis. *Journal of Cell Physiology* **219**: 449–458.

Fitzpatrick FA & Soberman R (2001). Regulated formation of eicosanoids. *Journal of Clinical Investigation* **107**: 1347–1351.

Fortes GB, Alves LS, De Oliveira R, Dutra FF, Rodrigues D, Fernandez PL, Souto-Padron T, De Rosa MJ, Kelliher M, Golenbock D, Chan FK and Bozza MT (2012). Heme induces programmed necrosis on macrophages through autocrine TNF and ROS production. *Blood* **119**: 2368–2375.

Friedrichs B, Toborek M, Hennig B, Heinevetter L, Muller C and Brigelius-Flohe R (1999). 13-HPODE and 13-HODE modulate cytokine-induced expression of endothelial cell adhesion molecules differently. *Biofactors* **9**: 61–72.

Gamble JR, Khew-Goodall Y and Vadas MA (1993). Transforming growth factor-beta inhibits E-selectin expression on human endothelial cells. *Journal of Immunology* **150**: 4494–4503.

Goeckeler ZM and Wysolmerski RB (1995). Myosin light chain kinase-regulated endothelial cell contraction: the relationship between isometric tension, actin polymerization, and myosin phosphorylation. *Journal of Cell Biology* **130**: 613–627.

Haendeler J, Tischler V, Hoffmann J, Zeiher AM and Dimmeler S (2004). Low doses of reactive oxygen species protect endothelial cells from apoptosis by increasing thioredoxin-1 expression. *FEBS Letters* **577**: 427–433.

Hafezi-Moghadam A, Noda K, Almulki L, Iliaki EF, Poulaki V, Thomas KL, Nakazawa T, Hisatomi T, Miller JW and Gragoudas ES (2007). VLA-4 blockade suppresses endotoxin-induced uveitis: *in vivo* evidence for functional integrin up-regulation. *FASEB Journals* **21**: 464–474.

Haug A, Hostmark AT and Harstad OM (2007). Bovine milk in human nutrition – a review. *Lipids in Health and Disease* **6**: 25.

Holst BD, Hurley WL and Nelson DR (1987). Involution of the bovine mammary gland: histological and ultrastructural changes. *Journal of Dairy Science* **70**: 935–944.

Hordijk PL, Anthony E, Mul FP, Rientsma R, Oomen LC and Roos D (1999). Vascular-endothelial-cadherin modulates endothelial monolayer permeability. *Journal of Cell Science* **112**: 1915–1923.

Ignotz RA and Massague J (1986). Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *Journal of Biological Chemistry* **261**: 4337–4345.

Ismail ZA and Dickinson C (2010). Alterations in coagulation parameters in dairy cows affected with acute mastitis caused by *E. coli* and *S. aureus* pathogens. *Veterinary Research Communications* **34**: 533–539.

Jasper J and Weary DM (2002). Effects of ad libitum milk intake on dairy calves. *Journal of Dairy Science* **85**: 3054–3058.

Jiang WG, Bryce RP, Horrobin DF and Mansel RE (1998). Regulation of tight junction permeability and occludin expression by polyunsaturated fatty acids. *Biochemical and Biophysical Research Communications* **244**: 414–420.

Kadl A and Leitinger N (2005). The role of endothelial cells in the resolution of acute inflammation. *Antioxidants & Redox Signaling* **7**: 1744–1754.

Kaplanski G, Farnarier C, Tissot O, Pierres A, Benoliel AM, Alessi MC, Kaplanski S and Bongrand P (1993). Granulocyte-endothelium initial adhesion. Analysis of transient binding events mediated by E-selectin in a laminar shear flow. *Biophysical Journal* **64**: 1922–1933.

Karlsson A, Markfjall M, Stromberg N and Dahlgren C (1995). Escherichia coli-induced activation of neutrophil NADPH-oxidase: lipopolysaccharide and formylated peptides act synergistically to induce release of reactive oxygen metabolites. *Infection and Immunity* **63**: 4606–4612.

Knepler JL Jr, Taher LN, Gupta MP, Patterson C, Pavalko F, Ober MD and Hart CM (2001). Peroxynitrite causes endothelial cell monolayer barrier dysfunction. *American Journal of Physiology: Cell Physiology* **281**: C1064–C1075.

Kobayashi M, Shimada K and Ozawa T (1990). Human recombinant interleukin-1 beta- and tumor necrosis factor alpha-mediated suppression of heparin-like compounds on cultured porcine aortic endothelial cells. *Journal of Cell Physiology* **144**: 383–390.

Kobayashi K, Oyama S, Numata A, Rahman MM and Kumura H (2013). Lipopolysaccharide disrupts the milk-blood barrier by modulating claudins in mammary alveolar tight junctions. *PLoS ONE* **8**: e62187.

Komatsu T, Itoh F, Kushibiki S and Hodate K (2005). Changes in gene expression of glucose transporters in lactating and nonlactating cows. *Journal of Animal Science* **83**: 557–564.

Lacasse P, Farr VC, Davis SR and Prosser CG (1996). Local secretion of nitric oxide and the control of mammary blood flow. *Journal of Dairy Science* **79**: 1369–1374.

Levi M, Van Der Poll T, Ten Cate H and Van Deventer SJ (1997). The cytokine-mediated imbalance between coagulant and anticoagulant mechanisms in sepsis and endotoxaemia. *European Journal of Clinical Investigation* **27**: 3–9.

Levick JR and Smaje LH (1987). An analysis of the permeability of a fenestra. *Microvascular Research* **33**: 233–256.

Levkau B, Kenagy RD, Karsan A, Weitkamp B, Clowes AW, Ross R and Raines EW (2002). Activation of metalloproteinases and their association with integrins: an auxiliary apoptotic pathway in human endothelial cells. *Cell Death & Differentiation* **9**: 1360–1367.

Ley K, Bullard DC, Arbones ML, Bosse R, Vestweber D, Tedder TF and Beaudet AL (1995). Sequential contribution of L- and P-selectin to leukocyte rolling *in vivo*. *Journal of Experimental Medicine* **181**: 669–675.

Linzell JL and Peaker M (1972). Day-to-day variations in milk composition in the goat and cow as a guide to the detection of subclinical mastitis. *British Veterinary Journal* **128**: 284–295.

Lombardo D, Fanni T, Pluckthun A and Dennis EA (1986). Rate-determining step in phospholipase A2 mechanism. 18 O isotope exchange determined by 13C NMR. *Journal of Biological Chemistry* **261**: 11663–11666.

Lubos E, Kelly NJ, Oldebeken SR, Leopold JA, Zhang YY, Loscalzo J and Handy DE (2011). Glutathione peroxidase-1 deficiency augments proinflammatory cytokine-induced redox signaling and human endothelial cell activation. *Journal of Biological Chemistry* **286**: 35407–35417.

Maddox JF, Aherne KM, Reddy CC and Sordillo LM (1999). Increased neutrophil adherence and adhesion molecule mRNA expression in endothelial cells during selenium deficiency. *Journal of Leukocyte Biology* **65**: 658–664.

Matsumoto M, Nishinakagawa H, Kurohmaru M, Hayashi Y and Otsuka J (1992). Pregnancy and lactation affect the microvasculature of the mammary gland in mice. *Journal of Veterinary Medical Science* **54**: 937–943.

Matsumoto M, Kurohmaru M, Hayashi Y, Nishinakagawa H and Otsuka J (1994). Permeability of mammary gland capillaries to ferritin in mice. *Journal of Veterinary Medical Science* **56**: 65–70.

Mattmiller SA, Corl CM, Gandy JC, Loor JJ and Sordillo LM (2011). Glucose transporter and hypoxia-associated gene expression in the mammary gland of transition dairy cattle. *Journal of Dairy Science* **94**: 2912–2922.

Mavangira V, Gandy JC, Zhang C, Ryman VE, Jones AD and Sordillo LM (2015). Polyunsaturated fatty acids influence differential biosynthesis of oxylipids and other lipid mediators during bovine coliform mastitis. *Journal of Dairy Science* **98**: 6202–6215.

Mayadas TN, Johnson RC, Rayburn H, Hynes RO and Wagner DD (1993). Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. *Cell* **74**: 541–554.

Mcever RP, Beckstead JH, Moore KL, Marshall-Carlson L and Bainton DF (1989). GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel–Palade bodies. *Journal of Clinical Investigation* **84**: 92–99.

Michel CC and Curry FE (1999). Microvascular permeability. *Physiological Review* **79**: 703–761.

Muller WA, Weigl SA, Deng X and Phillips DM (1993). PECAM-1 is required for transendothelial migration of leukocytes. *Journal of Experimental Medicine* **178**: 449–460.

Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, Ichikawa A, Aze Y, Tanaka T, Yoshida N, Ueno A, Oh-Ishi S and Narumiya S (1997). Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature* **388**: 678–682.

Nadjar A, Tridon V, May MJ, Ghosh S, Dantzer R, Amedee T and Parnet P (2005). NFkappaB activates *in vivo* the synthesis of inducible Cox-2 in the brain. *Journal of Cerebral Blood Flow & Metabolism* **25**: 1047–1059.

National Mastitis Council (2004). *National Mastitis Council*. Madison, WI.

Nawroth PP and Stern DM (1986). Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *Journal of Experimental Medicine* **163**: 740–745.

Nielsen MO, Fleet IR, Jakobsen K and Heap RB (1995). The local differential effect of prostacyclin, prostaglandin E2 and prostaglandin F2 alpha on mammary blood flow of lactating goats. *Journal of Endocrinology* **145**: 585–591.

Nishida K, Harrison DG, Navas JP, Fisher AA, Dockery SP, Uematsu M, Nerem RM, Alexander RW and Murphy TJ (1992). Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *Journal of Clinical Investigation* **90**: 2092–2096.

Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, Furuse M and Tsukita S (2003). Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *Journal of Cell Biology* **161**: 653–660.

Niu XF, Smith CW and Kubes P (1994). Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. *Circulation Research* **74**: 1133–1140.

Ohashi Y, Kawashima S, Hirata K, Yamashita T, Ishida T, Inoue N, Sakoda T, Kurihara H, Yazaki Y and Yokoyama M (1998). Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. *Journal of Clinical Investigation* **102**: 2061–2071.

Olson ST, Richard B, Izaguirre G, Schedin-Weiss S and Gettins PG (2010). Molecular mechanisms of antithrombin-heparin regulation of blood clotting proteinases. A paradigm for understanding proteinase regulation by serpin family protein proteinase inhibitors. *Biochimie* **92**: 1587–1596.

Parnham MJ, Bittner C and Leyck S (1987). Changes in glutathione peroxidase activities and the oxidative burst of leukocytes during inflammation in the mouse and rat. *Free Radical Research Communications* **4**: 183–188.

Pepper MS, Baetens D, Mandriota SJ, Di Sanza C, Oikemus S, Lane TF, Soriano JV, Montesano R and Iruela-Arispe ML (2000). Regulation of VEGF and VEGF receptor expression in the rodent mammary gland during pregnancy, lactation, and involution. *Developmental Dynamics* **218**: 507–524.

Pezeshki A, Stordeur P, Wallemacq H, Schynts F, Stevens M, Boutet P, Peelman LJ, De Spiegeleer B, Duchateau L, Bureau F and Burvenich C (2011). Variation of inflammatory dynamics and mediators in primiparous cows after intramammary challenge with Escherichia coli. *Veterinary Research* **42**: 15.

Piotrowska-Tomala KK, Siemieniuch MJ, Szostek AZ, Korzekwa AJ, Woclawek-Potocka I, Galvao AM, Okuda K and Skarzynski DJ (2012). Lipopolysaccharides, cytokines, and nitric oxide affect secretion of prostaglandins and leukotrienes by bovine mammary gland epithelial cells. *Domestic Animal Endocrinology* **43**: 278–288.

Poutrel B, Caffin JP and Rainard P (1983). Physiological and pathological factors influencing bovine serum albumin content of milk. *Journal of Dairy Science* **66**: 535–541.

Predescu S, Knezevic I, Bardita C, Neamu RF, Brovcovych V and Predescu D (2013). Platelet activating factor-induced ceramide micro-domains drive endothelial NOS activation and contribute to barrier dysfunction. *PLoS ONE* **8**: e75846.

Prosser CG, Davis SR, Farr VC and Lacasse P (1996). Regulation of blood flow in the mammary microvasculature. *Journal of Dairy Science* **79**: 1184–1197.

Quarrie LH, Addey CV and Wilde CJ (1994). Local regulation of mammary apoptosis in the lactating goat. *Biochemical Society Transactions* **22**: 178s.

Rabot A, Sinowatz F, Berisha B, Meyer HH and Schams D (2007). Expression and localization of extracellular matrix-degrading proteinases and their inhibitors in the bovine mammary gland during development, function, and involution. *Journal of Dairy Science* **90**: 740–748.

Rambeaud M, Almeida RA, Pighetti GM and Oliver SP (2003). Dynamics of leukocytes and cytokines during experimentally induced Streptococcus uberis mastitis. *Veterinary Immunology and Immunopathology* **96**: 193–205.

Ramirez RA, Lee A, Schedin P, Russell JS and Masso-Welch PA (2012). Alterations in mast cell frequency and relationship to angiogenesis in the rat mammary gland during windows of physiologic tissue remodeling. *Developmental Dynamics* **241**: 890–900.

Rao LV and Rapaport SI (1987). Studies of a mechanism inhibiting the initiation of the extrinsic pathway of coagulation. *Blood* **69**: 645–651.

Rippe B, Kamiya A and Folkow B (1979). Transcapillary passage of albumin, effects of tissue cooling and of increases in filtration and plasma colloid osmotic pressure. *Acta Physiologica Scandinavica* **105**: 171–187.

Romer LH, Mclean NV, Yan HC, Daise M, Sun J and Delisser HM (1995). IFN-gamma and TNF-alpha induce redistribution of PECAM-1 (CD31) on human endothelial cells. *Journal of Immunology* **154**: 6582–6592.

Rossiter H, Barresi C, Ghannadan M, Gruber F, Mildner M, Fodinger D and Tschachler E (2007). Inactivation of VEGF in mammary gland epithelium severely compromises mammary gland development and function. *FASEB Journal* **21**: 3994–4004.

Sans M, Panes J, Ardite E, Elizalde JI, Arce Y, Elena M, Palacin A, Fernandez-Checa JC, Anderson DC, Lobb R and Pique JM (1999). VCAM-1 and ICAM-1 mediate leukocyte-endothelial cell adhesion in rat experimental colitis. *Gastroenterology* **116**: 874–883.

Scalia R and Lefer AM (1998). *In vivo* regulation of PECAM-1 activity during acute endothelial dysfunction in the rat mesenteric microvasculature. *Journal of Leukocyte Biology* **64**: 163–169.

Schenkel AR, Mamdouh Z, Chen X, Liebman RM and Muller WA (2002). CD99 plays a major role in the migration of monocytes through endothelial junctions. *Nature Immunology* **3**: 143–150.

Schmitz S, Pfaffl MW, Meyer HH and Bruckmaier RM (2004). Short-term changes of mRNA expression of various inflammatory factors and milk proteins in mammary tissue during LPS-induced mastitis. *Domestic Animal Endocrinology* **26**: 111–126.

Scholz D, Devaux B, Hirche A, Potzsch B, Kropp B, Schaper W and Schaper J (1996). Expression of adhesion molecules is specific and time-dependent in cytokine-stimulated endothelial cells in culture. *Cell Tissue Research* **284**: 415–423.

Serhan CN, Gotlinger K, Hong S, Lu Y, Siegelman J, Baer T, Yang R, Colgan SP and Petasis NA (2006). Anti-inflammatory actions of neuroprotectin D1/protectin D1 and its natural stereoisomers: assignments of dihydroxy-containing docosatrienes. *Journal of Immunology* **176**: 1848–1859.

Serhan CN, Chiang N and Van Dyke TE (2008a). Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nature Reviews Immunology* **8**: 349–361.

Serhan CN, Yacoubian S and Yang R (2008b). Anti-inflammatory and pro-resolving lipid mediators. *Annual Review of Pathology* **3**: 279.

Shao Y, Wellman TL, Lounsbury KM and Zhao FQ (2014). Differential regulation of GLUT1 and GLUT8 expression by hypoxia in mammary epithelial cells. *American Journal of Physiology: Regulatory Integrative and Comparative Physiology* **307**: R237–R247.

Shuster DE, Kehrli ME Jr, Rainard P and Paape M (1997). Complement fragment C5a and inflammatory cytokines in neutrophil recruitment during intramammary infection with Escherichia coli. *Infection & Immunity* **65**: 3286–3292.

Sordillo LM, Weaver JA, Cao YZ, Corl C, Sylte MJ and Mullarky IK (2005). Enhanced 15-HPETE production during oxidant stress induces apoptosis of endothelial cells. *Prostaglandins and other Lipid Mediators* **76**: 19–34.

Sordillo LM, Streicher KL, Mullarky IK, Gandy JC, Trigona W and Corl CM (2008). Selenium inhibits 15-hydroperoxyoctadecadienoic acid-induced intracellular adhesion molecule expression in aortic endothelial cells. *Free Radicals Biology & Medicine* **44**: 34–43.

Sordillo LM, Contreras GA and Aitken SL (2009). Metabolic factors affecting the inflammatory response of periparturient dairy cows. *Animal Health Research Reviews* **10**: 53–63.

Spector AA (1975). Fatty acid binding to plasma albumin. *Journal of Lipid Research* **16**: 165–179.

Stirling JW and Chandler JA (1976). The fine structure of the normal, resting terminal ductal-lobular unit of the female breast. *Virchows Archives A: Pathology Anatomy & Histology* **372**: 205–226.

Swanson EW (1965). Comparing continuous milking with sixty-day dry periods in successive lactations. *Journal of Dairy Science* **48**: 1205–1209.

Tatarczuch L, Philip C and Lee CS (1997). Involution of the sheep mammary gland. *Journal of Anatomy* **190**: 405–416.

Thomas LH, Haider W, Hill AW and Cook RS (1994). Pathologic findings of experimentally induced Streptococcus uberis infection in the mammary gland of cows. *American Journal of Veterinary Research* **55**: 1723–1728.

Thompson RD, Noble KE, Larbi KY, Dewar A, Duncan GS, Mak TW and Nourshargh S (2001). Platelet-endothelial cell adhesion molecule-1 (PECAM-1)-deficient mice demonstrate a transient and cytokine-specific role for PECAM-1 in leukocyte migration through the perivascular basement membrane. *Blood* **97**: 1854–1860.

Trigona WL, Mullarky IK, Cao Y and Sordillo LM (2006). Thioredoxin reductase regulates the induction of haem oxygenase-1 expression in aortic endothelial cells. *Biochemical Journal* **394**: 207–216.

Van Nieuw Amerongen GP and Van Hinsbergh VW (2002). Targets for pharmacological intervention of endothelial hyperpermeability and barrier function. *Vascular Pharmacology* **39**: 257–272.

Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P and Pritchard KA Jr (1998). Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 9220–9225.

Walker NI, Bennett RE and Kerr JF (1989). Cell death by apoptosis during involution of the lactating breast in mice and rats. *American Journal of Anatomy* **185**: 19–32.

Weaver JA, Maddox JF, Cao YZ, Mullarky IK and Sordillo LM (2001). Increased 15-HPETE production decreases prostacyclin synthase activity during oxidant stress in aortic endothelial cells. *Free Radical Biology & Medicine* **30**: 299–308.

Weiss N, Zhang YY, Heydrick S, Bierl C and Loscalzo J (2001). Overexpression of cellular glutathione peroxidase rescues homocyst(e)ine-induced endothelial dysfunction. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 12503–12508.

Wilde CJ, Addey CV, Li P and Fernig DG (1997). Programmed cell death in bovine mammary tissue during lactation and involution. *Experimental Physiology* **82**: 943–953.

Winn RK and Harlan JM (2005). The role of endothelial cell apoptosis in inflammatory and immune diseases. *Journal of Thrombosis and Haemostasis* **3**: 1815–1824.

Wrighton CJ, Hofer-Warbinek R, Moll T, Eytner R, Bach FH and De Martin R (1996). Inhibition of endothelial cell activation by adenovirus-mediated expression of I kappa B alpha, an inhibitor of the transcription factor NF-kappa B. *Journal of Experimental Medicine* **183**: 1013–1022.

Xia Y, Tsai AL, Berka V and Zweier JL (1998). Superoxide generation from endothelial nitric-oxide synthase. A Ca^{2+}/calmodulin-dependent and tetrahydrobiopterin regulatory process. *Journal of Biological Chemistry* **273**: 25804–25808.

Yasugi T, Kaido T and Uehara Y (1989). Changes in density and architecture of microvessels of the rat mammary gland during pregnancy and lactation. *Archives of Histology and Cytology* **52**: 115–122.

Zhang W, Zheng S, Storz P and Min W (2005). Protein kinase D specifically mediates apoptosis signal-regulating kinase 1-JNK signaling induced by H2O2 but not tumor necrosis factor. *Journal of Biological Chemistry* **280**: 19036–19044.

Zhang X, Wang T, Gui P, Yao C, Sun W, Wang L, Wang H, Xie W, Yao S, Lin Y and Wu Q (2013). Resolvin D1 reverts lipopolysaccharide-induced TJ proteins disruption and the increase of cellular permeability by regulating IkappaBalpha signaling in

human vascular endothelial cells. *Oxidative Medicine and Cellular Longevity* **2013**: 185715.

Zhao FQ and Keating AF (2007). Expression and regulation of glucose transporters in the bovine mammary gland. *Journal of Dairy Science* **90** (suppl. 1): E76–E86.

Zia S, Giri SN, Cullor J, Emau P, Osburn BI and Bushnell RB (1987). Role of eicosanoids, histamine, and serotonin in the pathogenesis of Klebsiella pneumoniae-induced bovine mastitis. *American Journal of Veterinary Research* **48**: 1617–1625.

Zweier JL, Broderick R, Kuppusamy P, Thompson-Gorman S and Lutty GA (1994). Determination of the mechanism of free radical generation in human aortic endothelial cells exposed to anoxia and reoxygenation. *Journal of Biological Chemistry* **269**: 24156–24162.