Differential alterations in the ability of bovine neutrophils to generate extracellular and intracellular reactive oxygen species during the periparturient period

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Abstract

The periparturient period of a dairy cow is associated with increased incidence and/or severity of certain infectious diseases, including mastitis. It is believed that the heightened physiological demands of calving and initiation of milk production contribute to a state of immunosuppression during this period. Previous studies have indicated that neutrophil production of reactive oxygen species (ROS), which is a critical element of the host innate immune response to bacterial infection, is impaired in the 1–2 week period following calving. However, whether there is comprehensive inhibition of ROS production or selective inhibition of particular ROS remains unknown. The present study provides evidence that neutrophils isolated from cows \( n = 20 \) after calving have an increased capacity to generate intracellular ROS and an impaired ability to release extracellular superoxide anion and hydrogen peroxide.

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Introduction

The periparturient or transition period for a dairy cow begins 2–3 weeks before calving and lasts until 2–3 weeks after calving. This period is characterized by physiological, metabolic and nutritional changes that are often accompanied by the onset of metabolic disorders (Stevenson and Call, 1988; Goff and Horst, 1997) and the impairment of host immune function (Waller, 2000). It is believed that this impaired immune function contributes to the increased incidence and/or severity of certain infectious diseases, such as mastitis, in the period just after calving (Vangroenweghe et al., 2005; Sheldon et al., 2006).

Polymorphonuclear neutrophils (PMNs) are critical for the initial defense of the host against invading microbial pathogens. Impairment of PMN recruitment to the site of infection (Schalm et al., 1976; Nagahata, 2004) and PMN activity (Roth and Kaeberle, 1981; Cai et al., 1994; Tkalcevic et al., 2000) are associated with increased susceptibility to infection. Activated PMNs kill bacteria both through an oxygen-independent mechanism via the production of microbicidal peptides and proteases, and through an oxygen-dependent mechanism via the generation of reactive oxygen species (ROS) (Gudmundsson and Agerberth, 1999; Burg and Pillinger, 2001). ROS, which include superoxide anion \( \left( \text{O}_2^- \right) \), hydrogen peroxide \( \left( \text{H}_2\text{O}_2 \right) \), and hydroxyl radical \( \left( \text{OH}^- \right) \), are powerful oxidants that are produced in the phagosome. Furthermore, myeloperoxidase, a major component of the azurophilic granules that fuse with the phagosome, catalyzes the formation of hypochlorous acid \( \left( \text{HOCl} \right) \) from \( \text{H}_2\text{O}_2 \) in the presence of halogens (e.g., \( \text{Cl}^- \)). Other reactions between HOCl and \( \text{H}_2\text{O}_2 \) or amines can lead to the formation of singlet oxygen \( \left( ^1\text{O}_2 \right) \) or chloramines, respectively (El-Benna et al., 2005). The generation of ROS plays a critical role in PMN-mediated defenses
against bacteria, and defects in the ability to generate ROS are associated with impaired capacity to clear pathogens (Baehner, 1990; Dinauer, 1993).

Several studies have evaluated the activity of PMNs during the periparturient period in an attempt to elucidate whether the elevated incidence and/or severity of certain infectious diseases during this period can be correlated with impaired PMN function. Decreased PMN random migration (Detilleux et al., 1995), chemotaxis (Nagahata et al., 1988), and phagocytosis (Saad et al., 1989), have been observed around parturition. However, other studies have reported either no lactational stage-dependent differences in these responses or enhanced random migration and phagocytic activity by blood PMN during the early post-partum period (Kehrli et al., 1989; Hoedemaker et al., 1992; Cai et al., 1994; Detilleux et al., 1995; Dosogne et al., 1999). A number of studies have also investigated alterations in PMN respiratory burst activity during the periparturient period (Kehrli et al., 1989; Hoedemaker et al., 1992; Gilbert et al., 1993; Cai et al., 1994; Detilleux et al., 1995; Dosogne et al., 1999; Mehrzad et al., 2001) and the combined findings from these studies suggest a reduced ability to produce ROS immediately after calving. However, it remains unknown whether there is comprehensive inhibition of ROS production that affects both intracellular generation and extracellular release and/or whether there are particular ROS whose generation is selectively impaired.

Recently, several assays for the measurement of bovine PMN, ROS have been validated (Rinaldi et al., 2007). Using superoxide dismutase and catalase, which scavenge extracellular O$_2^-$ and H$_2$O$_2$, respectively, assays were established that could discriminate between intracellular production and extracellular release of ROS by PMN. The objective of the current study was to utilize these assays to delineate whether lactational stage-dependent changes in the production of specific ROS by bovine PMNs affected the production of several ROS or a limited subset (for example, intracellular versus extracellular).

Materials and methods

Animals

Blood was obtained from the coccygeal veins of 20 Holstein cows within 1–8 days after calving. All cows had normal calving deliveries and progressed onto lactation without complication. On the day that blood was collected from a periparturient (PP) cow, blood was also obtained from five randomly selected mid-lactating (ML) (100–200 days in milk) Holstein cows. All animals were free of visible clinical signs of disease on the day of sampling. The mean (±SE) age (1491 ± 68 days versus 1442 ± 133 days; $P = 0.3594$) and parity (2.26 ± 0.17 calvings versus 2.14 ± 0.17 calvings; $P = 0.7593$) of the ML and PP cows did not significantly differ. All cows were housed in a free-stall enclosed barn, milked twice daily, and subjected to the same husbandry conditions.

The use and care of all animals in this study were approved by the Beltsville Agricultural Research Center’s Animal Care and Use Committee.

Bovine blood PMNs

Blood was collected into Vacutainer glass tubes containing acid–citrate–dextrose (Becton Dickinson), inverted five times and stored on ice. PMNs were isolated using a Percoll gradient as previously described (Rinaldi et al., 2007). The purity of the isolated PMNs was >95% and viability was >90%.

To measure respiratory burst activity, PMNs were exposed to Hank’s balanced salt solution (HBSS), phorbol 12-myristate 13-acetate ([PMA]; 40 nM or 40 µM), or opsonized zymosan (250 µg/mL), and ROS production measured for 120 min by assaying the following: luminol- and isoluminol-dependent chemiluminescence; cytochrome c reduction; and Amplex Red- and 5,5′-dichloromethyll-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H$_2$DCFDA)-dependent fluorescence. All assays had been previously validated (Rinaldi et al., 2007) and were performed as described below.

Luminol chemiluminescence assay

Luminol-derived chemiluminescence, which is elicited in response to the generation of an array of ROS, including O$_2^-$, OH·, H$_2$O$_2$, peroxynitrite (ONOO$^-$), and HOCI (Saez et al., 2000; Munzel et al., 2002), was used to assess intracellular and extracellular PMN respiratory burst activity. For studies evaluating changes in luminol-derived chemiluminescence, 60 µL of HBSS, PMA, or zymosan, and 20 µL of luminol (5 mM) were added to 2 × 10$^6$ PMNs suspended in 100 µL of calcium- and magnesium-free (CMF) HBSS in wells of a 96-well plate. All reactions were adjusted to a final volume of 200 µL with HBSS. Chemiluminescence was measured at 5 min intervals with a microplate luminometer (Turner Biosystems). Background values, defined as the mean chemiluminescence values of luminol diluted in HBSS, were subtracted from all readings.

Isoluminol chemiluminescence and cytochrome c reduction assays

Isoluminol-dependent chemiluminescence and cytochrome c reduction, which are specific indicators of the generation of extracellular O$_2^-$ (Lundqvist and Dahlgren, 1996; Dahlgren and Karlsson, 1999; Munzel et al., 2002; Tarpey et al., 2004), were used to assess differences in extracellular O$_2^-$ production. Reactions with isoluminol were set up exactly as described for the luminol chemiluminescence assay with the exception that luminol was substituted with 20 µL of isoluminol (500 µM) and 5 µL of horseradish peroxidase (HRP; 160 U/mL) (Sigma). For studies evaluating changes in cytochrome c reduction, 50 µL of HBSS, PMA, or zymosan, and 10 µL of cytochrome c (1 mM) were added to 2 × 10$^6$ PMNs suspended in 100 µL of CMF-HBSS in wells of a 96-well plate. All reactions were adjusted to a final volume of 200 µL with HBSS. Absorbance was measured with a plate reader (Bio-Tec Instruments). Optical density (OD) was measured at 10 min intervals at a wavelength of 550 nm and the difference of the two ODs recorded. Background values, calculated from wells with cytochrome c diluted in HBSS, were subtracted from all values.

Amplex Red fluorescence assay

Amplex Red-dependent fluorescence, which is a highly specific indicator of extracellular H$_2$O$_2$ (Zhou et al., 1997; Fortezza et al., 2003), was used to measure the generation of extracellular H$_2$O$_2$. For studies evaluating Amplex Red-derived fluorescence, 20 µL of HBSS, PMA, or zymosan, and 80 µL of the Amplex Red reaction mixture, containing 75 µL of Amplex Red (10 mM) and 150 µL of horseradish peroxidase (10 U/mL) diluted in 11.8 mL of Krebs Ringer phosphate glucose solution (145 mM NaCl, 5.7 mM Na$_2$HPO$_4$, 4.86 mM KCl, 0.54 mM CaCl$_2$, 1.22 mM MgSO$_4$, 5.5 mM glucose), were added to 1.5 × 10$^5$ PMNs suspended in 10 µL of Krebs Ringer phosphate glucose solution in wells of a 96-well plate. All reactions were adjusted to a final volume of 120 µL with HBSS. Fluorescence was measured every 15 min with a plate reader (Bio-
Tec Instruments) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Background values, defined as the mean fluorescence values of the Amplex Red reaction mixture diluted in HBSS, were subtracted from all readings.

**CM-H$_2$DCFDA-fluorescence assay**

The oxidation of CM-H$_2$DCFDA, which is elicited in response to the generation of intracellular ONOO$^-$ and H$_2$O$_2$ (Rothe and Valet, 1990; Crow, 1997), was used to evaluate the intracellular production of ROS. For studies evaluating changes in CM-H$_2$DCFDA-fluorescence, 37.5 μL of HBSS, PMA, or zymosan, and 7.5 μL of CM-H$_2$DCFDA (200 μM), were added to $4 \times 10^5$ PMNs suspended in 50 μL of CMF-HBSS in wells of a 96-well plate. All reactions were adjusted to a final volume of 150 μL with HBSS. Fluorescence was measured every 15 min with a plate reader (Bio-Tec Instruments) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Background values, defined as the mean fluorescence values of CM-H$_2$DCFDA diluted in HBSS, were subtracted from all readings.

**Statistical methods**

To enable calculation of cumulative ROS production within a given assay, the area under the curve was calculated from plotted data points for each experimental condition. The D’Agostino and Pearson omnibus K2 normality test was used to evaluate whether the data followed a normal (Gaussian) distribution. For data following a normal distribution, a two-tailed t test was used to compare the mean responses between PMNs obtained from PP and ML cows. For data that did not follow a normal distribution, the Mann–Whitney test was used to compare responses. All calculations were performed using GraphPad Prism version 4.03 for Windows.

**Results**

PMNs obtained from cows just after calving demonstrated $\sim 20\%$ ($P = 0.0036$) and $34\%$ ($P = 0.0001$) increased luminol-dependent chemiluminescence following stimulation with either PMA or zymosan, respectively, than similarly treated PMNs obtained from ML cows (Fig. 1a). There were no differences ($P = 0.1711$) in the luminol-dependent chemiluminescence of unstimulated (control) PMNs obtained from cows in the two stages of lactation.

Relative to PMA-stimulated PMNs obtained from ML cows, PMNs isolated from cows just after calving demonstrated a decrease of $\sim 10\%$ in isoluminol-dependent chemiluminescence ($P = 0.059$) (Fig. 1b) and cytochrome c reduction ($P = 0.0069$) (Fig. 1c) following stimulation with PMA. In contrast, there were no statistically significant differences ($P = 0.3462$ and 0.4603) between zymosan-stimulated PMNs isolated from the two groups of animals in either assay.

Unstimulated, PMA-, and zymosan-stimulated PMNs from PP cows all demonstrated decreased Amplex Red-dependent fluorescence compared to similarly treated PMNs isolated from cows in mid-lactation (Fig. 1d). Relative to PMNs from ML cows, those obtained from cows just after calving demonstrated $\sim 56\%$ ($P = 0.0023$), 28% ($P = 0.0003$), and 24% ($P = 0.0165$) less Amplex Red-dependent fluorescence following exposure to HBSS (control), PMA, or zymosan, respectively.

Relative to PMNs isolated from ML cows, those obtained from PP cows showed $\sim 20\%$ ($P = 0.0659$) and 18% ($P = 0.0377$) higher CM-H$_2$DCFDA-dependent fluorescence, respectively, following stimulation with either PMA or zymosan (Fig. 1e). There were no statistically significant differences ($P = 0.7305$) in basal levels of CM-H$_2$DCFDA-dependent fluorescence between unstimulated PMNs isolated from the two groups of animals.

**Discussion**

The current study investigated lactational stage-dependent effects on bovine PMN respiratory burst activity. More specifically, we evaluated differences between intracellular generation and extracellular release of ROS, as well as influences on specific extracellular ROS. Luminol-dependent chemiluminescence is indicative of the production of intracellular and extracellular ROS (Briheim et al., 1984; Rest, 1994). Luminol has been commonly used as a comprehensive indicator of PMN respiratory burst activity and has been reported to measure O$_2^-$ (Faulkner and Fridovich, 1993; Lundqvist and Dahlgren, 1996), OH$^-$ (Yildiz and Demiryurek, 1998; Nemeth et al., 2002), H$_2$O$_2$ (Castro et al., 1996; Yildiz and Demiryurek, 1998), ONOO$^-$ (Radi et al., 1993), and HOCl (Brestel, 1985; Myhre et al., 2003). However, in a recent study using assay conditions identical to those used here, the majority of the measurable change in bovine PMN luminol-dependent chemiluminescence was shown to reflect either intracellular increases in ROS production and/or extracellular increases in ROS other than O$_2^-$ and H$_2$O$_2$ (Rinaldi et al., 2007).

Supporting the notion that increased luminol-dependent chemiluminescence observed at parturition in the current study (Fig. 1a) was reflective of increased generation of intracellular ROS, PMNs from PP cows demonstrated enhanced CM-H$_2$DCFDA-dependent fluorescence relative to PMNs obtained from ML cows (Fig. 1e). In fact, the magnitude of the differences in the luminol-dependent chemiluminescence and CM-H$_2$DCFDA-dependent fluorescence were comparable between PMNs isolated from cows in the two different stages of lactation. Together, these two independent assays suggest an increased capacity of PMNs obtained from PP cows to generate intracellular ROS.

In contrast to our findings, previous studies have reported a decrease in PMN luminol-dependent chemiluminescence after calving (Dosogne et al., 1999; Mehrzad et al., 2002). The discrepancy may be due to the fact that we compared PMN ROS production from cows after calving to cows in mid-lactation, whereas in previous studies the comparison was to PMNs obtained from cows before calving. Within 1–2 weeks before calving, PMN functions, including ROS production, are enhanced (Kehrli et al., 1989; Detilleux et al., 1995; Dosogne et al., 1999). Thus, the perceived impairment in ROS production at calving may actually reflect a return to baseline levels from the physiologically elevated state that exists prior to calving.
This notion is supported by published findings comparing PMN ROS activity at different stages of pregnancy (Hoedemaker et al., 1992). In the current study, we compared PMNs from PP and ML cows because of known differences in the incidence and severity of diseases, such as mastitis, that occur in cows in these two different stages of lactation (Vangroenweghe et al., 2005).

To elucidate changes in the PMN respiratory burst activity after calving more specifically, assays were utilized that detect only extracellular or intracellular ROS. Consistent with a previous study (Gilbert et al., 1993), PMA-induced PMN generation of extracellular O$_2^·$ was impaired after calving. The absolute finding, as well as the magnitude of the impairment, was consistent in two distinct assays used to specifically measure O$_2^·$ namely, isoluminol-dependent chemiluminescence (Fig. 1b) and cytochrome c reduction (Fig. 1c). Interestingly, there was no statistical lactational stage-dependent change in O$_2^·$ generation in response to zymosan that may be beyond the sensitivity of these assays.

Extracellular H$_2$O$_2$ is rapidly formed in activated PMN from O$_2^·$ by spontaneous or enzymatic dismutation by superoxide dismutase (Weiss, 1989; Hampton et al., 1998). Thus, one may hypothesize that a decreased ability to generate O$_2^·$ may impair corresponding generation of H$_2$O$_2$. Consistent with this hypothesis, PMNs isolated from cows just after calving demonstrated decreased generation of H$_2$O$_2$ relative to those obtained from cows in mid-lactation (Fig. 1d). To our knowledge, this is the first study specifically to evaluate the extracellular release of H$_2$O$_2$ in activated PMNs isolated from PP cows.

Conclusions

The current study has investigated the effect of lactational stage on ROS production of PMN's isolated from

Fig. 1. Respiratory burst activity of bovine peripheral blood neutrophils isolated from cows in two different stages of lactation. Blood neutrophils were isolated from cows in mid-lactation (100–200 days after calving) or at parturition (1–8 days after calving). Neutrophils were exposed to Hank’s balanced salt solution (control) or stimulated with phorbol 12-myristate 13-acetate (PMA) either 40 nM (a, b, d, e) or 40 μM (c), or opsonized zymosan (250 μg/mL a–c) in the presence of luminol (a), isoluminol (b), cytochrome c (c), Amplex Red (d), or CM-H$_2$DCFDA (e). Chemiluminescence (a, b), absorbance (c), and fluorescence (d, e) were measured for 120 min following neutrophil stimulation. Data are reported as the mean (±SE) of the area under the curve calculated from plotted data points for each assay during the 120 min measurement time. Responses of control and PMA-stimulated neutrophils are shown on the left y-axis and those of zymosan-stimulated neutrophils are shown on the right y-axis. * Significant different (P < 0.10, ** P < 0.05, and *** P < 0.01, respectively) relative to similarly treated neutrophils from cows in mid-lactation.
Holstein cows. Genetic differences between breeds of food-production animals are known to influence disease resistance (Kelm et al., 2001). Because PMNs play a critical role in host defense mechanisms, breed-dependent differences in their functioning, including ROS production, could explain genetic-dependent differential susceptibilities to infection. Whether the differences in ROS production between PP and ML Holstein cows reported here is mirrored in other breeds remains unknown.

Bacterial killing is a critical function of PMNs during infection. It is well established that intracellular generation of ROS is essential for PMN bactericidal activity. However, the contribution of extracellular ROS to PMN-mediated bacterial killing remains less clear (Hampton and Winterbourn, 1995; Hampton et al., 1998; Rinaldi et al., 2006). Inhibition of NADPH oxidase activity, which blocks intracellular and extracellular generation of ROS, impairs PMN bactericidal activity. However, agents that selectively inhibit extracellular O$_2^-$ and/or H$_2$O$_2$ fail to have a corresponding effect on PMN bactericidal activity (Johnston et al., 1975; Hampton et al., 1998; Rinaldi et al., 2006). Together, these data suggest that PMN bactericidal activity may be mediated by intracellular, and not extracellular, ROS. Our finding that impairment of ROS production at calving is limited to extracellular but not intracellular ROS, may suggest that critical ROS-mediated bacterial killing by bovine PMNs is preserved during the periparturient period.

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