Effect of *Cis*-Urocanic Acid on Bovine Neutrophil Generation of Reactive Oxygen Species

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ABSTRACT

Neutrophils play a fundamental role in the host innate immune response during mastitis and other bacterial-mediated diseases of cattle. One of the critical mechanisms by which neutrophils contribute to host innate immune defenses is through their ability to phagocytose and kill bacteria. The ability of neutrophils to kill bacteria is mediated through the generation of reactive oxygen species (ROS). However, the extracellular release of ROS can be deleterious to the host because ROS induce tissue injury. Thus, in diseases such as mastitis that are accompanied by the influx of neutrophils, the generation of large quantities of ROS may result in significant injury to the mammary epithelium. *cis*-Urocanic acid (*cis*-UCA), which is formed from the UV photoisomerization of the *trans* isoform found naturally in human and animal skin, is an immunosuppressive molecule with anti-inflammatory properties. Little is known about the effect of *cis*-UCA on neutrophils, although one report demonstrated that it inhibits human neutrophil respiratory burst activity. However, the nature of this inhibition remains unknown. Because of the potential therapeutic use that a molecule such as *cis*-UCA may have in blocking excessive respiratory burst activity that may be deleterious to the host, the ability of *cis*-UCA to inhibit bovine neutrophil production of ROS was studied. Further, because neutrophil generation of ROS is necessary for optimal neutrophil bactericidal activity, a response which is critical for the host innate immune defense against infection, the effects of *cis*-UCA on bovine neutrophil phagocytosis and bacterial killing were assayed. *cis*-Urocanic acid dose-dependently inhibited the respiratory burst activity of bovine neutrophils as measured by luminol chemiluminescence. Subsequently, the effect of *cis*-UCA on the production of specific oxygen radicals was investigated using more selective assays. Using 2 distinct assays, we established that *cis*-UCA inhibited the gen-

eration of extracellular superoxide. In contrast, *cis*-UCA had no effect on the generation of intracellular levels of superoxide or other ROS. At concentrations that inhibited generation of extracellular superoxide, bovine neutrophil phagocytosis and bacterial activity remained intact. Together, these data suggest that *cis*-UCA inhibits the tissue-damaging generation of extracellular ROS while preserving neutrophil bactericidal activity.

Key words: dairy cow, neutrophil, reactive oxygen species, respiratory burst

INTRODUCTION

Polymorphonuclear neutrophils (PMN) play an essential role in defending the host against invading microbial pathogens. Defects in PMN recruitment to the site of infection (Schalm et al., 1976; Nagahata, 2004) or reduced PMN functioning (Roth and Kaeblerle, 1981; Cai et al., 1994; Tkalecic et al., 2000) or both are associated with impaired host clearance of bacteria. Recruitment of PMN to the site of infection is mediated by chemokines, complement components, and arachidonate metabolites, and the rapidity with which PMN are recruited influences the outcome of infection (Burvenich et al., 2003; Paape et al., 2003). Once at the site of infection, activated PMN recognize, engulf, and kill bacterial pathogens, the latter of which is dependent upon the production of microbicidal peptides, proteases, and reactive oxygen species (ROS; Gudmundsson and Agerberth, 1999; Burg and Pillinger, 2001).

The generation of ROS, also referred to as respiratory or oxidative burst activity, is a characteristic property of phagocytes such as the PMN. Oxidative burst activity plays a critical role in PMN-mediated defenses against bacteria, because defects in the ability to generate ROS are associated with increased susceptibility to infection (Baehner, 1990; Dinanuer, 1993). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a membrane-associated, multicomponent protein enzyme complex that participates directly in the generation of ROS (Hampton et al., 1998; Babior, 1999). In unstimulated PMN, this enzyme complex remains dormant.

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Upon activation, the enzyme complex triggers the shuttling of electrons from cytosolic NADPH to oxygen present in the phagosomal compartment or in the extracellular milieu. In general, one molecule of oxygen acts as an acceptor for a single donated electron, leading to the generation of superoxide anion ($O_2^{\cdot-}$) (Weiss, 1989). Superoxide anion is the most proximally generated ROS by NADPH oxidase, and, in turn, serves as a precursor to the formation of other powerful oxidants (Weiss, 1989; Hampton et al., 1998). Superoxide anions can rapidly form hydrogen peroxide (H_2O_2) by spontaneous dismutation or enzymatic dismutation by superoxide dismutase. The interaction of superoxide anion and hydrogen peroxide in the presence of a transition metal catalyst can give rise to the formation of hydroxyl radical ($OH^{\cdot}$), a powerful oxidant. Myeloperoxidase (**MPO**), a major component of the azurophilic granules, catalyzes the transformation of hydrogen peroxide in the presence of halogens (e.g., Cl^-) into highly toxic molecules such as hypochlorous acid ($HOCl$). Hypochlorous acid can, in turn, react with hydrogen peroxide or amines to form singlet oxygen (1O_2) and chloramines, respectively. There is some controversy regarding the actual direct bactericidal activity of the individual ROS in vivo (Hampton et al., 1998; Segal, 2005); however, it is clear that NADPH oxidase-dependent generation of superoxide anion initiates a cascade resulting in the production of an array of ROS that mediate PMN bactericidal activity.

Although the ability to generate ROS is essential for optimal PMN bactericidal activity, ROS do not discriminate against pathogens and host tissue and induce injury to both. Increasing evidence suggests that ROS comprise some of the most injurious substances released from cells and that they exert their deleterious effect through a variety of mechanisms, including lipid peroxidation and the modification of DNA, which can include the induction of strand breaks (Henson and Johnston, 1987; Weiss, 1989; Hampton et al., 1998). In addition, there is evidence that the oxidizing environment created by the generation of ROS can enhance PMN-derived protease activity, the latter of which is also injurious to host tissues. In diseases such as mastitis, where PMN concentrations approach 50 million cells/mL of milk following IMI (Bannerman et al., 2004), the potential for tissue damage is great. In fact, several reports suggest that activated PMN induce direct injury to the mammary epithelium both in vitro and in vivo (Capuco et al., 1986; Ledbetter et al., 2001; Long et al., 2001; Burvenich et al., 2004; Lauzon et al., 2005). The increasing awareness of the extent to which tissue injury is induced by activated PMN continues to generate interest in the development of therapeutics that can limit extracellular generation of ROS without im-

pairing PMN microbicidal activity against phagocytosed bacteria.

Ultraviolet radiation is well established in a variety of model systems to exert both a local and systemic immunosuppressive effect (Norval, 2001). Urocanic acid, which is naturally present in human and animal skin in the stratum corneum of the epidermis, is a major cutaneous absorber of UV radiation (Norval and El-Ghorr, 2002). Urocanic acid is formed as a *trans* isomer from histidine and, upon UV exposure, is photoisomerized to *cis*-urocanic acid (**cis-UCA**). *cis*-Urocanic acid exerts an array of immunomodulatory properties both in vitro and in vivo and is believed to be partially responsible for the immunosuppressive effects of UV radiation. Evidence demonstrating an immunosuppressive role for *cis*-UCA include: 1) intradermal administration of *cis*-UCA inhibits the delayed-type hypersensitivity reaction to herpes simplex virus type 1 (Ross et al., 1986) and tumor antigens (Beissert et al., 2001); 2) *cis*-UCA suppresses the induction and elicitation of contact hypersensitivity (Lauerma et al., 1995; Hart et al., 1997; Reeve et al., 1998), possibly through the modulation of dermal mast cells (Wille et al., 1999) or epidermal Langerhans cells (Kurimoto and Streilein, 1992); 3) *cis*-UCA inhibits the proliferation and antigen-presenting activity of T cells from the spleen, and this inhibition correlates with IL-10 upregulation (Holan et al., 1998); and 4) allograft rejection and graft-vs.-host reactions are suppressed following *cis*-UCA administration (Gruner et al., 1992; Guymer and Mandel, 1993; Filipec et al., 1998). In addition to its suppressive effect on cell-mediated immunity associated with adaptive immune responses, *cis*-UCA also impairs cell-mediated innate immune responses. *cis*-Urocanic acid is reported to impair natural killer cell activity (Gilmour et al., 1993), monocyte production of TNF- α (Hart et al., 1993), and human PMN respiratory burst activity (Kivisto et al., 1996). Although there is a large body of evidence that *cis*-UCA suppresses cell-mediated innate and adaptive immune responses, whether this molecule acts directly to inhibit these immune or inflammatory responses or induces the activation of another immunosuppressive molecule remains unclear.

Based on the diverse immunosuppressive effects that *cis*-UCA has on different cells of the immune system, this molecule may be an attractive therapeutic agent for diminishing excessive inflammation that can be deleterious to the host. Because activated PMN are known to induce tissue injury, at least in part through ROS generation (Weiss, 1989), and *cis*-UCA has been demonstrated in one report to inhibit the respiratory burst activity of human PMN (Kivisto et al., 1996), the influence of this molecule on bovine PMN generation of ROS was assessed. In addition, because the actual mecha-

nism of *cis*-UCA-mediated inhibition of respiratory burst activity has not been reported for any cell type, selective assaying of the effect of *cis*-UCA on distinct ROS was performed. Finally, because intracellular killing of bacteria by PMN is essential for host control of infection, the effect of *cis*-UCA on PMN phagocytosis and intracellular killing were evaluated.

MATERIALS AND METHODS

Reagents

cis-Urocanic acid (BioCis Pharma, Ltd., Turku, Finland) was prepared as a 37.5 mM stock solution in Hanks' balanced salt solution (HBSS) or PBS at pH 6.5. Hanks' balanced salt solution and PBS, prepared at a pH 6.5 without *cis*-UCA, were used as vehicle controls in the various in vitro assays. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St. Louis, MO) was prepared as a 1 M stock solution in dimethyl sulfoxide (DMSO). Phorbol 12-myristate,13-acetate (PMA; Sigma Chemical Co.), dihydroethidium (DHE; Molecular Probes, Inc., Eugene, OR), and diphenyleneiodonium chloride (DPI; Calbiochem-Novabiochem Corp., San Diego, CA) were prepared as 10 mM stock solutions in DMSO. Methyl cypridina luciferin analog [MCLA; 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-a]pyrazin-3-one hydrochloride] and 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA; both from Molecular Probes, Inc.) were prepared as 1 mM stock solutions in DMSO. Cytochrome c from bovine heart and superoxide dismutase (SOD) from bovine erythrocytes (both from Sigma Chemical Co.) were prepared as 1 mM and 1,000 units/mL of stock solutions, respectively, in calcium- and magnesium-free HBSS (CMF-HBSS).

Cows

Clinically healthy lactating Holstein cows from the USDA-ARS Beltsville dairy herd were used as blood donors for all experiments. The use and care of all animals in this study were approved by the Beltsville Agricultural Research Center's Animal Care and Use Committee.

Isolation of Bovine Blood PMN

Blood was obtained from the tail veins of healthy lactating Holstein cows, collected into Vacutainer glass tubes containing acid-citrate-dextrose (Becton Dickinson Corp, Franklin, Lakes, NJ), inverted $\times 5$, and stored on ice. Polymorphonuclear neutrophils were isolated using a Percoll gradient as previously described (Weber et al., 2001). Briefly, 20 mL of blood was transferred

to 50-mL polypropylene conical tubes and centrifuged ($1,000 \times g$) for 20 min at 4°C. The plasma and buffy coat were aseptically aspirated and discarded. The remaining cells were suspended in 34 mL of ice-cold PBS and the suspension was slowly pipetted down the side of a clean 50-mL polypropylene conical tube containing 10 mL of 1.084 g/mL of Percoll (Sigma Chemical Co.). The tubes were centrifuged ($400 \times g$) for 40 min at 22°C. The supernatant, mononuclear cell layer, and Percoll were aseptically aspirated and a pellet composed of PMN and erythrocytes was retained. Erythrocytes were lysed by mixing 1 vol of cells with 2 vol of an ice-cold 0.2% NaCl solution and inverting the tube for 1 min. Tonicity was restored by the addition of 0.5 vol of a 3.7% NaCl solution. The tubes were centrifuged ($500 \times g$) for 2 min at 4°C. The cell pellet was washed twice by resuspension in CMF-HBSS and recentrifugation for 1 min at 4°C. Cells were enumerated using an electronic particle counter (Coulter Electronics, Inc., Hialeah, FL). Cell viability and differential cell counts were determined by trypan blue (Sigma Chemical Co.) exclusion and Wright staining, respectively. Purity of PMN was >90% and viability >95%. Cells suspended in either CMF-HBSS or PBS were maintained on ice until used in the various assays described below.

Luminol Chemiluminescence Assay

Polymorphonuclear neutrophils (2×10^5) suspended in 10 μ L of CMF-HBSS were incubated with 160 μ L of either HBSS or increasing concentrations of *cis*-UCA for 10 min at 37°C. Ten microliters of luminol (10 mM) and 20 μ L of PMA (400 nM) were added and chemiluminescence (CL) was measured every 5 min with a Veritas microplate luminometer (Turner Biosystems Inc., Sunnyvale, CA). Background values, defined as the mean CL values of unactivated PMN, were subtracted from all readings.

Caspase Activity Assay

Caspase activity was measured using a fluorimetric homogeneous caspase activity assay kit (Roche Diagnostics Corp., Indianapolis, IN). Polymorphonuclear neutrophils (4×10^5) suspended in 50 μ L of RPMI supplemented with 10% heat-inactivated (56°C for 30 min) newborn bovine calf serum (Cambrex Bio Science, Inc, Walkersville, MD) were incubated with 27 μ L of either PBS or *cis*-UCA (37.5 mM) and 10 μ L of either HBSS or PMA (400 nM). All reaction volumes were adjusted to 100 μ L with PBS. Fluorescence activity was measured after 0, 1, 2, 4, and 8 h on a Synergy HT multimodal plate reader (BioTek Instruments, Inc., Winooski, VT) at an excitation wavelength of 485 nm and an emission

wavelength of 528 nm. Plates were maintained at 37°C between readings.

Trypan Blue Exclusion Assay

Polymorphonuclear neutrophils (4×10^5) suspended in 50 μ L of RPMI supplemented with 10% heat-inactivated newborn bovine calf serum were incubated with 27 μ L of either PBS or *cis*-UCA (37.5 mM) and 10 μ L of either HBSS or PMA (400 nM). All reaction volumes were adjusted to 100 μ L with PBS. After various incubation times at 37°C, PMN viability was determined by adding 50 μ L of each sample to 50 μ L of trypan blue and scoring the first 100 cells encountered in the field of view of a light microscope as either alive or dead based on the uptake of trypan blue.

MCLA CL Assay

Polymorphonuclear neutrophils (4×10^5) suspended in 7.5 μ L of CMF-HBSS were incubated with 120 μ L of either HBSS or increasing concentrations of *cis*-UCA for 10 min at 37°C. A 7.5- μ L quantity of MCLA (2 μ M) and a 15- μ L quantity of PMA (400 nM) were added to the cells and CL was measured every 5 min with a microplate luminometer. The fold increase in superoxide production was determined by calculating the ratio of the arbitrary CL values of PMA-activated PMN to the CL values of unactivated PMN.

Cytochrome c Reduction Assay

Polymorphonuclear neutrophils (2×10^5) suspended in 20 μ L of CMF-HBSS were incubated with 160 μ L of either HBSS or increasing concentrations of *cis*-UCA for 10 min at 37°C. A 10- μ L quantity of the 1-mM stock solution of cytochrome c and a 10- μ L quantity of PMA (1 mM) were added to the cells and absorbance was measured on a plate reader (BioTek Instruments, Inc.). Optical density (OD) was measured at 10-min intervals at a wavelength of 550 nm. A background correction reading at 670 nm was subtracted from the 550-nm absorbance readings. Superoxide production was calculated as previously described (Sartorelli et al., 2000) using the following equation: superoxide (nmol) = $[(\text{OD}_{550} - \text{reference OD}_{670}) \times 100]/6.3$. Background values, defined as the mean amount of superoxide (nmol) produced by unactivated PMN, were subtracted from all values. To confirm the specificity of the assay to detect superoxide production, parallel reactions were run that included the addition of 2 μ L of SOD (1,000 units/mL) to wells containing PMA-stimulated PMN.

DHE Flow Cytometric Assay

Polymorphonuclear neutrophils (1×10^6) suspended in 500 μ L of CMF-HBSS were incubated with 100 μ L of DHE (200 μ M) and either 160 μ L of HBSS or *cis*-UCA (37.5 mM). All reaction volumes were adjusted to 1.8 mL with HBSS. As a positive control, reactions substituting the 160 μ L of HBSS with an equivalent volume of DPI (100 μ M), the latter of which inhibits the oxidation of DHE (Katsuyama et al., 2002), were run in parallel. Parallel reactions were also set up that included the addition of 20 μ L of SOD (1,000 units/mL) to PMA-stimulated PMN. Following a 15-min incubation at 37°C in a shaking (30 rpm) water bath, 200 μ L of PMA (400 nM) was added to the cells and the incubation was allowed to proceed. At 15 and 45 min after the addition of PMA, fluorescence was measured with a flow cytometer (Coulter Electronics, Inc.) equipped with an air-cooled argon ion laser. The laser was set at a 488-nm wavelength, 7.0 to 7.5 A of current, and 15 mW of power, and aligned using fluorospheres (Coulter fullbrite grade 2, 9.56 μ m diameter; Epics Division of Coulter Corp., Hialeah, FL). A 400- μ L quantity of 1% methylene blue was added to each sample immediately prior to assaying on the flow cytometer to quench extracellular fluorescence (Jain et al., 1991). A relative fluorescence index was calculated for each sample by multiplying the percentage of cells fluorescing by the mean channel fluorescence and dividing the resulting product by 100 (Salgar et al., 1991).

CM-H₂DCFDA Fluorescence Assay

Polymorphonuclear neutrophils (4×10^5) suspended in 7.5 μ L of CMF-HBSS were incubated with 120 μ L of either HBSS or increasing concentrations of *cis*-UCA for 10 min at 37°C. As a positive control, reactions substituting the 120 μ L of HBSS with an equivalent volume of DPI (10 μ M), the latter of which inhibits NADPH oxidase and corresponding generation of ROS detected with CM-H₂DCFDA (Chandel et al., 1998; Andersen et al., 2003; Jackson et al., 2004), were run in parallel. A 7.5- μ L quantity of CM-H₂DCFDA (200 μ M) and 15 μ L of PMA (400 nM) were added to the cells and fluorescence was measured every 15 min on a fluorescent plate reader (BioTek Instruments, Inc.) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm as previously described (Wang and Joseph, 1999). Background values, defined as the mean fluorescent values of unactivated PMN, were subtracted from all readings.

PMN Phagocytosis and Killing

Staphylococcus aureus strain 305 (American Type Culture Collection, Manassas, VA) was inoculated on a

blood agar plate (Becton Dickinson Diagnostic Systems, Inc., Sparks, MD) and incubated overnight at 37°C. Ten colonies were transferred from the plate to 10 mL of brain–heart infusion broth (Becton Dickinson Diagnostic Systems, Inc.) and incubated overnight at 37°C at 225 rpm. The tube was subsequently placed in an ice-water bath and mixed by swirling. A 0.1-mL aliquot from the tube was serially diluted in PBS and 0.1 mL quantities of the resulting dilutions were spread on blood agar plates. The plates were incubated overnight at 37°C and the stock culture was maintained at 4°C. After determining the concentration (cfu/mL) of the stock culture based on the colony counts of the spread plates, the stock culture was diluted in PBS to yield a final concentration of 1.55×10^8 cfu/mL.

To assess PMN phagocytosis and killing, 200 μ L of *Staph. aureus* (3.1×10^7 cfu) was added to tubes with or without 1×10^6 PMN suspended in 1 mL of PBS, 400 μ L of pooled ($n = 5$ cows), heat-inactivated bovine serum, and 160 μ L of either *cis*-UCA (37.5 mM), DPI (100 μ M), or HBSS. The ratio of bacteria to PMN was 31:1. The reaction volume was adjusted to 2 mL with PBS and the samples were placed on an orbital shaker for 60 min at 39°C. All reactions were set up in duplicate.

To determine the percentage of PMN containing phagocytosed bacteria and the actual number of phagocytosed bacteria per PMN, Wright-stained cytospin centrifuge slides were prepared using a 50- μ L aliquot of each reaction (Dulin et al., 1982). 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 *cis*-UCA affected the bactericidal activity of PMN, 1.9 mL of the remaining reaction were sonicated with a Virsonic disrupter (Virtis Co., Gardiner, NY) at a power setting of 35 for 60 s (Paape and Guidry, 1977). Rupture of the PMN was verified by microscopic examination. Sonication had no effect on bacterial viability (data not shown). A 0.1-mL aliquot from each sonicated reaction was serially diluted in PBS and 0.1 mL quantities of the resulting dilutions were spread on blood agar plates. The plates were incubated for 18 h 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 PMN and dividing this difference by the number of bacteria incubated in the absence of PMN.

Statistical Methods

For luminol-, MCLA-, cytochrome *c*-, and CM-H₂DCFDA-based assays involving multiple measure-

ments of CL, absorbance, or fluorescence over time, the area under the curve was calculated from plotted data points for each experimental condition using GraphPad Prism, version 4.00 for Windows (GraphPad Software, Inc., San Diego, CA). For all in vitro assays, a one-way ANOVA was used to compare the mean responses between experimental groups and activated PMN (i.e., PMN incubated with PMA alone). The Tukey post hoc comparison test was used to determine between which groups significant differences existed. All statistical analyses were performed using GraphPad Prism software. A *P*-value of <0.05 was considered significant.

RESULTS

cis-UCA Inhibits Bovine PMN Respiratory Burst Activity

Luminol-derived CL, which is elicited in response to the generation of an array of intracellular and extracellular ROS, including superoxide anion, hydroxyl radicals, hydrogen peroxide, peroxynitrite, and hypochlorous acid (Saez et al., 2000; Munzel et al., 2002), was used to assess the effect of *cis*-UCA on PMN-induced respiratory burst (Figure 1). Increases in luminol-dependent CL were evident within 45 min of stimulation with PMA and continued to increase until reaching a plateau 60 min later (Figure 1A). *cis*-Urocanic acid dose-dependently inhibited the luminol-dependent CL of PMA-stimulated PMN. Overall peak luminescence for each experimental condition was calculated as the area under the respective curve for the last 30 min of CL measurements (Figure 1B). Concentrations as low as 30 μ M *cis*-UCA partially blocked PMN generation of ROS by ~23%. Intermediate concentrations of *cis*-UCA (0.3 to 1 mM) inhibited ~63% of this response, whereas the highest concentration tested (10 mM) completely abrogated luminol-dependent CL.

The Onset of Spontaneous Bovine PMN Apoptosis and Injury Is Not Influenced by *cis*-UCA

To determine whether the ability of *cis*-UCA to inhibit respiratory burst activity in PMN could be attributed to injury or cell death or both that would nonspecifically impair PMN functioning, including respiratory burst activity, the effect of *cis*-UCA on PMN apoptosis and viability was assayed (Figure 2). Changes in caspase activity were used to assess whether *cis*-UCA influenced the onset of apoptosis in unstimulated and PMA-stimulated PMN apoptosis (Figure 2A). Within 4 h of incubation, unstimulated and PMA-stimulated PMN demonstrated increased caspase activity relative to freshly isolated PMN exposed to HBSS or PMA and immediately assayed for caspase activity (time 0). Fur-

Figure 1. Effect of *cis*-urocanic acid (*cis*-UCA) on bovine neutrophil oxidative burst activity. Blood neutrophils isolated from 5 cows were exposed to increasing concentrations of *cis*-UCA for 10 min. Neutrophils were subsequently stimulated with phorbol 12-myristate,13-acetate (PMA, 40 nM) in the presence of luminol, and chemiluminescence values were measured every 5 min. Background values, defined as the chemiluminescence of unstimulated neutrophils incubated with luminol, were subtracted from all readings, and the difference is reported as the mean ($\pm$ SE) chemiluminescence in arbitrary units (A). To analyze the effect of *cis*-UCA on peak generation of reactive oxygen species, the area under the curve was calculated from plotted data points for each experimental condition for the last 30 min of chemiluminescence measurements (B). ***Decreased ($P < 0.05$ and $P < 0.01$, respectively) relative to neutrophils stimulated with PMA in the absence of *cis*-UCA.

ther increases in caspase activity were evident after 8 h of incubation. Regardless of the time point, comparable levels of caspase activity were observed among activated and unactivated PMN regardless of exposure to *cis*-UCA (10 mM).

To evaluate the ability of *cis*-UCA to induce cell injury, PMN treated in parallel as above were assessed for their ability to exclude trypan blue (Figure 2B). In contrast to the early onset of apoptosis, loss of membrane integrity as assessed by trypan blue uptake was not observed until 24 h after incubation. The percentage

Figure 2. Effect of *cis*-urocanic acid (*cis*-UCA) on bovine neutrophil apoptosis and viability. Blood polymorphonuclear neutrophils (PMN) isolated from 5 cows were stimulated with phorbol 12-myristate,13-acetate (+PMA, 40 nM) in the presence or absence of 10 mM *cis*-UCA. Parallel studies were also performed with unstimulated (-PMA) neutrophils. Neutrophils were either immediately (time 0) assayed for caspase activity (A) and trypan blue exclusion (B) or incubated at 37°C for increasing time intervals and subsequently assayed. Caspase activity is reported in mean ($\pm$ SE) arbitrary fluorescence units (A) and viability is reported as the mean ($\pm$ SE) percentage of neutrophils that excluded trypan blue (B). ***Different ($P < 0.05$ and $P < 0.01$, respectively) relative to time 0 measurements of neutrophils exposed to identical conditions.

of injured cells at this time (~31 to 43%) was comparable regardless of whether PMN were activated with PMA or exposed to *cis*-UCA or both.

cis-UCA Inhibits Bovine PMN Generation of Extracellular Superoxide Anion

The MCLA-derived CL, which is a specific indicator of the generation of extracellular superoxide anion (Skatchkov et al., 1998; Munzel et al., 2002), was used

Figure 3. Effect of *cis*-urocanic acid (*cis*-UCA) on bovine neutrophil generation of extracellular superoxide as measured by 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one hydrochloride (MCLA)-dependent chemiluminescence. Blood neutrophils isolated from 11 cows were exposed to increasing concentrations of *cis*-UCA for 10 min. Neutrophils were subsequently stimulated with phorbol 12-myristate,13-acetate (PMA, 40 nM) in the presence of MCLA, and chemiluminescence values were measured every 5 min. The mean ($\pm$ SE) fold increase in chemiluminescence relative to unstimulated neutrophils incubated with MCLA is shown (A). To analyze the effect of *cis*-UCA on peak generation of extracellular superoxide, the area under the curve was calculated from plotted data points for each experimental condition for the first 3 measurements (0 to 10 min). **Decreased ($P < 0.01$) relative to neutrophils stimulated with PMA in the absence of *cis*-UCA.

to determine whether *cis*-UCA could inhibit generation of PMN extracellular superoxide (Figure 3). Increases in MCLA-dependent CL peaked within 5 min of stimulation with PMA and rapidly decreased thereafter (Figure 3A). *cis*-Urocanic acid dose-dependently inhibited the MCLA-dependent CL of PMA-stimulated PMN. Overall peak luminescence for each experimental condition, calculated as the area under each respective curve

during the first 10 min of activation, demonstrated that concentrations of *cis*-UCA as low as 10 μ M could inhibit extracellular superoxide generation (Figure 3B). The highest concentration of *cis*-UCA (1 mM) assayed inhibited ~48% of the PMA-induced MCLA CL.

To confirm the ability of *cis*-UCA to inhibit extracellular superoxide, reduction of cytochrome *c*, which occurs in response to the generation of extracellular superoxide anion (Munzel et al., 2002; Tarpey et al., 2004), was also assayed (Figure 4). When using this assay, increases in superoxide anion production were evident within 10 min of PMA stimulation and reached a maximum 10 min later (Figure 4A). *cis*-Urocanic acid inhibited extracellular superoxide anion production of PMA-stimulated PMN as assessed by cytochrome *c* reduction. Overall superoxide generation for each experimental condition was calculated as the area under each respective curve (Figure 4B). Similar to findings in the MCLA-based assay, the lowest concentration of *cis*-UCA that could inhibit PMA-induced extracellular superoxide generation was 10 μ M. Concentrations of *cis*-UCA ranging from 10 μ M to 10 mM inhibited PMA-stimulated PMN generation of superoxide anion by ~40 to 50%. To confirm the specificity of this assay to detect extracellular superoxide, PMA-stimulated PMN were also incubated with SOD (10 units/mL) and cytochrome *c* reduction was assessed. Superoxide dismutase reduced extracellular levels of superoxide generated from PMA-stimulated PMN by ~90%.

***cis*-UCA Does Not Affect Bovine PMN Generation of Intracellular Superoxide, Peroxynitrite, and Hydrogen Peroxide**

Dihydroethidium oxidation, which is specifically elicited in response to the generation of intracellular superoxide anion (Munzel et al., 2002; Walrand et al., 2003; Tarpey et al., 2004), was used to assess the effect of *cis*-UCA on PMN generation of intracellular superoxide (Figure 5). Increased oxidation of DHE, as indicated by increasing fluorescence, was evident within 15 min of stimulation with PMA and remained unchanged 30 min later. Incubation with *cis*-UCA (3 mM) or SOD (10 units/mL) had no effect on PMA-induced generation of intracellular superoxide. In contrast, the cell-permeable NADPH oxidase inhibitor, DPI (8 μ M), completely abrogated the PMA-stimulated PMN generation of intracellular superoxide, as reflected by the absence of DHE oxidation and corresponding generation of fluorescence.

Because *cis*-UCA had no apparent effect on the generation of intracellular superoxide, the oxidation of CM-H₂DCFDA, which is elicited in response to the generation of intracellular peroxynitrite anion and hydrogen

Figure 4. Effect of *cis*-urocanic acid (*cis*-UCA) on bovine neutrophil generation of extracellular superoxide as measured by cytochrome *c* reduction. Blood neutrophils isolated from 5 cows were exposed to increasing concentrations of *cis*-UCA for 10 min. Neutrophils were subsequently stimulated with phorbol 12-myristate,13-acetate (PMA, 50 μ M) in the presence of cytochrome *c*, absorbance values were measured, and the amount of superoxide anion production calculated. To demonstrate specificity of the assay, parallel reactions were set up in which PMA-activated neutrophils were incubated with superoxide dismutase (SOD, 10 U/mL). Background values, defined as the mean amount of superoxide (nmol) produced by unactivated neutrophils, were subtracted from all experimental conditions and the difference is reported as the mean ($\pm$ SE) superoxide production in nmol units (A). To analyze the effect of *cis*-UCA on the generation of superoxide, the area under the curve was calculated from plotted data points for each experimental condition (B). ***Decreased ($P < 0.05$ and $P < 0.01$, respectively) relative to neutrophils stimulated with PMA in the absence of *cis*-UCA.

peroxide (Rothe and Valet, 1990; Crow, 1997), was used to assess whether *cis*-UCA could affect the generation of other intracellular ROS (Figure 6). Increases in CM-H₂DCFDA oxidation were evident within 30 min of stimulation with PMA and continued to increase up to 45 min later (Figure 6A). *cis*-Urocanic acid had no apparent effect on PMA-stimulated PMN generation of

Figure 5. Effect of *cis*-urocanic acid (*cis*-UCA) on bovine neutrophil generation of intracellular superoxide. Blood neutrophils isolated from 5 cows were exposed to *cis*-UCA (3 mM), superoxide dismutase (SOD; 10 U/mL), or diphenyleneiodonium chloride (DPI, 8 μ M) for 15 min. Neutrophils were subsequently stimulated with phorbol 12-myristate,13-acetate (PMA, 40 nM) in the presence of dihydroethidium and the numbers of cells fluorescing and the intensity of fluorescence were recorded 15 and 45 min later on a flow cytometer. The mean ($\pm$ SE) relative fluorescence index values for each experimental group are shown. ***Decreased ($P < 0.05$ and $P < 0.01$, respectively) relative to neutrophils stimulated with PMA in the absence of any inhibitor.

intracellular peroxynitrite anion and hydrogen peroxide, whereas the NADPH oxidase inhibitor DPI partially blocked this response. Overall oxidation of CM-H₂DCFDA for each experimental condition was calculated as the area under each respective curve (Figure 6B). All concentrations of *cis*-UCA tested (10 μ M to 1 mM) had no significant effect on PMN oxidation of intracellular CM-H₂DCFDA, whereas DPI (8 μ M) inhibited oxidation by ~66%.

DPI, but Not *cis*-UCA, Inhibits PMN-Mediated Killing of *Staph. aureus*

To determine whether the ability of *cis*-UCA to selectively block extracellular generation of ROS could interfere with PMN functions required for effective host responses to infection, the influence of *cis*-UCA on PMN phagocytosis and bacterial killing was assessed (Table 1). Polymorphonuclear neutrophils treated with or without *cis*-UCA (3 mM) or DPI (8 μ M) were incubated with *Staph. aureus* for 60 min. The percentage of PMN containing phagocytosed bacteria and the number of phagocytosed bacteria within each PMN were subse-

Figure 6. Effect of *cis*-urocanic acid (*cis*-UCA) on bovine neutrophil generation of intracellular peroxynitrite and hydrogen peroxide. Blood neutrophils isolated from 5 cows were exposed to increasing concentrations of *cis*-UCA or a fixed concentration of diphenyleneiodonium chloride (8 μ M) for 10 min. Neutrophils were subsequently stimulated with phorbol 12-myristate,13-acetate (PMA, 40 nM) in the presence of 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester, and fluorescence values were measured every 15 min. Background values, defined as the mean fluorescent values of unactivated neutrophils, were subtracted from all readings and the difference is reported as the mean ($\pm$ SE) fluorescence in arbitrary units (A). To analyze the effect of *cis*-UCA on generation of reactive oxygen species, the area under the curve was calculated from plotted data points for each experimental condition (B). **Decreased ($P < 0.01$) relative to neutrophils stimulated with PMA in the absence of inhibitor.

quently enumerated. Equivalent percentages of PMN that phagocytosed *Staph. aureus* and equivalent numbers of bacteria phagocytosed were observed regardless of treatment. Relative to untreated (control) PMN, those exposed to *cis*-UCA demonstrated an equivalent capability to kill *Staph. aureus*. In contrast, PMN incubated with DPI demonstrated a ~50% impairment in

the ability to kill *Staph. aureus* relative to control or *cis*-UCA treated PMN.

DISCUSSION

The current study investigated the inhibitory effect of *cis*-UCA on bovine PMN respiratory burst activity. Consistent with a previous study of human PMN (Kivisto et al., 1996), *cis*-UCA inhibited luminol-dependent respiratory burst activity in a dose-dependent manner (Figure 1). Whereas the lowest reported concentration of *cis*-UCA to partially inhibit human PMN respiratory burst activity was 700 μ M, concentrations as low as 30 μ M were able to block the same response in bovine PMN to a similar extent (i.e., ~23% inhibition). At the highest concentration assayed (10 mM), *cis*-UCA completely abrogated bovine PMN respiratory burst activity. The differences in minimum effective inhibitory concentrations between the 2 studies may be due to differential species-dependent sensitivity to *cis*-UCA. Alternatively, because the 2 studies used different agonists to induce respiratory burst activity, namely, zymosan and PMA, the differences in MIC may be due to the differential activation elicited by these agonists.

Within 6 to 12 h of isolation, PMN begin to undergo spontaneous apoptosis (Savill et al., 1989). Correspondingly, within 4 h of in vitro incubation following the 2 to 3 h PMN isolation procedure, bovine PMN demonstrated an increase in caspase activity that reflected the onset of apoptosis (Figure 2A). Because all the functional assays investigating PMN responses were conducted within 2 h of PMN isolation, the onset of spontaneous apoptosis did not present a major concern. However, the possibility that the *cis*-UCA-mediated decrease in luminol-dependent CL was due to enhancement of PMN apoptotic death by *cis*-UCA needed to be ruled out. As shown in Figure 2A, the onset and level of spontaneous apoptosis were unaffected in PMN exposed to the highest concentration of *cis*-UCA (10 mM) tested in any assay of ROS production. Disruption of membrane integrity, which was assessed by trypan blue exclusion and is an indicator of cell injury, was not observed until 24 h after in vitro incubation of untreated PMN (Figure 2B). Similar to apoptosis, *cis*-UCA had no effect on the percentage of cells that lost membrane barrier function or on the time at which the onset of this process occurred relative to PMN not exposed to *cis*-UCA. Together, these data suggest that the *cis*-UCA-mediated inhibition of respiratory burst activity cannot be ascribed to nonspecific impairment of PMN functioning due to *cis*-UCA-induced injury or apoptotic cell death or both.

Luminol is a cell-permeable compound; thus, CL generated by this molecule reflects the production of both

Table 1. Effect of *cis*-urocanic acid (*cis*-UCA, 3 mM) and diphenyleneiodonium chloride (DPI, 8 μ M) on bovine polymorphonuclear neutrophil (PMN) phagocytosis and killing of *Staphylococcus aureus*¹

Indicator	Control	<i>cis</i> -UCA	DPI
PMN with phagocytosed bacteria, %	93.0 $\pm$ 2.0	92.4 $\pm$ 1.4	94.6 $\pm$ 1.4
Number of phagocytosed bacteria:PMN	15.3 $\pm$ 1.0	16.0 $\pm$ 0.6	16.0 $\pm$ 0.4
Killing of bacteria, %	56.3 $\pm$ 4.3	58.2 $\pm$ 3.4	28.2 $\pm$ 4.8*

¹All data presented as mean $\pm$ standard error; n = 5.**P* < 0.01.

intracellular and extracellular ROS (Briheim et al., 1984; Rest, 1994). Luminol has been used to measure an array of ROS, including superoxide (Faulkner and Fridovich, 1993; Lundqvist and Dahlgren, 1996); hydroxyl radical (Yildiz and Demiryurek, 1998; Nemeth et al., 2002); hydrogen peroxide (Castro et al., 1996; Yildiz and Demiryurek, 1998); peroxyxynitrite (Radi et al., 1993); and hypochlorous acid (Brestel, 1985; Myhre et al., 2003). However, the findings of many of these studies are contradictory in terms of the specificity of luminol for the various ROS. The contradictions arise, in part, from the difficulty in identifying whether a given ROS directly reacts with luminol or whether it is converted to another ROS, the latter of which is responsible for eliciting luminol-derived CL. Therefore, although luminol-dependent CL is a sensitive indicator of respiratory burst activity, luminol is limited in its ability to discriminate among individual ROS.

Superoxide anion is the most proximally generated ROS by NADPH oxidase and serves as a precursor to the direct or indirect formation of other ROS (Weiss, 1989; Hampton et al., 1998). Because of its critical role in the generation of downstream ROS, the ability of *cis*-UCA to inhibit generation of superoxide was investigated using 2 distinct assays based on MCLA-dependent CL and cytochrome *c* reduction. Several studies have established that MCLA-dependent CL is a specific marker of extracellular superoxide production (Nakano et al., 1986; Nishida et al., 1989; Nakano, 1990; Pronai et al., 1992). Measurement of cytochrome *c* reduction is also a widely used and accepted technique for the detection of extracellular superoxide generation (Munzel et al., 2002). Reduction of cytochrome *c* is not an absolute specific marker of superoxide production because cellular reductants, such as glutathione, have been reported to reduce cytochrome *c* (Tarpey et al., 2004). However, the extent of specificity of the assay for superoxide can be readily ascertained by the inclusion of control reactions containing exogenous SOD.

Using 2 distinct assays of extracellular superoxide production, MCLA-dependent CL and cytochrome *c* reduction, we established that *cis*-UCA inhibited PMA-induced superoxide generation (Figure 3 and 4). For both assays, the lowest concentration of *cis*-UCA that

inhibited superoxide production was 10 μ M and the extent of inhibition (~40 to 50%) was equivalent. The specificity of the cytochrome *c* assay to detect superoxide was confirmed by the ability of exogenously added SOD to inhibit ~90% of the reduction of cytochrome *c* (Figure 4). Thus, 2 independent assays confirmed that *cis*-UCA inhibits PMN generation of extracellular superoxide.

2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol [1,2-*a*]pyrazin-3-one hydrochloride-dependent CL and cytochrome *c* are limited to the detection of extracellular superoxide, and both of these assays established that *cis*-UCA inhibited extracellular superoxide generation. To investigate whether *cis*-UCA inhibited global generation of this ROS, intracellular superoxide generation was specifically measured with the fluorescent dye, DHE. Dihydroethidium is a cell-permeable compound and its oxidation is a specific indicator of superoxide generation (Rothe and Valet, 1990; Benov et al., 1998; Walrand et al., 2003). At a concentration that maximally inhibited extracellular superoxide production, *cis*-UCA had no effect on intracellular generation of superoxide in PMA-stimulated PMN (Figure 5). The finding that addition of exogenous SOD, which is cell impermeable, had no effect on DHE-mediated fluorescence emitted from PMA-activated PMN confirmed that the assay detected only intracellular superoxide. The cell-permeable NADPH oxidase inhibitor DPI, which was included as a positive control, completely abrogated the PMA-stimulated generation of intracellular superoxide, consistent with previous reports (Ellis et al., 1988; Hampton and Winterbourn, 1995; Katsuyama et al., 2002). Together, these data suggest that *cis*-UCA inhibits extracellular, but not intracellular, superoxide production.

To determine whether *cis*-UCA influenced the generation of other intracellular ROS, the cell-permeable probe CM-H₂DCFDA was used to monitor their production in activated PMN. This probe is not oxidized by superoxide (Bass et al., 1983; LeBel et al., 1992) but is reportedly oxidized by other ROS, including peroxyxynitrite anion and hydrogen peroxide (Rothe and Valet, 1990; Crow, 1997; Kooy et al., 1997). By using CM-H₂DCFDA, *cis*-UCA was determined to have no effect

on the production of these ROS (Figure 6). Consistent with its role in inhibiting NADPH oxidase, the cell-permeable compound DPI significantly inhibited CM-H₂DCFDA-mediated fluorescence in PMA-activated PMN. Although one cannot rule out that the generation of other intracellular ROS not detected by CM-H₂DCFDA is impaired by *cis*-UCA, these findings suggest that the ability of *cis*-UCA to inhibit respiratory burst activity is largely mediated by its ability to block extracellular ROS production.

cis-Urocanic acid inhibited extracellular, but not intracellular, superoxide production. Interestingly, *cis*-UCA was able to completely inhibit luminol-dependent CL, which measures both intracellular and extracellular ROS. Thus, if *cis*-UCA only blocks the generation of extracellular ROS, one might expect that *cis*-UCA should only partially inhibit luminol-dependent CL. However, there is considerable controversy regarding the actual identity of the ROS that are directly responsible for evoking luminol-dependent CL. There is evidence that luminol-dependent CL is dependent upon both superoxide generation and myeloperoxidase activity and that reactants formed by the reaction of superoxide with myeloperoxidase-derived radicals are responsible for eliciting CL (Edwards, 1987; Suematsu et al., 1988; Ginsburg et al., 1993). Further, there are reports that luminol-dependent CL is also dependent upon the generation of nitric oxide (Wang et al., 1991; Radi et al., 1993; Catz et al., 1995). Whether *cis*-UCA inhibits nitric oxide production or myeloperoxidase-derived intracellular ROS not detected by CM-H₂DCFDA, or both, remains unknown. Thus, the ability of *cis*-UCA to completely inhibit luminol-dependent CL may reflect not only the inhibition of extracellular ROS, but also the inhibition of intracellular ROS and NO that contribute to luminol-dependent CL. Further, the ratio of the amount of ROS released from bovine PMN extracellularly vs. that generated intracellularly remains unknown. If the ROS responsible for luminol-dependent CL are predominantly extracellular, then one may expect that *cis*-UCA may inhibit the vast majority of luminol-dependent CL regardless of the fact that luminol measures both intracellular and extracellular ROS. This latter hypothesis is supported by a report that sodium butyrate inhibits up to 80% of PMA-induced luminol-dependent CL in the absence of detectable decreases in intracellular superoxide (Liu et al., 2001). The definitive finding of the current study, based on 2 distinct assays, is that *cis*-UCA inhibits extracellular generation of superoxide. Whether the ability of *cis*-UCA to completely block luminol-dependent CL reflects *cis*-UCA inhibition of intracellular and extracellular ROS other than superoxide remains unclear.

The finding that *cis*-UCA exerts an inhibitory effect on extracellular generation of ROS, which are known to induce host tissue injury, suggests a possible therapeutic use for *cis*-UCA in limiting injury induced by the presence of large numbers of activated PMN. However, enthusiasm for implementation of *cis*-UCA as a therapeutic to limit host tissue injury would be diminished if *cis*-UCA impaired the bactericidal activity of PMN. It is known that DPI, which inhibits NADPH oxidase-mediated generation of ROS, impairs PMN bactericidal activity (Ellis et al., 1988; Hampton and Winterbourn, 1995). Therefore, the effect of *cis*-UCA on phagocytosis and killing of *Staph. aureus* was investigated. The percentage of PMN that phagocytosed bacteria and the number of bacteria phagocytosed were unaffected by exposure to the highest concentration of *cis*-UCA demonstrated to inhibit extracellular superoxide production (Table 1). Further, *cis*-UCA had no effect on the ability of PMN to kill *Staph. aureus*. Consistent with previous reports (Ellis et al., 1988; Hampton and Winterbourn, 1995), DPI had no effect on PMN phagocytosis of *Staph. aureus*, but did impair bacterial killing. It is well established that intracellular generation of ROS is critical to PMN bactericidal activity; however, the contribution of extracellular ROS to PMN-mediated bacterial killing remains less clear (Hampton et al., 1998). The impairment of bactericidal activity by DPI is consistent with its ability to inhibit intracellular generation of ROS. Conversely, the lack of impairment of the bactericidal activity of PMN exposed to *cis*-UCA is consistent with the findings that *cis*-UCA does not impair intracellular generation of ROS.

Two independent assays have established that *cis*-UCA inhibits production of extracellular superoxide, whereas intracellular generation of ROS was unaffected. The finding that *cis*-UCA did not impair bactericidal activity triangulates the finding that much, if not all, of the inhibitory activity of *cis*-UCA is exerted at the extracellular level. The mechanism of this action remains unknown. One possibility is that *cis*-UCA scavenges extracellular superoxide similar to SOD. However, unlike SOD, there is evidence that *cis*-UCA is cell permeable. Thus, if this hypothesis were true, the scavenging ability of *cis*-UCA would be expected to be preserved intracellularly. The anti-inflammatory properties of *cis*-UCA extend beyond that of inhibiting respiratory burst activity and include the ability to down-regulate IL-1 and IL-2 production and diminish MHC expression (Rasanen et al., 1989). Thus, rather than simply scavenging ROS, it is more likely that the ability of *cis*-UCA to impair extracellular ROS generation is mediated through a common mechanism that results in abrogation of an array of inflammatory processes.

In summary, *cis*-UCA was identified as reducing the extracellular levels of superoxide generated by activated PMN without impairing PMN phagocytotic and bactericidal activity. To our knowledge, this is the first study to investigate the effects of *cis*-UCA on bovine PMN respiratory burst activity. Further, this is the first study to demonstrate with any cell type that the inhibitory effect of *cis*-UCA on respiratory burst activity is exerted at the level of extracellular production of ROS and that superoxide is one ROS whose generation is specifically blocked. These findings suggest that *cis*-UCA may have therapeutic value in disease settings accompanied by massive PMN recruitment, such as mastitis, by limiting the production of extracellular ROS that are potentially damaging to host tissue while preserving PMN activity critical to bacterial clearance.

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