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Effects of Insulin and 2,4-Thiazolidinedione on Bovine Neutrophil Function In Vitro

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EFFECTS OF INSULIN AND 2,4-THIAZOLIDINEDIONE ON BOVINE NEUTROPHIL FUNCTION IN VITRO

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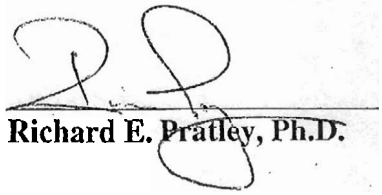

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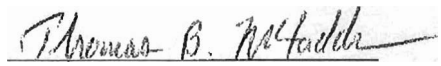

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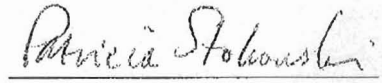

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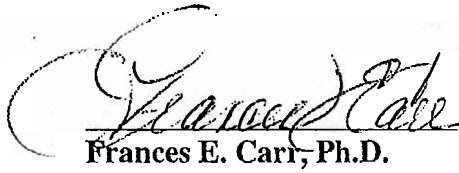

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ABSTRACT

The dairy cow experiences a period of immunosuppression around the time of calving that contributes to the increased incidence and severity of infectious diseases observed during this period. This reduction in immune capacity is due in part to the impairment of neutrophil function, a key component of the innate immune system. In fact, the success of the host defense mechanisms against infection depends on the ability of neutrophils to reach the site of the infection, recognize, engulf and ultimately destroy the pathogen using several mechanisms such as the generation and release of reactive oxygen species (ROS) and the recently described neutrophil extracellular traps or NETs. The alteration in some of these functions and the overall killing ability of neutrophils during the periparturient period has been widely described. However, the physiological mechanisms underlying the period of immunosuppression are not completely elucidated. Interestingly, the impairment of these immune defense mechanisms coincides with the profound metabolic changes associated with parturition and lactogenesis. Changes in several hormones and metabolites have been proposed to be the cause of the reduction in neutrophil function, but the effect of insulin on the functional capacity of these cells has not been investigated. Not only does the concentration of plasma insulin fall as parturition approaches, but also the animal experiences a period of impaired insulin action, termed insulin resistance, during this same time-frame. Therefore, we isolated circulating neutrophils from periparturient and midlactating cows and incubated them with insulin alone or in combination with the insulin-sensitizing agent 2,4-thiazolidinedione (TZD). Subsequently, we measured the total, extracellular, and intracellular generation of ROS, NETs release, phagocytic and killing ability. Insulin did not improve any of the parameters used to assess neutrophil function. In contrast, TZD had a potent inhibitory effect on the total ROS generation, despite an increase in extracellular superoxide anion production. Surprisingly, TZD did not alter the ability of neutrophils to phagocytose and/or kill *Staphylococcus aureus* during an in vitro co-culture. Results suggest that TZD can reduce the oxidative stress that neutrophils experience during their respiratory burst and diminish the damage that ROS cause to the surrounding tissue without compromising the capacity of neutrophils to eliminate the invading pathogen.

CITATION

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CHAPTER ONE: REVIEW OF LITERATURE

INTRODUCTION

Infectious diseases such as mastitis and metritis reduce the profitability of dairy farming due to treatment cost, decreased milk production, and extra labor (Bartlett et al., 1990). For instance, the losses due to mastitis alone are estimated at over 2 billion dollars annually in the US (NMC, 1996). Remarkably, 75% of disease in dairy cows occurs during the periparturient period which is considered to last from 3 weeks before to 3 weeks after parturition (LeBlanc et al., 2006). This increased incidence of diseases is due in part to a period of immunosuppression that occurs for several weeks around the time of calving (Mallard et al., 1998). Furthermore, the resolution of clinical cases of mastitis is determined by cow factors and depends in particular on the capacity of polymorphonuclear cells (PMN) or neutrophils to migrate into the mammary gland, and subsequently phagocytose and kill bacteria (Vangroenweghe et al., 2005). Impairment of these processes results in a period of reduced immunological capacity or immunosuppression around the time of calving (Shuster et al., 1996). Despite considerable research, the understanding of the physiological mechanisms that control this reduced immunocompetence and the increased severity of infections during the periparturient period remains unclear (Paape et al., 2002), although changes in endocrine and metabolic factors that occur during this period seem to have a negative effect on neutrophil number and functionality (Burvenich et al., 1999, Scalia et al., 2006, Suriyasathaporn et al., 2000). Interestingly, this period of immune suppression coincides with a period of reduced circulating insulin concentration and development of insulin

resistance. There is evidence from studies with humans that insulin can improve the ability of neutrophils to kill pathogens (Walrand et al., 2006), but the effects of this hormone on neutrophil function have not been investigated in dairy cows. Thus, the elucidation of endocrine factors that contribute to periparturient immunosuppression and the development of novel strategies to modulate these factors, may offer promise for preventing infectious diseases in dairy cows.

ROLE OF NEUTROPHILS DURING INFECTION

Mammary gland defense mechanisms can be placed into two categories: adaptive and innate immunity. In the case of clinical mastitis, innate immunity attempts to immediately control the intramammary infection (IMI) during the early stages of infection whereas the adaptive immune system responds to the challenge with a high degree of specificity several weeks after initial exposure to the antigen if the innate defense mechanisms fail to completely eliminate the pathogen. The components of innate immunity include primary barriers that protect the host (e.g. skin, mucosal membranes, acidity of the stomach, perspiration, etc.), soluble factors (including the complement system, the enzyme lysozyme, and interferon proteins) and cellular components such as neutrophils, macrophages, and natural killer cells. Importantly, the total number and activity of neutrophils and macrophages play an early role in determining the severity and duration of the infection (Sordillo and Streicher, 2002). Following bacterial invasion, TOLL-like receptors (TLR), expressed on the cellular membrane of macrophages and other antigen-presenting cells, recognize and bind the pathogen-associated molecular patterns (PAMP) in order to release chemoattractants and initiate the migration of blood neutrophils to the

site of infection (Werling et al., 2006). In fact, Saad and Ostensson (1990) infused endotoxin through the teat canal of one udder quarter of healthy cows and determined by cytofluorometric analysis that 74% of the total leukocytes present in milk before infusion were macrophages and monocytes whereas neutrophils are the predominant leukocyte population between 6 and 59 hours after the endotoxin challenge increasing up to 91% at 27 hours post-infusion. In the presence of bacteria, activated phagocytes and epithelial cells produce and release endotoxin, complement-cleavage product C5a, TNF-α, interleukin-1 (Il-1), Il-2, Il-8, and interferons that trigger the initiation of the inflammatory reaction. Once circulating neutrophils sense these chemotactic compounds, they migrate up the concentration gradient of these chemoattractants, roll and adhere to vascular endothelium with the assistance of adhesion molecules. Adhesion molecules such as E-selectin, leukocyte integrin (ICAM-1), CD11a:CD18 (LFA-1) and S-Le are critical in the migration of the PMN through the vessel wall (Paape et al., 2002, Suriyasathaporn et al., 2000). Extravasation or diapedesis of phagocytes is a sequential process beginning with tethering mediated by low-affinity binding of selectin molecules that slow the neutrophils at the vessel wall. This low-affinity binding causes neutrophils to roll along the endothelial lining until L-selectin and β_2-integrin become activated, enhancing affinity that promotes firm adhesion, transendothelial migration and finally de-adhesion (Diez-Fraile et al., 2002).

Once the activated neutrophils cross the blood-milk barrier, they migrate up the gradient of the chemoattractant and express increased levels of Fc receptors for antibody and complement that enable them to bind more effectively to pathogens coated with such

opsonins. Although not essential for phagocytosis, opsonization promotes the uptake of pathogens by neutrophils (Paape et al., 2002). For instance, Guidry et al. (1993) reported that in-vitro phagocytosis of *Staphylococcus aureus* by neutrophils isolated from blood was 57.9 and 32.1% increased when the bacteria were opsonized with IgM and IgG_2, respectively, compared to PMN phagocytosis of non-opsonized *S. aureus*. Neutrophils use their TLR and other pattern recognition receptors to bind and engulf opsonized pathogens and then rely on several killing mechanisms such as the action of potent oxidants (e.g. intracellular and extracellular release of reactive oxygen species), granular enzymes-peptides (Paape et al., 2002) and neutrophil extracellular trap destructive machinery (Brinkmann et al., 2004) to eliminate the invading microorganisms. Therefore, during an IMI, the success of the innate immune response depends on rapid recognition of the pathogen, migration of PMN to the mammary gland and the effectiveness of the cellular and humoral killing mechanisms (Burvenich et al., 2007).

Neutrophil killing mechanisms

Neutrophils play a key role in the resolution of intrammary infection by host defense mechanisms and have been the focus of intense research (Paape et al., 2003). Several lines of investigation have demonstrated that neutrophils are critical to fight a pathogenic insult. For example, Schalm et al. (1976) infused *S. aureus* in mammary quarters of 4 lactating cows to establish chronic mastitis and subsequently administered anti-bovine leukocyte serum that resulted in severe neutropenia in blood and depletion of neutrophil reserves in the bone marrow. Although this observational study lacked an

appropriate control group, the disappearance of neutrophils from blood and bone marrow in these animals was followed by the development of gangrenous mastitis that even resulted in the death of one of the cows as soon as 16 hours after neutropenia. Impairement of the neutrophil's ability to adhere to and cross the endothelial cell lining as well as migrate to the site of the infection favors uncontrolled pathologic processes. In fact, the genetic disorder known as bovine leukocyte adhesion deficiency (BLAD; characterized by reduced expression of the adhesion molecule integrin and a lower ability of PMN to migrate across bovine endothelial cell monolayers), usually results in chronic bacterial infections and premature death (Olchowy et al., 1994). Although the mammary gland posses several antimicrobial defense systems, it is the massive influx of PMNs that will resolve the infection through their killing mechanisms, including the production and release of reactive oxygen species (ROS), reactive nitrogen species (RNS) and the recently described extracellular traps or NETS (Burvenich et al., 2003).

Production of reactive oxygen species (ROS)

The neutrophil is a phagocytic granulocyte generated by the multistep processes of granulopoiesis beginning from a common myeloid precursor and progressing through myeloblast, promyelocyte, myelocyte, metamyelocyte and band cell stages. Granulopoiesis occurs in the bone marrow and includes changes in cell size, nuclear shape and development of granular proteins (Burvenich et al., 2003). Once in circulation, the neutrophil has a short half-life of approximately 8 or 9 hours (Paape et al., 2002). Mature neutrophils have a polymorphic segmented nucleus that allows the cell to line up its nuclear lobes to facilitate the rapid migration between endothelial cells. Within its

cytoplasm, the neutrophil has large islets of glycogen and several types of granules that contain antimicrobial compounds. Bovine neutrophils have azurophilic or primary granules that contain the enzyme peroxidase involved in the production of reactive oxygen species (ROS), specific or secondary granules (less electron dense than the primary granules) and "novel" granules with the majority of antimicrobial protein activity (Paape et al., 2003).

Neutrophils have a dramatic increase in the turnover of the hexose monophosphate shunt and the production of several ROS after cell activation. This respiratory burst activity is non-mitochondrial and is mediated by the enzyme complex NADPH oxidase (Shepherd, 1986). The core component of the NADPH oxidase, the flavocytochrome b_{558}, is distributed between the plasma membrane and the membrane of specific granules when the cell is in rest. Upon activation, this component is incorporated into the wall of the phagocytic vacuole where it carries electrons from NADPH in the cytoplasm to O_2 inside the vacuole. In order to facilitate electron transport through the flavocytochrome, it must interact with other components of the complex such as the proteins p67phox, p47phox, p40phox and p21rac, that also translocate to the membrane of the vacuole (Segal, 2005). Thus, the NADPH oxidase complex catalyzes the consumption of O_2 via electron transfer from NADPH to O_2 resulting in the generation of superoxide anion (O_2^-). In turn, superoxide dismutase converts O_2^- to H_2O_2 which is the main precursor of neutrophil ROS (Hodgson et al., 2006). The enzyme myeloperoxidase forms a complex with H_2O_2 that attacks the pathogen directly or reacts with nitrogen intermediates to form highly reactive compounds (Shepherd, 1986). In fact, the enzyme

nitric oxidase synthase catalyzes the conversion of L-arginine to citrulline with the concomitant release of reactive nitrogen species (RNS) such as nitric oxide, which is oxidized to nitrite and nitrate. The lysosomal vacuoles containing ROS and RNS will fuse with phagosomes, which are vacuoles originating from the extracellular surface containing engulfed (phagocytosed) bacteria and extracellular fluid components. The fusion of the lysosome with the phagosome creates a phagolysosome in which the bacteria can be killed.

Polymorphonuclear neutrophils have the capacity to kill bacteria intracellularly or extracellularly by oxygen-independent or -dependent mechanisms (oxidative burst). Neutrophil killing via extracellular release of ROS is a rather non-specific and inefficient method of bacterial killing because these toxic products are released somewhat indiscriminately into the infected tissue and may or may not actually come in contact with bacteria. Even though these extracellular reactive oxygen species are toxic to bacteria, they also result in severe oxidative stress to mammalian cells. Indeed, neutrophils may promote tissue injury and affect mammary function via either ROS production or granular enzyme release (Zhao and Lacasse, 2007). In fact, Capuco et al. (1986) obtained mammary tissue explants at slaughter from noninfected quarters of 5 lactating cows and incubated them with intact, lysed or zymozan-activated neutrophils isolated from mammary secretions of nulliparous heifers. They reported that after 8 hr in culture, control tissue examined by light microscopy was morphologically normal whereas PMN-treated explants had degenerated, presenting less alveolar luminal area, swollen milk secretory cells with nuclear pyknosis, epithelial vacuolation and filling of

luminal areas with cell debris. No differences in tissue morphology were found among explants treated with lysed, intact or activated neutrophils, but the magnitude of epithelial lysis was greater in those explants incubated with activated PMN.

Neutrophil extracellular traps (NETs)

Neutrophil extracellular traps or NETs were first described by Brinkmann et al. (2004) as a novel antimicrobial mechanism of neutrophils. The biology of these NETs is not yet completely understood, but it is known that after activation, nuclear components with bactericidal properties including the chromatin-histones H1, H2A, H2B, H3 and H4 as well as degradative enzymes such as elastase, myeloperoxidase and the bactericidal permeability increasing protein (BPI) are secreted into the extracellular matrix to physically trap the microorganism and provide a high local concentration of molecules that effectively kill the pathogen. Brinkmann and colleagues also suggested that DNA is a major structural component of NETs since treatment with deoxyribonuclease resulted in disintegration of NETs. Recently, Fuchs et al. (2007) reported that NETs emerge from dying neutrophils in a process different from apoptosis and necrosis. Approximately 2 hours after activation, the cells lose their nuclear and granular membranes allowing the mixing of granular components and chromatin followed by the rupture of the cell membrane and release of NETs into the intracellular space. These neutrophils neither presented a necrotic morphology nor showed signs of DNA fragmentation. However, it is still in debate whether this mechanism used by neutrophils to form and release NETs is similar to that of cell division (Brinkmann and Zychlinsky, 2007). Importantly, Urban et al. (2006) found direct evidence that NETs have anti-microbial properties. They

incubated PMN with the yeast *Candida albicans* for 2 hours and determined colony-forming units to estimate total and extracellular killing by neutrophils, and reported a reduction of total killing from 84% to 48% when DNase was added to digest any formed NETs.

Recent studies have also indicated that bovine neutrophils may set extracellular traps (NETs) that effectively kill bacteria in milk (Lippolis et al., 2006b). Interestingly, NETs secreted by bovine PMN collected from blood are as effective in eliminating bacteria in milk as they are in media. This unchanged killing capacity in milk versus blood is contrary to other killing mechanisms of neutrophils, and suggests that NETs may be an important mechanism of defense of the bovine mammary gland.

PERIPARTURIENT IMMUNOSUPPRESSION

The periparturient period between late pregnancy and early lactation, also known as the transition period, has dramatic effects on the metabolism and health of the dairy cow. The main challenge faced by the animal during this time is the increase of nutrient requirements for milk production at a time when nutrient intake is not sufficient to meet these requirements (Bell, 1995). Remarkably, most infectious diseases and metabolic disorders occur during this short period of time considered to last from 3 wk before parturition until 3 wk after parturition (Drackley, 1999). One of the most important advances in the last 25 years of disease investigation in dairy cattle is the recognition of this transition period as critical for disease prevention in dairy cattle (LeBlanc et al., 2006). Indeed, it has been established that the alterations in immune defense mechanisms

are centered in this period (Mallard et al., 1998) coincide with the metabolic challenges associated with parturition and the onset of lactation. In fact, the number of new cases of clinical mastitis seems to be considerably higher during the first week of lactation compared to those detected from the second week onward in primiparous and multiparous cows (Wilson et al., 2004).

Dairy cows are especially susceptible to intramammary infections during the periparturient period not only because the number of circulating mature neutrophils is lower, but also due to a reduction of polymorphonuclear neutrophil functions including phagocytosis, respiratory burst activity, superoxide anion production, random migration and chemotaxis (Cai et al., 1994). In fact, Shuster et al. (1996) challenged cows at 6-10 days after calving or midlactation with an intramammary infusion of *E. coli* and reported that the peripariparturient animals had more severe clinical signs of mastitis as well as more rapid intramammary bacterial growth (~35 fold greater) after 10 hours of inoculation compared to the midlactating group. The decrease of neutrophil number and functionality during this phase is not well understood and may be partially due to multiple endocrine and metabolic factors (Burvenich et al., 1999, Scalia et al., 2006, Suriyasathaporn et al., 2000), mineral status (Goff, 2006) or underlying changes in expression of key cellular genes (Madsen et al., 2002). Furthermore, periparturient immunosuppression is characterized by a greater percentage of immature neutrophils released into the blood from the bone marrow (Cai et al., 1994) and these immature cells have decreased functional capacity relative to mature neutrophils (Van Merris et al., 2002).

Several lines of research indicate that the respiratory burst activity of neutrophils is diminished around the time of calving. Hoeben et al. (2000) isolated neutrophils twice weekly from healthy cows (n=8) for a period of 14 weeks around parturition and reported that luminol-dependent chemiluminescence (CL) decreased beginning approximately 1 week before parturition, reached lowest values during the first week postpartum and remained low until 5 weeks after parturition. These findings were confirmed by Mehrzad et al. (2002) who reported that the production of reactive oxygen species (ROS) quantified by a similar CL assay, sharply decreased approximately 3 weeks before parturition and stayed low during the first weeks of lactation.

In addition to the reduction in neutrophil respiratory burst activity, the mechanisms regulating the PMN apoptotic pathway may also be altered around the time of calving. The percentage of apoptotic neutrophils in blood is higher during the periparturient period compared to mid-lactation suggesting that more PMN's undergo apoptosis around parturition (Van Oostveldt et al., 2001). Also, apoptotic cells have a decreased respiratory burst and phagocytic activity suggesting that this process might contribute to some extent to the reduction of PMN functionality (Van Oostveldt et al., 2002). In summary, it has been well established that specific mechanisms used by PMNs to kill bacteria are impaired during the periparturient period. Several studies have reported that PMNs obtained from cows during the periparturient period have a reduced capacity to kill gram positive and negative bacteria compared to neutrophils collected from midlactating cows (Cai et al., 1994; Daniel et al., 1991; Detilleux et al., 1995;

Dosogne et al., 2001; Kehrli et al., 1989) confirming that bovine neutrophil function is diminished during this period.

METABOLIC ADAPTATIONS OF THE PERIPARTURIENT COW

The periparturient period represents a considerable metabolic challenge to the dairy cow due to increased nutrient requirements to support the growth and metabolism of the gravid uterus (fetus, fetal membranes, uterine tissues and placenta) and the onset of milk secretion. In late pregnancy, maternal amino acids, glucose, lactate and acetate supply the fetus with the carbon and nitrogen required for its growth, however, more dramatic increases in mammary requirements for glucose, amino acids and fatty acids occur after parturition to support milk synthesis (Bell, 1995). In fact, Overton (1998) estimated the glucose demand of lactating cows to be approximately 1100 g/d at day 21 before parturition whereas 21 days after calving, it increased up to 2500 g/d. Despite the higher nutrient requirements during the periparturient period, voluntary dry matter intake does not increase to the same degree to meet these requirements. Thus, the animal enters a state of negative energy balance and must undergo substantial metabolic adjustments to support pregnancy and lactation (Bell and Bauman, 1997).

In the dairy cow, the supply of glucose to extrahepatic tissues depends primarily on gluconeogenesis because in ruminants little glucose is absorbed from the gastrointestinal tract. As a metabolic adaptation to higher glucose demands, the whole-body oxidation of glucose decreases whereas gluconeogenesis from endogenous sources is enhanced after parturition (Bell, 1995). Reynolds et al. (2003) reported a net glucose

release by the liver of 294, 317, 627, 777 and 810 mmol/h at days -19, -9, 11, 21 and 33 relative to parturition, respectively. Even though liver mass changes and transfer of nutrients across membranes are also upregulated, most of the increased flux of nutrients relies on a higher hepatic metabolic activity (Drackley et al., 2001).

Changes in fatty acid metabolism, including the massive mobilization of triacylglycerols (TG) from fat tissue, are necessary when energy intake is insufficient to support maintenance and milk production. The hydrolysis of stored TG yields one molecule of glycerol and three fatty acids which can be released into the bloodstream as non-esterified fatty acids (NEFA). In fact, plasma NEFA increases approximately twofold between 17 and 2 days before parturition and twofold again at calving (Grummer, 1993). Since hepatic fatty acid uptake is dependent upon NEFA concentration in blood, the liver is challenged around parturition with an increased supply of NEFA. Drackley et al. (2001) estimated a NEFA entry rate of 13.45 mol/day into the circulation of a cow producing 31.9 kg of milk a day at 4 wk of lactation, of which approximately 25% may be taken up by the liver. Assuming that a certain amount of these NEFA is oxidized by the hepatocytes, they concluded that 525 g/d of NEFA would be available for re-esterification or ketogenesis. Plasma NEFA are highly correlated with lipolysis and can be used as an indicator of energy balance (Mashek et al., 2001). Thus, NEFA are oxidized by the liver to produce reducing equivalents, used for hepatic TG synthesis and export as VLDL or converted to ketone bodies. These ketone bodies can be ultimately utilized for oxidation by the heart, kidney, skeletal tissue and gastrointestinal tract or as substrates for mammary gland *de-novo* fatty acid synthesis (Drackley et al., 2001).

Endocrine regulation

Bauman and Currie (1980) defined homeorhesis as the coordinated changes in metabolism to support a physiological state such as pregnancy and/or lactation. These physiological adaptations must be mediated by endocrine regulation including changes in hormones as well as altered tissue responses to these humoral factors. Thus, several hormones regulate the key non-mammary adaptations during the transition period (suppression of peripheral glucose utilization, increase in hepatic gluconeogenesis and mobilization of fat stores). Insulin is one of the major regulators of these adaptations, as described in the next section. However, different hormones also have either a direct effect on glucose and lipid metabolism or indirect effects through alterations in the tissues responses to insulin (Bell and Bauman, 1997).

Growth hormone (GH), also known as somatotropin, plays a pivotal role in the development of the mammary gland (ductal morphogenesis), milk synthesis and metabolic regulation of periparturient dairy cows. The plasma concentration of GH increases during late pregnancy, with a sharp peak at parturition and a post-partum decline to moderate levels through early lactation (Bell, 1995). GH binds to its specific cell surface receptor which is highly expressed in liver but is also present in other tissues. GH can exert its effects directly or via the insulin-like growth factors (IGF-1 and -II) released from target tissues upon binding of GH to its receptor. These effects include an increase in milk synthesis by the mammary gland, enhanced gluconeogenesis and synthesis of protein by the liver, increased lipolysis/reduced lipogenesis and decreased utilization of glucose by muscle tissue (Renaville et al., 2002). Exogenous administration

of bovine somatotropin (bST) to midlactation cows that results in an increase in milk yield, dramatically reduced the rate of lipogenesis and the mRNA expression of the lipogenic enzymes acetyl CoA carboxylase and fatty acid synthase (Lanna et al., 1995). Similarly, Bauman et al. (1988) injected cows with bST between days 63 and 146 of lactation and reported an increase in plasma NEFA (+104 μmol/day) and a reduction in whole body glucose oxidation (-0.4 mol/day), compared to control cows injected with an excipient. Thus, exogenous GH administration results in marked changes in partitioning of nutrients to support higher milk production. Importantly, the GH actions on liver are reduced during the periparturient period limiting IGF-1 production whereas its effects on muscle and adipose tissue remain intact (Bauman and Vernon, 1993). In fact, the expression of GH receptors in liver early in lactation is reduced compared to that of midlactation, resulting in a fall of approximately 70% in plasma IGF-1, which is primarily produced by hepatic tissues (Wook Kim et al., 2004).

Epinephrine, norepinephrine, and glucagon to some extent, stimulate lipolysis by binding β-adrenergic receptors in adipocytes, resulting in stimulation of adenylate cyclase activity and increased production of cyclic AMP with the subsequent activation of protein kinase A. In turn, this kinase phosphorylates and activates hormone-sensitive lipase which is responsible for the breakdown of TGs into free fatty acids and glycerol (Vernon and Pond, 1997). In response to the necessity to mobilize energy from fat stores, the sensitivity and responsiveness of adipose tissue to these adrenergic agents is increased during the periparturient period (Bell, 1995). Thus, increased lipolysis during lactation is mediated in part by decreased serum insulin and increased response to catecholamines.

The marked periparturient increase in plasma prolactin and the increase in prolactin receptors in the mammary gland that occurs with the onset of lactation are essential for the initiation of lactogenesis, including direct effects on ion transport, amino acid transport, golgi volume, and synthesis and secretion of milk fat and proteins (Tucker, 2000). In addition to its role in lactogenesis, prolactin may also regulate the metabolic adaptations of transition cows, supporting the partitioning of nutrients towards the mammary gland by decreasing synthesis of lipid reserves and increasing mobilization of fat stores (Bauman and Currie, 1980).

Plasma levels of estradiol in dairy cows increase progressively during late pregnancy, peaking several days prior to parturition whereas progesterone concentrations decrease just before parturition (Bell, 1995). Although the mechanisms are not completed elucidated, higher progesterone during pregnancy might increase the sensitivity of adipose tissue to insulin resulting in increased glucose uptake and lipogenesis. In contrast, during late pregnancy and early lactation, estrogen decreases insulin sensitivity and responsiveness to allow increased hepatic gluconeogenesis, reduced lipogenesis and reduced protein synthesis in muscle (Bell and Bauman, 1997).

Role of insulin in the partitioning of nutrients during the periparturient period

Insulin is a peptide hormone composed of 51 amino acid residues that is synthesized and secreted by the β-pancreatic cells located in clusters of cells known as islets of Langerhans. It consists of two peptide chains, termed A and B (21 and 30 amino acids, respectively) joined by two disulfide bridges (Owerbach et al., 1980). The

production of insulin begins with the synthesis of mRNA in the nucleus of the β-pancreatic cell followed by the formation of the insulin precursor preproinsulin in the rough endoplasm reticulum. This precursor is transferred to the Golgi apparatus and then to the secretory granules in the cytoplasm where insulin is stored. Several factors can stimulate insulin secretion including nutrients, gastrointestinal hormones, parasympathetic stimuli and certain therapeutics (Hayirli, 2006).

Insulin can influence metabolism indirectly by regulating substrate supply from extrahepatic tissues or by controlling homeorhetic adaptations such as gluconeogenesis and lipolysis. It promotes the uptake of glucose into muscle and fat tissue, enhances incorporation of amino acids into muscle and increases lipogenesis by adipocytes (Brockman, 1978). As described earlier, the last stage of pregnancy and the onset of lactation require significant metabolic adaptations to meet the increased glucose requirements of the gravid uterus or lactating mammary gland. In the dairy cow this adaptation is partially accomplished via decreased circulating concentrations of insulin during lactation. Prepartum plasma insulin concentration is higher than postpartum (Bell, 1995, Blum et al., 1973); Smith (2006) reported a decline in plasma insulin from approximately 0.67 ng/mL at day 21 before parturition to 0.1 ng/mL on the day of calving. After parturition, the hormone levels slowly increase to reach 0.2 ng/mL 3 weeks after calving. This lower periparturient insulin concentration is due to a diminished pancreatic output rather than any increase in hepatic uptake (Lomax et al., 1979) and is one of the metabolic regulations that allow mobilization of stored nutrients to support the increased energy demands of lactation.

Insulin is the major hormone favoring fatty acid synthesis and lipid accumulation in bovine adipose tissue by up-regulation of transcription of genes encoding enzymes involved in lipid synthesis such as lipoprotein lipase and acetyl CoA carboxylase and stimulation of glucose transport through the translocation of glucose transporters (Vernon and Pond, 1997). Thus, the drop in plasma insulin together with the development of insulin resistance during the periparturient period, allows the mobilization of fat stores in the dairy cows. In fact, exogenous insulin has a profound effect on fatty acid metabolism in cows during the transition period. For instance, Mashek et al. (2001) infused insulin at a constant rate of 1 μg/kg/hr for 4 days, while maintaining glucose concentration at previously determined baseline levels, into cows at weeks 4 and 17 of lactation to determine its effects on plasma metabolites and hormones. They reported a decrease of 259 uEq/L in plasma NEFA in early lactating cows, as well as a reduction in β-hydroxybutyrate of 0.64 mM regardless of stage of lactation, compared to the pre-infusion levels.

Insulin signaling

Insulin signaling involves several events initiated by hormone binding to its receptor which is located in the cell surface. The insulin receptor is a tetramer with two alpha and two beta subunits with intrinsic tyrosine kinase activity. After binding, the tyrosine residue of the beta subunits are autophosphorylated at several intracellular tyrosine residues resulting in a conformational change of the membrane with the subsequent phosphorylation of insulin receptor substrate proteins (IRS), and other targets such as Shc, Cbl, p62dok and Gab-1, by the insulin receptor tyrosine kinase (Lizcano and

Alessi, 2002). Following phosphorylation, these substrates act as docking molecules for proteins that contain src homology region 2 (SH2) domains that become activated or associate with other downstream molecules resulting in a complicated cascade of events (Biddinger and Kahn, 2006). Several intracellular kinases, including the PtdIns 3, MAP, S6 and protein kinase C (downstream of PDK-1) are components of the insulin signaling and mediate the coupling of the receptor to its downstream effectors to yield numerous biological responses (Kim et al., 2003, Wilden and Kahn, 1994). Insulin is a mediator of nutrient partitioning, it plays a major role in the regulation of glucose metabolism, promotes the cellular utilization of glucose, and also regulates protein and lipid metabolism (Hayirli et al., 2002).

Insulin resistance

Insulin resistance develops when normal levels of insulin produce a less than normal response either because of decreased sensitivity to the hormone (associated with alterations before the interaction with its receptor), or due to a decrease in the maximal response to the hormone, related to post-receptor alterations (Kahn, 1978). Even though the assessment of insulin resistance focuses on its effects on glucose homeostasis, insulin resistance also affects other metabolic processes such as the synthesis and storage of fat, protein synthesis and cell growth and differentiation. Furthermore, not all the processes and tissues regulated by the hormone become equally resistant to insulin. In fact, it has been suggested from studies with insulin receptor knockout mice that insulin resistance in fat and muscle is not always detrimental to overall glucose homeostasis, in contrast with

the diabetic-ketoacidosis effects of insulin resistance in brain, liver and β-cells (Biddinger and Kahn, 2006).

Reduced sensitivity to insulin of glucose utilization, lipolysis and nonesterified fatty acid (NEFA) mobilization, has been observed during late pregnancy in humans, laboratory animals and sheep (Bell, 1995) indirectly mediating the enhanced glucose synthesis and attenuated glucose utilization in nonuterine and nonmammary tissues (Bell and Bauman, 1997). The molecular mechanism of the insulin resistance in lactation, including the transcription of genes encoding metabolic enzymes have not been totally elucidated (Vernon and Pond, 1997). In fact, most of the evidence on insulin resistance in ruminants comes from studies with pregnant and lactating sheep. For instance, Vernon et al. (1981) biopsied adipose tissue from the rump of sheep at different physiological stages of lactation and found a reduced number of high-affinity and total insulin receptors (both receptors per cell and receptors per area of cell surface) in adipocytes by day 105 of pregnancy, compared to samples obtained from animals between 70 to 100 days of pregnancy. The authors also reported a fall of circulating insulin from 4.29 to 0.87 ng/mL, suggesting that the changes in the hormone levels and its response machinery facilitate the switch to lipid mobilization observed during late pregnancy in sheep. Furthermore, muscle insulin resistance has been documented in lactating sheep; Vernon et al. (1990) reported that the ability of insulin to stimulate glucose utilization by muscle tissue, assessed by glucose arterial-vein difference across the hindlimb, is diminished in lactating ewes at ~18 days postpartum compared to non-pregnant non-lactating animals. Similarly, Petterson et al. (1994) performed a hyperinsulinemic euglycemic clamp to

assess insulin resistance by means of quantifying its antilipolytic effect. They reported a reduced responsiveness to the hormone in pregnant ewes compared to nonpregnant sheep since the maximal insulin-suppressed concentrations of NEFA and glycerol (R_{max}), were 148 and 64.5 μmol/L, respectively. In beef cows, peripheral tissue responsiveness to insulin was lower in late pregnant and lactating animals than in nonpregnant nonlactating and dry cows, respectively (Sano et al., 1990) but tissue responsiveness to insulin remained unchanged during lactation in dairy cattle, perhaps as a result of greater weight loss in dairy than beef animals during lactation (Sano et al., 1993).

2,4-Thiazidionelidione, a PPARγ agonist, enhances insulin sensitivity

PPARγ is a member of the nuclear receptor family highly expressed in adipose tissue and immune system. It contains N-terminal, DNA-binding and ligand-binding domains. Once the ligand binds to the receptor causing a conformational change, it binds to other transcription factors such as the 9-cis-retinoic acid receptor (RXR) to form a heterodimer. This complex in turn binds to peroxisome proliferator response elements (PPRE) on DNA resulting in the activation of transcription. PPARγ activation has a role in many physiological events such as adipocyte differentiation, stimulation of insulin action, regulation of lipid metabolism and effects on inflammatory processes. However, the mechanisms underlying these effects have not been completely elucidated (Houseknecht et al., 2002).

Thiazolidinediones (TZDs) are synthetic PPARγ ligands that have anti-inflammatory effects, improve insulin resistance and lower plasma glucose levels in

diabetic humans (Pascual et al., 2007). In dairy cows, Smith et al. (2007) administered 4 mg/kg BW of 2,4-TZD during the last 25 days of pregnancy and reported that treated cows had lower plasma NEFA concentrations compared to controls (70 vs. 83 μEq/L, repectively) during the prepartum period and a tendency to be decreased ($P<0.06$) from day 7 before parturition through day 7 postpartum (113 μEq/L for cows treated with TZD vs. 205 μEq/L for control animals). Similarly, Kushibiki et al. (2001) found that 2,4-TZD administration to steers with recombinant bovine tumor necorsis factor (rbTNF)-induced insulin resistance restored insulin-mediated glucose utilization since the calculated area under the curve for changes of plasma glucose concentrations after a single dose of i.v. insulin was 39.5 and 45.65% smaller in the TNF-treated steers compared to control and TNF + TZD steers, respectively.

Both PPARα and PPARγ are expressed in human mononuclear cells and macrophages. In addition to their role in fatty acid metabolism, these PPARs also exert direct anti-inflammatory activities (Chinetti et al., 2000). Therefore, their synthetic ligand TZD, has been shown to induce a potent anti-inflammatory effect by binding to PPARγ, which through DNA-independent mechanisms, interfere with transcription factors involved in inflammatory pathways (Yki-Jarvinen, 2004). As an example, Jiang et al. (1998) incubated activated human blood peripheral mononuclear cells from 6 donors with troglizatone and reported a dose-dependent inhibition of PMA-induced synthesis of the inflammatory cytokines TNF-γ, IL-6 and IL-1β. Similarly, incubation of macrophages with several types of thiazolidinediones inhibited the mRNA expression of nitric oxidase synthase (iNOS), responsible for the generation of reactive nitrogen species (Ricote et al.,

1998). Consistent with these effects during in vitro incubations, administration of TZDs to healthy subjects has suppressed ROS generation by mononuclear cells and decreased the plasma concentrations of TNF-α and IL-6 and the adhesion molecule sICAM (Ghanim et al., 2001). These findings suggest a modulatory role for PPARs in the control of the inflammatory response with potential therapeutic applications in inflammation-related diseases (Chinetti et al., 2000).

EFFECT OF PERIPARTURIENT ENDOCRINE AND METABOLIC CHANGES ON IMMUNE CELL FUNCTION

The periparturient period of dairy cows has the highest incidence and severity of infectious diseases due to a period of immunosuppression which coincides with the appearance of structural changes in the mammary gland and a metabolic shift from anabolism to catabolism, both modulated through changes in concentrations of systemic and local hormones (Vangroenweghe et al., 2005). This period of reduced immune capacity may not be explained by the reduction of a single independent immune factor. Instead, the humoral and metabolic changes associated with the physiological adaptations that occur around parturition, seem to affect several functions of various types of immune cells (Mallard et al., 1998, Overton and Waldron, 2004). A strong immune response can affect the metabolic status of periparturient cows and therefore, it is possible that immunosuppression might be an adaptation to lactation, despite its detrimental effects on immune processes (Overton and Waldron, 2004).

The postpartum negative energy balance (NEB) and the related endocrine changes have been proposed to contribute to periparturient immunosuppression. For instance, Kimura et al. (1999) hypothesized that removing the nutrient demands of the mammary gland via mastectomy while maintaining endocrine and other changes associated with parturition would eliminate the reduction of neutrophil function after calving. There was no difference in leukocyte counts between mastectomized and intact cows and the elimination of milk production didn't prevent the decline of myeloperoxidase activity during the last 3 weeks of pregnancy, but mastectomized animals had a rapid recovery of myeloperoxidase activity after parturition compared to controls. In contrast with these results, Perkins et al. (2001) fed Holstein steers either a positive or negative energy balance diet (210 and 60% of maintenance requirements) in a crossover design with two treatment periods of 3 weeks separated by an adaptation period of 2 weeks. They reported that the negative energy balance had no effect on the mean fluorescence intensity associated with the expression of the adhesion molecules CD11b, CD18 and MHC class I, but modestly increased the expression of CD62L and MHC class II on neutrophils and leukocytes, respectively. The same investigators (2002) infused endotoxin into one mammary quarter of midlactating cows fed ad libitum or at 80% of maintenance energy requirements. Intramammary infusion elicited a local and systemic inflammatory response including a 60% reduction in blood leukocyte counts and an increase in milk somatic cell counts, but there was no effect of feed restriction on blood total leukocyte counts, rumen motility, serum TNF-α or milk IgG concentrations. Furthermore, Stabel et al. (2003) increased the dietary energy intake of periparturient cows with Johne's disease

by mechanically stuffing refused feed into fistulated cows. They reported no differences in chemotaxis and iodination of neutrophils collected from these cows compared to neutrophils isolated from cows fed ad libitum. Together, these findings suggest that the negative energy balance per se that dairy cows experience during the periparturient period has little or no effect on immunosuppression. However the presence of the mammary gland and the metabolic demands of lactogenesis seem to contribute to this period of reduced immune competence.

There is also evidence that the changes in fatty acid metabolism around parturition associated with high levels of non-esterified fatty acids (NEFAs) and ketone bodies impair immunity even though the mechanisms of this effect is still unknown (Suriyasathaporn et al., 2000). For example, the myeloperoxidase activity of blood neutrophils, and cytochrome *c* reduction, both indexes of ROS production, were impaired in cows with puerperal metritis and subclinical endometritis that showed higher NEFA levels during the same period (Hammon et al., 2006). The authors reported a negative correlation (R = 0.44) between plasma NEFA levels and myeloperoxidase activity for these animals. Although these results do not establish a cause-effect relationship, the negative effect of NEFA on neutrophil function has been confirmed by Scalia et al. (2006) who reported that incubation of bovine neutrophils with high (2 and 1m*M*), moderate (0.5 and 0.25m*M*) or low (0.125 and 0.0625m*M*) concentrations of NEFA resulted in a ~11% reduction in oxidative burst activity in those cells incubated with low and moderate levels of NEFA whereas the highest concentration of NEFA resulted in an increase of necrosis with a concomitant decrease in neutrophil viability of 51%.

As discussed earlier, the metabolic profile of periparturient dairy cows also includes high circulating concentrations of ketone bodies. To test the hypothesis that this increase in ketones is partially responsible for immunosuppression, Suriyasathaporn et al. (1999) compared the chemotaxis ability of lymphocytes isolated from cows between their 5-10 weeks of lactation with low, medium or high plasma levels of β-hydroxybutyrate (BHB). Cows with high levels of BHB (>1.6mM) had a lower chemotactic activity compared to those with low and medium BHB levels (<0.8 and 0.8-1.6 mM, respectively). Consistently, when they isolated blood lymphocytes from subclinically and clinically ketotic cows and incubated them with BHBA, acetoacetate, acetone or a mixture of these, they reported an impairment of in-vitro migration ability in an environment with ketone bodies. Similarly, 1 and 2.5 m*M* concentrations of β-hydroxybutyric acid in vitro have been shown to inhibit the chemiluminescence associated respiratory burst (10 and 15%, respectively) of neutrophils isolated from healthy periparturient cows (Hoeben et al., 1997).

In addition to metabolites, endocrine factors seem to be partially responsible for the diminished neutrophil activity during the periparturient period. For example, glucocorticosteroids, widely used as anti-inflammatory agents, suppress several functions of immunity. Hoeben et al (1998) measured the respiratory burst activity of bovine neutrophils in-vitro after exposure to one of eight glucocorticosteroids and reported a decrease in the chemiluminescence response from cells treated with 7 out of 8 drugs, including cortisone and hydrocortisone which at a concentration of 2×10^{-4} mM reduced the respiratory burst activity by 7 and 13%, respectively. However, the peak of cortisol

that occurs at calving lasts only hours and might not explain the prolonged state of immunosupression observed around (especially prior to) parturition (Overton and Waldron, 2004).

Lactogenic hormones and factors associated with structural and functional changes during the dry period and onset of lactation have been related to periods of highest incidence of infections (Vangroenweghe et al., 2005). In fact, the effect of the lactogenic hormone prolactin (PRL) and growth hormone (GH), both necessary for the transition from a proliferative to a lactating mammary gland, on periparturient immunity have been studied. Dairy steers on long day photoperiods have higher concentrations of circulating PRL but reduced expression of PRL receptor on peripheral blood mononuclear cells (PBMCs), compared to those under short day photoperiod exposure (Auchtung and Dahl, 2004) and neutrophils collected from cows assigned to a short day photoperiod of 8 hours of light during the dry period had greater migration towards the chemoattractants IL-8 and C5a, while their lymphocytes showed enhanced cellular proliferation (Auchtung et al., 2004). Treatment of cows with bovine somatotropin (bST) may enhance neutrophil function; Hoeben et al. (1999) treated healthy lactating cows (6-15 weeks postpartum) with 500 mg of bST 7 days before and after experimental *S. uberis* infection and reported reduced fever, tachycardia, swelling, pain and firmness of infected glands but little effect on the luminol-dependent luminescence of blood neutrophils compared to control cows. In summary, all the metabolic and endocrine changes during this period seem to contribute to some extent to the reduction in neutrophil function. Insulin, a key endocrine regulator of these changes might have an effect on immunity

either by altering plasma and tissue concentrations of metabolites and other hormones or by a direct effect on PMNs.

Effect of insulin on neutrophil activity

Insulin receptors have been detected in low levels on neutrophils in dairy cattle by indirect immunofluorescence staining and flow cytometry (Nielsen et al., 2003), but the effects of insulin on immune function have not been investigated in dairy cows. It is conceivable that insulin could act upon neutrophils in multiple ways. For example, changes in insulin concentration or resistance to the hormone might result in a lack of stored glycogen within the neutrophil that could limit the production of NADPH and the subsequent bacteriocidal peroxidase activity of these cells (Hammon et al., 2006), modulation of the activity of key glycolytic enzymes such as glucose-6-phospate dehydrogenase (G6PDH) and phosphofructokinase (PFK) (Alba-Loureiro et al., 2006) or could alter neutrophil granulopoiesis and differentiation.

In humans, it has been recognized that patients with diabetes have impaired neutrophil phagocytosis, bactericidal activity, release of lysosomal enzymes and production of ROS (Alba-Loureiro et al., 2007, Bilgic et al., 2008). Also, neutrophils isolated from the peritoneal cavity of induced diabetic rats had reduced phagocytosis and hydrogen peroxide content (17 and 31%, respectively) compared to control rats. Interestingly, insulin treatment restored these neutrophil functions to the values found in control animals (Alba-Loureiro et al., 2006). In view of these findings, the therapeutic use of insulin to improve neutrophil function has been further investigated, at least in

humans. For example, incubation of neutrophils with insulin at physiologically relevant concentrations has been shown to increase their chemotaxis ability in a dose-response manner (Cavalot et al., 1992) whereas Rassias et al. (1999) reported that non-diabetic patients receiving insulin treatment during cardiac surgery had enhanced phagocytic activity due to a 41% increased absolute number of circulating neutrophils.

Interestingly, infusion of insulin into healthy patients with controlled plasma glucose levels has yielded contrasting results. Walrand et al. (2004) infused 0.7 mU/kg/min of insulin into non-diabetic, non-obese subjects while maintaining strict euglycemia for a period of 4 hours. They collected neutrophils from blood before and after the infusion and reported that insulin increased the total neutrophil counts by 66%, the directed migration of PMNs towards a chemoattractant by approximately 150% as well as the overall phagocytosis, and bactericidal capacity, when compared to neutrophils isolated before the insulin clamp. Contrary to these results, Fejfarova et al. (2006) performed a similar hyperinsulinemic euglycemic clamp on healthy individuals and reported no effect of insulin infusion on neutrophil phagocytic or respiratory burst activity, compared to neutrophils isolated from subjects receiving a saline infusion. They suggested that these findings contrast to those reported by the study of Walrand and others (2004) because in that study, the authors did not compare the neutrophil function indexes with an appropriate control such as the infusion of saline.

CONCLUSION

Dairy cows are more susceptible to mastitis during the periparturient period because of the development of a state of immunosuppression characterized by a reduction of number and function of neutrophils around the time of calving. The mechanisms responsible for this decrease in neutrophil function are not completely understood, but it is possible that several factors contribute to this phenomenon. Remarkably, the periparturient cow experiences a tremendous metabolic challenge due to the increased nutrient requirements to support pregnancy and lactogenesis. Thus, the animal undergoes a series of physiological changes carefully coordinated by the endocrine system that result in partitioning of nutrients towards the mammary gland. In view of this time-coincidence between the periparturient metabolic changes and this period of immunosuppression, the effect of several metabolites and hormones on immune function has been investigated. For example, the presence of the mammary gland (Kimura et al., 1999), the negative energy balance during the periparturient period (Perkins et al., 2002, Perkins et al., 2001), high levels of NEFA and ketone bodies (Hoeben et al., 1997, Scalia et al., 2006, Suriyasathaporn et al., 2000), cortisol (Hoeben et al., 1998) and sex steroids (Winters et al., 2003) have been proposed to contribute to the reduction in the immune capacity of transition cows. Despite these considerable efforts, the physiology of the periparturient immunosuppression has not been elucidated (Paape et al., 2002b).

Part of the homeorhetic control in cows during the transition period includes a decrease in the plasma concentration of insulin and the development of insulin resistance beginning several weeks before parturition that allows a shift from anabolism to

catabolism typical of the periparturient period. However, whether insulin modulates immune function in dairy cows is unknown. In humans, diabetes has been associated with decreased neutrophil function and there is some evidence that exogenous insulin can improve this condition. Furthermore, a class of antidiabetic drugs known as thiazolidinediones exert insulin-sensitizing effects by binding to the nuclear receptor PPARγ. Interestingly, recent studies have reported a potent anti-inflammatory effect of these TZDs, at least on human macrophages and peripheral mononuclear cells. Together, these findings highlight the potential for therapeutic intervention on insulin metabolism of bovine neutrophils to improve the immune competence of dairy cows during the periparturient period.

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CHAPTER TWO: EFFECTS OF IN VITRO INSULIN AND 2,4-THIAZOLIDINEDIONE ON THE FUNCTION OF NEUTROPHILS HARVESTED FROM BLOOD OF COWS IN DIFFERENT PHYSIOLOGICAL STATES

ABSTRACT

Neutrophils (PMN) were isolated from twenty six Holstein cows in different physiological states (12 ± 1.7 d prepartum, n = 8; 7 d postpartum, n = 9; 253 ± 25.2 d postpartum, n = 9) and incubated in vitro for 120 min in a factorial arrangement of treatments with 0, 1.5 or 15 ng/mL of bovine insulin and 0 or 300 μg/mL of the PPAR-γ ligand 2,4-thiazolidinedione (TZD). Following the incubations, PMN functional assays were performed to determine treatment effects on total, extracellular, and intracellular generation of reactive oxygen species (ROS), neutrophil extracellular trap (NETs) formation and phagocytic-killing abilities. The respiratory burst activity of PMN collected from cows at 7d postpartum was reduced compared to that of PMN from midlactating and pre-partum cows, but NETs expression was 23 and 36% higher in PMN from pre-partum cows compared to mid-lactating and post-partum cows, respectively. Furthermore, insulin had no effect on any of the assays used to assess PMN function. In contrast, TZD inhibited total ROS production by ~89%, increased extracellular superoxide generation by ~43% but had no effect on intracellular ROS. Interestingly, TZD did not alter the ability of the PMN to release NETs and engulf or kill *S. aureus*. These findings suggest an anti-inflammatory effect of TZD that may result in reduced extracellular oxidative damage with maintainance of PMN antimicrobial activity.

Key words: neutrophil, immunosuppression, insulin, thiazolidinedione

INTRODUCTION

The periparturient dairy cow is more susceptible to infectious diseases in part due to a period of immunosuppression that occurs for several weeks around the time of calving (Mallard et al., 1998). As an example, the number of first cases of clinical mastitis is higher during the first week of lactation compared to those detected from the second week onward (Wilson et al., 2004). Also, Shuster et al. (1996) challenged periparturient cows with an intramammary infusion of *E. coli* and reported that they had more severe clinical signs of mastitis as well as a more rapid bacterial growth after 10 hours of inoculation compared to midlactating animals. Despite considerable progress in the understanding of the biology of the periparturient cow in the last 25 years, the incidence and severity of the most important infectious disease has remained unchanged (LeBlanc et al., 2006b). A recent survey by the National Animal Animal Health Monitoring Service reported that clinical mastitis is prevalent in 16.5% of the cows in the U.S. and that from the total number of cows that are permanently removed from the herd, 23% are because of mastitis or udder problems (USDA, 2008).

During an intramammary infection, patrolling macrophages recognize the pathogen and trigger an inflammatory response resulting in the migration of polymorphonuclear cells (PMN) or neutrophils from circulation to the site of infection. Thus, during the early stages of the insult, neutrophils are the predominant leukocyte present in the gland accounting for up to 91% of the total leukocyte population (Saad and Ostensson, 1990). Neutrophils play a key role in the resolution of mastitis and have been the focus of intense research (Paape et al., 2003). These PMN, by means of their TLR receptors and

other pattern recognition receptors, bind and engulf opsonized bacteria in a process known as phagocytosis. Subsequently, PMN use several killing mechanisms such as generation and release of potent oxidants (e.g. intracellular and extracellular release of reactive oxygen species or ROS) and antimicrobial enzymes and peptides (Paape et al., 2002). Neutrophils are also capable of mixing these toxic compounds with their own DNA and histones and release these structures termed neutrophil extracellular traps (NETs) into the extracellular matrix where the pathogens are physically trapped and destroyed (Brinkmann et al., 2004).

The total number and function of neutrophils are reduced during the periparturient period (Cai et al., 1994, Detilleux et al., 1995, Kehrli et al., 1989), but the physiological mechanisms responsible for these decreases remain unclear (Paape et al., 2002). Remarkably, there is a co-incidence between this period of reduced neutrophil function and the periparturient fall of plasma insulin together with the occurrence of insulin resistance, typical of lactation (Bell and Bauman, 1997). Moreover, there is evidence that insulin can regulate neutrophil function in humans. For instance, neutrophils collected from healthy individuals had improved chemotaxis, phagocytosis and bactericidal ability after the subjects received a 4-hour insulin infusion under strict euglycemia, relative to the neutrophils collected before the infusion (Walrand et al., 2004). However, the effects of this hormone on neutrophil function has not been investigated in dairy cows, despite the fact that neutrophils isolated from dairy heifers express insulin receptors (Nielsen et al., 2003). Furthermore, 2,4 thiazolidinedione (TZD), a synthetic PPARγ ligand, has been shown to restore insulin response in steers with insulin resistance induced by recombinant

tumor necrosis alpha (Kushibiki et al., 2001). PPARγ is a member of the nuclear receptor family highly expressed in adipose tissue and immune system that upon activation can regulate adipocyte differentiation, stimulation of insulin action, regulation of lipid metabolism and inflammatory processes (Houseknecht et al., 2002). Therefore, we hypothesized that periparturient cows have reduced neutrophil function compared to midlactating animals and that incubation of PMN with insulin or the PPAR-γ ligand TZD would improve PMN functionality as assessed by the production and release of ROS, NETs formation, and their phagocytotic and killing ability.

MATERIALS AND METHODS

Animal Procedures

The use and care of all animals were approved by The University of Vermont Animal Care and Use Committee. Twenty-six multiparous Holstein cows entering second or greater lactation were used in this experiment. All cows were free of obvious clinical infection or inflammation and their rectal temperatures were less than 39.5°C. Individual milk samples were collected the day of the experiments and somatic cell counts (SCCs) were determined using a DeLaval® cell counter (DeLaval International AB, Sweden). SCCs were not different between midlactation and postpartum cows (181 ± 54 and 145 ± 57 x 10^3 SCC/mL, respectively; $P > 0.20$). Blood (80 mL) was collected via coccygeal vessel puncture into evacuated tubes containing acid-citrate dextrose (Vacutainer®, Becton Dickinson, Franklin Lakes, NJ), inverted 3 times and stored on ice until laboratory processing (<45 min). Samples were taken from cows 12 ± 1.7 days before calving (n=8), 7 days postpartum (n=9) and during mid-lactation (n=9; 253 ± 25.2 days

postpartum) to evaluate the differential effects of physiologic state and/or in vitro insulin and TZD incubation on blood neutrophil function.

Reagents

Percoll® was obtained from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). Bovine insulin at a concentration of 10mg/ml in a 25 m*M* HEPES, pH 8.2 solution and Ionomycin from *Streptomyces conglobatus* were obtained from Sigma Chemical Co. (St. Louis, MO). The 2,4-Thiazolinedione (TZD; Sigma Chemical Co.) was prepared as a 100 mg/mL stock solution in dimethyl sulfoxide (DMSO). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co.), methyl cypridina luciferin analog [MCLA; 2-methyl-6-(4-methoxyphenyl)-3,7dihydroimidazol[1,2-a]pyrazin-3-one hydrochloride; Sigma Chemical Co.] and Phorbol 12-myristate,13-acetate (PMA; Sigma Chemical Co.) were prepared as 89.4 m*M*, 1371.1 μ*M* and 162.12 μ*M* stock solutions, respectively, in DMSO. 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein di-acetate acetyl ester (CM-H2DCFDA; Invitrogen Co., Eugene, OR) was prepared as 1730.7 μ*M* stock solution in DMSO. Sytox® Orange nucleic acid stain was obtained from Invitrogen Co. as a 5 m*M* solution in DMSO. All stock solutions were stored at -20°C. All working solutions and cell suspensions were prepared using sterile calcium and magnesium-free Hanks' Balanced Salt Solution (CMF-HBSS; Sigma Chemical Co.) or RPMI-1640 (GIBCO, Grand Island, NY) and were pre-warmed to 37°C immediately prior to their use in the assays.

Isolation of Bovine Neutrophils

Neutrophils were isolated using procedures modified from Weber et al. (2001) as validated in our laboratory. Blood (10 mL) was aliquotted into to 50-mL polypropylene conical tubes (Corning Inc., NY) and centrifuged at 1000 × *g* for 20 min at 4°C to separate plasma from the cell pack. Plasma was discarded and the remaining cells were suspended in 35 mL of ice-cold PBS. The suspension was gently pipetted down the side of a 50-mL polypropylene conical tube containing 10 mL of 1.084 g/mL of Percoll®. The cells were then subjected to gradient centrifugation (400 x *g* for 40 min at 22°C). The supernatant, mononuclear cell layer and Percoll® were aseptically aspirated and discarded. The pellet (PMN and erythrocytes) was retained and erythrocytes were lysed by hypotonic shock. For this procedure, 2 volumes of an ice-cold 10.56 m*M* Na_2HPO_4, 2.67 m*M* NaH_2PO_4 solution were added to 1 volume of cell suspension and the solution was mixed by gentle aspiration and inversion. Tonicity was restored by the addition of 1 volume of a 10.56 m*M* Na_2HPO_4, 2.67 m*M* NaH_2PO_4 0.43 *M* NaCl solution. The cell solution was then centrifuged (500 x *g*) for 4 min at 4°C and the pellet washed twice by resuspension in 35 mL of CMF-HBSS (Sigma Chemical Co.) and recentrifugation (500 x *g* for 3 min at 4°C). Cell viability and differential cell counts were determined using light microscopy by Trypan Blue (Sigma Chemical Co.) exclusion and Giemsa stained smears, respectively. Purity of PMN was > 95% and viability > 97%. Cells were suspended either in CMF-HBSS or RPMI-1640, the concentration of cells was adjusted for every assay and the suspensions were kept at 4 °C until use in functional assays.

Insulin and TZD Incubations

Incubations and functional assays were performed in white-walled, clear-bottomed 96-well microplates (Corning, Costar Inc., NY), except for the killing-phagocytosis assay which was conducted in 5 mL polystyrene round-bottomed tubes (Becton Dickinson Labware, Franklin Lakes, NJ). For all the assays, working solutions of insulin and/or TZD in CMF-HBSS or RPMI-1640 + 2% heat-inactivated FBS (NETs assay) were pipetted into the wells or tubes to reach final concentrations of 0, 1.5 or 15 ng/mL of insulin with 300 μg/mL of TZD either alone or in combination with the insulin treatments. Suspensions of 2 x 10^5 (Luminol assay; final volume 200 μL/well), 4 x 10^5 (MCLA and CM-H_2DCFDA assays; final volume 150 μL/well), 5 x 10^5 (NETs assay; final volume 100 μL/well) and 2 x 10^5 neutrophils (killing-phagocytosis assay; final volume 1000 μL/tube) in CMF-HBSS or RPMI 1640 were added to the treatment solutions. The plates and tubes were then incubated with lids in a humidified incubator (37°C, 5% CO_2) for 120 minutes while gently shaking. After incubation, the cell suspensions were mixed by aspiration and the functional assays performed.

Luminol Chemiluminescence Assay

After incubation with insulin and TZD, the total neutrophil ROS production was immediately assessed by a luminol chemiluminescence assay as described by Rinaldi et al. (2006b) with some modifications. Luminol is a cell permeable compound that reacts with several ROS including superoxide (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hypochlorous acid (HClO); thus, the derived luminescence product of these reactions in the presence of luminol is an indicator of

intracellular and extracellular ROS produced by PMN (Rinaldi et al., 2006b). Twenty microliters of 5m*M* 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol) and 20 µL of either 400 n*M* PMA (stimulated cells) or HBSS (control) were added to all wells. All reactions were then adjusted to 200 uL using CMF-HBSS. Chemiluminescence (CL) was measured every 5 or 10 min for 190 min with a Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT). All reactions were carried out in quadruplicates. Background values, defined as the mean CL values of unactivated PMN, were subtracted from all readings.

MCLA Chemiluminescence Assay

After incubation, the extracellular neutrophil superoxide anion radical (O_2^-) production was measured by a MCLA chemiluminescence assay. The specificity of this probe for O_2^- has been confirmed by total inhibition by superoxide dismutase which catalyzes the conversion of O_2^- to H_2O_2 (Rinaldi et al., 2006b). Ten microliters of 2 µ*M* MCLA and 30 µL of either 400 n*M* PMA (stimulated cells) or HBSS (control) were added to all wells. The reactions were then adjusted to a volume of 200 uL using CMF-HBSS. The CL was measured every 10 min for 80 min with a Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT). Since MCLA is rapidly consumed, 10 µL of 2 µ*M* MCLA were added to each well immediately before each 10-min reading throughout the course of CL measurements. All reactions were carried out in quintuplicates. Background values, defined as the mean CL values of unactivated PMN, were subtracted from all readings.

CM-H2DCFDA Fluorescence Assay

The intracellular neutrophil ROS production was assessed a the CM-H_2DCFDA fluorescence assay with some modifications. CM-H_2DCFDA is a cell-permeant probe that reacts only with ROS that are trapped inside the cell, including hydrogen peroxide (H_2O_2), nitric oxide (NO) and peroxynitrite anion ($ONOO^-$) (Rinaldi et al., 2006b). After incubation, 10 µL of 200 µ*M* CM-H_2DCFDA and 30 µL of either 400 n*M* PMA (stimulated cells) or HBSS (control) were added to all wells. All reactions were then adjusted to 200 uL using CMF-HBSS. Fluorescence was measured every 10 min for 200 min, except between min 30 and 70 when it was measured every 5 min, with a Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. All reactions were carried out in quadruplicates. Background values, defined as the mean fluorescence values of unactivated PMN, were subtracted from all readings.

Neutrophil Extracellular Trap (NETs) Assay

Neutrophil extracellular trap formation was quantified using Sytox® Orange as described by Lippolis et al. (2006b) with some modifications. After PMN incubation with treatments in 96-well plates for 120 min, 20 uL of PMA and 20 uL of ionomycin were added to the stimulated set of wells to obtain a final concentration of 10 ng/ml and 1 µ*M*, respectively. Forty microliters of RPMI-1640 + 2% heat-inactivated FBS were added to the control wells. Subsequently, the plates were centrifuged (10 min at 650 × *g*) and incubated (37 °C and 5% CO_2 for 60 min). After incubation, supernatant from the plates was removed, and the wells were stained and protected from light with 50 µl of 5 µ*M*

Sytox Orange in CMF-HBSS (10 min at room temperature). The stain was poured off, and the wells washed once with 100 µl of CMF-HBSS. All reactions were performed in sixtuplicates. A Synergy HT plate reader (BioTek Instruments, Inc) was used to detect fluorescence with an excitation wavelength of 530 nm and emission wavelength of 580 nm.

Killing and Phagocytosis Assay

The assay was performed as described by Rinaldi et al. (2006a) with modifications. *Staphylococcus aureus* strain 305 (American Type Culture Collection, Manassas, VA) was inoculated on a blood agar plate (Northeast Laboratory Services Inc., ME) and incubated overnight at 37°C. Ten colonies were transferred from the plate to a sterile tube containing 10 mL of brain–heart infusion broth (Becton Dickinson Diagnostic Systems, Inc.) and incubated overnight at 37°C at 225 rpm in an orbital shaker. After the overnight incubation, the tube was placed in an ice-water bath and mixed using a vortex mixer. One hundred microliters from the culture were serially diluted in PBS and 100 µL of the resulting dilutions were spread on blood agar plates. The plates were incubated overnight at 37°C and the stock culture maintained at 4°C. After determining the concentration [colony forming units (cfu)/mL] of the stock culture based on the colony counts of the spread plates, the stock culture was diluted in HBSS to yield a final concentration of 4 x 10^7 cfu/mL.

To assess PMN phagocytosis and killing, 200 µL of *S. aureus* (8 x 10^6 cfu) were added to sterile tubes with or without 2 x 10^6 neutrophils previously incubated (1.4 mL) and 400 µL of pooled, heat-inactivated bovine serum. The ratio of bacteria to neutrophils

was 4:1. The samples were placed on an orbital shaker for 60 min at 37°C. All reactions were performed in duplicate. To determine the percentage of PMN containing phagocytosed bacteria and the actual number of phagocytosed bacteria per PMN, a 50-μL aliquot of each reaction was used to prepare a smear that was stained with Giemsa stain. The first 100 PMN encountered in the field of view of a light microscope were scored as either positive or negative for intracellular bacteria. For those PMN scored as positive, the number of intracellular bacteria was enumerated.

To evaluate whether insulin and TZD incubations affected the bactericidal activity of PMN, 1.95 mL of the remaining reaction were placed in ice and sonicated with a sonic dismembrator (Model 100, Thermo Fisher Scientific Inc.) at a power setting of 1 for 15 s. Rupture of the PMN was verified by microscopic examination. Sonication did not affect bacterial viability (Rinaldi et al., 2006b; unpublished observation). A 100 μL aliquot from each sonicated reaction was serially diluted in PBS and 100 μL quantities of the resulting dilutions was spread on blood agar plates. The plates were incubated overnight at 37°C and the colonies enumerated. The percentage of bacteria killed were determined by calculating the difference in the number of bacteria incubated in the absence and presence of PMN and dividing this difference by the number of bacteria incubated in the absence of PMN.

Statistical Analysis

The experiment was conducted as a completely randomized design with three levels of treatment (insulin, TZD and physiological state) applied to the incubation well/tube within cow. Data was analyzed by analysis of variance as a mixed model using

the mixed procedure of SAS (2001). The variables in the model statement included animal, the treatments and their interactions. For the luminol-, MCLA-, and CM-H_2DCFDA-based assays involving multiple measurements of CL or fluorescence over time, the area under the curve (AUC) was calculated from plotted data points for each experimental condition using the expand procedure of SAS with cubic spline interpolation and the trapezoid rule (SAS, 2001). The analyzed area between the curves was generated using the least squares estimates for CL or fluorescence at each time point in the assay and this area was used as the value tested for analysis of variance. For the CM-H_2DCFDA assay, a mixed model analysis of variance with repeated measures was also used as a more sensitive analysis of treatment differences than the calculation of AUC. Least squares means and standard errors were generated using the LSMeans statement in conjunction with the pdiff option of SAS (2001). Tukey's multiple comparison test was used to assess specific treatment differences for those variables shown to have significant overall treatment effects in the analysis of variance. All reported means are the adjusted least squares means ± standard error of the mean (SEM) and the significance was declared when $P < 0.01$.

RESULTS

Reactive Oxygen Species Production

Changes in luminol-derived CL were evident 30 min after activation with PMA. For those cells incubated with 0, 1.5 or 15 ng/mL of insulin without TZD, CL values peaked 100 min after activation and then decreased. In contrast, the luminol-derived CL values of PMN treated with 300 μg/mL of TZD were only modestly increased from baseline

with a peak at 70 min after activation and a much more rapid decline back to baseline compared to insulin-only treatments or control wells (Figure 2.1A). To determine any differential effects of in vitro insulin and TZD treatment on PMN, the areas under the curve (AUCs) were calculated from these plotted data points. Insulin treatment had no effect on the luminol-dependant CL associated with total ROS production in PMA-stimulated cells as luminol-derived CL was not different between control and insulin-only treated wells (Figure 2.1B; insulin treatment effect, $P > 0.20$). Incubation of cells with 300 μg/mL of TZD inhibited total ROS by approximately 88.9%, compared to control and insulin-only treated wells (Figure 2.1B; TZD treatment effect, $P < 0.01$). The overall effect of physiological state on luminol-derived CL is presented in Figure 2.2. The luminol-derived CL of neutrophils isolated from postpartum cows was 37 and 32% lower compared to midlactation and prepartum animals, respectively (physiological state effect, $P < 0.01$). However, the luminol-derived CL of PMN collected from midlactation was similar to that of PMN isolated from prepartum cows (midlactation vs. prepartum, $P > 0.20$). Furthermore, there was no insulin by physiological state interaction effect ($P > 0.20$), but the magnitude of the reduction in the luminol-derived CL observed in postpartum cows relative to midlactation and prepartum cows with 0 μg/mL TZD treatment was lower than the magnitude of this reduction in PMN incubated with 300 μg/mL (Figure 2.3; TZD by stage of lactation interaction effect, $P < 0.01$).

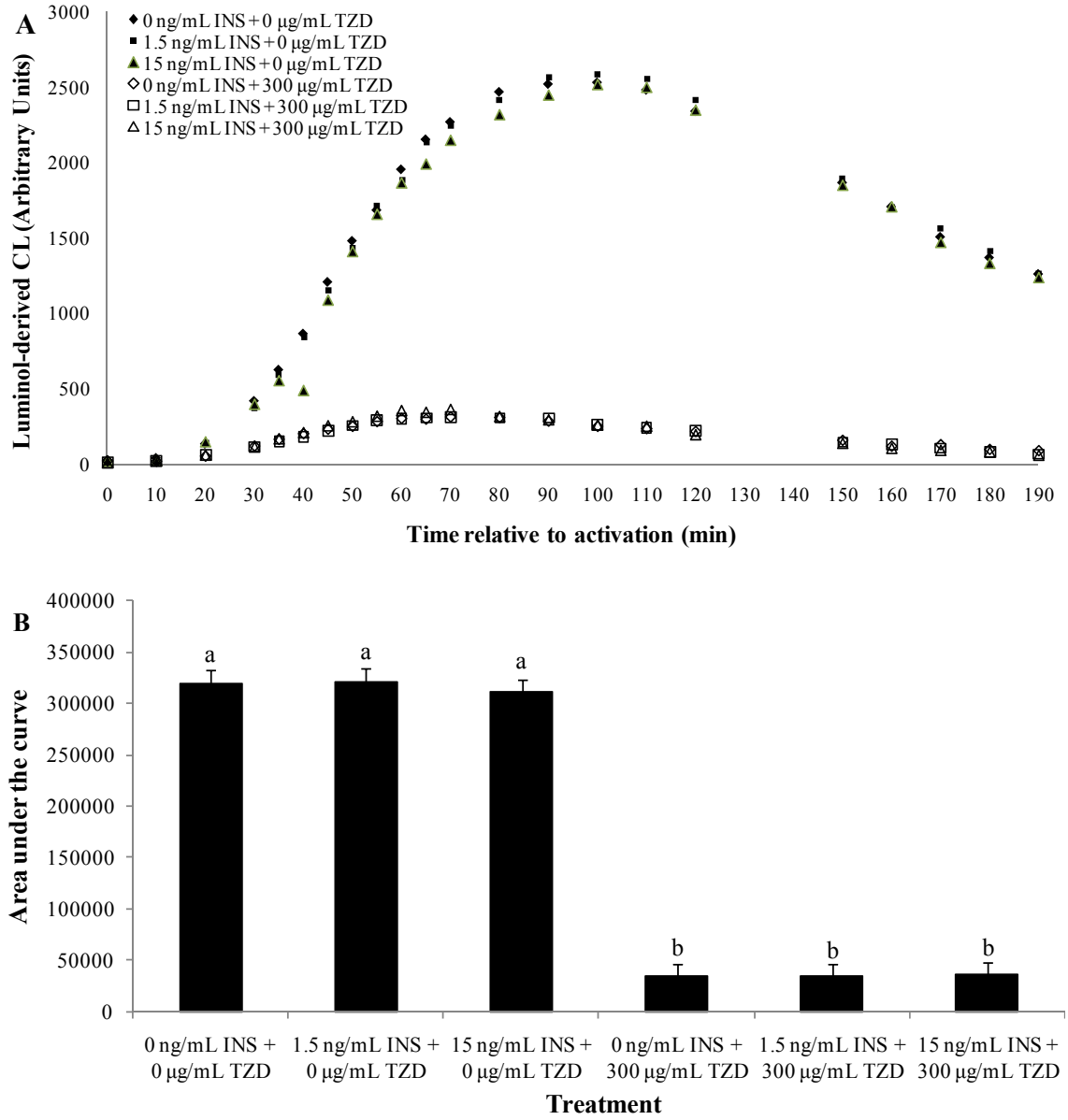

Figure 2.1 Effect of Insulin (INS) and 2,4-Thiazolinedione (TZD) on total ROS production by bovine neutrophils.

Neutrophils were collected from cows (n = 25) and incubated with 0, 1.5 or 15 ng/mL of insulin and 0 or 300 µg/mL of TZD either alone or in combination with the insulin treatments. Data represent the mean luminol-derived chemiluminescence (CL) in arbitrary units measured every 5 or 10 min over a 190 min period after activation of neutrophils with Phorbol 12-myristate,13-acetate (PMA). The CL values of non-activated neutrophils were substracted from all the CL measurements at every time point (A). To assess the overall magnitude of the luminol-dependant CL response over time, the area under the curve (± SEM) calculated from plotted luminol-derived measurements (B). ** TZD treatment effect, $P < 0.01$. [a,b] Bars with different letters differ ($P < 0.01$).

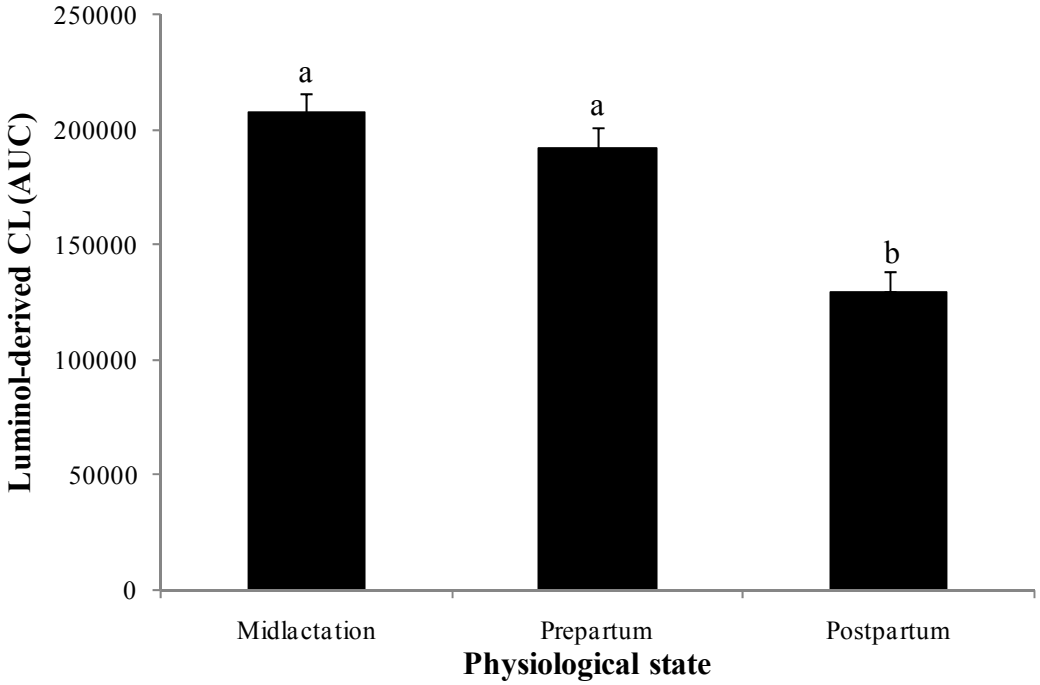

Figure 2.2 Effect of physiological state on total ROS production by bovine neutrophils. Neutrophils were collected from pregnant midlactation (253 ± 25.2 d postpartum; n = 9), prepartum (-12 ± 1.7 d; n = 8) and postpartum (+7d; n = 8) cows. Data represent the area under the curve (± SEM) calculated from plotted luminol-derived measurements over a 190 min period after activation of neutrophils with Phorbol 12-myristate,13-acetate (PMA). The CL values of non-activated neutrophils were substracted from all the CL measurements at every time point. **Physiological state effect, $P < 0.01$. [a,b] Bars with different letters differ ($P < 0.01$).

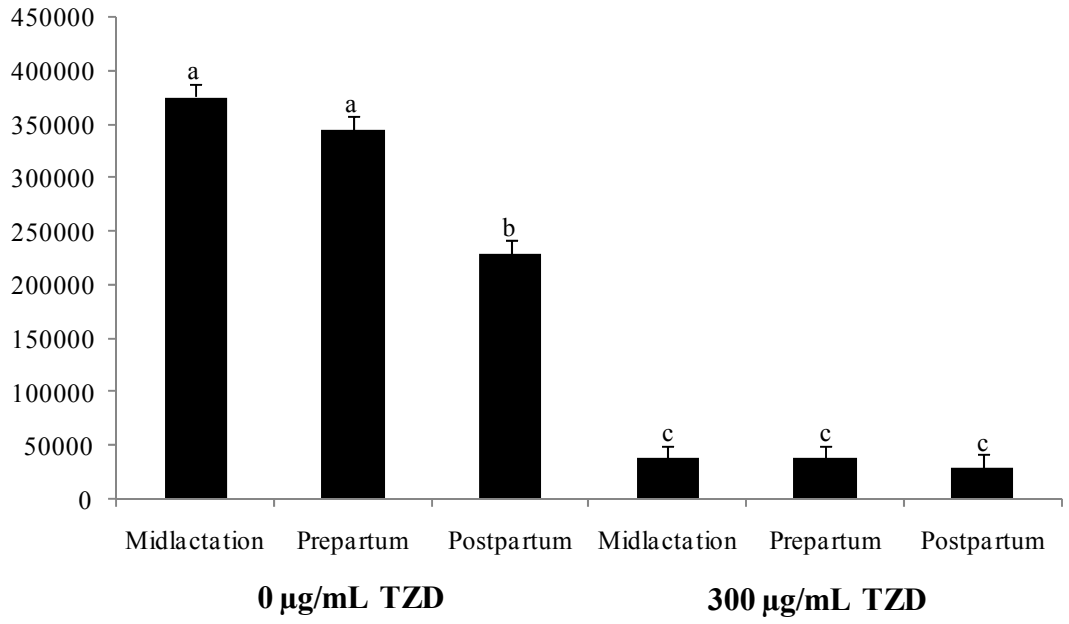

Figure 2.3 Effects of TZD and physiological state on total ROS production by bovine neutrophils.
Neutrophils were collected from pregnant midlactation (253 ± 25.2 d postpartum; n = 9), prepartum (-12 ± 1.7 d; n = 8) and postpartum (+7d; n = 8) cows. Data represent the area under the curve (± SEM) calculated from plotted luminol-derived measurements over a 190 min period after activation of neutrophils with Phorbol 12-myristate,13-acetate (PMA). The CL values of non-activated neutrophils were substracted from all the CL measurements at every time point. **TZD by physiological state effect, $P < 0.01$. [a,b,c] Bars with different letters differ ($P < 0.01$).

The MCLA-derived CL in all treatment and control groups peaked at the 21 min measurement time-point after PMA activation and then declined. The PMN treated with 300 μg/mL of TZD had a higher maximal response after cell stimulation and this difference was maintained throughout the rest of CL measurements, compared to insulin-only treatments or control wells (Figure 2.4A). To determine any differential effects of in vitro insulin and TZD treatment on PMN, the areas under the curve (AUCs) were calculated from these plotted data points. Insulin treatment had no effect relative to control wells on the MCLA-dependant CL associated with extracellular O_2^- ROS production in PMA-stimulated cells (Figure 2.4B; insulin treatment effect, $P > 0.20$). In contrast, TZD enhanced the O_2^- generation in approximately 43% compared to control wells (Figure 2.4B; TZD treatment effect, $P < 0.01$). Neutrophils from postpartum cows had a 43 and 37% lower MCLA-dependant CL, compared to midlactating and prepartum animals, respectively (Figure 2.5; physiological state effect, $P < 0.01$). The MCLA-derived CL of PMN collected from midlactating was similar to that of PMN isolated from prepartum cows (Figure 2.5; $P > 0.20$). No interaction effects with either in vitro treatment or physiological state were evident for PMN MCLA-derived CL (all interactions, $P > 0.20$).

Changes in CM-H_2DCFDA-derived fluorescence were observed after 30 min after activation with PMA. The fluorescence values from the cells incubated with 300 μ/mL of TZD increased and reached a plateau approximately 140 min after stimulation whereas those of PMN incubated with 0, 1.5 or 15 ng/mL of insulin without TZD continued to increase (Figure 2.6A).

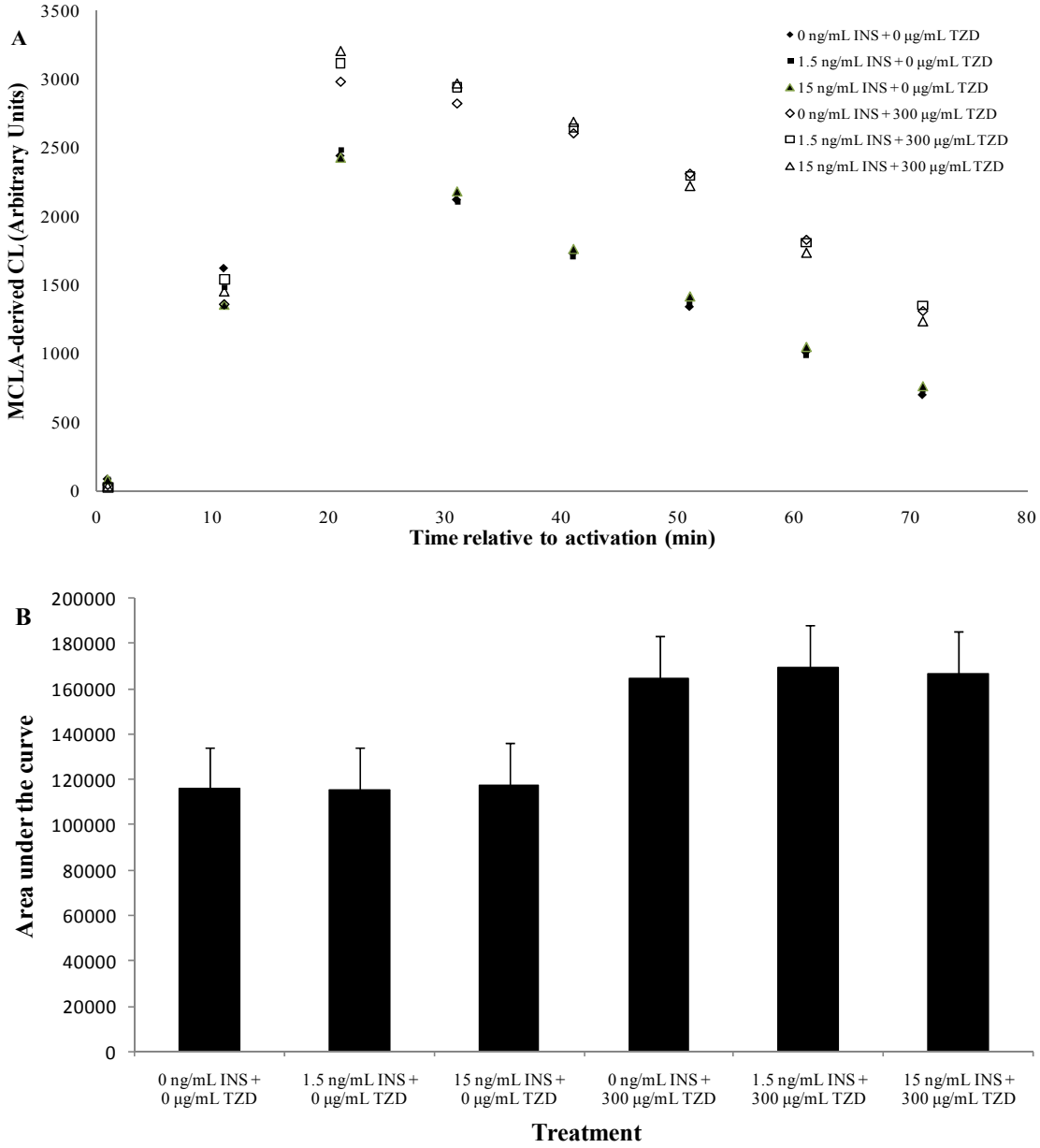

Figure 2.4 Effect of Insulin (INS) and 2,4-Thiazolinedione (TZD) on extracellular O_2^- production by bovine neutrophils.

Neutrophils were collected from cows (n = 26) and incubated with 0, 1.5 or 15 ng/mL of insulin and 0 or 300 µg/mL of TZD either alone or in combination with the insulin treatments. (A) Mean MCLA-dependant chemiluminescence (CL) (arbitrary units) measured every 10 min over a 80 min period after activation of neutrophils with Phorbol 12-myristate,13-acetate (PMA). The CL values of non-activated neutrophils were substracted from all the CL measurements at every time point (A). To assess the overall magnitude of the luminol-dependant CL response over time, the area under the curve (± SEM) calculated from plotted luminol-derived measurements (B). ** TZD treatment effect, $P < 0.01$.

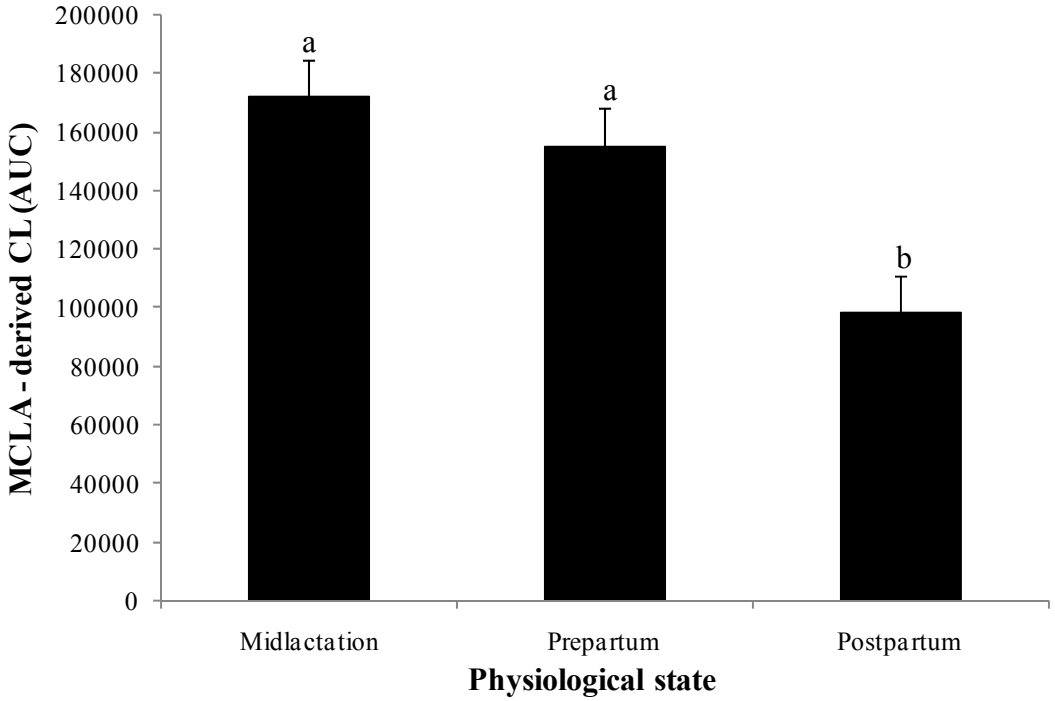

Figure 2.5 Effect of physiological state on extracellular O_2^- production by bovine neutrophils.

Neutrophils were collected from pregnant midlactation (253 ± 25.2 d postpartum; n = 9), prepartum (-12 ± 1.7 d; n = 8) and postpartum (+7d; n = 9) cows. Data represent the area under the curve (± SEM) calculated from plotted MCLA-derived measurements over a 70 min period after activation of neutrophils with Phorbol 12-myristate,13-acetate (PMA). The CL values of non-activated neutrophils were substracted from all the CL measurements at every time point. **Physiological state effect, $P < 0.01$. ^{a,b} Bars with different letters differ ($P < 0.01$).

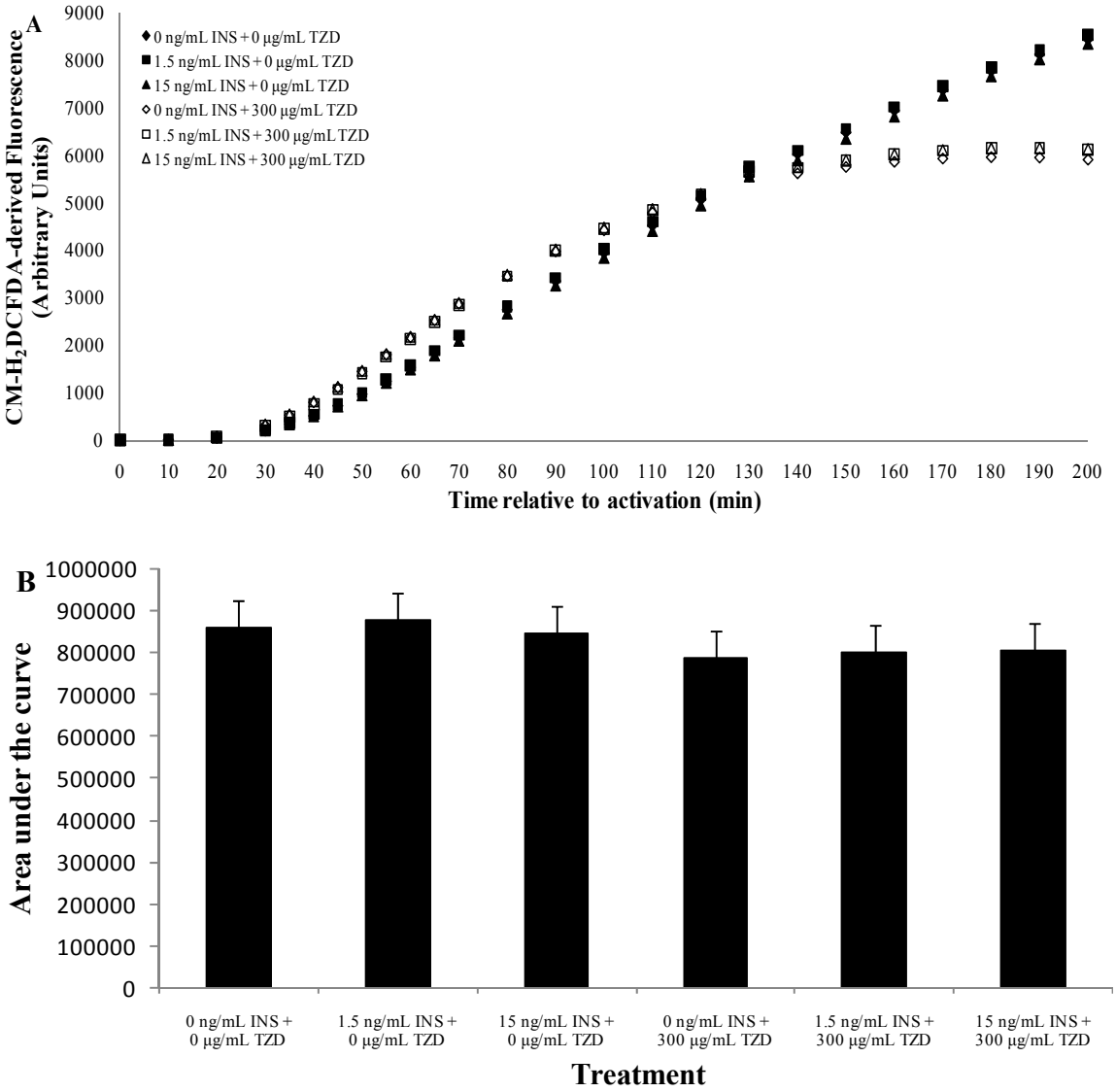

Figure 2.6 Effect of Insulin (INS) and 2,4-Thiazolinedione (TZD) on intracellular ROS production by bovine neutrophils.

Neutrophils were collected from cows (n = 24) and incubated with 0, 1.5 or 15 ng/mL of insulin and 0 or 300 µg/mL of TZD. Data represent the mean CM-H_2DCFDA-derived fluorescence in arbitrary units measured every 10 min over a 200 min period after activation of neutrophils with Phorbol 12-myristate,13-acetate (PMA). The fluorescence values of non-activated neutrophils were substracted from all measurements at every time point. **TZD by incubation time relative to PMA activation effect, $P < 0.01$. (A). To assess the overall magnitude of the CM-H_2DCFDA-derived fluorescence response over time, the area under the curve (± SEM) was calculated from plotted measurements (B).

There was no effect of insulin or TZD on the intracellular ROS production based on calculated AUCs (Figure 2.6B; insulin or TZD effect, $P > 0.20$). However, when the data were analyzed by analysis of variance with repeated measures for each incubation measurement time-point, the interaction of TZD by incubation time relative to PMA activation was significant (Figure 2.6A; $P < 0.01$). Furthermore, the intracellular ROS production of neutrophils isolated from postpartum cows was 43 and 37% lower, compared to midlactation and prepartum animals, respectively (Figure 2.7; physiological state effect, $P < 0.01$). However, the fluorescence values of PMN collected from midlactation was similar to that of PMN isolated from prepartum cows (Figure 2.7; $P > 0.20$). There were no insulin by TZD or in vitro treatment by physiological state interaction effects on the PMN intracellular generation of ROS as measured by CM-H_2DCFDA-derived fluorescence (all interactions, $P > 0.20$).

Neutrophil Extracellular Trap Release (NETs)

There was no effect of insulin or TZD on NETs release relative to control wells (Figure 2.8; effect of insulin or TZD, $P > 0.20$). However, the mean fluorescence associated with NETs released from neutrophils collected from prepartum cows was 23 and 36% higher than that of PMN collected from midlactating and postpartum cows, respectively (Figure 2.9; day of lactation effect, $P < 0.01$). Neutrophil NETs expression from midlactation cows was similar to that of PMN isolated from postpartum cows (Figure 2.9; $P > 0.20$). There were no insulin by TZD or in vitro treatment by physiological state interaction effects on PMN NETs release ($P > 0.20$).

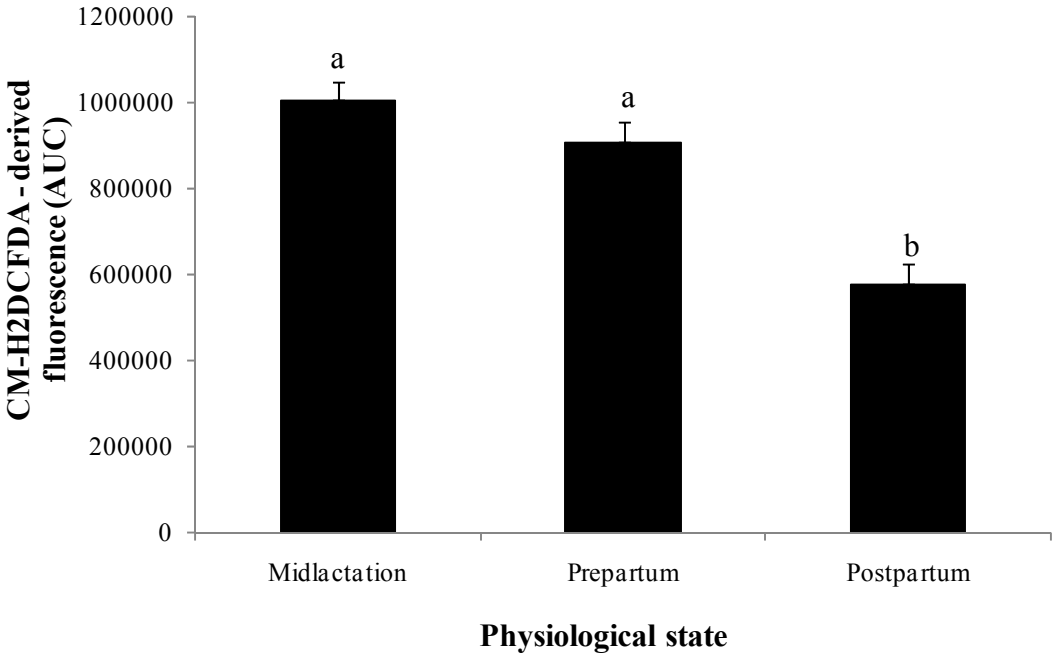

Figure 2.7 Effect of physiological state on intracellular ROS production measured by bovine neutrophil.
Neutrophils were collected from pregnant midlactation (253 ± 25.2 d postpartum; n = 9), prepartum (-12.7 d; n = 8) and post-partum (+7d; n = 7) cows. Data represent the area under the curve (± SEM) calculated from plotted CM-H_2DCFDA-derived measurements over a 200 min period after activation of neutrophils with Phorbol 12-myristate,13-acetate (PMA). The CL values of non-activated neutrophils were substracted from all the CL measurements at every time point. **Physiological state effect, $P < 0.01$. [a,b] Bars with different letters differ ($P < 0.01$).

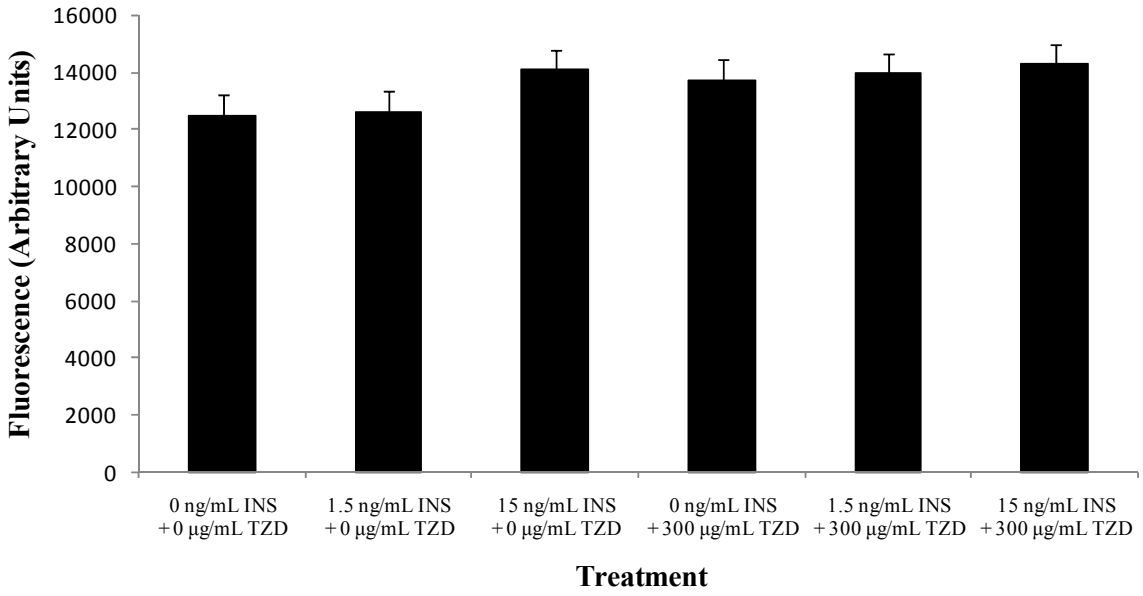

Figure 2.8 Effect of Insulin (INS) and 2,4-Thiazolinedione (TZD) on NETs expression by bovine neutrophils.
Neutrophils were collected from cows (n = 24) and incubated with 0, 1.5 or 15 ng/mL of insulin and 0 or 300 µg/mL of TZD. Data represent the mean fluorescence in arbitrary units measured after 1 hour-incubation of neutrophils with Phorbol 12-myristate,13-acetate (PMA).

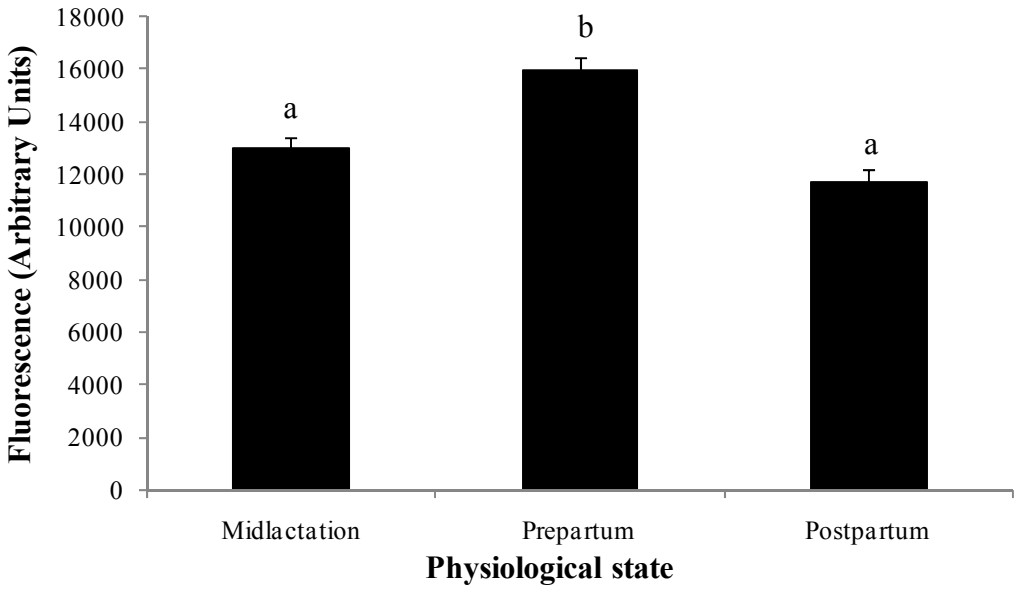

Figure 2.9 Effect of physiologcal state on NETs expression by bovine neutrophils.
PMN were collected from pregnant midlactation (253 ± 25.2 d postpartum; n = 8), prepartum (-12 ± 1.7 d; n = 8) and postpartum (+7d; n = 8) cows. Data represents mean fluorescence (± SEM) of cells stained with Sytox Orange after 1-hour incubation with Phorbol 12-myristate,13-acetate (PMA). **Physiological state effect, $P < 0.01$. [a,b] Bars with different letters differ ($P < 0.01$).

Killing and Phagocytosis

There was no effect of insulin or TZD or their combination on the percentage of cells positive for intracellular bacteria or the number of phagocytosed *S. aureus* per PMN (Table 2.1; insulin or TZD effect, $P > 0.20$). Neutrophils collected from postpartum cows had higher percentage of cells with phagocytosed bacteria and number of bacteria per neutrophil, compared to prepartum and midlactation animals (Table 2.2; physiological state effect, $P < 0.01$). There was no effect of insulin or TZD on the killing ability of PMN (Table 2.1; insulin or TZD effect, $P > 0.20$). However, the killing ability of neutrophils isolated from postpartum animals was 61 and 97% higher compared to prepartum and midlactation animals, respectively (Table 2.2; physiological state effect, $P < 0.01$). No in vitro treatment or physiological sate interaction effects were evident (all interactions, $P > 0.20$)

Table 2.1 Effect of insulin (INS) and 2,4-Thiazolinedione (TZD) on the bactericidal and phagocytic abilities of neutrophils against *Staphylococcus aureus**

Index	**Treatment (ng/mL INS + µg/mL TZD)**					
	0 + 0	**1.5 + 0**	**15 + 0**	**0 + 300**	**1.5 + 300**	**15 + 300**
PMN with phagocytosed bacteria, %	27.3 ± 4	26.0 ± 4	25.5 ± 4	23.7 ± 4	24.1 ± 4	23.4 ± 4
Number of engulfed bacteria per PMN	13.0 ± 1	13.4 ± 1	12.3 ± 1	12.6 ± 1	12.9 ± 1	12.4 ± 1
Killing ability, %	23.3 ± 4	24.8 ± 4	23.7 ± 4	22.7 ± 4	21.7 ± 4	20.5 ± 4

* Data are means (± SEM; n=24)

Table 2.2 Effect of physiological state on the bactericidal and phagocytic abilities of neutrophils against *Staphylococcus aureus**

Index	**Mid-lactation**	**Pre-partum**	**Post-partum**
PMN with phagocytosed bacteria, %	13.1 ± 2.8^{a}	21.7 ± 2.8^{a}	40.2 ± 2.8^{b}
Number of engulfed bacteria per PMN	11.5 ± 0.6^{a}	11.4 ± 0.6^{a}	15.4 ± 0.6^{b}
Killing ability, %	16.3 ± 2.6^{a}	19.9 ± 2.7^{a}	32.1 ± 2.7^{b}

*Data are means (± SEM; n=8) Effect of physiological state, $P < 0.01$

[a,b] Values in the same row with different letter differ ($P < 0.01$)

DISCUSSION

The total, intracellular and extracellular ROS production of blood neutrophils collected from prepartum and postpartum cows was reduced compared to that of PMN isolated from midlactation animals although the difference between prepartum and postpartum cows was not significant. These results are in concordance with previous reports (Dosogne et al., 1999, Hoeben et al., 2000, Mehrzad et al., 2001, 2002). However, there are conflicting reports regarding the changes in ROS generation between the prepartum and early lactation period. For instance, Hoeben et al. (2000) and Dosogne et al. (1999) reported a reduction in luminol-dependant CL beginning ~3 weeks before parturition with minimum levels 1 week after calving, but only the second group reported a significant decrease in PMA-induced CL of neutrophils between -1 and 1 week after parturition. Furthermore, this reduction in the capacity of the PMN to produce ROS can be partially explained by the decrease in the protein abundance (Lippolis et al., 2006a) and activity (Cai et al., 1994) of the enzyme myeloperoxidase reported in early lactation. The results of the current study confirm that the respiratory burst activity of blood neutrophils from cows in early lactation, independently of the cellular location of ROS, is impaired and perhaps contributes to the period of high incidence and severity of infectious diseases that cows experience around the time of calving (Mallard et al., 1998, Vangroenweghe et al., 2005).

PMN from prepartum cows expressed higher levels of NETs compared to mid and early lactation animals. Contrary to the oxygen-dependent killing mechanisms, NETs may not experience a period of reduced functionality during the periparturient period. In

fact, Lippolis et al. (2006b) reported that incubation of PMN in milk, in contrast with other aspects of neutrophil function, did not reduce their ability to release NETs suggesting that when other PMN killing mechanisms are impaired, neutrophils might rely on the antimicrobial activity of NETs. The same group (2006a), performed a proteomic analysis of bovine PMN collected during midlactation and 28 days before calving and reported that those from prepartum cows, had higher levels of the histones H2B.e and H2B.f. Because these histones are structural components of NETs (Brinkmann et al., 2004), their enhanced expression may contribute to the increased NETs synthesis in prepartum vs. midlactating cows observed in our study.

The phagocytic capacity assessed by the percentage of PMN with engulfed *S. aureus* and the number of intracellular bacteria of neutrophils from early lactation cows was increased compared to prepartum and midlactating animals. Increases in neutrophil phagocytic activity during the periparturient period have also been reported in several studies (Detilleux et al., 1995, Dosogne et al., 1999, Guidry et al., 1976, Kehrli et al., 1989). This inverse relationship between the neutrophil ROS generation and the phagocytic ability during early lactation has been attributed to the ability of the PMN to use the extra energy available for phagocytic processes in a period when the consumption of energy for production of ROS is reduced (Detilleux et al., 1995). Conversely, Cai et al. (1994) and Daniel et al. (1991) reported no change in neutrophil phagocytosis during the periparturient period whereas Dosogne et al. (2001) found no difference in phagocytosis between PMN collected from midlactation, prepartum and postpartum cows. This inconsistency in findings may be attributed to the different pathogens and neutrophil to

pathogen ratio used in the in vitro co-cultures. However, in contrast with other aspects of neutrophil function, none of these reports suggest that the phagocytic ability of neutrophils is impaired during the periparturient period, suggesting that decreased phagocytic ability may not contribute to periparturient immunosuppression.

In this study, the killing ability of PMN from early lactation cows against *S. aureus* was higher than that of prepartum and midlactation animals. On the other hand, most studies that have measured the bactericidal activity of neutrophils during the periparturient period have reported a decrease in the overall killing capacity of PMN against gram positive and negative bacteria (Cai et al., 1994, Daniel et al., 1991, Detilleux et al., 1995, Dosogne et al., 2001, Kehrli et al., 1989). The conflicting results of our study may be attributed to the lower 4:1 bacteria to PMN ratio that we used in our co-culture in an effort to reduce the number of intracellular bacteria and improve light microscopy-counting. This might be reflected in the lower killing percentages obtained in our assays (~21%), compared to the ~57% reported by Rinaldi et al. (Rinaldi et al., 2006a) who performed a killing assay under similar conditions, except for a higher 16:1 bacteria to neutrophil ratio. In fact, Detilleux et al. (2004) applied mathematical models that describe the changes in bacteria number during exposure to PMN in vitro and determined that the bacteria to neutrophil ratio should be within the range of 12:1 and 175:1 for effective phagocytosis and killing.

Incubation of PMN with a high physiological (1.5ng/mL) or a supraphysiologycal (15 ng/mL) concentration of insulin had no effect on the respiratory burst activity, NETs release, phagocytosis and killing ability of neutrophils isolated from cows in different

physiological states. In contrast, experiments with human neutrophils indicate that insulin stimulated in a dose-response manner the generation of hydrogen peroxide (Spagnoli et al., 1995) and chemotactic ability (Cavalot et al., 1992), whereas 100 μU/mL of insulin increased the expression of the neutrophil intercellular adhesion molecule-1 (ICAM-1) by approximately 80% (Okouchi et al., 2002). Also, it has been reported that patients with diabetes mellitus have increased susceptibility to and severity of infections (Bertoni et al., 2001), in part as a result of alterations in neutrophil phagocytosis, respiratory burst activity and H_2O_2 production (Alba-Loureiro et al., 2006, Bilgic et al., 2008). Insulin treatment improved neutrophil phagocytic capacity of diabetic patients with foot infections (Top et al., 2007) and after cardiac surgery (Rassias et al., 1999). However, studies looking at the effect of experimental hyperinsulinemia on neutrophil function in non-diabetic subjects have yielded conflicting results. For instance, Walrand et al. (2004) reported an increase in chemotaxis, phagocytosis and bactericidal activities in PMN collected from healthy individuals after a 4-hour hyperinsuliemic euglycemic clamp whereas Fejfarova et al. (2006) and Stegenga (2008) did not find any alterations of phagocytosis, migration and ROS production during induced hyperinsulinemia with strict euglycemia when compared to subjects receiving saline infusion. In summary, there is evidence from in vitro and in vivo studies in humans that insulin modulates neutrophil functionality, at least in individuals with defective insulinemia. In dairy cows, plasma insulin levels and the peripheral tissue sensitivity and responsiveness to insulin is lower in the periparturient period compared to midlactation, as part of the homeorhetic adaptations that support the increased influx of nutrients towards pregnancy and lactation

(Bell and Bauman, 1997). Thus, we hypothesized that in vitro incubation with higher insulin levels, alone or in combination with the PPAR-γ agonist and insulin sensitizing agent TZD would have a differential effect on the functionality of periparturient PMN, relative to PMN isolated from midlactating cows. The lack of a direct effect of insulin on bovine neutrophil activity reported in this study may be explained by the low levels of insulin receptors detected on bovine neutrophils (Nielsen et al., 2003). Perhaps, in dairy cows, insulin does not regulate neutrophil function directly, but might act indirectly via improvement of metabolic control during the periparturient period. For instance, insulin may reduce the level of circulating non-esterified fatty acids (NEFA) and ketone bodies that have detrimental effects on neutrophil and leukocyte activity (Scalia et al., 2006, Suriyasathaporn et al., 1999). Thus, the in vivo effect of insulin on periparturient neutrophil function requires investigation.

Incubation of PMN with TZD resulted in a potent inhibitory effect on the neutrophil luminol-dependant CL, an indicator of the PMN generation of a wide array of ROS including extracellular and intracellular hydroxyl radical (OH^-), peroxynitrite ($ONOO^-$), hypochlourus acid (HClO), intracellular superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Rinaldi et al., 2006b). Furthermore, TZD had no effect on the generation of intracellular NO, $ONOO^-$ and H_2O_2 assesed by the CM-H_2DCFDA fluorescence assay, when the differences were assessed by calculated AUCs. However, when the data was analyzed by ANOVA with repeated measures, the effect of TZD treatment by incubation time was significant. Perhaps TZD decreases the CM-H_2DCFDA-derived fluorescence of PMN, but the duration of our assay might have not

been enough for TZD to affect the generation of intracellular ROS. These findings are consistent with the emerging evidence regarding the anti-inflammatory effects of thiazolidinediones and other PPAR-γ ligands. Importantly, these effects are thought to be mediated by the inhibition of the nuclear factor κB (NFκB), which during inflammation, enters the nucleus and activates the expression of several pro-inflammatory genes, including cytokines and enzymes necessary for the production of ROS (Karin and Ben-Neriah, 2000, Rutledge and Adeli, 2007). Indeed, Ghanim et al. (2001) treated obese subjects with troglitazone, a insulin sensitizer of the TZD class, and reported a marked fall in ROS generation by mononuclear cells together with a decrease in the expression of NADPH oxidase, NFκB and IκB proteins. These TZD anti-inflammatory effects might be independent of metabolic alterations because administration of a lower dose of a TZD to diabetic patients resulted in decreased inflammatory markers without changes in glucose homeostasis, plasma insulin and free fatty acids (Ghanim et al., 2006). Since excess of ROS production can damage the host cell membrane by peroxidation of the components of the lipid bilayer (Fialkow et al., 2007), cause alterations in several regulatory pathways that modulate apoptosis (Fadeel et al., 1998), expression of cytokines and phagocytosis (Zhang et al., 2003) or ultimately promote injury to the surrounding tissue (Capuco et al., 1986), TZD treatment may be beneficial to the dairy cow during an inflammatory process by means of reducing the host cell oxidative stress or by decreasing the damage that ROS cause to the surrounding tissue. Importantly, the reduction of ROS generation by TZD observed in this study was not accompanied by an impaired ability of the neutrophils to release NETs or engulf and kill bacteria, suggesting

that TZD may reduce inflammation while leaving the essential antimicrobial capacity of neutrophils intact. Furthermore, it is conceivable that TZD has different effects on individual ROS produced by bovine PMN. The fact that neutrophils incubated with TZD had increased concentrations of extracellular O_2^- measured by MCLA-derived CL can be explained by the lower generation of H_2O_2, OH^-, $ONOO^-$ and HClO. Since O_2^- is the main precursor of these compounds that result from reactions catalyzed by the enzymes superoxide dismutase and myeloperoxidase (Hodgson et al., 2006, Shepherd, 1986), the inhibition of these reactions may result in higher concentrations of O_2^- available for dismutation to hydrogen peroxide and oxygen.

In vivo administration of TZD to periparturient cows might improve their immune function directly by reducing the oxidative stress that the immune cells experience themselves during the generation of ROS or by decreasing the damage that ROS cause to the surrounding tissue during inflammation. These effects can be exerted on neutrophils, as reported in our study, or on other leukocytes, as suggested by experiments involving human peripheral mononuclear cells (Jiang et al., 1998) and macrophages (Ricote et al., 1998). Furthermore, in vivo administration of TZD restored the insulin-mediated glucose utilization of steers with rbTNF-induced insulin resistance (Kushibiki et al., 2001) and infusion of TZD to cows during the last 25 days of pregnancy resulted in a 15% reduction of plasma prepartum NEFA concentrations and a decrease of β-hydroxybutyrate (BHBA) as parturition approached, compared to control animals (Smith et al., 2007). Thus, it is possible that TZD may also improve the immunity of periparturient cows indirectly by improving glucose metabolism of leukocytes or by reducing the plasma concentration of

NEFA and β-hydroxybutyrate (BHBA) that have been shown to have a negative effect on PMN function (Hoeben et al., 1997, Scalia et al., 2006).

CONCLUSIONS

As indicated by three assays to measure PMN ROS production, the respiratory burst of PMN is impaired in early lactation, and may contribute to the state of immunosuppression. Interestingly, NETs formation was not impaired postpartum and was actually increased in prepartum dairy cows. Our results suggest that insulin has no direct effect on bovine neutrophil function, but TZD increased the generation of intracellular superoxide anion and strongly inhibited the release of other intracellular and extracellular ROS. Interestingly, these anti-inflammatory effects of TZD did not interfere with the overall neutrophil phagocytosis and killing ability, suggesting that TZD may reduce the cell oxidative stress and diminish the damage that ROS cause to the surrounding tissue. Furthermore, TZD did not reduce NETs expression, a killing mechanism that may be particularly important to the health of the mammary gland. These direct effects of TZD on PMN function coupled with potential indirect effects of TZD on immunity via circulating metabolites suggest a potential important role for TZD in improving periparturient immune function and health.

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APPENDICES

APPENDIX A: ISOLATION OF BOVINE NEUTROPHILS FROM BLOOD

Principle

A prerequisite for experiments using bovine neutrophils is an adequate method of isolation of these cells from blood to obtain highly purified and functional cell populations (Soltys et al., 1999). Blood collection was performed using tubes containing acid citrate dextrose (ACD) because when different anticoagulants such as heparin are used, the buffy coat has a fibrin mesh-like membrane that interferes with leukocyte separation and may change their morphology and functionality (Carlson and Kaneko, 1973). The most frequent technique used for separating granulocytes (also known as polymorphonuclear cells or PMNs), from peripheral blood is by discontinuous density centrifugation, first described by Boyum (1968). In this method, mononuclear leucocytes are first removed from peripheral blood by slow centrifugation using a density gradient medium. During centrifugation, cell subtypes will sediment to an equilibrium position in the gradient where the density gradient is equal to the density of the cells. To separate mononuclear cells from PMNs, a target density of 1.084 g/mL is used so that mononuclear cells, which have a density less than 1.084 g/mL will sediment on top of the density gradient medium while heavier cells such as PMNs (neutrophils, basophils, eosonophils) and erythrocytes cross the gradient. In a second step granulocytes are separated from erythrocytes by hypotonic shock (Figure 3.1). Purity of the neutrophil population, assessed by Giemsa staining and differential cell counting, was > 95% with

eosonophils being the major contaminant cell type. Neutrophil viability was > 97% as determined by Trypan Blue® exclusion.

Reagents

- Sterile PBS, pH 7.4

  8g of NaCl (Sigma Chemical cat. # S6191), 0.2g of KCl (Sigma Chemical cat. # P5405), 1.44g of Na_2HPO_4 (Sigma Chemical cat. # S5136) and 0.24g of KH_2PO_4 (Sigma Chemical cat. # P5655) per L of solution

- 1.5 *M* NaCl

  300 mL of 5 M NaCl (Fisher cat. # 9385658) per L of solution

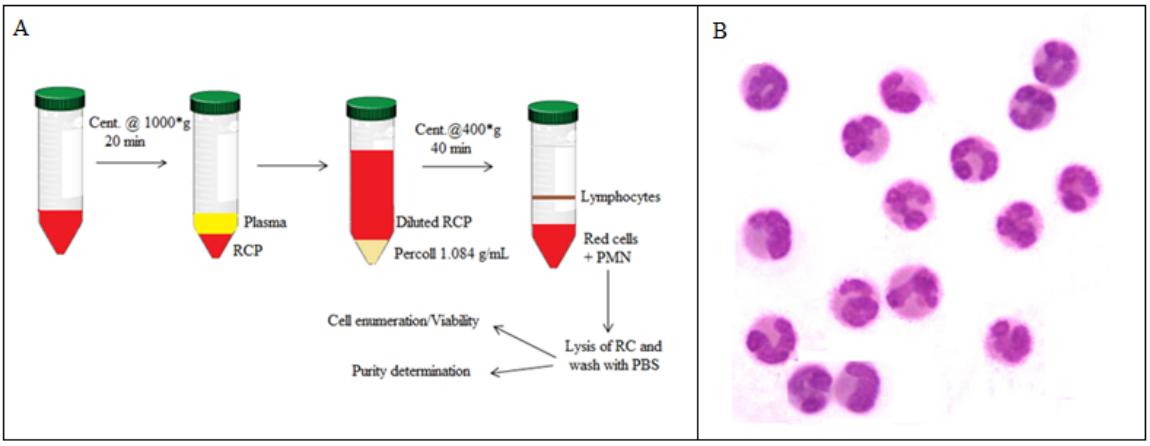
- 1.084 g/mL Percoll® (GE Healthcare Bio-Sciences cat. # 17-0891-01)

  601.54L of Percoll®, 298.46 mL of $_{dd}H_2O$ and 100 mL of 1.5 M NaCl per L of solution

- Lysing solution 10.56 m*M* Na_2HPO_4, 2.67 m*M* NaH_2PO_4 (Sigma Chemical cat. # S3139)
- Restoring solution 10.56 m*M* Na_2HPO_4, 2.67 m*M* NaH_2PO_4 0.43 *M* NaCl
- Ca-Mg-free Hank's balanced salt solution (CMF-HBSS, Sigma Chemical cat. # H6648)
- Trypan Blue (Sigma Chemical cat. # T8154)
- Giemsa® stain (Sigma Chemical cat. # G9641)

Procedure

1. Transfer 10 mL of blood to a 50-mL polypropylene conical tube and centrifuge at 1000×g for 20 min at 4°C to separate plasma from the cell pack.
2. Discard plasma by aspiration and suspend the remaining cells in 35 mL of ice-cold PBS.
3. Slowly pipette down this suspension to the side of a 50-mL polypropylene conical tube containing 10 mL of 1.084 g/mL of Percoll® without mixing.
4. Centrifuge at 400 x g for 40 min at 22°C (slow acceleration, no brake).
5. Discard by aspiration the supernatant, mononuclear cell layer and Percoll®
6. Retain the pellet (PMN and erythrocytes) and lyse erythrocytes by adding 2 volumes of an ice-cold lysing solution for each volume of cell suspension. Mix gentle by aspiration and inversion. Restore tonicity by adding 1 volume of restoring solution.
7. Centrifuge (500 x g) for 4 min at 4°C and wash the pellet washed twice by resuspension in 35 mL of CMF-HBSS and recentrifugation (500 x g for 3 min at 4°C).
8. Resuspend cells in 2-3 mL CMF-HBSS. Determine cell viability by Trypan Blue® following the instructions provided by the manufacturer.
9. For differential counting, make a smear by placing a small drop of the cell suspension near the end of a glass slide and spread the sample using another slide or a coverslip so that the angle between them is 30-40 degrees.
10. Fix the smear by immersion in 100% Methanol for 4 min. Let the smear dry and stain it with a Giemsa stain following the manufacturer's instructions.



Percoll isolation of bovine neutrophils (A) and purified blood PMN (100 X, B)

APPENDIX B: TOTAL REACTIVE OXYGEN SPECIES PRODUCTION ASSAY (LUMINOL)

Principle

Luminol-derived chemiluminescence (CL) is elicited by several reactive oxygen species (ROS) including superoxide (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hypochlourus acid (HClO). Thus, the selective use of various scavengers, such as ebselen, uric acid, catalase, and superoxide dismutase (SOD), is necessary to determine which of these species is responsible for the signal produced by luminol (Munzel et al., 2002, Rinaldi et al., 2006b). Because luminol is a cell permeable compound, the ROS measured reflects their extracellular as well as intracellular production even though Rinaldi et al. (2006b) suggested that changes in PMN luminol-dependant CL may not detect extracellular release of O_2^- and OH^-. Upon neutrophil activation by PMA, the membrane-bound NADPH oxidase complex catalyze the consumption of O_2 via electron transfer from NADPH to O_2 resulting in the generation of O_2^- which can be converted to H_2O_2, the main precursor of other neutrophil ROS. The enzyme myeloperoxidase then forms a complex with H_2O_2 that is either going to attack the pathogen directly or react with nitrogen intermediates. After exposure to ROS, the chemiluminescent probe luminol is oxidized under basic conditions with the release energy in the form of photons that can be detected by a luminometer:

$$\text{Luminol} + [\text{ROS generated by PMNs}] \xrightarrow{\text{oxidation}} [\text{Products}] + \text{light}$$

For example:

$C_8H_7N_3O_2 + 2\ OH^- \rightarrow C_8H_5NO_4^{2-} + N_2 + 2H_2O + light$

Reagents

- Ca-Mg-free Hank's balanced salt solution (CMF-HBSS, Sigma Chemical cat. # H6648)
- Dimethyl sulfoxide ≥ 99.5% GC (DMSO, Sigma Chemical cat. # D5879)
- 5m*M* Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma Chemical cat. # A8511)

  Stock solution (89.4 m*M*): 3960 mg of luminol in 10 mL of DMSO

  Working solution (5 m*M*): 559.2 µL of stock in 10 mL of CMF-HBSS

- 400 n*M* phorbol 12-myristate, 13-acetate ≥ 98.0% TLC (PMA, Sigma Chemical cat. # 79346)

  Stock solution (162.117 µ*M*): 1 mg of PMA in 10 mL of DMSO

  Working solution (400 n*M*): 24.67 µL of stock in 10 mL of CMF-HBSS

Procedure

1. Pre-warm all reagents at 37°C
2. Pipette 2 x 10^5 cells per well suspended in CMF-HBSS in white/clear-bottom 96-well plates with non-binding surface (Corning cat. #3604)
3. Add 20 µL of 5m*M* Luminol

4. Adjust reaction volumes to 180 uL with CMF-HBSS

5. Add 20 μL of either 400 n*M* phorbol 12-myristate, 13-acetate (PMA) (Stimulated cells) or CMF-HBSS (Control)

6. Read plate every 5 or 10 for 190 min under the following parameters for every reading:

   Temperature setpoint: 37°C
   Shake: fast for 0:05
   Reading detection method: luminescence
   Read type: endpoint
   Integration time: 0.3SS.s
   Emission: hole
   Optics position: top
   Sensitivity: 115
   Top probe vertical offset: 1mm

7. Substract background values, defined as the mean CL values of unactivated PMN.

APPENDIX C: EXTRACELLULAR O_2^- PRODUCTION ASSAY (MCLA)

Principle

Cypridina Luciferin methoxy-analogue [2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo [1,2-a]pyrazin-3(7H)-one hydrochloride, MCLA] dependant luminescence is a highly sensitive assay used to detect extracellular production of superoxide anion radical (O_2^-). The specificity of MCLA for O_2^- has been confirmed by complete inhibition of MCLA-derived CL by superoxide dismutase (SOD) (Rinaldi et al., 2006b). On exposure to O_2^-, the chemiluminescent probe MCLA releases a photon that can be detected by a scintillation counter or a luminometer:

$C_{14}H_{13}N_3O_2 \cdot HCl + O_2^- \rightarrow C_{14}H_{13}N_3O_2 \cdot HCl \cdot 2OOH + light$

In CL measurements over time, the addition of MCLA to the reaction before each reading is necessary since it is rapidly consumed.

Reagents

- Ca-Mg-free Hank's balanced salt solution (CMF-HBSS, Sigma Chemical cat. # H6648)
- Dimethyl sulfoxide ≥ 99.5% GC (DMSO, Sigma Chemical cat. # D5879)
- 2 µ*M* MCLA (Cypridina Luciferin methoxy-analogue, Sigma Chemical cat. # 87787)
- Stock solution (1371.1 µ*M*): 10 mg of luminol in 25 mL of DMSO
- Working solution (2 µ*M*): 14.587 µL of stock in 10 mL of CMF-HBSS

- 400 n*M* phorbol 12-myristate, 13-acetate ≥ 98.0% TLC (PMA, Sigma Chemical cat. # 79346)
- Stock solution (162.117 µ*M*): 1 mg of PMA in 10 mL of DMSO
- Working solution (400 n*M*): 24.67 µL of stock in 10 mL of CMF-HBSS

Procedure

1. Pre-warm all reagents at 37°C
2. Pipette 4 x 10^5 cells per well suspended in CMF-HBSS in white/clear-bottom 96-well plates with non-binding surface (Corning cat. #3604)
3. Add 10 µL of 2 µ*M* MCLA
4. Adjust reaction volumes to 160 uL with CMF-HBSS
5. Add 40 µL of either 400 n*M* phorbol 12-myristate, 13-acetate (PMA) (Stimulated cells) or CMF-HBSS (Control)
6. Read the plate every 10 min for 80 min under the following parameters for every reading:

   Temperature setpoint: 37 C
   Shake: fast for 0:05
   Reading detection method: luminescence
   Read type: endpoint
   Integration time: 0.3SS.s
   Emission: hole
   Optics position: top
   Sensitivity: 115
   Top probe vertical offset: 1mm

7. Add 10 µL of 2 µ*M* MCLA 1 min before each reading
8. Substract background values, defined as the mean CL values of unactivated PMN

APPENDIX D: INTRACELLULAR REACTIVE OXYGEN SPECIES PRODUCTION ASSAY (CM-H_2DCFDA)

Principle

CM-H_2DCFDA is a cell-permeant probe for ROS detection that are trapped intracellularly. It is nonfluorescent until removal of the acetate groups by intracellular esterases and oxidation that occurs within the cell (Rinaldi et al., 2006b). Fluorescence occurs when light is absorbed from an external source (485 nm excitation wavelength) by the fluorescent probe CM-H_2DCFDA that undergoes its florescent properties due to its interaction with the ROS. In turn, the emitted light by the reaction has a wavelength of 528 nm.:

CM-H_2DCFDA + [ROS released by PMNs] + light (485 nm) → [Products] + photon energy (528 nm)

The intracellular ROS by measured by CM-H_2DCFDA include hydrogen peroxide (H2O2), peroxynitrite anion ($ONOO^-$) and nitric oxide. It has been shown that superoxide dismutase has no inhibitory effect on CM-H_2DCFDA-dependant fluorescence suggesting that it specifically oxidizes intracellular ROS produced by bovine PMNs.

Reagents

- Ca-Mg-free Hank's balanced salt solution (CMF-HBSS, Sigma Chemical cat. # H6648)
- Dimethyl sulfoxide ≥ 99.5% GC (DMSO, Sigma Chemical cat. # D5879)

- 200 μ*M* 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein di-acetate acetyl ester (CM-H2DCFDA Invitrogen cat. # C6827)

  Stock solution (1730.7 μ*M*): 1 mg of CM-H2DCFDA in 1 mL of DMSO

  Working solution (200 μ*M*): 1 mL of stock in 8.654 mL of CMF-HBSS

- 400 nM phorbol 12-myristate, 13-acetate ≥ 98.0% TLC (PMA, Sigma Chemical cat. # 79346)

  Stock solution (162.117 μ*M*): 1 mg of PMA in 10 mL of DMSO

  Working solution (400 n*M*): 24.67 μL of stock in 10 mL of CMF-HBSS

Procedure

1. Pre-warm all reagents at 37°C
2. Pipette 4 x 10^5 cells per well suspended in CMF-HBSS in white/clear-bottom 96-well plates with non-binding surface (Corning cat. #3604)
3. Add 10 μL of 200 μ*M* CM-H2DCFDA
4. Adjust reaction volumes to 170 uL with CMF-HBSS
5. Add 30 μL of either 400 n*M* phorbol 12-myristate, 13-acetate (PMA) (Stimulated cells) or CMF-HBSS (Control)
6. Read the plate every 5 or 10 min for 200 min under the following parameters for every reading:

   Temperature setpoint: 37 C
   Shake: fast for 0:05
   Reading detection method: fluorescence
   Read type: endpoint
   Excitation : 485/20
   Emission: 528/20

Optics position: top
Sensitivity: 40

7. Top probe vertical offset: 1mm

8. Substract background values, defined as the mean CL values of unactivated PMN

APPENDIX E: NEUTROPHIL EXTRACELLULAR TRAPS (NETS) ASSAY

Principle

Neutrophil extracellular traps (NETs) are extracellular structures released by activated neutrophils. DNA is the major structural component of NETs but they also contain several antimicrobial proteins including elastase, cathepsin and myeloperoxidase (Brinkmann et al., 2004). NETs active formation can be quantified in a fluorometer with the use of a high-affinity DNA binding stain, such as Sytox® Orange, that will not cross the cell membrane of live cells. After incubation, the nucleic acids fluoresce bright orange when excited with a 520-550 nm source. Fluorescence can then be measured using a plate reader.

Reagents

- Ca-Mg-free Hank's balanced salt solution (CMF-HBSS, Sigma Chemical cat. # H6648)
- RPMI-1640 + 2% heat-inactivated FBS

  490 mL of Advanced RPMI 1640 Reduced Serum Medium 1X (+ non-essential aminoacids, + 110 mg/L sodium pyruvate, - L-glutamine; Invitrogen cat. #12633) + 10 mL of heat inactivated Fetal Bovine Serum (FBS; Invitrogen cat. #10091)
- 0.07 µg/mL phorbol 12-myristate, 13-acetate ≥ 98.0% TLC (PMA, Sigma Chemical cat. # 79346)

  Stock solution (162.117 µ*M*): 1 mg of PMA in 10 mL of DMSO

Working solution (0.07 µg/mL): 28 µL of stock in 40 mL of RPMI-1640 + 2% heat-inactivated FBS

- 7 µ*M* Ionomycin from *Streptomyces conglobatus* ≥98% HPLC (Sigma cat. #I9657)

  Stock solution (0.1mg/mL): 1 mg of Ionomycin in 10 mL of DMSO

  Working solution (7 µ*M*): 1985.2 µL of stock in 40 mL of RPMI-1640 + 2% heat-inactivated FBS

- 5 µ*M* Sytox® Orange (5m*M* Invitrogen cat. # S11368)

  10 µL of Sytox® Orange 5m*M* in 10 mL of CMF-HBSS

Procedure

1. Pre-warm all reagents at 37°C
2. Pipette 5 x 10^5 cells per well (100 µL) suspended in RPMI-1640 + 2% heat-inactivated FBS in non-treated white/clear-bottom 96-well plates (Corning cat. #3632)
3. Add 20 uL of either 0.07 µg/mL PMA and 7 µ*M* ionomycin (stimulated cells) or RPMI-1640 + 2% heat-inactivated FBS (controls)
4. Centrifuge the plates (10 min at 650 × g) and incubate at 37 °C and 5% CO2 for 60 min. - Remove supernatant from the plates
5. Stain the wells by adding 50 µl of 5 µ*M* Sytox® Orange protected from light for 10 min at room temperature
6. Pour off the stain and wash the wells once with 100 µl of CMF-HBSS
7. Read plate under the following parameters:

Shake: fast for 0:05
Reading detection method: fluorescence
Read type: endpoint
Excitation : 530/25
Emission: 590/35
Optics position: bottom
Sensitivity: 40
Top probe vertical offset: 1mm

8. Substract background values, defined as the mean CL values of unactivated PMN

APPENDIX F: PHAGOCYTOSIS AND KILLING ASSAY

Principle

Anti-microbial activity of neutrophils can be measured by colony-counting techniques after lysis of cells that contain viable bacteria. In a first step, neutrophils are incubated with a bacterial suspension, which concentration was previously adjusted, and heat-inactivated bovine serum for a period of 1 hr. During this period, the phagocytes engulf and kill the pathogens by their intracellular and extracellular mechanisms. Then, an aliquot from this reaction is obtained to prepare smears to be analyzed under light microscopy to determine the number of intracellular bacteria (Figure 3.2). The reaction is further sonicated to disrupt the cell membrane and liberate the intracellular bacteria. This suspension can be serially diluted and used to determine the number of bacteria present in the reactions with neutrophils in comparison with those reactions only with bacteria.

Reagents

- Ca-Mg-free Hank's balanced salt solution (CMF-HBSS, Sigma Chemical cat. # H6648)
- Sterile PBS, pH 7.4

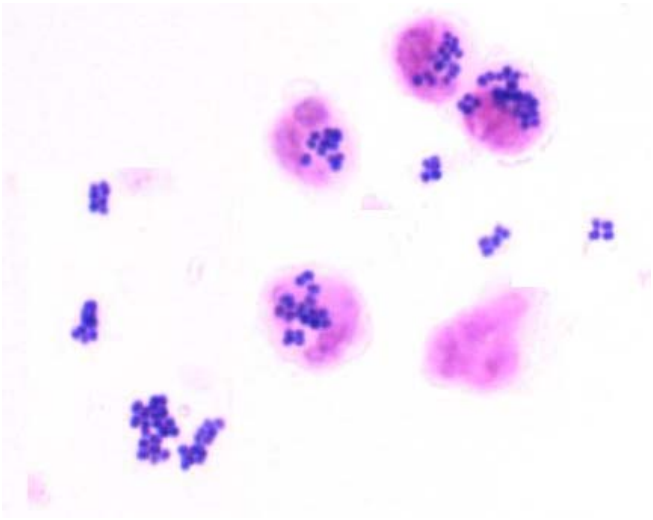
  8g of NaCl (Sigma Chemical cat. # S6191), 0.2g of KCl (Sigma Chemical cat. # P5405), 1.44g of Na_2HPO_4 (Sigma Chemical cat. # S5136) and 0.24g of KH_2PO_4 (Sigma Chemical cat. # P5655) per L of solution
- Brain-heart infusion broth (Sigma Chemical cat. # 53286)
- Bovine Serum (Heat inactivated, Fisher Scientific cat. # 19135280)

- Giemsa® stain (Sigma Chemical cat. # G9641)

Procedure

1. Inoculate *Staphylococcus aureus* strain 305 on a blood agar plate and incubate overnight at 37°C.
2. Transfer 10 colony forming units (cfu) from the plate to a sterile tube containing 10 mL of brain–heart infusion broth (prepared according to instructions provided by the manufacturers) and incubate overnight at 37°C at 225 rpm in an orbital shaker.
3. After incubation, place the tube in an ice-water bath and mixed by vortexing.
4. Serially dilute 100 μL from the culture in PBS and spread 100 μL of the resulting dilutions on blood agar plates. Incubate the plates overnight at 37°C and maintain the stock culture at 4°C.
5. Determine the concentration (cfu/mL) of the stock culture based on the colony counts of the spread plates.
6. Dilute the stock culture in HBSS to yield a final concentration of 4×10^7 cfu/mL.
7. Add 200 μL of this culture (8×10^6 cfu) to sterile tubes with or without 2×10^6 neutrophils (1.4 mL) and 400 μL of pooled, heat-inactivated bovine serum (ratio of bacteria to neutrophils 4:1).
8. Place the samples on an orbital shaker for 60 min at 37°C.
9. After incubation, prepare a smear with a 50-μL aliquot of each reaction and stain with Giemsa stain (score the first 100 PMN encountered in the field of view of a light microscope as either positive or negative for intracellular bacteria; for those scored as positive, count the number of intracellular bacteria).

10. Place 1.95 mL of the remaining reaction in ice and sonicate with a sonic dismembrator (Model 100, Fisher Scientific) at a power setting of 1 for 15 s (Verify rupture of PMN by microscopic examination).
11. Serial dilute 100 μL aliquot from each sonicated reaction in PBS and spread 100 μL quantities of the resulting dilutions on blood agar plates.
12. Incubate the plates overnight at 37°C and enumerate the colonies.
13. Determine the percentage of bacteria killed by calculating the difference in the number of bacteria incubated in the absence and presence of PMN and dividing this difference by the number of bacteria incubated in the absence of PMN.



Neutrophils with engulfed *S. aureus*

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