Induction of Nitric Oxide Production by Bovine Mammary Epithelial Cells and Blood Leukocytes

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ABSTRACT

A recent study from our laboratory has shown that significant amounts of nitric oxide are released by somatic cells recovered during endotoxin-induced mastitis. The present study was undertaken to investigate which cell type(s) among milk somatic cell population can produce nitric oxide under inflammatory conditions. Nitric oxide release from mammary epithelial cell lines and from bovine neutrophils and monocytes extracted from blood was measured in response to cytokines and Escherichia coli lipopolysaccharides. An epithelial cell line isolated from bovine mammary gland, FbE cells, was found to release nitric oxide after exposure to interleukin-1β. This nitric oxide production was completely abolished by addition of L-N6-(1-iminoethyl) lysine, a potent inducible nitric oxide synthase inhibitor. Bovine monocytes produced nitric oxide in response to recombinant bovine interferon-γ alone or in combination with E. coli lipopolysaccharides. In these cells, nitric oxide release was reduced by the addition of inducible nitric oxide synthase inhibitors L-N6-(1-iminoethyl) lysine and aminoguanidine. Lipopolysaccharides and recombinant bovine interferon-γ increased nitric oxide synthase mRNA in neutrophils, but nitric oxide release could not be detected under any of the experimental conditions used. These results show that bovine epithelial cells and mononuclear phagocytes produce nitric oxide under inflammatory conditions and suggest that these cell populations are responsible for nitric oxide release observed during mastitis. (Key words: nitric oxide, somatic cells, mastitis, inducible nitric oxide synthase)

INTRODUCTION

Mastitis is a devastating disease affecting the dairy cow and represents a significant economic loss to dairy producers. Mastitis is an inflammatory response of the mammary tissue to physiological and(or) metabolic changes, traumas or, more frequently, to injuries caused by microorganisms. During inflammatory response, the animal immune system is mobilized to protect the host and eliminate the pathogen. Macrophages, which are the first to encounter and phagocytose the pathogen, release chemotactical substances, including cytokines, which massively recruit neutrophils to the infected gland (Burvenich et al., 1994; Craven, 1983; Shuster et al., 1997). Mechanisms that normally protect the host from infection and eliminate bacteria can also cause extensive tissue injury in some situations. The inflammatory reaction accompanying mastitis causes mammary secretory cell damage, resulting in reduced milk production (Oliver and Calvino, 1995; Rajala-Shultz et al., 1999).

Nitric oxide (NO) is a potent biological effector regulating blood vessel dilatation, serving as a neuronal messenger and playing a complex role in inflammatory response (Dawson and Dawson, 1995). A family of three enzymes termed NO synthases produces NO from L-arginine. Two isozymes are calcium/calmodulin-dependent and are constitutively expressed. The inducible isozyme of NO synthase (iNOS) is expressed in a wide
array of cell types after stimulation with inflammatory mediators and bacterial products (Bochsler et al., 1996; Nath and Powledge, 1997). Involvement of cytokines such as interleukin-1β, tumor necrosis factor α (TNF-α), and interferon-γ during mastitis has been documented (Shuster et al., 1993; Sordillo and Peel, 1992). Interleukin-1 and TNF-α cause a rapid translocation of NF-κB a nuclear factor, implicated in the control of several inflammatory genes like iNOS (Gilad et al., 1998). Overproduction of NO was observed in several inflammatory diseases (Dawson and Dawson, 1995; Hobbs et al., 1999). Toxic effects of NO occur through the formation of peroxynitrite, a powerful oxidant that causes diverse chemical reactions in biological systems, including protein and DNA nitrosylation as well as lipid peroxidation (Maeda and Akaike, 1998; Murphy, 1999). It is presently not known whether peroxynitrite is a damage-causing agent during bovine mastitis. Recently, our laboratory clearly showed that significant amounts of NO are released in milk during clinical mastitis. Bovine monocytes from peripheral blood were isolated (Lacasse et al., 1997) and endotoxin-induced mastitis is involved in mastitis. Interleukin-1 and TNF-α cause a rapid translocation of NF-κB a nuclear factor, implicated in the control of several inflammatory genes like iNOS (Gilad et al., 1998). Overproduction of NO was observed in several inflammatory diseases (Dawson and Dawson, 1995; Hobbs et al., 1999). Toxic effects of NO occur through the formation of peroxynitrite, a powerful oxidant that causes diverse chemical reactions in biological systems, including protein and DNA nitrosylation as well as lipid peroxidation (Maeda and Akaike, 1998; Murphy, 1999). It is presently not known whether peroxynitrite is a damage-causing agent during bovine mastitis. Recently, our laboratory clearly showed that significant amounts of NO are released in milk during clinical mastitis (Lacasse et al., 1997) and endotoxin-induced mastitis (Bouchard et al., 1999). We have also shown that somatic cells recovered from lipopolysaccharide (LPS)-infused quarters can release NO into the culture medium. The cell type(s) responsible for this NO production is(are) still unknown.

Milk somatic cells consist of several cell types, including macrophages, neutrophils, lymphocytes, and a smaller percentage of epithelial cells. During mastitis, neutrophils become the major cell type found in mammary secretions. The cell type(s) responsible for the release of NO in milk during mastitis is not known. During mastitis, neutrophils are the major cell type found in mammary secretions and can constitute more than 90% of the total somatic cells (Kehrl and Shuster, 1994; Paape et al., 1981). In the study reported here, two mammary epithelial cell lines (FbE and MAC-T) and blood leukocytes (neutrophils and monocytes) were used to verify involvement of these cell types in NO production observed during mastitis. Bovine monocytes from peripheral blood can be induced by cytokines and phagocytic stimulus to produce NO (Goff et al., 1996; Zhao et al., 1996). However, there is no report of NO release by bovine neutrophils and mammary epithelial cells. The objectives of this study were to determine whether mammary epithelial cell lines or blood leukocytes would release NO upon stimulation with LPS and cytokines involved in mastitis.

MATERIALS AND METHODS

Mammary Epithelial Cell Culture

Two different bovine cell lines were used to study NO production by mammary epithelial cells: MAC-T and FbE. The epithelial cell line MAC-T is widely used (Huynh et al., 1991), and FbE cells are spontaneously immortal cells positive for vimentin and ZO-1 (Woodward, 1996). Cells were grown on tissue culture plastic in complete Dulbecco’s modified Eagle medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% (vol/vol) fetal bovine serum, 1 μg/ml of insulin, and 1% (vol/vol) antibiotic/antimycotic.

Reagents for In Vitro Activation

All reagents, including recombinant bovine interferon-γ (rBoIFN-γ, generously supplied by Novartis, Basel, Switzerland), recombinant bovine interleukin-1β (rBoIL-1β), interleukin-2 (rBoIL-2, American Cyanamid Co., Princeton, NJ), and LPS (Escherichia coli 055:B5, Sigma) were dissolved in PBS. Aminoguanidine (Sigma) and L-N6-(1-iminoethyl) lysine (L-NIL, Calbiochem, San Diego, CA) were used as competitive inhibitors of L-arginine to demonstrate the specificity of the NO synthesis pathway.

Isolation of Leukocytes from Peripheral Blood

Leukocytes were isolated from whole blood according to a method described by Carlson and Kaneko (1973) with some modifications. Briefly, blood samples were collected from the caudal vein of healthy lactating Holstein cows into EDTA-coated Vacutainer tubes. Whole blood was layered on Ficoll-Paque Plus (Amersham Pharmacia, Montreal, Canada) and centrifuged 40 min at 500 × g. For monocytes isolation, interface (containing monocytes and lymphocytes) between plasma and Ficoll-Paque Plus was washed in Hanks balanced salt solution (HBSS) and centrifuged, and cells were resuspended in DMEM-F12. This cell suspension was layered on tissue culture plates previously treated with fetal bovine serum for 1 h at 37°C. Monocytes were allowed to adhere for 1 h at 37°C in a 5% CO2 atmosphere. Nonadherent cells were removed, and adherent cells (monocytes) were rinsed with warm DMEM-F12 and removed from plastic by incubating in DMEM-F12 containing 0.01 M EDTA for 20 min at 37°C followed with vigorous shaking. Monocytes were washed twice in HBSS and suspended (1 × 10⁶ cells/ml) in DMEM-F12 for NO production assays.

Neutrophils were isolated by hypotonic lysis of erythrocytes in the red pellet with Tris-buffered 0.15 M ammonium chloride. Cells were washed twice in HBSS with centrifugation at 350 × g for 10 min and suspended (1 × 10⁶ cells/ml) in DMEM-F12 for NO production assays. This procedure typically produced cell fractions with >99% viability as determined by trypan blue dye exclusion.

NOx Determination

Nitric oxide production was evaluated by measuring its more stable metabolites, nitrite and nitrate (NOx),
in cell-free supernatants. For NO\textsubscript{x} assays, cells were subcultured in 24-well culture plate in DMEM-F12 without serum as it contains nitrates that interfere with the assay. Cells were exposed to cytokines and/or LPS for various periods of time (indicated in the figure legend) and conditioned media were collected and centrifuged at 400 \times g for 5 min. Using a spectrometric method based on the Greiss reaction (Green et al., 1982), we evaluated NO\textsubscript{x} concentration. First, nitrate was reduced to nitrite with nitrate reductase (nitrate oxidoreductase from \textit{Aspergillus} species: Boehringer Mannheim Canada: EC 1.6.6.2) as described (Gillam et al., 1993). Briefly, 75-\mu l samples were incubated with 25 \mu l of nitrate reductase buffer (0.4 U/ml of nitrate reductase, 420 \mu M NADPH, 160 mM potassium phosphate buffer, pH 7.5) at room temperature for 1 h in a microplate. An equal volume (100 \mu l) of Greiss reagent (1% sulfanilamide, 0.1% n-(1-naphthyl)-ethylenediamine, 2% phosphoric acid) was added, the reaction was allowed to proceed 10 min, and total nitrite was evaluated by reading optical density of samples at 540 nm. The final NO\textsubscript{x} concentration was determined by comparison with a NaNO\textsubscript{2} standard curve (0 to 100 \mu M in DMEM-F12) freshly prepared before each experiment. Basal level of NO\textsubscript{x} in DMEM-F12 alone was measured and subtracted from sample concentration.

### Detection of Inducible Nitric Oxide Synthase Gene Expression by Reverse-Transcription (RT)-PCR

Cellular RNA was isolated from bovine monocytes and neutrophils using TRIzol reagent (Gibco BRL, Grand Island, NY). Preparations of RNA were treated with RNase-free DNase (Gibco BRL). First strand DNA synthesis was performed using 1.0 to 1.5 \mu g of total RNA with 0.5 \mu g oligo(dT)\textsubscript{12–18} as a primer and Superscript II RNase H\textsuperscript{−} reverse transcriptase (Gibco BRL). The RT reaction was done in a 50-\mu l volume using 1 \times RT buffer (50 mM Tris-HCl, pH 8.3, 3 mM MgCl\textsubscript{2}, 75 mM KCl, and 10 mM DTT), 0.5 mM dNTP, 30 U of RNAguard, and 200 U Superscript II. The reaction was incubated for 50 min at 42\textdegree C, followed by inactivation of the reverse transcriptase at 70\textdegree C for 5 min. First-strand DNA was used as PCR template with sense (5'-GGAACGC-GTACAAAGGAGATA-3') and antisense (5'-CATAGC-GGATGAGCTGGGCG-3') primers that were specific for bovine iNOS gene (designed from GenBank using PC1 Gene software). For PCR, 1.5 \mu l of the RT reaction was used in a volume of 50 \mu l (1 \times PCR Pharmacia buffer, 30 pmol of each primer, 1.25 U of Taq polymerase (QIA-GEN, Ontario, Canada), and 0.4 mM dNTP). The preparation was overlaid with mineral oil and amplified in Perkin-Elmer DNA thermal cycler after determination of optimal condition for linearity [around 31 denaturation cycles for iNOS and 24 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)]. The denaturation cycles were performed at 94\textdegree C for 1 min (duration of the first cycle was 2 min), primer annealing at 60\textdegree C for 1 min and extension at 72\textdegree C for 1 min. An aliquot of 10 \mu l of PCR product was electrophoresed on 1% agarose gel containing 1 \mu g of ethidium bromide per milliliter. Bands were visualized and photographed by UV transillumination. The level of mRNA expression for iNOS was determined by densitometric image analysis (Bio-Rad GS-670 imaging densitometer and Molecular Analyst 1.5 software, Hercules, CA), where the constitutively expressed GAPDH gene expression was also used. The mRNA expression levels for iNOS are presented as relative units after normalization to the observed GAPDH level.

### Statistical Analysis

All data are expressed as mean \pm SEM. Data were analyzed with the GLM procedure of SAS (SAS, 1985). Means were separated by least significant differences protected by an overall F value (P < 0.05). The number of replicates for each treatment and number of cow used in each experiment is included in the figure.

### RESULTS

#### NO Production by Mammary Epithelial Cells

The NO\textsubscript{x} release by two bovine mammary epithelial cell lines, FbE and MAC-T, was studied. FbE cells consistently produced significant amounts of NO\textsubscript{x} in response to rBoIL-1\textbeta in a dose-dependent manner (P < 0.01; Figure 1). This rBoIL-1\textbeta-induced NO\textsubscript{x} release was completely abolished (P < 0.01) when the cells were exposed to L-NIL\textsubscript{2}, a potent iNOS inhibitor. However, rBoIL-2 (200 ng/ml) and LPS (40 \mu g/ml) failed to induce NO\textsubscript{x} production by FbE cells (data not shown). In experiments with MAC-T cells, no detectable amounts of NO\textsubscript{x} were measured when incubated with LPS or cytokines (rBoIFN-\gamma, rBoIL-1\textbeta, rBoIL-2; data not shown).

#### NO Production by Bovine Monocytes and Neutrophils

As shown is Figure 2, moderate amounts of NO\textsubscript{x} were released when monocytes were stimulated with rBoIFN-\gamma alone (P < 0.01). No significant amount of NO\textsubscript{x} (P > 0.25) was detected in the conditioned media from unstimulated monocytes or cells stimulated with LPS alone. The addition of LPS to rBoIFN-\gamma-induced monocytes had variable effects on NO\textsubscript{x} release. Indeed, in some animals, LPS increased rBoIFN-\gamma-induced NO production, whereas in other, LPS had the opposite
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Figure 1. Nitrite plus nitrate (NO\textsubscript{x}) release by bovine mammary epithelial cell line FbE. Confluent cells were incubated with graded concentrations of recombinant bovine interleukin (rBoIL)-1\textbeta (0, 20, and 200 ng/ml) or 20 ng rBoIL-1\textbeta + 100 \mu M L-N\textsuperscript{6}-(iminoethyl) lysine (L-NIL) per milliliter for 24 h at 37°C and 5% CO\textsubscript{2}. Supernatants were assayed for the accumulation of NO\textsubscript{x}. Individual experiments were performed in quadruplicate. \textit{a} \textit{P} < 0.01 compared with control. \textit{b} \textit{P} < 0.01 compared with treatment with 200 ng rBoIL-1\textbeta per milliliter.

Figure 2. Effect of lipopolysaccharide (LPS) and recombinant bovine interferon (rBoIFN)-\gamma on nitrite plus nitrate (NO\textsubscript{x}) release by bovine monocytes. Cells (1 × 10\textsuperscript{6} monocytes: 1 × 10\textsuperscript{6} monocytes) were isolated from bovine blood and incubated in the presence of medium alone (Dulbecco’s modified Eagle’s medium/F12), LPS, or the combination rBoIFN-\gamma and LPS. None of the stimulatory conditions used in this study induced NO production by bovine monocytes (Figure 4), even after 96 h in culture. The addition of the enzyme superoxide dismutase (SOD; Calbiochem; EC 1.15.1.1) to the media did not result in measurable accumulation of NO\textsubscript{x} (data not shown). In other experiments, NO\textsubscript{x} accumulation was not detected when bovine monocytes were exposed to phorbol 12-myristate 13-acetate (PMA), formyl-methyl-leucine-phenylalanine, rBoIL-1\textbeta, or rBoIL-2 (data not shown). Moreover, incubation of neutrophils with different concentration of serum (1, 2.5, 5, and 10%) and LPS resulted in a nondetectable increase in NO\textsubscript{x} level (data not shown).

Surprisingly, the addition of neutrophils to rBoIFN-\gamma-induced monocytes inhibited NO\textsubscript{x} accumulation in media (\textit{P} < 0.05). When both cell types were exposed to rBoIFN-\gamma and LPS, more NO\textsubscript{x} was released compared with monocytes alone (\textit{P} < 0.05).

iNOS mRNA Levels

Expression of iNOS, the enzyme responsible for NO production, was investigated in both neutrophils and monocytes (Figure 5). In response to rBoIFN-\gamma with or without LPS, iNOS mRNA level increased in bovine monocytes. Surprisingly, combination of rBoIFN-\gamma and LPS increased similar levels of iNOS mRNA in both cell types (Figures 5 and 6). In unstimulated monocytes, iNOS mRNA was detected, whereas it was undetectable in nonactivated neutrophils.

DISCUSSION

In this experiment, FbE cells, a bovine mammary epithelial cell line, produced NO\textsubscript{x} when stimulated with rBoIL-1\textbeta. This rBoIL-1\textbeta-induced NO\textsubscript{x} release was completely abolished when cells were exposed to L-NIL, an L-arginine-dependent pathway. Other proinflammatory agents such as rBoIL-
Figure 3. Dose-dependent effect of aminoguanidine and L-N^6-(iminoethyl) lysine (L-NIL) on recombinant bovine interferon (rBoIFN-γ)-induced nitrite plus nitrate (NOx) release by bovine monocytes. 1 × 10^6 cells/ml were isolated from bovine blood and incubated for 48 h in Dulbecco’s modified Eagle’s medium F12 supplemented with rBoIFN-γ (100 U/ml) in the absence or presence of A) aminoguanidine or B) L-NIL. Data represent the mean for two cows, and each treatment was done in duplicate.

Figure 4. Effect of lipopolysaccharide (LPS) and recombinant bovine interferon (rBoIFN-γ) on nitrite plus nitrate (NOx) produced by (white) neutrophils (1 × 10^6/ml), (black) monocytes (1 × 10^6/ml), or (gray) monocytes (1 × 10^6/ml) and neutrophils (1 × 10^6/ml) coculture. Cells were isolated from bovine blood and incubated for 48 h with Media alone (Dulbecco’s modified Eagle’s medium/F12), and (per ml) 2 µg of LPS, 100 U of rBoIFN-γ, or 2 µg of LPS + 100 U of rBoIFN-γ for 48 h at 37°C and 5% CO2. Data represent the mean for four cows, and each treatment was done in triplicate. ^P < 0.01 compared with control. *P < 0.05 compared with monocytes alone with the same treatment.
Detection of inducible nitric oxide synthase (iNOS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA by reverse transcription PCR. mRNA was extracted from bovine blood neutrophils or monocytes. Cells were incubated for 6 h at 37°C with culture medium alone; lipopolysaccharide (LPS) (2 µg/ml); recombinant bovine interferon (rBoIFN-γ) (100 U/ml); LPS (2 µg/ml) + rBoIFN-γ (100 U/ml). In first lane, RNA was extracted from freshly isolated neutrophils or monocytes (no incubation). The number under each band represents the expression level relative to the housekeeping gene GAPDH. The optimal cycle number was 25 for amplification of GAPDH fragment and 43 cycles for amplification of iNOS fragment. The experiment was performed on four cows. Results from one typical cow are shown.

Expression of iNOS in both monocytes and neutrophils was investigated. Results showed that rBoIFN-γ but not LPS increased iNOS mRNA levels in bovine monocytes. Despite variable effects on NOx accumulation, combination of LPS and rBoIFN-γ consistently increased iNOS mRNA. Crespo et al. (2000) have reported that pretreatment of mouse bone marrow culture-derived macrophages with LPS dramatically reduces their ability to produce NO in response to subsequent stimulation with IFN-γ. In our case, LPS and rBoIFN-γ induced iNOS mRNA increase, but LPS might have variable effects on protein synthesis that could explain variable NOx accumulation.

In freshly isolated monocytes, iNOS mRNA was expressed. After 6 h of incubation in media alone, no iNOS expression was observed. Expression of iNOS in freshly isolated monocytes could be attributed to stimulation with cytokines secreted by lymphocytes during the adherence step of monocyte isolation. The majority of the lymphocytes present in the adherence step are gamma-delta T (γδT) cells capable of producing IFN-γ (Goff et al., 1996). Results indicated that contaminating γδT cells in monocyte cultures might influence monocyte iNOS mRNA levels. After 6 h in culture media without lymphocytes, iNOS mRNA in monocytes might have been down regulated, and no band would have been observed.

Surprisingly, LPS and rBoIFN-γ increase iNOS mRNA in bovine blood neutrophils, whereas they did not produce a significant amount of NOx when incubated with LPS and rBoIFN-γ, even after 96 h in culture. To our knowledge, iNOS expression in bovine neutrophils has not been reported. Nitric oxide production...
was studied in bovine neutrophils but not iNOS expression (Goff et al., 1996). We have demonstrated here that LPS and rBoIFN-γ can increase iNOS mRNA in bovine neutrophils without stimulating NOx release. Mature neutrophils have a small Golgi apparatus and some mitochondria but very few ribosomes or rough endoplasmic reticulum (Tizard, 1996), thus they cannot synthesize large amounts of protein. Therefore, the translation of iNOS mRNA might have been impaired. Undetectable NOx accumulation might also be due to superoxide anion production. Some NO might be released by neutrophils but react with superoxide anions to form peroxynitrite (ONOO−). Superoxide anions are produced by PMA-activated bovine neutrophils under these conditions (Boulanger, 2000). To study NO release by rodent neutrophils, cells need to be incubated with SOD, a scavenger of superoxide anion (Rodenas et al., 1996). However, we repeated the experiment, adding SOD, and observed no difference in NOx concentration measured.

A reaction of superoxide anion with NO might explain the reduction of NOx accumulation in rBoIFN-γ-stimulated coculture of neutrophils and monocytes compared with monocytes alone. However, when both cell types were exposed to rBoIFN-γ and LPS, an increase in NOx accumulation was observed. In this experiment, the highest levels of iNOS mRNA in neutrophils occurred after rBoIFN-γ plus LPS stimulation. Pigatto et al. (1990) reported that LPS-stimulated monocytes and psoriatic human monocytes can stimulate neutrophil chemotaxis, phagocytosis, and superoxide anion production. Similarly, it is possible that in presence of monocytes, some neutrophil iNOS mRNA gets translated to proteins and leads to NO production. The potential contribution of lymphocytes, which are important components of milk somatic cell population, to NO production has not been investigated. However, Schuberth et al. (1998) found that NO production by cells isolated from bovine blood was correlated with the proportion of CD14+ monocytes but not with CD4+ and CD8+ lymphocytes.

We investigated NO production by bovine monocytes, neutrophils, and mammary epithelial cells in order to identify which cell type(s) among milk somatic cells can release NO during endotoxin-induced mastitis. In conclusion, mammary epithelial cells and/or mononuclear phagocytes contribute to NO production upon stimulation with LPS and cytokines involved in mastitis (IL-1 and IFN-γ). Nevertheless, the possibility that some NO release could be attributed to neutrophils or lymphocytes cannot be excluded. These results suggest that during mastitis, NO released by mammary epithelial cells and monocytes may damage mammary tissue. Keeping up with our long-term objective to reduce mastitis-associated tissue damage, the effect of NO and other free radicals on mammary epithelial cell damage are currently studied.

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