Effects of in vitro insulin and 2,4-thiazolidinedione on the function of neutrophils harvested from blood of cows in different physiological states

X. S. Revelo and M. R. Waldron¹

Division of Animal Sciences, University of Missouri-Columbia, Columbia 65211

ABSTRACT

Neutrophils [polymorphonuclear neutrophilic leukocytes (PMNL)] were isolated from 26 Holstein cows in different physiological states (12 ± 1.7 d prepartum, $n = 8$; 7 ± 0 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 μ g/mL of the peroxisome proliferator-activated receptor- γ ligand 2,4-thiazolidinedione (TZD). Following the incubations, PMNL functional assays were performed to determine treatment effects on proxies for total, extracellular, and intracellular generation of reactive oxygen species (ROS), neutrophil extracellular trap formation, and phagocytic killing abilities. The ROS production of PMNL collected from cows at 7 d postpartum was reduced compared with that of PMNL from midlactation and prepartum cows, but neutrophil extracellular trap expression was 23 and 36% greater in PMNL from prepartum cows compared with that in PMNL from midlactation and postpartum cows, respectively. Insulin had no effect on PMNL functional assay results. In contrast, TZD inhibited a measurement of total ROS production by 89%, increased extracellular superoxide generation by 43%, but had no effect on the intracellular ROS measured. Interestingly, TZD did not alter the ability of the PMNL to release neutrophil extracellular traps and engulf or kill *Staphylococcus aureus*. These findings suggest a possible anti-inflammatory effect of TZD that may result in reduced extracellular oxidative damage with maintenance of PMNL 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 was greater during the first week of lactation compared with those detected from the second week onward (Wilson et al., 2004). Shuster et al. (1996) challenged cows with an intramammary infusion of *Escherichia coli* and reported that periparturient cows had more severe clinical signs of mastitis as well as a more rapid bacterial growth after 10 h of inoculation compared with midlactation animals. Despite considerable progress in the last 25 yr in the understanding of the biology of the periparturient cow, the incidence and severity of the most important infectious disease has remained unchanged (LeBlanc et al., 2006). A recent survey by the National Animal Health Monitoring Service reported that clinical mastitis is prevalent in 16.5% of the cows in the United States (USDA, 2008). Furthermore, this survey revealed that of the total number of cows that are permanently removed from the herd, 23% are culled because of mastitis or udder-related problems.

During an IMI, patrolling macrophages recognize the pathogen and trigger an inflammatory response resulting in the migration of PMNL 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 PMNL, by means of their toll-like receptors and other pattern-recognition receptors, bind and engulf opsonized bacteria in a process known as phagocytosis. Subsequently, PMNL use several killing mechanisms such as generation and release of potent oxidants [e.g., intracellular and extracellular reactive oxygen species (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 releasing structures termed neutrophil extracellular traps (NET) 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 (Kehrli et

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¹Corresponding author: waldronm@missouri.edu

al., 1989; Cai et al., 1994; Detilleux et al., 1995), but the physiological mechanisms responsible for these decreases remain unclear (Paape et al., 2002). Remarkably, there is a coincidence between this period of reduced neutrophil function and the periparturient fall of plasma insulin together with the onset of insulin resistance during late gestation as discussed by 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-h 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 have not been investigated in dairy cows, despite the fact that neutrophils isolated from dairy heifers (Nielsen et al., 2003) and multiparous cows [S. Kato (University of Vermont, Burlington) and M. R. Waldron; unpublished observation] express insulin receptors. Relative to insulin function, the synthetic peroxisome proliferator-activated receptor (PPAR)- γ ligand 2,4 thiazolidinedione (TZD) has been shown to restore insulin response in steers with insulin resistance induced by recombinant tumor necrosis α (Kushibiki et al., 2001). Peroxisome proliferator-activated receptor- γ is a member of the nuclear receptor family highly expressed in adipose tissue and the immune system that upon activation can regulate adipocyte differentiation, stimulate insulin action, regulate lipid metabolism, and affect inflammatory processes (Houseknecht et al., 2002). Therefore, we hypothesized that periparturient cows have reduced neutrophil function compared with midlactation animals and that incubation of PMNL with insulin or the PPAR- γ ligand TZD would improve PMNL functionality as assessed by the production and release of ROS, NET 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 SCC were determined using a DeLaval cell counter (DeLaval International AB, Tumba, Sweden). Milk SCC were not different between

midlactation and postpartum cows (181 ± 54 and $145 \pm 57 \times 10^3$ 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 $\pm$ 1.7 d before calving ($n = 8$), 7 d postpartum ($n = 9$), and during midlactation ($n = 9$; 253 $\pm$ 25.2 d postpartum, 138 $\pm$ 9.9 d pregnant) to evaluate the differential effects of physiologic state and in vitro insulin and TZD incubation on blood neutrophil function. All functional assays were performed simultaneously on the same day for any given cow.

Reagents

Percoll was obtained from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). Bovine insulin at a concentration of 10 mg/mL in a 25 mM HEPES, pH 8.2, solution and Ionomycin from *Streptomyces conglobatus* were obtained from Sigma Chemical Co. (St. Louis, MO). The 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)-3,7-dihydroimidazol[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 mM, 1371.1 μ M, and 162.12 μ M stock solutions, respectively, in DMSO. The 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate 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 mM solution in DMSO. All stock solutions were stored at -20°C. All working solutions, cell suspensions, and reactions 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 prewarmed to 37°C immediately before their use in the assays. In our in vitro cellular system, CMF-HBSS and RPMI-1640 (Table 1) were used to maintain a balanced environment (intra- and extracellular osmolarity, pH, [O₂], and temperature) and provide the cells with water and inorganic ions essential for normal cell metabolism. The use of HBSS as a buffering medium during PMNL-chemiluminescence (CL) assays results in maximal light emission and cell activation compared with PBS and Tris (Freitas et al., 2009).

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 50-mL polypropylene conical tubes (Corning Inc., Corning, NY) and centrifuged at $1,000 \times g$ for 20 min at 4°C to separate plasma from the cell pack. The 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 Percoll. The cells were then subjected to gradient centrifugation ($400 \times g$ for 40 min at 22°C). The supernatant, mononuclear cell layer, and Percoll were aseptically aspirated and discarded. The pellet (PMNL and erythrocytes) was retained, and erythrocytes were lysed by hypotonic shock. For this procedure, 2 volumes of an ice-cold 10.56 mM Na₂HPO₄, 2.67 mM NaH₂PO₄ 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 mM Na₂HPO₄, 2.67 mM NaH₂PO₄, 0.43 M NaCl solution. The cell solution was then centrifuged ($500 \times g$) for 4 min at 4°C, and the pellet was washed twice by resuspension in 35 mL of CMF-HBSS and recentrifugation ($500 \times g$ 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 PMNL was >95% and viability >97%. Cells were suspended either in CMF-HBSS or in 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). For all the assays, working solutions of insulin, TZD, or insulin and TZD in CMF-HBSS or RPMI-1640 + 2% heat-inactivated fetal bovine serum (NET assay) were pipetted into the wells or tubes to reach final concentrations of 0, 1.5, or 15 ng/mL of insulin, alone or in combination with 300 µg/mL of TZD. Suspensions of 2×10^5 (luminol assay; final volume 200 µL/well), 4×10^5 (MCLA and CM-H₂DCFDA assays; final volume 150 µL/well), 5×10^5 (NET assay; final volume 100 µL/well), and 2×10^5 neutrophils (killing-phagocytosis assay; final volume 1,000 µL/tube) in CMF-HBSS or RPMI-1640 were added to the treatment solutions. The

Table 1. Composition of calcium- and magnesium-free Hanks' Balanced Salt Solution (CMF-HBSS) and RPMI-1640¹ used in the vitro cellular systems

Component	Medium	
	CMF-HBSS (g/L)	RPMI-1640 (g/L)
KCl	0.40	0.40
KH ₂ PO ₄	0.06	—
NaHCO ₃	0.35	2.00
NaCl	8.00	6.00
Na ₂ HPO ₄	0.05	0.80
Ca(NO ₃) ₂ 4H ₂ O	—	0.10
MgSO ₄	—	0.05
D-glucose	1.00	2.00

¹RPMI-1640, GIBCO, Grand Island, NY.

plates and tubes were then incubated with lids in a humidified incubator (37°C, 5% CO₂) for 120 min while being gently shaken. After incubation, the cell suspensions were mixed by aspiration, and the functional assays were performed.

Luminol CL Assay

After incubation with insulin and TZD, a proxy of the total neutrophil ROS production was immediately assessed by a luminol CL assay as described by Rinaldi et al. (2007) with some modifications validated in our laboratory. Luminol is a cell-permeable compound that reacts with superoxide (O₂⁻), hydroxyl radical (OH⁻), hydrogen peroxide (H₂O₂), peroxynitrite (ONOO⁻), and hypochlorous acid (HClO; Rinaldi et al., 2007). Although the derived luminescence product of these reactions in the presence of luminol is not all-inclusive, it is a satisfactory indicator of the intracellular and extracellular ROS generation by the PMNL. Twenty microliters of 5 mM 5-amino-2,3-dihydro-1,4-phthalazine-dione (luminol) and 20 µL of either 400 nM PMA (stimulated cells) or HBSS (control) were added to all wells. All reactions were then adjusted to 200 µL using CMF-HBSS. Chemiluminescence 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 quadruplicate. Background values, defined as the mean CL values of unactivated PMNL, were subtracted from all readings.

MCLA CL Assay

After incubation, the extracellular neutrophil superoxide anion radical (O₂⁻) production was measured by a MCLA CL assay. The specificity of this probe for O₂⁻ was confirmed by Rinaldi et al. (2007), who reported that superoxide dismutase, the enzyme that catalyzes

the conversion of O_2^- to H_2O_2 , completely abrogated the MCLA-dependent CL. Ten microliters of 2 μM MCLA and 30 μL of either 400 nM PMA (stimulated cells) or HBSS (control) were added to all wells. The reactions were then adjusted to a volume of 200 μL using CMF-HBSS. The CL was measured every 10 min for 80 min with a Synergy HT plate reader (BioTek Instruments Inc.). Because MCLA is rapidly consumed, 10 μL of 2 μM MCLA was added to each well immediately before each 10-min reading throughout the course of CL measurements. All reactions were carried out in quintuplicate. Background values, defined as the mean CL values of unactivated PMNL, were subtracted from all readings.

CM-H₂DCFDA Fluorescence Assay

A proxy for the intracellular neutrophil ROS production was assessed using the CM-H₂DCFDA fluorescence assay with some modifications. The cell-permeant probe CM-H₂DCFDA reacts only with ROS that are trapped inside the cell, including hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxyxynitrite anion (ONOO⁻) (Rinaldi et al., 2007). After incubation, 10 μL of 200 μM CM-H₂DCFDA and 30 μL of either 400 nM PMA (stimulated cells) or HBSS (control) were added to all wells. All reactions were then adjusted to 200 μL 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.) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. All reactions were carried out in quadruplicate. Background values, defined as the mean fluorescence values of unactivated PMNL, were subtracted from all readings.

NET Assay

Neutrophil extracellular trap formation was quantified using Sytox Orange as described by Lippolis et al. (2006b) with some modifications. After PMNL incubation with treatments in 96-well plates for 120 min, 20 μL of PMA and 20 μL of ionomycin were added to the stimulated set of wells to obtain final concentrations of 10 ng/mL and 1 μM , respectively. Forty microliters of RPMI-1640 + 2% heat-inactivated fetal bovine serum were added to the control wells. Subsequently, the plates were centrifuged ($650 \times g$ for 10 min at 23°C) and incubated (37°C and 5% CO₂ 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 μM 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 sextuplicate. 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. Background values, defined as the mean fluorescence values of unactivated PMNL, were subtracted from all readings.

Killing and Phagocytosis Assay

The phagocytosis and killing assay was performed as described by Rinaldi et al. (2006) with modifications validated in our laboratory. *Staphylococcus aureus* strain 305 (American Type Culture Collection, Manassas, VA) was inoculated on a blood agar plate (Northeast Laboratory Services Inc., Winslow, 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 $0.54 \times g$ in an orbital shaker (model G2, New Brunswick Scientific Inc., Edison, NJ). After the overnight incubation, the tube was placed in an ice-water bath and subsequently mixed using a vortex mixer. One hundred microliters from the culture was serially diluted in PBS, and 100 μL of the resulting dilutions was spread on blood agar plates. The stock culture was maintained at 4°C, and the plates were incubated overnight at 37°C to determine the concentration (cfu/mL) of the stock culture based on the colony counts of the spread plates. Immediately before the initiation of the phagocytosis and killing assay, the stock culture was placed on ice and diluted in HBSS to yield a final concentration of 4×10^7 cfu/mL.

To assess PMNL phagocytosis and killing, 200 μL of *Staph. aureus* (8×10^6 cfu) and 400 μL of pooled, heat-inactivated bovine serum were added to sterile tubes with or without 2×10^6 PMNL previously incubated with the insulin and TZD treatments (1.4 mL). The ratio of bacteria to PMNL was 4:1. The samples were placed on a tube rotator for 60 min at 37°C. All reactions were performed in duplicate. To determine the percentage of PMNL containing phagocytosed bacteria and the actual number of phagocytosed bacteria per PMNL, a 50- μL aliquot of each reaction was used to prepare a smear that was stained with Giemsa stain. The first 100 PMNL encountered in the field of view of a light microscope were scored as either positive or negative for intracellular bacteria. For those PMNL scored as positive, the number of intracellular bacteria was enumerated.

To evaluate whether insulin and TZD incubations affected the bactericidal activity of PMNL, 1.95 mL of the remaining reaction was placed in ice and sonicated with a sonic dismembrator (model 100, Thermo Fisher

Scientific Inc., Pittsburgh, PA) at a power setting of 1 for 15 s. Rupture of the PMNL was verified by microscopic examination. Sonication did not affect bacterial viability (Rinaldi et al., 2007; X. S. Revelo and M. R. Waldron, unpublished observation). A 100- μ L aliquot from each sonicated reaction was serially diluted in PBS, and 100- μ L quantities of the resulting dilutions were spread on blood agar plates. The plates were incubated overnight at 37°C and the colonies enumerated. The percentage of bacteria killed was determined by calculating the difference in the number of bacteria incubated in the absence and presence of PMNL and dividing this difference by the number of bacteria incubated in the absence of PMNL.

Statistical Analysis

The experiment was conducted as a completely randomized design with a factorial arrangement of treatments (insulin, TZD, and physiological state) applied to the incubation well or tube within cow. Data was analyzed by ANOVA 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-H₂D-DCFDA-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 ANOVA. For the CM-H₂DCFDA assay, a mixed model ANOVA 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 ANOVA. All reported means are the adjusted least squares means $\pm$ standard error of the mean, and the significance was declared when $P < 0.01$.

RESULTS

ROS 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 PMNL treated with 300 μ g/mL of TZD were only modestly increased from baseline with a peak at 70 min after activation and displayed a much more rapid decline back to baseline compared with insulin-only treatments or control wells (Figure 1A). To determine any differential effects of in vitro insulin and TZD treatment on PMNL, the AUC were calculated from these plotted data points. Insulin treatment had no effect on the luminol-dependent CL associated with total ROS production in PMA-stimulated cells; luminol-derived CL was not different between control and insulin-only treated wells (Figure 1B; insulin treatment effect, $P > 0.20$). Incubation of cells with 300 μ g/mL of TZD inhibited total ROS by approximately 88.9% compared with control and insulin-only treated wells (Figure 1B; TZD treatment effect, $P < 0.01$). The overall effect of physiological state on luminol-derived CL is presented in Figure 2. The luminol-derived CL of neutrophils isolated from postpartum cows was 37 and 32% less compared with that of midlactation and prepartum animals, respectively (physiological state effect, $P < 0.01$). However, the luminol-derived CL of PMNL collected from midlactation cows was not different from that of PMNL 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 μ g/mL TZD treatment was less than the magnitude of this reduction in PMNL incubated with 300 μ g/mL of TZD (Figure 3; TZD-by-physiological state interaction effect, $P < 0.01$).

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 PMNL treated with 300 μ g/mL of TZD had a greater maximal response after cell stimulation, and this difference was maintained throughout the rest of the MCLA-derived CL measurements compared with insulin-only treatments or control wells (Figure 4A). To determine any differential effects of in vitro insulin and TZD treatment on PMNL, the AUC were calculated from these plotted data points. Insulin treatment had no effect ($P > 0.20$) relative to control wells on the MCLA-dependent CL associated with extracellular O₂⁻ ROS production in PMA-stimulated cells (Figure 4B). In contrast, TZD enhanced the O₂⁻ generation approximately 43% compared with control wells (Figure 4B; TZD treatment effect, $P < 0.01$). Neutrophils from postpartum cows had a 42.9 and 36.6% lower MCLA-dependent CL compared with those from midlactation and prepartum ani-

mals, respectively (Figure 5; physiological state effect, $P < 0.01$). The MCLA-derived CL of PMNL collected from midlactation cows was similar to that of PMNL isolated from prepartum cows (Figure 5; $P > 0.20$). No interaction effects with either in vitro treatment or physiological state were evident for PMNL MCLA-derived CL (all interactions, $P > 0.20$).

Changes in CM-H₂DCFDA-derived fluorescence were observed at 30 min after activation with PMA. The fluorescence values from the cells incubated with 300 $\mu\text{g/mL}$ of TZD increased and reached a plateau approximately 140 min after stimulation, whereas those

of PMNL incubated with 0, 1.5, or 15 ng/mL of insulin without TZD continued to increase (Figure 6A). There was no effect of insulin or TZD on the intracellular ROS production based on calculated AUC (Figure 6B; insulin or TZD effect, $P > 0.20$). However, when the data were analyzed by ANOVA with repeated measures for each incubation measurement time point, the interaction of TZD by incubation time relative to PMA activation was significant (Figure 6A; $P < 0.01$). Furthermore, the intracellular ROS production of neutrophils isolated from postpartum cows was 42.9 and 36.7% less compared with that of neutrophils isolated from midlactation and

Figure 1. Effect of insulin (INS) and 2,4-thiazolidinedione (TZD) on a proxy for the total reactive oxygen species production by bovine neutrophils. Neutrophils were collected from cows ($n = 25$) and incubated for 120 min with 0, 1.5, or 15 ng/mL of insulin and 0 or 300 $\mu\text{g/mL}$ of TZD either alone or in combination with the insulin treatments. (A) 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. The CL values of nonactivated neutrophils were subtracted from all the CL measurements at every time point. (B) To assess the overall magnitude of the luminol-dependent CL response over time, the area under the curve ($\pm$ SEM) was calculated from plotted luminol-derived measurements. Area under the curve differed by TZD treatment, $P < 0.01$. ^{a,b}Bars with different letters differ ($P < 0.01$).

Figure 2. Effect of physiological state on a proxy for the total reactive oxygen species 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 ($+7 \pm 0$ d; $n = 8$) cows. Data represent the area under the curve (AUC; $\pm$ SEM) calculated from plotted luminol-derived measurements over a 190-min period after activation of neutrophils with phorbol 12-myristate,13-acetate. The chemiluminescence (CL) values of nonactivated neutrophils were subtracted from all the CL measurements at every time point. Luminol-derived CL differed by physiological state, $P < 0.01$. ^{a,b}Bars with different letters differ ($P < 0.01$).

prepartum animals, respectively (Figure 7; physiological state effect, $P < 0.01$). However, the fluorescence values of PMNL collected from midlactation cows were similar to those of PMNL isolated from prepartum cows

(Figure 7; $P > 0.20$). There were no insulin-by-TZD or in vitro treatment-by-physiological state interaction effects on the PMNL intracellular generation of ROS as measured by CM-H₂DCFDA-derived fluorescence (all interactions, $P > 0.20$).

NET Release

There was no effect of insulin or TZD on NET release relative to control wells (Figure 8; effect of insulin or TZD, $P > 0.20$). However, the mean fluorescence associated with NET released from neutrophils collected from prepartum cows was 23 and 36% greater than that of PMNL collected from midlactation and postpartum cows, respectively (Figure 9; physiological state effect, $P < 0.01$). Neutrophil NET expression from midlactation cows was similar to that of PMNL isolated from postpartum cows (Figure 9; $P > 0.20$). There were no insulin-by-TZD or in vitro treatment-by-physiological state interaction effects on PMNL NET release ($P > 0.20$).

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 *Staph. aureus* per PMNL (Table 2; insulin or TZD effect, $P > 0.20$). Neutrophils collected from postpartum cows had

Figure 3. Effects of 2,4-thiazolidinedione (TZD) and physiological state on a proxy for the total reactive oxygen species 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 ($+7 \pm 0$ d; $n = 8$) cows. Data represent the area under the curve (AUC; $\pm$ SEM) calculated from plotted luminol-derived measurements over a 190-min period after activation of neutrophils with phorbol 12-myristate,13-acetate. The chemiluminescence (CL) values of nonactivated neutrophils were subtracted from all the CL measurements at every time point. Luminol-derived CL differed by TZD $\times$ physiological state, $P < 0.01$. ^{a-c}Bars with different letters differ ($P < 0.01$).

Figure 4. Effect of insulin (INS) and 2,4-thiazolidinedione (TZD) on extracellular O_2^- production by bovine neutrophils. Neutrophils were collected from cows ($n = 26$) and incubated for 120 min 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 methyl cypridina luciferin analog (MCLA)-dependent chemiluminescence (CL; arbitrary units) measured every 10 min over an 80-min period after activation of neutrophils with phorbol 12-myristate,13-acetate. The CL values of nonactivated neutrophils were subtracted from all the CL measurements at every time point. (B) To assess the overall magnitude of the luminol-dependent CL response over time, the area under the curve ($\pm$ SEM) was calculated from plotted luminol-derived measurements. Area under the curve differed by TZD treatment, $P < 0.01$.

a greater percentage of cells with phagocytosed bacteria and number of bacteria per neutrophil compared with those from prepartum and midlactation animals (Table 3; physiological state effect, $P < 0.01$). There was no effect of insulin or TZD on the killing ability of PMNL (Table 2; insulin or TZD effect, $P > 0.20$). However, the killing ability of neutrophils isolated from postpartum animals was 61 and 97% greater compared with that of neutrophils isolated from prepartum and midlactation animals, respectively (Table 3; physiological state effect, $P < 0.01$). No in vitro treatment or physiological

state interaction effects were evident (all interactions, $P > 0.20$).

DISCUSSION

A 2-h incubation of PMNL with a high physiological (1.5 ng/mL) or a supraphysiological (15 ng/mL) concentration of insulin had no effect on the respiratory burst activity, NET release, phagocytosis and killing ability of neutrophils isolated from cows in different physiological states. In contrast, in vitro experiments involving hu-

man neutrophils indicate that insulin at physiological concentrations can influence some aspects of their function. For instance, a 30-min incubation with 5.8 and 58 ng/mL of insulin stimulated the generation of hydrogen peroxide in a dose-responsive manner (Spagnoli et al., 1995), 1.4 ng/mL of insulin enhanced PMNL chemotaxis toward formyl-methionyl-leucyl-phenylalanine by approximately 50% (Cavalot et al., 1992), and 3.7 ng/mL of insulin increased the expression of the neutrophil intercellular adhesion molecule-1 by approximately 80% after 48 h of incubation with the hormone (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). A standard insulin 2-wk 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 investigating the effect of experimental hyperinsulinemia on neutrophil function in nondiabetic subjects have yielded conflicting results. For instance, Walrand et al. (2004) reported an increase in chemotaxis, phagocytosis, and bactericidal activities in PMNL collected from healthy individuals after a 4-h hyperinsulinemic euglycemic clamp, whereas Fejfarová et al. (2006) and Stegenga et al. (2008) did not find any alterations of phagocytosis, migration, and ROS production during induced hyperinsulinemia with strict euglycemia when compared with 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 are reduced in the periparturient period compared with midlactation, as part of the homeorhetic adaptations that support the increased flux of nutrients toward pregnancy and lactation (Bell and Bauman, 1997). Thus, we hypothesized that in vitro incubation with greater 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 PMNL relative to PMNL isolated from midlactation cows. The lack of a direct effect of insulin on bovine neutrophil activity may be an artifact of the experimental methods such as short incubation time or exposure to insulin at an insensitive time point of the PMNL life cycle. Alternatively, the lack of insulin effect may be explained by the relatively 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

Figure 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 ($+7 \pm 0$ d; $n = 9$) cows. Data represent the area under the curve (AUC; $\pm$ SEM) calculated from plotted methyl cypridina luciferin analog (MCLA)-derived measurements over a 70-min period after activation of neutrophils with phorbol 12-myristate,13-acetate. The chemiluminescence (CL) values of non-activated neutrophils were subtracted from all the CL measurements at every time point. MCLA-derived CL differed by physiological state, $P < 0.01$. ^{a,b}Bars with different letters differ ($P < 0.01$).

via improvement of metabolic control during the periparturient period. For instance, insulin may reduce the level of circulating NEFA and ketone bodies that have detrimental effects on neutrophil and leukocyte activity (Suriyasathaporn et al., 1999; Scalia et al., 2006). Thus, the in vivo effect of insulin on periparturient neutrophil function requires investigation.

Incubation of PMNL with TZD resulted in a potent inhibitory effect on the neutrophil luminol-dependent CL, an indicator of the PMNL generation of a wide array of ROS including extracellular and intracellular hydroxyl radical (OH^-), peroxynitrite ($ONOO^-$), hypochlorous acid ($HClO$), intracellular superoxide (O_2^-), and hydrogen peroxide (H_2O_2) (Rinaldi et al., 2007). Furthermore, TZD had no effect on the generation of intracellular NO, $ONOO^-$, and H_2O_2 assessed by the CM- H_2 DCFDA fluorescence assay, when the differences were assessed by calculated AUC. However, when the data were analyzed by ANOVA with repeated measures, treatment of PMNL with TZD resulted in a modest decrease in the generation of CM- H_2 DCFDA-derived intracellular ROS 140 min after cell activation. Perhaps TZD has a delayed inhibitory effect on the generation of these intracellular ROS relative to luminol-derived ROS, but the duration of our assay might not have allowed us to observe the decrease in these intracellular ROS to its full extent. Overall, the decreased respiratory burst activity is consistent with the emerging evidence regarding the anti-inflammatory effects of thiazolidin-

Figure 6. Effect of insulin (INS) and 2,4-thiazolidinedione (TZD) on a proxy for the intracellular reactive oxygen species production by bovine neutrophils. Neutrophils were collected from cows ($n = 24$) and incubated for 120 min with 0, 1.5, or 15 ng/mL of insulin and 0 or 300 µg/mL of TZD. Data represent the mean 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein di-acetate acetyl ester (CM-H₂DCFDA)-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 nonactivated neutrophils were subtracted from all measurements at every time point. Incubation with TZD affected neutrophil CM-H₂DCFDA-derived fluorescence over time (panel A; treatment by time effect, $P < 0.01$), but did not affect the overall magnitude of CM-H₂DCFDA-derived fluorescence from neutrophils as measured by area under the curve analysis (panel B, insulin or TZD effect, $P > 0.20$).

ediones and other PPAR- γ ligands. Importantly, these effects are thought to be mediated by the inhibition of the nuclear factor κ B, which during inflammation, enters the nucleus and activates the expression of several proinflammatory 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, an insulin sensitizer and PPAR- γ ligand 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, nuclear factor κ B, and I κ B proteins. These TZD anti-inflammatory effects

might be independent of metabolic alterations because administration of a lesser dose of a TZD to diabetic patients resulted in decreased inflammatory markers without changes in glucose homeostasis, plasma insulin, and FFA (Ghanim et al., 2006). Reactive oxygen species produced during neutrophil phagocytosis are essential to trigger apoptosis of neutrophils (Zhang et al., 2003). Thus, imbalances in PMNL intracellular ROS generation may reduce their viability. Importantly, the reduction of ROS generation by TZD observed in this study was not accompanied by an impaired ability of the neutrophils to release NET or engulf and kill bacteria, suggesting that TZD may reduce inflammation

Figure 7. Effect of physiological state on a proxy for the intracellular reactive oxygen species production by bovine neutrophil. Neutrophils were collected from pregnant midlactation (253 ± 25.2 d postpartum; $n = 9$), prepartum (-12 ± 1.7 d; $n = 8$), and postpartum ($+7 \pm 0$ d; $n = 7$) cows. Data represent the area under the curve (AUC; $\pm$ SEM) calculated from plotted 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA)-derived measurements over a 200-min period after activation of neutrophils with phorbol 12-myristate,13-acetate. The chemiluminescence (CL) values of nonactivated neutrophils were subtracted from all the CL measurements at every time point. CM-H₂DCFDA-derived fluorescence differed by physiological state, $P < 0.01$. ^{a,b}Bars with different letters differ ($P < 0.01$).

while leaving the essential antimicrobial capacity of neutrophils intact.

Excess ROS production can damage the host cell membrane by peroxidation of the components of the lipid bilayer (Fialkow et al., 2007) or ultimately promote injury to the surrounding tissue (Capuco et al.,

1986). It is conceivable that TZD has different effects on individual ROS produced by bovine PMNL. Neutrophils incubated with TZD had increased concentrations of extracellular O₂⁻ measured by MCLA-derived CL, yet displayed lesser generation of H₂O₂, OH⁻, ONOO⁻, and HClO. The reason for these inconsistent results

Figure 8. Effect of insulin (INS) and 2,4-thiazolidinedione (TZD) on neutrophil extracellular traps expression. Neutrophils were collected from cows ($n = 24$) and incubated for 120 min 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 a 1-h incubation of neutrophils with phorbol 12-myristate,13-acetate. The fluorescence values of nonactivated neutrophils were subtracted from all measurements.

Figure 9. Effect of physiological state on neutrophil extracellular traps expression. Neutrophils were collected from pregnant midlactation (253 ± 25.2 d postpartum; $n = 8$), prepartum (-12 ± 1.7 d; $n = 8$), and postpartum ($+7 \pm 0$ d; $n = 8$) cows. Data represent mean fluorescence ($\pm$ SEM) of cells stained with Sytox Orange (Invitrogen Co., Eugene, OR) after a 1-h incubation with phorbol 12-myristate,13-acetate. The fluorescence values of nonactivated neutrophils were subtracted from all measurements. Fluorescence differed by physiological state, $P < 0.01$. ^{a,b}Bars with different letters differ ($P < 0.01$).

is not obvious, but differential regulation of mitochondrial, cytosolic, and extracellular superoxide dismutase isozymes has been reported by others (Marklund, 1992; Miao and St. Clair, 2009). Furthermore, results from our luminol assay indicated that TZD inhibited the production of O_2^- , H_2O_2 , OH^- , $ONOO^-$, and $HClO$ by 89%. Although the specific identity or cellular location of the remaining 11% ROS is uncertain, extracellular O_2^- contributed to 41% of the ROS detected by luminol-dependent CL (Rinaldi et al., 2007). Even if we assume that TZD augments both intra- and extracellular O_2^- , this increase does not represent more than 4.5% of the total ROS because O_2^- should account for 41% of the 11% ROS that was noninhibited by TZD. In addition, O_2^- is relatively nonreactive with most biological substrates and has less oxidative potential compared with other ROS. In fact, O_2^- is between 100 and 1,000 times less toxic than other powerful oxidants produced by the PMNL such as $HOCl$ (Conner and Grisham, 1996). Therefore, the increase in extracellular O_2^- in response to TZD observed in our MCLA assay is less striking

relative to the large decrease in total ROS determined by luminol-derived CL.

In vivo administration of TZD to periparturient cows might enhance immune function directly by acting on PMNL as discussed above. 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). It is possible that TZD can improve the immunity of periparturient cows indirectly by improving glucose metabolism of leukocytes. For instance, in vivo administration of TZD restored the insulin-mediated glucose utilization of steers with insulin resistance induced by recombinant bovine tumor necrosis factor- α (Kushibiki et al., 2001). Although glucose uptake by PMNL is not insulin dependent (Maratou et al., 2007), TZD could enhance glucose metabolism by acting on intracellular events that regulate its utilization. In rats, administration of 15 mg/kg per d of rosiglitazone, a PPAR- γ agonist of the TZD family, enhanced inguinal fat glucose oxidation and its incorporation into glycogen by 112% and 85%, respectively, an effect accompanied by increased mRNA levels of phosphofructokinase, pyruvate dehydrogenase, lactate dehydrogenase, cytochrome oxidase, citrate synthase, and isocitrate dehydrogenase (Festuccia et al., 2009). Interestingly, TZD also seems to enhance glucose utilization in tissues in which glucose uptake is not dependent on insulin. For example, in vitro incubation of brain glial cells from rats with pioglitazone, also a member of the TZD subclass of antidiabetic drugs, increased glucose consumption and lactate production via the cAMP-protein kinase A pathway in a time- and dose-dependent manner (Dello Russo et al., 2003). In dairy cows, infusion of TZD during the last 25 d of pregnancy resulted in a 15% reduction of prepartum plasma NEFA concentration and a decrease of plasma BHBA concentration as parturition approached compared with control animals (Smith et al., 2007). Thus, TZD might contribute to mammary gland health by reducing the plasma concentration of NEFA and BHBA that have been shown to have negative effects on PMNL function (Hoeben et al., 1997; Scalia et al., 2006).

Table 2. Effect of insulin (INS) and 2,4-thiazolidinedione (TZD) on the bactericidal and phagocytic abilities of neutrophils against *Staphylococcus aureus*¹

Index	Treatment (ng/mL of INS + μ g/mL of TZD)					
	0 + 0	1.5 + 0	15 + 0	0 + 300	1.5 + 300	15 + 300
PMNL with phagocytosed bacteria (%)	27.3 $\pm$ 4	26.0 $\pm$ 4	25.5 $\pm$ 4	23.7 $\pm$ 4	24.1 $\pm$ 4	23.4 $\pm$ 4
Number of engulfed bacteria per PMNL	13.0 $\pm$ 1	13.4 $\pm$ 1	12.3 $\pm$ 1	12.6 $\pm$ 1	12.9 $\pm$ 1	12.4 $\pm$ 1
Killing ability (%)	23.3 $\pm$ 4	24.8 $\pm$ 4	23.7 $\pm$ 4	22.7 $\pm$ 4	21.7 $\pm$ 4	20.5 $\pm$ 4

¹Data are means $\pm$ SEM ($n = 24$).

Table 3. Effect of physiological state on the bactericidal and phagocytic abilities of neutrophils against *Staphylococcus aureus*¹

Index	Prepartum	Postpartum	Midlactation
PMNL with phagocytosed bacteria (%)	21.7 ± 2.8 ^a	40.2 ± 2.8 ^b	13.1 ± 2.8 ^a
Number of engulfed bacteria per PMNL	11.4 ± 0.6 ^a	15.4 ± 0.6 ^b	11.5 ± 0.6 ^a
Killing ability (%)	19.9 ± 2.7 ^a	32.1 ± 2.7 ^b	16.3 ± 2.6 ^a

^{a,b}Values in the same row with different superscripts differ ($P < 0.01$).

¹Data are means ± SEM (n = 8). Effect of physiological state, $P < 0.01$.

As with any in vitro system, it is impossible to completely duplicate the in vivo physiologic environment, and it is possible that in vitro conditions may have affected basal cellular metabolism or PMNL ability to respond to treatment. For example, the calcium-free environment of the HBSS incubation media increases the precision of the CL assays; however, it is possible that the low calcium environment could influence cellular metabolism. Doré et al. (1990) reported that the level of cytosolic Ca^{2+} , intracellular Ca^{2+} flux, and apparent activation of bovine PMNL incubated in Ca^{2+} free buffer media was less than when cells were incubated in the presence of extracellular Ca^{2+} . Experimentally induced hypocalcemia in periparturient cows had no effect on in vitro ROS generation, ingestion of *Staph. aureus*, and antibody-dependent cell-mediated cytotoxicity by PMNL, at least when these assays were performed in medium buffer containing physiological levels of Ca^{2+} (Kehrli and Goff, 1989). In contrast, Kimura et al. (2006) reported that the flux of intracellular ionized calcium in peripheral mononuclear cells stimulated with an anti-CD3-secondary antibody complex was reduced in periparturient cows diagnosed with milk fever compared with healthy animals. It is unknown whether the low Ca^{2+} concentration in our in vitro system may have attenuated PMNL activity similar to that reported by Doré et al. (1990); however, all assay activators utilized resulted in dramatic and sustained cellular responses, and several significant effects of treatment are reported, most of which are consistent with other peer-reviewed research.

The luminol-, CM- H_2DCFDA -, and MCLA-derived ROS production of blood neutrophils collected from prepartum and postpartum cows was reduced compared with that of PMNL isolated from midlactation animals. These results are in concordance with previous reports (Dosogne et al., 1999; Hoebe et al., 2000; Mehrzad et al., 2001, 2002) and 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. However, no difference in ROS production was detected between prepartum and postpartum cows. There are conflicting reports regarding the changes in ROS generation

between the prepartum and early lactation period. For instance, Hoebe et al. (2000) and Dosogne et al. (1999) reported a reduction in luminol-dependent CL beginning ~3 wk before parturition with minimum levels 1 wk after calving, but only the second group reported a significant decrease in PMA-induced CL of neutrophils between -1 and 1 wk after parturition. 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).

The PMNL from prepartum cows expressed greater levels of NET compared with mid- and early-lactation animals. Contrary to the oxygen-dependent killing mechanisms, NET may not experience a period of reduced functionality during the periparturient period. Lippolis et al. (2006b) reported that incubation of PMNL in milk, in contrast with other aspects of neutrophil function, did not reduce their ability to release NET, suggesting that when other PMNL killing mechanisms are impaired, neutrophils might rely on the antimicrobial activity of NET. Lippolis et al. (2006a) also performed a proteomic analysis of bovine PMNL collected during midlactation and 28 d before calving and reported that those from prepartum cows had greater levels of the histones H2B.e and H2B.f. Because these histones are structural components of NET (Brinkmann et al., 2004), their enhanced expression may contribute to the increased NET synthesis in prepartum versus midlactation cows observed in our study.

The phagocytic capacity assessed by the percentage of PMNL with engulfed *Staph. aureus* and the number of intracellular bacteria of neutrophils from early-lactation cows was increased compared with that of prepartum and midlactation animals. Increases in neutrophil phagocytic activity during the periparturient period have also been reported in several studies (Guidry et al., 1976; Kehrli et al., 1989; Detilleux et al., 1995; Dosogne et al., 1999). Detilleux et al. (1995) suggested that periparturient PMNL phagocytosis is increased because PMNL use the extra energy avail-

able for phagocytic processes in a period when the consumption of energy for production of ROS is reduced. 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 PMNL collected from midlactation, prepartum, and postpartum cows. This inconsistency in findings may be attributed to the different pathogens and neutrophil-to-pathogen ratios used in the in vitro cocultures. However, in contrast with other aspects of neutrophil function, none of these reports indicated 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 PMNL from early-lactation cows against *Staph. aureus* was greater than that of prepartum and midlactation animals. Studies that have measured the bactericidal activity of blood neutrophils during the periparturient period have reported a decrease (Daniel et al., 1991; Detilleux et al., 1995; Dosogne et al., 2001) or no change (Kehrli et al., 1989; Cai et al., 1994) in the killing capacity of PMNL during early lactation compared with prepartum and midlactation. Daniel et al. (1991) and Detilleux et al. (1995) also observed a transient increase in the killing capacity of PMNL during the last 4 wk before calving followed by a decrease during the first week of lactation. Interestingly, the decrease in PMNL cell-mediated cytotoxicity during the first week of lactation reported by Cai et al. (1994) was observed only in cows with retained placenta, mastitis, or metritis, whereas the cytotoxicity of PMNL from clinically normal cows did not change from weeks -1.5 to 4 relative to parturition. Furthermore, PMNL purified from milk did not experience a reduction in the ability to kill *Staph. aureus* during the periparturient period (Daniel et al., 1991; Detilleux et al., 1995), and it is possible that the increase in immature PMNL in blood that occurs around parturition (Guidry et al., 1976) might be responsible for the differences in bactericidal capacities between blood and milk PMNL populations. Perhaps, changes in the population of mature and juvenile PMNL in blood might help explain the discrepancy of results regarding the antimicrobial capacity of PMNL during the periparturient period. Differences in methodologies used to determine bactericidal killing could also justify the inconsistency of the findings. Detilleux (2004) applied mathematical models that described the changes in bacteria number during exposure to PMNL 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. The same author suggested that ratios outside these limits will result

in defective killing of bacteria. Cognizant of these results, we initially attempted a bacteria-to-PMNL ratio ranging from 32:1 down to 12:1; however, validation of the assay in our laboratory was not possible until a bacteria-to-PMNL ratio of 4:1 was achieved. Therefore, the conflicting results of our study may be attributed to the lower 4:1 bacteria-to-PMNL ratio that we used in our coculture in an effort to reduce the number of intracellular bacteria and improve counting precision during light microscopy. Although admittedly counterintuitive, this ratio may also be responsible for the overall lesser killing percentages obtained in our assays (21%) compared with the 57% reported by Rinaldi et al. (2006), who performed a killing assay under similar conditions, except for a greater 16:1 bacteria-to-neutrophil ratio. In addition, the lesser killing percentage obtained in our experiments may have resulted in an accumulation of phagocytosed bacteria within the cell that may have been responsible for our inability to accurately and precisely enumerate bacteria within PMNL when coincubated at greater ratios.

CONCLUSIONS

Our results suggest that insulin has no direct effect on bovine neutrophil function, but TZD increased the generation of extracellular 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 without diminishing antimicrobial activity. Furthermore, TZD did not reduce NET expression, a killing mechanism that may be particularly important to the health of the mammary gland. These direct effects of TZD on PMNL 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. As indicated by 3 assays to measure PMNL ROS production, the respiratory burst of PMNL is impaired in early lactation and may contribute to the state of immunosuppression. Interestingly, NET formation was not impaired postpartum and was actually increased in prepartum dairy cows.

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