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Exogenous phospholipase A2 affects inflammatory gene expression in primary bovine mammary epithelial cells

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Research Article

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Abstract

This Research Communication addresses the hypothesis that exogenously administered phospholipase A2 (PLA2) affects the inflammatory responses of bovine mammary epithelial cells (bMEC) in vitro with the aim of providing preliminary justification of investigation into the uses of exogenously administered PLA2 to manage or treat bovine mastitis. Primary bMEC lines from 11 lactating Holstein dairy cows were established and the expression of 14 pro-inflammatory genes compared under unchallenged and lipopolysaccharide (LPS)-challenged conditions, with and without concurrent treatment with bovine pancreatic PLA2G1B, a secreted form of PLA2. No differences in the expression of these genes were noted between PLA2-treated and untreated bMEC under unchallenged conditions. Following LPS challenge, untreated bMEC exhibited significant downregulation of *CXCL8*, *IL1B*, *CCL20*, and *CXCL1*. In contrast, PLA2-treated bMEC exhibited significant downregulation of *IL1B* and *CCL20* only. These findings indicate that exogenous PLA2 affects the expression of some pro-inflammatory factors in immune-stimulated bMEC, but does not influence the constitutive expression of these factors. Further investigation of the influence of exogenous PLA2 in the bovine mammary gland is justified.

Bovine mastitis has a major impact on the dairy industry worldwide, in large part due to the substantial costs associated with decreased milk yield and altered milk production among affected cows. Changes in the production capacity of the bovine mammary gland and alterations in milk composition result from damage to bovine mammary epithelial cells (bMEC), the milk-producing compartment. Tissue damage during mastitis can be caused by direct effects of mastitis pathogens on bMEC, but host immune response-induced cellular injury often contributes substantially to tissue damage during inflammation. Thus, in addition to pathogen clearance, minimizing host-induced tissue damage is an important factor in the resolution of inflammatory diseases such as bovine mastitis.

Modulation of the endogenous enzyme phospholipase A2 (PLA2) has been proposed as a therapeutic approach in the treatment of a number of inflammatory diseases (Yedgar *et al.*, 2000). The PLA2 superfamily of enzymes is involved in several cellular processes in many tissue types, including promotion and regulation of inflammation *via* generation of lipid mediators. Because of the involvement of PLA2 in cellular processes such as normal phospholipid metabolism, universal PLA2 modulation has potentially detrimental effects, including impairment of cell viability. Therefore, selective modulation of specific PLA2 subtypes involved most heavily in inflammation, such as secreted PLA2 (sPLA2), may be a more optimal approach (Yedgar *et al.*, 2000).

Secreted PLA2 has both pro-inflammatory and anti-inflammatory effects, but little information is available on the role of endogenous or exogenously administered PLA2 in the pathogenesis of bovine mastitis. Effects of sPLA2 noted in other species and tissues may be extrapolated to the bovine mammary gland only tentatively, as variation in PLA2-receptor-binding activity is noted among different mammalian species (Hanasaki, 2004). Although pro-inflammatory PLA2 activity contributes to inflammation-induced damage in many diseases, there is some evidence for an overall anti-inflammatory effect of sPLA2 in mastitis. In a mouse model of bovine mastitis, intramammary administration of bovine PLA2G1B, an sPLA2, in mice with experimental, LPS-induced mastitis reduced inflammation despite displaying no bactericidal activity (Seroussi *et al.*, 2013). Whether PLA2 in the bovine mammary gland during mastitis has an overall pro- or anti-inflammatory effect has yet to be described.

The aim of this study was to determine the effects of exogenously administered PLA2 on inflammatory responses of primary bMEC. We hypothesized that exogenously administered

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PLA2 would alter the expression of inflammatory genes by bMEC in the absence of an inflammatory stimulus, bMEC exposed to an inflammatory stimulus, or both.

Methods

The use of animals in this study was approved by the Utah State University Institutional Animal Care and Use Committee (protocol IACUC-2282), and permission was obtained from the cattle owner.

Bovine MEC collection, isolation, and establishment

Milk-derived primary bMEC lines were established from adult lactating Holstein dairy cows from a commercial dairy farm in northern Utah. All cows were free from clinical mastitis at the time of cell collection. Criteria for an absence of clinical mastitis were an absence of visible inflammation of the mammary gland and an absence of visible changes in the color or consistency of the milk at the time of cell collection, as assessed by a veterinarian.

Bovine MEC isolation and primary cell line establishment were carried out as previously reported (Wellnitz *et al.*, 2006), with modifications. Four hundred mL of hand-stripped milk was subjected to a series of wash and centrifugation steps (3000 rpm for 10 min each) followed by passage of the re-suspended cell pellet through a 100 µm pore size cell strainer to separate cell pellets from debris and other milk components. Following a final centrifugation step, the cell pellet was suspended in 5 ml of growth medium consisting of HuMEC Ready Medium (Life Technologies, Grand Island, NY) supplemented with penicillin (60 µg/ml), streptomycin (200 µg/ml), gentamycin (120 µg/ml), nystatin (50 µg/ml), and 10% fetal bovine serum, and seeded into a T-25 culture flask. Cells were incubated at 38.5 °C with 5% CO_2. After 12–18 h, the media was exchanged for fetal bovine serum-free growth media. Growth medium was changed every 2–3 d thereafter.

Primary bMEC were passaged when confluence was reached using trypsin 0.05%/EDTA 0.02% (Sigma-Aldrich, St. Louis, MO). At the second passage, cell lines were split into 8, 34.8 mm diameter cell culture wells and assigned in duplicate to four groups: untreated, unchallenged; untreated, LPS-challenged; PLA2-treated, unchallenged; and PLA2-treated, LPS-challenged. Treatments with PLA2 and/or challenge with LPS and RNA isolation were performed once bMEC reached 50–100% confluence at the third passage.

Lipopolysaccharide from *Escherichia coli* was used to stimulate an inflammatory response by bMEC. Lipopolysaccharide challenge methods were similar to those described previously (Pareek *et al.*, 2005), with some modifications. Briefly, growth medium was exchanged for medium of the same composition but with or without 50 µg/ml LPS from *E. coli* (Sigma-Aldrich, St. Louis, MO) and/or 50 µg/ml PLA2G1B from bovine pancreas (product P8913, Sigma-Aldrich, St. Louis, MO). Cells were incubated at culture conditions for 6 h, after which medium was removed. Cells were lysed, and total RNA was isolated and purified using the Purelink RNA isolation kit (Life Technologies). Quality of RNA was determined using the BioAnalyzer (Agilent Technologies, Santa Clara, CA). Complimentary DNA was generated using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), with RT-PCR conditions as follows: primer annealing at 25 °C for 10 min, reverse transcription at 42 °C for 1 h, and enzyme inactivation at 85 °C for 5 min.

Cell lineage verification

Verification of epithelial lineage of bMEC was achieved using observation of cell morphology and expression of *KRT8*. Observations of cell morphology were made at P_0 and subsequent passages by a veterinary pathology resident using phase-contrast microscopy. Colonies that demonstrated a cobblestone pattern comprised of polygonal cells with round to oval nuclei typical of epithelial cells were considered likely to be of bMEC origin. Expression of *KRT8* was determined *via* polymerase chain reaction (PCR). Primers were designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) based on NCBI reference sequence NM_001033610.1 and were as follows: forward primer 5′-AATCAAGTATGAGGAGCTGC-3′; reverse primer 5′-CATCCTTAACAGCCATCTCA-3′. Polymerase chain reactions were as follows: initial denaturation at 98 °C for 30 s followed by 32 cycles of 98 °C for 10 s, 57 °C for 15 s, and 72 °C for 30 s. Gel electrophoresis was used to confirm the presence of PCR product. Sequencing of PCR product to confirm *KRT8* amplification was carried out by the Center for Integrated Biotechnology, Utah State University (Logan, UT).

Quantitative PCR for gene expression

Relative expression of 14 pro-inflammatory genes were assessed *via* quantitative PCR (qPCR). Genes assessed included *TLR4*, *IL6*, *IL33*, *CXCL8*, *IL1A*, *IL1B*, *IL2RG*, *IL1RN*, *IL1R1*, *CXCL5*, *CXCL8*, *CCR2*, *CCL20*, and *CXCL1*. Primers were designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) (online Supplementary Table S1). Efficiency of all primers was between 80–110%.

Quantitative PCR was performed by the Utah State University Center for Integrated Technology (Logan, UT) using Fluidigm Biomark technology and EvaGreen detection chemistry. The ΔΔCt normalization method was used for relative quantification as implemented by Fluidigm Real-Time PCR Analysis software using *GAPDH* as a reference gene.

Statistical analysis

Statistical analyses were performed using the mixed model procedure of SAS (Version 9.4, SAS Institute, Cary, NC, USA). The model included the dependent variable of the mean CT (cycle threshold) value of two experimental replicates for each treatment group of each cell line individually. Independent variables included gene, treatment, and a two way interaction of cell line * trt. The LSMEANS function and a pre-planned pairwise comparisons procedure were used to evaluate if significant differences existed between treatments and the means of the three way interaction. Significance in the model was set at $P < 0.05$.

Results and discussion

Cell lines and qPCR

Primary bMEC lines were successfully established from 11 lactating Holstein cows at various stages of lactation. The cellular morphology of all cell lines was consistent with epithelial cells on monolayer culture, and all cell lines expressed *KRT8* as

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Table 1. Mean CT (cycle threshold) values and *P*-values for genes differentially expressed between unchallenged and LPS-challenged bMEC with and/or without PLA2 treatment

Gene	*n*	CT, Control	CT, LPS	*P*-Value	SE	CT, PLA2	CT, PLA2 and LPS	*P*-Value	SE
IL1B	9	20.7	16.2	<0.01	0.7	20.9	16.2	<0.01	0.7
CXCL8	10	12.0	9.7	0.03	1.0	11.89	9.4	0.35	1.3
CCL20	10	17.5	11.2	<0.01	1.0	16.4	10.9	<0.01	1.0
CXCL1	10	12.7	10.3	<0.01	0.9	11.9	10.1	0.06	0.9

bMEC: milk-derived bovine mammary epithelial cells.
LPS: 50 µg/ml lipopolysaccharide from *E. coli*.
PLA2: 50 µg/ml phospholipase A2G1B.

determined by PCR. Quality of RNA for all cell lines had an RNA integrity number (RIN) > 7.0. For each gene, cell lines for which qPCR reactions failed within any treatment group were excluded from analysis of expression of that gene, but at least 9 cell lines were used for each gene. Table 1 shows the sample size for each gene along with mean CT values and *P*-values for each of the treatment groups.

Effects of PLA2 on pro-inflammatory gene expression by bMEC

Based on mean CT values, 4 of the 14 genes were significantly differentially expressed between unchallenged and LPS-challenged bMEC among non PLA2-treated cell lines, and 2 genes among PLA2-treated cell lines. Both *IL1B* ($n = 9$) and *CCL20* ($n = 10$) were significantly ($P < 0.05$) lower in LPS-challenged bMEC relative to unchallenged bMEC, regardless of PLA2 treatment, while *CXCL8* ($n = 10$) and *CXCL1* ($n = 10$) were significantly ($P < 0.05$) lower in only LPS-challenged, untreated bMEC. Downregulation, rather than upregulation, of these pro-inflammatory genes in LPS-challenged bMEC was somewhat unexpected, as other studies have demonstrated their upregulation during bovine mastitis in the mammary gland or other tissues (Persson Waller *et al.*, 2003; Jiang *et al.*, 2008; Rinaldi *et al.*, 2010). Nevertheless, these findings demonstrate an effect of exogenously administered sPLA2 on the expression of several pro-inflammatory genes following LPS challenge but not on constitutive expression of these genes.

The presence of the pro-inflammatory chemokine ligand CCL20 has been demonstrated in human milk early in lactation (Lourenço *et al.*, 2017), suggesting a potential role in mammary gland defences. In cattle, upregulation of *CCL20* by the liver occurs in conjunction with leukocytosis following intramammary LPS challenge (Jiang *et al.*, 2008). The present study demonstrates basal expression of *CCL20* by primary bMEC as well as its downregulation by LPS challenge, regardless of the presence of exogenously administered sPLA2. Similarly, the expression of *IL1B*, a pro-inflammatory cytokine present in the bovine mammary gland during the acute stages of mastitis caused by a number of mastitis pathogens (Persson Waller *et al.*, 2003), was not affected by PLA2 treatment.

Downregulation of *CXCL8* in the current study did not occur following LPS challenge in PLA2-treated bMEC, in contrast to untreated bMEC, where downregulation of this gene did occur following LPS challenge. Interleukin-8 is upregulated in the mammary gland during mastitis (Persson Waller *et al.*, 2003) and in bMEC in vitro in response to mastitis pathogens (Lahouassa *et al.*, 2007). Correlations between endogenous PLA2 and CXCL8 levels may exist in some tissues during inflammatory diseases. For example, suppression of PLA2-II during pancreatitis in rats resulted in decreased serum levels of CXCL8 and other pro-inflammatory cytokines (Zhang *et al.*, 2013). Studies investigating a potential similar correlation are lacking in bovine mammary tissue. Our results show that exposure to increased levels of PLA2 *via* exogenous administration may affect *CXCL8* expression by bMEC following LPS challenge. However, PLA2 treatment alone, in the absence of LPS, was not sufficient to alter *CXCL8* expression in these cells. Further studies are needed to demonstrate whether endogenous levels of PLA2 are correlated to levels of cytokines such as CXCL8 in the bovine mammary gland.

Similarly, downregulation of *CXCL1* in responses to LPS challenge did not occur in PLA2-treated bMEC. Upregulation of *CXCL1* has been demonstrated in the bovine mammary gland during mastitis (Rinaldi *et al.*, 2010) and in a murine model of bovine mastitis following LPS challenge (Zheng *et al.*, 2006). Similar to *CXCL8*, *CXCL1* appears to be an important factor in neutrophil chemotaxis during bovine mastitis and its expression by primary bMEC following LPS challenge is influenced by treatment with exogenous PLA2.

A reason for downregulation of these pro-inflammatory genes following LPS challenge is unknown. These findings are in contrast to other studies discussed above, where these cytokines were found to increase during mastitis. However, many of these studies examined cytokine production within the mammary gland as a whole rather than among bMEC alone. Other cell types, such as resident leukocytes and mesenchymal cells, contribute to cytokine production within the mammary gland during mastitis and may explain these divergent results. Our finding of decreased cytokine production among bMEC exposed to an inflammatory stimulus may reflect differences in the role of bMEC in the production of these specific cytokines during mastitis when compared to other cell types. Given our finding that PLA2 may affect the expression of select cytokines by bMEC, further studies on the effects of PLA2 on other mammary gland cell types or the mammary gland as a whole are warranted.

In support of our hypothesis, the results of this study show that exogenously administered sPLA2 may inhibit the differential expression of two neutrophil chemotactic factors, *CXCL8* and *CXCL1*, by primary bMEC following exposure to LPS. Based on mean CT values, these cytokines were downregulated following LPS challenge in cell lines not treated with PLA2, but did not show differential expression following LPS challenge in PLA2-treated cell lines. This finding indicates a role for sPLA2 in the modulation of inflammation by an important cell type in the bovine mammary gland, and further studies into its possible uses in the prevention, management, or treatment of bovine mastitis are justified.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029919000232.

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