Ethyl pyruvate diminishes the endotoxin-induced inflammatory response of bovine mammary endothelial cells

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ABSTRACT

The endotoxin-induced inflammatory response during coliform mastitis is difficult to control with the currently available therapeutics. Endothelial cells are among the first cell type to be engaged in the inflammatory response and can modulate the severity of inflammation by producing proinflammatory mediators upon endotoxin exposure. Ethyl pyruvate, an ethyl ester of pyruvic acid, can ameliorate endotoxin-induced inflammatory responses by inhibiting the production of proinflammatory mediators in several *in vitro* and *in vivo* endotoxemia models. The objective of this study was to determine the effect of ethyl pyruvate on the production of vascular proinflammatory mediators that are associated with the pathogenesis of coliform mastitis. The ability of ethyl pyruvate to reduce the expression of proinflammatory mediators was evaluated in cultured bovine mammary endothelial cells (BMEC) stimulated with endotoxin. Treatment of endotoxin-stimulated BMEC with ethyl pyruvate significantly reduced gene expression of IL-6, IL-8, and intercellular adhesion molecule 1 as well as expression of eicosanoid-producing enzymes, including cyclooxygenase 2 and 15-lipoxygenase 1. This is the first time that the effect of ethyl pyruvate was evaluated in an *in vitro* BMEC model of coliform mastitis. The ability of ethyl pyruvate to effectively inhibit gene and protein expression of potent vascular proinflammatory mediators *in vitro* warrants further investigations to assess *in vivo* efficacy. Ethyl pyruvate is safe for human consumption, and it may be an attractive candidate as a therapeutic in ameliorating the severe pathogenesis associated with coliform mastitis.

Key words: ethyl pyruvate, inflammation, endothelium, coliform mastitis

INTRODUCTION

Coliform mastitis is a serious problem affecting the dairy industry because it is severe, sometimes fatal,

and difficult to control with antibiotics currently formulated for intramammary use. The acute symptoms associated with coliform mastitis result from rapid growth of the bacteria, release of endotoxin, and the subsequent development of an unlimited inflammatory reaction (Smith et al., 1984; Hogan and Smith, 2003). Endotoxin, derived from the outer membrane of gram-negative bacteria, initiates the acute inflammatory phase response by inducing the synthesis of eicosanoids, cytokines, and other proinflammatory molecules at the foci of infection. Extensive necrosis of mammary parenchymal tissue and loss of milk production may result from an exaggerated acute or protracted local inflammatory response (Hill et al., 1984). These potent mediators not only regulate local inflammatory reactions, but also can gain access to the circulation and induce systemic responses. Some of the acute systemic symptoms of coliform mastitis that are directly or indirectly induced by the acute phase molecules include fever, depression, respiratory distress, reduced rumen motility, metabolic disturbances, and death (Lohuis et al., 1990). Several acute phase mediators produced during endotoxemia are associated with the severity of disease. In fact, both milk and sera concentrations of tumor necrosis factor- α (TNF- α), IL-1 β , IL-8, prostaglandin E₂ (PGE₂), and thromboxane B₂ (TXB₂) are associated closely with the manifestation of acute symptoms of coliform mastitis (Sordillo and Peel, 1992; Shuster et al., 1996; Riollot et al., 2000; Vangroenweghe et al., 2005).

Endothelial cells are both targets for and producers of these proinflammatory mediators and are among the first to be engaged in an inflammatory response as they control the extravasation of leukocytes from the circulation and into the tissues. Under normal physiological conditions, the endothelium maintains a delicate balance between vasodilation and vasoconstriction, coagulation and blood fluidity, and leukocyte adhesion and migration. The vascular endothelium, however, is also a primary target for endotoxin during the pathogenesis of gram-negative bacteria. Endotoxin interacts with transmembrane toll-like receptor-4 on endothelial cells, with the aid of LPS-binding protein and CD14, to activate the transcriptional regulation of endothelial

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cell-derived inflammatory mediators via nuclear factor- κ B (**NF- κ B**; Riollot et al., 2000; Tak and Firestein, 2001; Dauphinee and Karsan, 2006). The vascular-derived proinflammatory cytokines and adhesion molecules facilitate the recruitment of activated leukocytes promoting prompt pathogen elimination. Prolonged exposure to large quantities of endotoxin, however, can cause a hyperactive proinflammatory response by endothelial cells that can lead to severe tissue damage and endotoxic shock (Grandel and Grimminger, 2003). Therefore, strategies that would effectively contain the uncontrolled proinflammatory response during coliform mastitis may prevent or diminish the pathological consequences of endotoxemia.

Ethyl pyruvate, an aliphatic ester derived from pyruvic acid, has proven to be a promising antiinflammatory agent in several in vitro and in vivo models of endotoxemia (Ulloa et al., 2002; Venkataraman et al., 2002; Yang et al., 2002; Sappington et al., 2003; Song et al., 2004; Johansson et al., 2008). Ethyl pyruvate was first discovered to have antiinflammatory properties by Yang et al. (2002) in murine liver and colonic mucosa following resuscitation from hemorrhagic shock. Treatment with ethyl pyruvate decreased gene expression of inducible nitric oxide synthetase, TNF- α , cyclooxygenase (**COX**)2, and IL-6, as well as activation of the transcription factor NF- κ B in liver and colonic mucosa (Yang et al., 2002). Following that initial study, the therapeutic value of ethyl pyruvate was further verified in murine and porcine endotoxemia models in which survival was increased even when ethyl pyruvate treatment was delayed until 12 and 24 h after the onset of severe infection (Ulloa et al., 2002; Venkataraman et al., 2002; Yang et al., 2002; Sappington et al., 2003; Hauser et al., 2005). The effectiveness of ethyl pyruvate in lessening an endotoxin-induced inflammatory reaction was confirmed in vitro using the RAW 264.7 murine macrophage-like cell line, as ethyl pyruvate treatment decreased TNF- α , IL-6, and IL-10 at both the gene transcription and protein levels following endotoxin stimulation (Ulloa et al., 2002; Song et al., 2004). Inhibition of NF- κ B is at least partially responsible for the ability of ethyl pyruvate to decrease the inflammatory response as NF- κ B transcriptionally regulates over 200 genes including inflammatory genes TNF- α , IL-6, IL-8, COX2, and inducible nitric oxide synthetase (Yang et al., 2002).

The ability of ethyl pyruvate to modulate vascular inflammatory responses was investigated recently and was found to dose-dependently decrease expression of intercellular adhesion molecule 1 (**ICAM1**), vascular cell adhesion molecule 1 (**VCAM1**), E-selectin, and IL-8 by endotoxin-stimulated human umbilical vein endothelial cells (**HUVEC**; Johansson et al., 2008).

However, the vascular endothelium is not homogeneous, and endothelial cells isolated from various species and different vascular beds can vary in both their physiological and functional characteristics. For example, bovine endothelial cell monolayers were more permeable in response to endotoxin and had increased direct sensitivity to endotoxin-induced apoptosis compared with endotoxin-challenged human endothelial cells (Harlan et al., 1983a,b; Bannerman and Goldblum, 1999, 2003). Therefore, it is important to use bovine mammary endothelial cells to study the pathogenesis of mastitis. The goal of this study was to determine the effect of ethyl pyruvate on the production of vascular proinflammatory mediators that may contribute to the pathogenesis associated with coliform mastitis. Strategies that could decrease the otherwise uncontrolled inflammatory response of mammary tissues during coliform mastitis may affect the pathogenesis of endotoxemia in cattle.

MATERIALS AND METHODS

Chemicals and Reagents

Antibiotics and antimycotics, trypsin-EDTA, HEPES buffer, and Ham's F-12K medium (Kaighn's Nutrient Mixture F-12) were purchased from Cellgro (Herndon, VA). Hyclone Laboratory (Logan, UT) supplied fetal bovine serum (**FBS**). The CellTiter-Glo Luminescent Cell Viability Assay was purchased from Promega Corporation (Madison, WI). The RNeasy Plus Mini Kit for isolation of RNA was purchased from Qiagen (Valencia, CA). Reagents for real-time PCR (**qPCR**) were purchased from Applied Biosystems (Foster City, CA). All other chemicals, including ethyl pyruvate, were purchased from Sigma (St. Louis, MO).

Bovine Mammary Endothelial Cell Culture System

Endothelial cells were isolated from the bovine mammary artery by collagenase digestion and purified through cloning techniques using the limited dilution method as described previously (Aherne et al., 1995). Briefly, isolated cells were grown in a T25 culture flask at 37°C with 5% CO₂. Once cells reached 75 to 90% confluence, they were harvested and transferred into a 96-well tissue culture plate at a dilution of 0 to 2 cells/well. Once confluent, these cells were consecutively passed in 24-well plates, 6-well plates, and T25 culture flasks before cryopreservation. The purified bovine mammary endothelial cells (**BMEC**) were then cultured and stimulated in Ham's F-12K medium containing 10% FBS, 20 mM HEPES, antibiotics, and antimycotics (100 U/mL consisting of penicillin, streptomycin, and amphotericin B), heparin (100 μ g/mL),

insulin (10 µg/mL), transferrin (5 µg/mL), and sodium selenite (10 ng/mL). For experimental use, BMEC were used between passages 5 and 10. The day before stimulation, BMEC were seeded so they would be 75 to 85% confluent by the time of stimulation (i.e., 2.5×10^6 cells per 100-mm dish). Initial experiments treated the cells with 0 to 80 mM ethyl pyruvate for up to 6 h. In subsequent experiments evaluating the effect of ethyl pyruvate treatment on endotoxin-stimulated BMEC, cells were stimulated with 50 ng/mL of endotoxin (from *Escherichia coli* O111:B4) for an initial 1 h followed by an additional 1 h of incubation with ethyl pyruvate (0, 5, or 10 mM ethyl pyruvate) for RNA isolation or 2 h for protein isolation.

Trypan Blue Exclusion

Trypan blue exclusion was used to evaluate cell death. Following treatment of BMEC with 0, 5, 10, 20, 40, or 80 mM ethyl pyruvate for 4 and 6 h, cells were collected using trypsin and diluted 1:4 in trypan blue. Viable and nonviable cells were counted using a hemocytometer to obtain the percentage of viable cells.

CellTiter-Glo Luminescent Cell Viability Assay

Metabolic activity, and thereby cell viability, was measured using CellTiter-Glo Viability Assay (Promega) that quantifies the amount of ATP as an indicator of metabolically active cells. Endothelial growth factor (30 µg/mL) was used as a control for this assay to ensure that no significant changes in proliferation or metabolism occurred within the 6-h stimulation period. The mixture of substrate and buffer results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of viable cells. Luminescence was measured by using a Wallac Victor3 1420 Multilabel Plate Reader (PerkinElmer, Waltham, MA). Data were expressed as percentage relative luminescence versus 0 mM ethyl pyruvate.

Gene Transcript Quantification by qPCR

Following stimulation, BMEC RNA was extracted for quantification of IL-1 β , IL-6, IL-8, ICAM1, COX1, COX2, 15-lipoxygenase 1 (**15-LOX1**), and β -actin mRNA transcript expression by qPCR. Briefly, total RNA was extracted utilizing the RNeasy Plus Mini Kit (Qiagen), which included an in-column genomic DNA elimination treatment. Purified RNA was converted to cDNA using the Applied Biosystems High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). All qPCR assays were conducted utilizing Taq-

Man gene expression assays from Applied Biosystems. Briefly, TaqMan primer and probe sets (Table 1) were designed from bovine sequences with the Applied Biosystems Pipeline software and synthesized by Applied Biosystems. Samples were assayed in triplicate with 100 ng of cDNA per reaction along with 10 µL of TaqMan Fast Universal PCR Master Mix (2 $\times$), and 1 µL of the appropriate TaqMan Gene Expression Assay Mix (20 $\times$) on the Applied Biosystems 7500 Fast Real-Time PCR System. The relative quantification of each gene was calculated utilizing the 7500 Fast SDS Software (version 1.3.1; Livak and Schmittgen, 2001). Data were calculated based on the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta C_t}$) of relative quantification, using β -actin as the control gene (Steibel et al., 2009). The calibrator was set as the 0 mM ethyl pyruvate sample in dose-titration experiments, whereas the endotoxin-stimulated sample was used as the calibrator in the remaining experiments.

Western Blot Analysis for ICAM1

Whole cell lysates were harvested in M-Per reagent (Pierce, Rockford, IL) and centrifuged at $10,000 \times g$ for 10 min at 4°C to remove membrane fractions. Equal amounts of protein (20 to 30 µg) were resolved on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The Millipore Snap i.d. Protein Detection System (Millipore, Billerica, MA) was utilized to carry out the remaining steps. The membrane was blocked in 5% dry milk in Tris-buffered saline (TBS) with 0.01% Tween-20 at room temperature and washed 3 times with TBS. Next, the membrane was incubated for 10 min with anti-human ICAM1 (1:100 dilution, Invitrogen, Carlsbad, CA) in 5% BSA in TBS with 0.01% Tween-20. Following 3 washes with TBS, the membrane was incubated with goat anti-mouse IgG secondary antibody labeled with horseradish peroxidase (HRP; 1:3,000 dilution, Pierce) for 10 min at room temperature, washed 3 times with TBS, exposed to HRP substrate (Pierce), and visualized by chemiluminescence using the ChemiDoc XRS (Bio-Rad, Hercules, CA) and Quantity One software (Bio-Rad). The membrane was stripped with Restore Plus Western Blot Stripping Buffer (Pierce) and reblotted under similar conditions using anti-human actin (1:3,000 dilution, Millipore) to serve as the loading control. Density of the bands was determined using Quantity One software. The ratio of ICAM1:actin was calculated and the values were expressed as a fold change over the endotoxin-stimulated BMEC.

Lipid Mediator ELISA

The activities of 15-LOX1, COX1, and COX2 were determined by measuring the production of their

Table 1. Gene targets and primer sequences for real-time quantitative PCR

Target ¹	Accession no.		Sequence (5' to 3')
ICAM1	NM-174348	Forward	GCAGGTGGTCCACAAACAC
		Reverse	GCAATCCCCGCTGGTCTAGTC
		Probe	ATGTCCTGTACGGCCCC
IL-1 β	NM-174093	Forward	GCTCTCCACCTCCTCTCACA
		Reverse	CTCTCCTTGCACAAAGCTCATG
		Probe	CAGAACACCACTTCTCG
IL-6	X57317	Forward	AGGACGGATGCTTCCAATCTG
		Reverse	GAAGACCAGCAGTGGTTCTGAT
		Probe	CAATCAGGCGATTTGC
IL-8	NM-173925	Forward	GCTCTCTTGGCAGCTTTTCT
		Reverse	GGCATCGAAGTTCTGTACTCATTCT
		Probe	CAGAACTGCAGCTTCAC
COX1	NM-001105323.1	Forward	ATGGAGTTCAACCAGCTTTACCA
		Reverse	CAGAACTGCTCGTAGCTGTAGTC
		Probe	CATGCCCCGACTCCTTC
COX2	NM-174445.2	Forward	GGCGATGAGCAGTTGTTCCA
		Reverse	TGCTGTACGTAGTCTTCAATCACAAT
		Probe	CAAGCAGGCTAATCCT
β -Actin	NM-173979	Forward	CCGCCCCGCTAGCA
		Reverse	AACTGGTTGCGGTGTGCA
		Probe	CCTTCGCCGCTCCGC
15-LOX1	NM-174501	Forward	GTGCCTTCCGTCTATACATCCTATG
		Reverse	CCCGGATGTTAATTTCCATGGTGTA
		Probe	CCCGGATGTTAATTTCCATGGTGTA

¹ICAM1 = intercellular adhesion molecule 1; COX1 and COX2 = cyclooxygenase 1 and 2; 15-LOX1 = 15-lipoxygenase 1.

predominant downstream metabolites 15-hydroxy-5,8,11,13-eicosatetraenoate (**15-HETE**), 13-hydroxyoctadecadienoic acid (**13-HODE**), TXB₂, and PGE₂. The specific COX1 and COX2 metabolites were chosen as they are the primary downstream metabolites and known to be produced during coliform mastitis (Anderson et al., 1985; Peter et al., 1990). The immediate downstream metabolites of 15-LOX1, 15-HETE and 13-HODE, were selected because they were previously shown to be produced by endotoxin-stimulated BMEC (Olson et al., 1995; Cao et al., 2000). The metabolites were measured using enzyme immunoassays (Assay Designs, Ann Arbor, MI). Briefly, cells were seeded at 3.5×10^5 cells per well of a 6-well plate the day before treatment with endotoxin or endotoxin and ethyl pyruvate. Following stimulation, culture supernatant and cells were collected by scraping tissue culture plates before being flash frozen in liquid nitrogen and stored at -80°C until analysis. Samples were thawed on ice and 0.3% of a reducing/antioxidant mixture (0.2 mg/mL butylated hydroxytoluene, 0.2 mg/mL EDTA, 2 mg/mL indomethacin, 2 mg/mL triphenyl phosphate in methanol:ethanol:water, 2:1:1) was added before sonication using a Branson Sonifier 250 (Branson Ultrasonics Corp., Danbury, CT) for 30 s at an output of 10%. Samples were acidified to pH 3.5 with 2 N HCl and incubated on ice for 15 min before centrifugation at $3,000 \times g$ for 10 min at 4°C . Solid-phase extraction was completed using the Strata X C18 columns

(Phenomenex, Torrance, CA). Briefly, columns were prepared with 2 mL of methanol followed by 2 mL of water before addition of the sample. The column was washed with 2 mL of water, 2 mL of 15% methanol, and 2 mL of hexane before elution with 1.2 mL of ethyl acetate (2×0.6 mL). The samples were dried under a stream of nitrogen and resuspended in 30 μL of ethanol. After a 15-min incubation, a total of 750 μL of assay buffer was added to each sample by adding 250 μL each time, vortexing, and incubating for 15 min between each addition. The samples were used at the following final dilutions: 13-HODE (neat), 15-HETE (1:2), PGE₂ (1:10), and TXB₂ (1:10).

The standard curves were prepared immediately before setting up the assays (13-HODE: 3.9 to 1,000 ng/mL, 15-HETE: 78.12 to 20,000 pg/mL, PGE₂: 7.81 to 1,000 pg/mL, TXB₂: 13.7 to 10,000 pg/mL). Utilizing the antibody-coated plate, 100 μL of standard or sample, 50 μL of conjugate, and 50 μL of antibody were combined along with the appropriate assay controls. The 13-HODE and TXB₂ plates were incubated for 2 h at room temperature on an orbital shaker, whereas the 15-HETE and PGE₂ plates were incubated for 18 to 24 h at 4°C . Following their respective incubation periods, the wells were washed 3 times with wash buffer before the addition of 200 μL of p-nitrophenyl phosphate (pNpp) substrate. Additional incubations of 2 h for 13-HODE, and 1 h for 15-HETE and PGE₂ at 37°C as well as 45 min for TXB₂ at room temperature were required

Figure 1. The effect of ethyl pyruvate (EP) on bovine mammary epithelial cell (BMEC) metabolic activity, hence viability, was monitored by measuring ATP. Isolated BMEC were treated with 0, 5, 10, 20, or 40 mM EP. Viability was evaluated after 4 and 6 h exposure to EP utilizing the CellTiter-Glo Viability Assay (Promega Corp., Madison, WI). Data are expressed as percentage relative luminescence versus 0 mM EP and are representative of means $\pm$ SEM of 5 separate experiments. Means marked with an * differ significantly ($P < 0.05$) from their respective 0 mM EP.

after which 50 μ L of stop solution was added to all wells and the plates were read at 405 nm along with a correction reading at 590 nm. All metabolites were expressed as nanograms per 5×10^5 cells. All samples were run on a single 96-well plate for each metabolite with the following intraassay CV and detection limits: 13-HODE, 7.6%, 14 to 248 ng/mL; 15-HETE, 11.4%, 416 to 5,129 pg/mL; PGE₂, 6.7%, 7 to 125 pg/mL; and TXB₂, 3.1%, 32 to 990 pg/mL.

Statistical Analysis

Statistical evaluation of viability, assessed via trypan blue exclusion and ATP production, for each of the treatments and time points measured was conducted using Proc Mixed (SAS version 9.1, SAS Institute Inc., Cary, NC) and Prism 4 (GraphPad Software Inc., La Jolla, CA). Comparisons of the means were made by ANOVA followed by Tukey-Kramer's Honestly Significant Difference post hoc test as necessary. Data are shown as means $\pm$ SEM.

RESULTS

In Vitro Cytotoxicity of Ethyl Pyruvate

The maximum tolerable dose of ethyl pyruvate that could be used in a cell culture model was assessed by monitoring in vitro BMEC viability. No significant changes in BMEC viability occurred following a 4-h treatment with 0, 5, 10, 20, or 40 mM ethyl pyruvate,

with viability counts consistently $>87\%$ as determined by trypan blue exclusion. After a 6-h incubation with ethyl pyruvate, however, viability decreased to 75.83 ± 8.5 and $54.8 \pm 15.5\%$ at the 20 and 40 mM doses, respectively (data not shown). Treatment of BMEC with 80 mM ethyl pyruvate after 4 and 6 h of exposure decreased viability to a point where they could not be counted. Additionally, BMEC were determined to be metabolically active, and thereby viable, by measuring ATP production following ethyl pyruvate treatment. A significant decrease in cellular ATP, which directly relates to cell viability, occurred with 20 and 40 mM ethyl pyruvate at 4 and 6 h (Figure 1). No significant decrease in BMEC viability was observed with 5 or 10 mM ethyl pyruvate at any of the time points investigated. Incubating BMEC with endothelial growth factor also did not change cellular ATP activity for the 4- or 6-h period. Based on the above measures of viability in BMEC, it appears that 10 mM ethyl pyruvate is the maximum tolerable in vitro concentration.

Ethyl Pyruvate Reduces Endotoxin-Induced Expression of IL-6 and IL-8

To evaluate the effects of ethyl pyruvate on proinflammatory cytokine expression, BMEC were first stimulated with endotoxin followed by treatment with ethyl pyruvate. Before beginning these experiments, the ability of ethyl pyruvate alone to modify the expression of selected proinflammatory genes was monitored by qPCR and resulted in no significant induction of proinflammatory genes for ICAM1, IL-1 β , IL-6, or IL-8 following treatment with 0, 5, or 10 mM ethyl pyruvate for 1, 4, and 6 h (Figure 2). Endotoxin stimulation, however, significantly increased IL-1 β , IL-6, and IL-8 mRNA expression in BMEC compared with unstimulated BMEC (Figure 3). Ethyl pyruvate treatment was able to ameliorate the endotoxin-induced cytokine response by significantly inhibiting IL-6 and IL-8 gene expression (Figure 3). Treatment of endotoxin-stimulated BMEC with 5 and 10 mM ethyl pyruvate resulted in 70.4 and 84.8% reductions, respectively, for IL-6 compared with endotoxin-stimulated BMEC. However, only the 10 mM dose of ethyl pyruvate significantly decreased IL-8 expression with a 61.7% reduction compared with endotoxin-stimulated BMEC. Treatment with ethyl pyruvate did decrease endotoxin-induced IL-1 β gene expression, but the change in expression was not significantly different compared with the peak expression observed in the endotoxin-stimulated group. Viability of BMEC was continuously monitored and the results (not shown) confirmed that no changes in cell viability occurred during endotoxin stimulation and ethyl pyruvate treatment.

Figure 2. Effect of ethyl pyruvate (EP) on proinflammatory gene expression in bovine mammary epithelial cells (BMEC). The BMEC were cultured with 0, 5, or 10 mM ethyl pyruvate for 1, 4, and 6 h before RNA isolation. The expression of intercellular adhesion molecule 1 (ICAM1), IL-1 β , IL-6, and IL-8 mRNA transcripts was evaluated by real-time quantitative PCR. Data are expressed as $2^{-\Delta\Delta C_t}$ value using 0 h BMEC as the calibrator and are representative of means $\pm$ SEM for 3 separate experiments.

Ethyl Pyruvate Inhibits Endotoxin-Induced Expression of ICAM1

The ability of ethyl pyruvate to modulate expression of ICAM1 by endotoxin-stimulated BMEC was examined at both the gene and protein level. The 10 mM dose of ethyl pyruvate significantly decreased the endotoxin-induced gene and protein expression of ICAM1 by approximately 50% (Figure 4A and 4B).

Effects of Ethyl Pyruvate on Expression of COX

The ability of ethyl pyruvate to modulate COX1 and COX2 mRNA expression and COX enzyme activities was evaluated. No significant changes in COX1 gene expression were observed after either endotoxin stimulation or ethyl pyruvate treatment (Figure 5A). In contrast, COX2 mRNA increased significantly following endotoxin challenge and ethyl pyruvate was able to decrease COX2 gene expression in a dose-dependent manner (Figure 5A). To determine if changes at the gene level were representative of modifications in COX activity, select downstream metabolites of both COX1 and COX2 were measured. Concentrations of TXB₂ and PGE₂ were assessed because they are the major downstream metabolites of the COX pathway in BMEC (Cao et al., 2000). No significant changes in TXB₂ concentrations were detected following endotoxin stimulation or detected following ethyl pyruvate treatment of endotoxin-stimulated BMEC (Figure 5B). In contrast, the production of PGE₂ increased significantly following endotoxin stimulation (Figure 5B). Treating BMEC with ethyl pyruvate resulted in a significant decrease in PGE₂ concentrations compared with the endotoxin-stimulated group (Figure 5B).

Effects of Ethyl Pyruvate on Expression of 15-LOX1

The ability of ethyl pyruvate to modulate endotoxin-induced changes in 15-LOX1 gene expression and en-

Figure 3. Ability of ethyl pyruvate (EP) to diminish endotoxin-induced proinflammatory cytokine gene expression in bovine mammary epithelial cells (BMEC). The BMEC were unstimulated/untreated or stimulated with 50 ng/mL endotoxin for 1 h and treated with 0, 5, or 10 mM EP for an additional 1 h before isolation of RNA. The expression of IL-1 β , IL-6, and IL-8 mRNA transcripts was evaluated by real-time quantitative PCR. Data are expressed as $2^{-\Delta\Delta C_t}$ value using the endotoxin-stimulated BMEC as the calibrator and are representative of means $\pm$ SEM for 7 separate experiments. ^{a-c}Means without a common letter differ significantly ($P < 0.05$) within treatments for individual cytokines.

A. ICAM1 gene expression

B. ICAM1 protein expression

Figure 4. Ability of ethyl pyruvate (EP) to decrease endotoxin-induced adhesion molecule expression by bovine mammary epithelial cells (BMEC). (A) The BMEC were unstimulated/untreated or stimulated with 50 ng/mL endotoxin for 1 h and treated with 0, 5, or 10 mM EP for an additional 1 h before isolation of RNA. The mRNA transcript expression of intercellular adhesion molecule 1 (ICAM1) was evaluated by real-time quantitative PCR. Data are expressed as $2^{-\Delta\Delta C_t}$ using the endotoxin-stimulated BMEC as the calibrator and are representative of means $\pm$ SEM for 7 separate experiments. ^{a,c}Means without a common letter differ significantly ($P < 0.05$) between treatments. (B) The BMEC were unstimulated/untreated or stimulated with 50 ng/mL endotoxin for 1 h and treated with 0, 5, or 10 mM EP for an additional 2 h before isolation of whole cell lysates. Protein expression of ICAM1 was determined by Western blot utilizing a bovine cross-reactive ICAM1 antibody. Data are expressed as the fold increase of ICAM1:actin ratio over the endotoxin-stimulated BMEC ICAM1:actin ratio and are representative of means $\pm$ SEM for 4 separate experiments. ^{a,b}Means without a common letter differ significantly ($P < 0.05$) between treatments.

zyme activity by BMEC was determined via qPCR and ELISA, respectively. A significant increase in 15-LOX1 gene expression was observed following endotoxin stimulation. Treatment of endotoxin-stimulated BMEC with 10 mM ethyl pyruvate significantly reduced 15-LOX1 gene expression to values observed in the control group (Figure 6A). The production of predominant 15-LOX metabolites (15-HETE and 13-HODE) increased only slightly up to 3 h following endotoxin stimulation, peaking at 1 h, whereas ethyl pyruvate treatment yielded no significant effect with these specific 15-LOX1 metabolites (Figure 6B).

DISCUSSION

Ethyl pyruvate is an antiinflammatory agent that was investigated in 2 phase-I human studies showing the ability to inhibit cytokine secretion following endotoxin administration and with few adverse effects (Bennett-Guerrero et al., 2009). The antiinflammatory capabilities of ethyl pyruvate combined with the fact that ethyl pyruvate is safe for human consumption as a food additive (FAO/WHO, 2001) may make it a particularly interesting candidate to reduce the severity of bovine coliform mastitis without severely affecting milk withdrawal times. Coliform mastitis is characterized by an acute and exaggerated inflammatory response due to the release of endotoxin. Endothelial cells are exposed to circulating endotoxin and subsequently play an integral part in the ensuing proinflammatory response as BMEC are not only exposed to proinflammatory mediators produced by activated leukocytes, but are also potent producers of proinflammatory molecules during endotoxin exposure (Harlan et al., 1983b; Braquet et al., 1989; Bannerman and Goldblum, 1999; Corl et al., 2008). Therefore, the effect of ethyl pyruvate treatment on BMEC following endotoxin exposure is of particular interest in the context of controlling bovine coliform mastitis.

This is the first study to examine the potential of ethyl pyruvate as an antiinflammatory agent in a bovine vascular model of endotoxemia. Previous studies showed that ethyl pyruvate is toxic to HUVEC at concentrations greater than 10 mM, but the maximum tolerable dose of ethyl pyruvate was determined for BMEC because of the possibility of different cellular responses dependent upon species and cellular location (Johansson et al., 2008). Based on viability and proliferation assay results, the current study corroborated with the previously mentioned HUVEC study, as 10 mM was the maximum tolerable concentration of ethyl pyruvate in BMEC (Johansson et al., 2008). In vitro studies utilizing other cell types, such as murine macrophages (RAW264.7), also used ethyl pyruvate at

a maximum concentration of 10 mM, suggesting that the tolerable level of ethyl pyruvate may be consistent across species and cell types when evaluated in vitro (Ulloa et al., 2002).

The role of endothelial cells during the pathogenesis of coliform mastitis is complicated because an adequate response is necessary to expedite bacterial clearing, but an exaggerated response may damage the endothelium and surrounding tissues. Several previous coliform mastitis studies documented increased production of proinflammatory cytokines, such as IL-6, IL-8, and IL-1 β , in mammary epithelial cells, mammary leukocyte populations, sera, and milk secretions (Sordillo and Peel, 1992; Shuster et al., 1996; Riollot et al., 2000; Rainard and Riollot, 2006). The data presented here are consistent with previous studies because endotoxin stimulation increased the transcript levels of several acute phase cytokines, including IL-6, IL-8, and IL-1 β in BMEC (Corl et al., 2008). This study showed for the first time that ethyl pyruvate treatment was effective in reducing the endotoxin-induced gene expression of these vascular-derived proinflammatory cytokines. Although not evaluated at the protein level in this study, the potential inhibition of these cytokines is especially important in terms of the vasculature homeostasis considering the ability of both IL-6 and IL-8 to persist in the circulation for extended periods, contributing to both the acute and chronic inflammatory responses (Bannerman, 2009). In humans, IL-6 is considered an accurate marker for inflammation and is known to activate T cells, induce B-cell differentiation, and enhance neutrophil responses (Barton and Jackson, 1993; Jones et al., 2001; Song and Kellum, 2005; Bannerman, 2009). The chemokine functions of IL-8 can attract circulating neutrophils to the site of infection, whereas IL-1 β increases the adhesion of leukocytes by upregulating the expression of vascular adhesion molecules, including ICAM1 and VCAM1. Expression of adhesion molecules is necessary in the extravasation of leukocytes to the site of infection, but their enhanced expression following endotoxin exposure may lead to damage to the microvasculature as a result of a hyperactive proinflammatory response (Bannerman and Goldblum, 1999). Ethyl pyruvate may be a means of controlling the vascular dysfunction that results from enhanced ICAM1 expression because treatment with ethyl pyruvate significantly decreased the endotoxin-induced ICAM1 gene and protein expression in BMEC. Collectively, the changes in cytokine and adhesion molecule gene expression observed in this study agree with several other in vitro and in vivo studies that showed ethyl pyruvate dose-dependently decreased proinflammatory gene expression in the RAW264.7 murine macrophage cell line, HUVEC, and murine mucosa following resuscitation from hemorrhagic shock (Ulloa

A. COX relative gene expression

B. COX metabolite production

Figure 5. Effect of ethyl pyruvate (EP) treatment on cyclooxygenase (COX) gene expression and activity in endotoxin-stimulated bovine mammary epithelial cells (BMEC). (A) The BMEC were unstimulated/untreated or stimulated with 50 ng/mL endotoxin for 1 h and treated with 0, 5, or 10 mM EP for an additional 1 h before RNA collection. The mRNA transcript expression of COX1 (white bars) and COX2 (black bars) was evaluated by real-time quantitative PCR. Data are expressed as 2^{-ΔΔCt} using the endotoxin-stimulated BMEC as the calibrator and are representative of means ± SEM for 7 separate experiments. (B) The BMEC were unstimulated/untreated or stimulated with 50 ng/mL endotoxin for 2 h and treated with 0 or 10 mM EP for an additional 1 h before collection. The activity of COX was measured by evaluating the production of thromboxane B₂ (TXB₂) and prostaglandin E₂ (PGE₂) by enzyme immunoassay. Data are expressed as nanograms of metabolite/5 × 10⁵ cells and are representative of means ± SEM for 4 separate experiments. ^{a-c}Means without a common letter differ significantly (*P* < 0.05) between treatments.

A. 15-LOX1 relative gene expression **B. 15-LOX1 metabolite production**

Figure 6. Effect of ethyl pyruvate (EP) on 15-lipoxygenase 1 (15-LOX1) gene expression and activity in endotoxin-stimulated bovine mammary epithelial cells (BMEC). The BMEC were unstimulated/untreated or stimulated with 50 ng/mL endotoxin for 1 h and treated with 0 or 10 mM EP for an additional 1 h before sample collection. (A) The mRNA transcript expression of 15-LOX1 was evaluated by real-time quantitative PCR. Data are expressed as $2^{-\Delta\Delta C_t}$ using the endotoxin-stimulated BMEC as the calibrator and are representative of means $\pm$ SEM for 5 separate experiments. ^{a,b}Means without a common letter differ significantly ($P < 0.05$) between treatments. (B) The activity of 15-LOX1 was measured by evaluating the production of 15-hydroxy-5,8,11,13-eicosatetraenoate (15-HETE) and 13-hydroxyoctadecadienoic acid (13-HODE) by enzyme immunoassay. Data are expressed as nanograms of metabolite/ 5×10^5 cells and are representative of means $\pm$ SEM for 4 separate experiments.

et al., 2002; Venkataraman et al., 2002; Yang et al., 2002; Johansson et al., 2008). Most previous studies that evaluated the antiinflammatory effects of ethyl pyruvate, however, either pretreated the cells with ethyl

pyruvate or concurrently incubated ethyl pyruvate with proinflammatory agonists (Ulloa et al., 2002; Reade and Fink, 2005; Fink, 2007; Johansson et al., 2008). The current study treated endotoxin-stimulated BMEC

in an attempt to mimic what would occur in a clinical situation, as ethyl pyruvate would likely be administered in response to symptoms of a proinflammatory assault. Our findings are consistent, however, with an *in vivo* murine endotoxemia model in which ethyl pyruvate administration occurred up to 24 h after endotoxin injection and was found to significantly increase survival. The ability of ethyl pyruvate to inhibit the endotoxin-induced upregulation of acute phase cytokines and adhesion molecule expression by BMEC could play a role in avoiding the hyperactive inflammatory response, vascular dysfunction, and tissue damage associated with endotoxemia. These preliminary *in vitro* findings provide the scientific underpinning to explore in more detail how ethyl pyruvate may be used in the treatment of acute coliform mastitis in cows.

Eicosanoids also modulate the inflammatory response and their production is the target of several therapies currently utilized to ameliorate the proinflammatory response associated with endotoxemia. Eicosanoids derived from the COX pathways are readily produced during coliform mastitis (Vangroenweghe et al., 2005). Metabolites of the COX1 pathway are generally responsible for the maintenance of various physiological properties, whereas most COX2 metabolites are considered proinflammatory. Ethyl pyruvate treatment significantly decreased the endotoxin-induced gene expression of COX2 in BMEC, agreeing with previous findings in murine mucosa following resuscitation from hemorrhagic shock (Yang et al., 2002; Hauser et al., 2005; Fink, 2007). As expected, the transcript expression of the constitutively expressed COX1 remained stable in BMEC following endotoxin stimulation and ethyl pyruvate treatment. The endotoxin-induced increase in transcript expression of COX2 coincided with a significant increase in the production of a downstream metabolite, PGE₂. The production of PGE₂ is increased during coliform mastitis and is important in regulating vascular function by inducing vasodilation (Anderson et al., 1985). Ethyl pyruvate significantly reduced the production of PGE₂ compared with peak concentrations in the endotoxin-stimulated BMEC. The ability of ethyl pyruvate to selectively inhibit COX2 mRNA expression and some downstream metabolites is exciting because most current nonsteroidal antiinflammatory drugs used in food animals, such as carprofen, are nonselective COX1 and COX2 inhibitors or require pretreatment to be effective in the inhibition of COX2 in coliform challenge models (Vangroenweghe et al., 2005). Most current antiinflammatory therapeutics focus on inhibition of the COX pathways without regard to subsequent effects on the LOX pathways. Of specific interest to the vascular model of endotoxemia used for this study is 15-LOX1, which is the predominant lipoxygenase in

endothelial cells and whose metabolites were increased in a porcine model of endotoxemia (Olson et al., 1995). In the BMEC model, ethyl pyruvate treatment significantly decreased the endotoxin-induced upregulation of 15-LOX1 gene expression. Some metabolites of 15-LOX1 have detrimental proinflammatory effects on the vasculature because they increase the expression of proinflammatory mediators and may initiate apoptosis at increased concentrations (Sordillo et al., 2005, 2008). The immediate downstream metabolites of 15-LOX1, 15-HETE and 13-HODE, are especially associated with vascular dysfunction in several inflammatory-based disease models. Although endotoxin-induced production of 15-HETE and 13-HODE did not increase significantly in this study, it is possible that other downstream mediators are increased or that these metabolites are affected at times different from the limited *in vitro* sample schedule. Future studies would benefit from a full lipidomics analysis in response to ethyl pyruvate to identify the specific vascular-derived proinflammatory and resolving eicosanoids that may be modulated following endotoxin stimulation over an extended period.

This is the first study to demonstrate the antiinflammatory capabilities of ethyl pyruvate in an *in vitro* model of coliform mastitis utilizing mammary endothelial cells. These preliminary studies suggest that ethyl pyruvate may have an effect on the severity of acute coliform mastitis as both an antiinflammatory agent and a selective COX2 and 15-LOX1 inhibitor. The ability of ethyl pyruvate to decrease the gene expression of several proinflammatory mediators produced by endotoxin-stimulated BMEC provides the scientific underpinning to assess *in vivo* efficacy in the future. Ethyl pyruvate may prove to be a novel therapeutic to ameliorate the exaggerated inflammatory response and subsequent pathology associated with coliform mastitis.

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