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Quantification of bovine oxylipids during intramammary *Streptococcus uberis* infection

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ABSTRACT

Streptococcus uberis mastitis results in severe mammary tissue damage in dairy cows due to uncontrolled inflammation. Oxylipids are potent lipid mediators that orchestrate pathogen-induced inflammatory responses, however, changes in oxylipid biosynthesis during *S. uberis* mastitis are unknown. Thus, the current objective was to determine how oxylipid concentrations change in milk and mammary tissues during different stages of *S. uberis* mastitis. Increased arachidonic acid and linoleic acid-derived oxylipids were significantly increased in *S. uberis*-infected bovine mammary tissue. Linoleic acid metabolites, hydroxyoctadecadienoic acid (HODE) and oxooctadecadienoic acid, predominated in tissue and milk. Furthermore, in vitro exposure of bovine mammary endothelial cells to 13-hydroperoxyoctadecadienoic acid, upstream metabolite of HODE, significantly increased cyclooxygenase-2 expression, but 13-HODE exposure had no effect. The findings in the current study indicate lipidomic profiling may explain some of the dynamics of inflammation during bacterial challenge, however continued research is necessary to determine sample compartments which best reflect disease pathogenesis.

1. Introduction

Mastitis, an inflammation of the mammary gland, negatively impacts the U.S. dairy industry by reducing milk yield and quality [1]. Whereas many pathogens cause bovine mastitis, *Streptococcus uberis* is a major pathogen of concern as it results in severe tissue damage and significant milk production losses related to an ineffective inflammatory response [2]. Mastitis causes by *S. uberis* infections present as acute or subclinical, which may persist as chronic infections with associated inflammation [3]. The major cause of *S. uberis*-induced pathology is the sustained migration of leukocytes into the secretory tissue resulting in irreversible damage. Histopathological data from *S. uberis*-infected mammary tissue indicate that colonization of *S. uberis* can survive aggressive neutrophil infiltration into mammary tissues and cause the development of chronic disease [4,5]. The underlying mechanisms regulating the initiation and resolution of mammary gland inflammatory responses to *S. uberis* are unclear. Previous research in bovine mastitis suggests that the composition of oxylipid profiles within affected tissues may regulate the severity and duration of the localized inflammatory response. For example, the milk lipoxin A_4 (LXA_4) to leukotriene B_4 (LTB_4) ratio was lower in cows suffering from chronic mastitis compared to healthy cows [6]. Similarly, the concentration of arachidonic acid (AA)-derived oxylipids, such as prostaglandins and thromboxane, significantly increased during clinical mastitis [7,8]. As such, the ability of the host to respond quickly and eliminate mastitis-causing pathogens may be directly influenced by the production of pro- and anti-inflammatory oxylipids [1,6,9]. Though previous research has quantified a limited number of oxylipids during clinical mastitis, it is now recognized that the lipidome is extensive and complex. Advancing the current understanding of the bovine lipidome can be used to develop novel, targeted therapies to control tissue damage related to an unregulated inflammatory response, such as mastitis.

Predominant fatty acid substrates used for oxylipid biosynthesis in both humans and dairy cows are polyunsaturated fatty acids (PUFA), including AA and linoleic acid (LA) [10,11]. Fatty acid substrates are cleaved from the phospholipid membrane by phospholipase A_2, and may undergo non-enzymatic and enzymatic oxidation. Non-enzymatic pathways are primarily mediated by reactive oxygen species (ROS) and free radicals formed as end products of the electron transport chain and as byprod-

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ucts of phagocyte-associated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [12]. In general, non-enzymatic oxidation of lipids occurs by a hydroxyl radical attack of hydrogen and subsequent O_2 interaction to form a lipid peroxyl radical, which can damage cell membranes and propagate lipid peroxidation [13,14]. Enzymatic pathways that predominantly catalyze oxylipid biosynthesis are cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome (CYP). The COX and LOX enzymes oxidize PUFA by removing a hydrogen, allowing 2 O_2 to interact forming a lipid hydroperoxide [15]. Intrinsic peroxidase activity of COX enzymes reduces AA-derived PGG_2 to the hydroxyl, PGH_2 and subsequent enzymatic metabolism yields several different prostaglandins and thromboxanes [15]. Linoleic acid-derived hydroperoxides are reduced to hydroxyl oxylipids by antioxidant mechanisms, such as glutathione peroxidases or non-enzymatic quenching [16–18]. Hydroxyl oxylipids can then be enzymatically dehydrogenated to ketones [19,20]. Oxidation of AA by LOX also synthesizes leukotrienes and lipoxins, which require the coordination of multiple enzymes, i.e., LXA_4 biosynthesis requires 15-LOX, 5-LOX, and epoxide hydrolases [21]. Lastly, CYP catalyzes a NADPH-dependent monooxygenation of AA and subsequent metabolism to form several oxylipids including 12-HETE, epoxyeicosatrienoic acids, and dihydroxyeicosatrienoic acids.

Given the breadth of oxylipid species and limited research in bovine models, it is difficult to pinpoint predominant pathways during bovine disease. Inflammatory-based disease models in humans suggest that oxylipid therapy may be used to control unregulated inflammation in veterinary species as well [22]. Determining how a targeted bovine lipidome changes during disease may be used to assess disease progression and severity. Previous research supports the predominance of LA as a substrate for oxidation, suggesting an abundance of LA-derived oxylipids during health and disease [10,11]. The initial product of LA oxidation is hydroperoxyoctadecadienoic acid (HPODE) and the sequential metabolism of HPODE to hydroxyoctadecadienoic acid (HODE) to oxooctadecadienoic acid (oxoODE) synthesizes oxylipids that may have very different inflammatory functions. For example, 13-HPODE is associated with increased adhesion molecules and apoptosis, whereas 13-oxoODE is a potent ligand for an anti-inflammatory nuclear receptor [19]. Additionally, different isoforms of LA-derived metabolites are synthesized depending on the mode of oxidation. Oxidation of LA by 15-LOX-1 primarily yields 13-HPODE, whereas 9-HPODE is primarily produced during non-enzymatic oxidation [23]. However, HPODE is rapidly reduced to stable HODE, and thus, are measured during disease. For example, 9-HODE and 13-HODE are predominant in plasma from healthy individuals and may be increased during oxidative stress and inflammatory-based diseases, such as atherosclerosis [11,24]. The purpose of this study was to document oxylipid changes in the milk and mammary tissue of dairy cows during different stages of *S. uberis* mastitis. The hypothesis was that LA metabolites, 9- and 13-HODE, are increased during *S. uberis* mastitis and contribute to an inflammatory phenotype in endothelial cells.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade acetonitrile, HPLC-grade methanol, formic acid, sodium selenite, insulin, heparin, transferrin, ethylenediaminetetraacetic acid (EDTA), triphenylphosphine (TPP) were purchased from Sigma–Aldrich (St. Louis, MO). Diethyl ether and butylated hydroxy toluene (BHT) were purchased from ACROS Organics (Fair Lawn, NJ). Antibiotic/antimycotic and all bovine Taqman® primers were purchased from Thermo Fisher Scientific (Waltham, MA). Deuterated and nondeuterated standards were purchased from Cayman Chemical (Ann Arbor, MI). Indomethacin was from Cayman Chemical (Ann Arbor, MI). Magnesium sulfate was purchased from Avantor Performance Materials, Inc. (Central Valley, PA) and sodium borate from Fisher Science Education (Nazareth, PA). Fetal bovine serum was purchased from Hyclone Laboratories, Inc. (Logan, Utah).

Table 1
Bovine primers for qRT-PCR.

Gene[a]	Reference sequence	TaqMan® assay ID[b]
RPS9	NM_001101152.2	Bt03272016_m1
PGK1	NM_001034299.1	Bt03225857_m1
ACTB	NM_173979.3	Bt03279174_g1
ICAM-1	NM_174348.2	Bt03213911_m1
VCAM-1	NM_174484.1	Bt03279189_m1
E-selectin	NM_17418.2	Bt03213082_m1
IL-6	NM_173923.2	Bt03211905_m1
IL-8	NM_173925.2	Bt03211906_m1
IL-1β	NM_174093.1	Bt03212741_m1
15-LOX-1	NM_174501.2	Bt03214775_m1
15-LOX-2	NM_001205703.1	Bt04284773_m1
5-LOX	NM_001192792.1	Bt04297609_m1
COX-1	NM_001105323.1	Bt03817775_m1
COX-2	NM_174445.2	Bt03214492_m1

[a] RPS9 = ribosomal protein S9; PGK1 = phosphoglycerate kinase; ACTB = β-actin; ICAM-1 = intercellular adhesion molecule-1; VCAM-1 = vascular adhesion molecule-1; 15-LOX-1 = 15-lipoxygenase-1; 15-LOX-2 = 15-lipoxygenase-2; 5-LOX = 5-lipoxygenase; COX-1 = cyclooxygenase-1; COX-2 = cyclooxygenase-2.
[b] Life Technologies, Carlsbad, CA.

2.2. Streptococcus uberis *0140J challenge for quantification of oxylipids in mammary tissue*

The National Animal Disease Center animal care and use committee approved all animal-related procedures used in this study (Protocol ARS-2620). Five healthy, mid-lactation Holstein dairy cows were infused with an average of approximately 600 CFU of *S. uberis*, strain 0140J into 1 mammary gland. The dose of *S. uberis* was based on previous preliminary experiment with this strain, which gave a reproducible infection rate of >95%. The mammary gland was cleaned with water to remove all debris and then dried thoroughly. The cow was milked and then the teat end was disinfected with a 70% ethanol solution and allowed to dry. Using a sterile teat cannula, bacteria were infused into one mammary gland. After infusion, the teat end was held closed and the bacterial inoculum was massaged upward into the main cistern. The mammary gland was further massaged to ensure distribution of the inoculation. All teats were then dipped in a standard iodine-based teat dip. Challenged cows were monitored symptoms of clinical disease including increased rectal temperature, increased somatic cell count in milk, and abnormalities in mammary gland and milk appearance. Mammary gland abnormalities included pain, swelling, heat, redness, or hardening of the mammary gland and milk abnormalities included a watery consistency, flakes, clots, or pus. At the onset of clinical symptoms, cows were euthanized and parenchymal mammary tissue was excised and either placed in a sterile vial alone (oxylipid quantification) or in RNAlater (mrNA quantification) and snap frozen in liquid nitrogen (Qiagen, Venlo, Limburg). Samples were stored at −80 °C (oxylipid quantification) or −20 °C until processing. Adhesion molecule, proinflammatory cytokine, and oxylipid biosynthetic enzyme mRNA expression was quantified by qRT-PCR using the RNeasy Mini Kit (Qiagen, Venlo, Limburg) following the manufacturer's protocol. Samples were run in triplicate with ribosomal protein S9 (RPS9) as the endogenous control. Gene expression was calculated using the ΔCt method for statistical analysis and also using the $2^{-\Delta\Delta Ct}$ method for graphical purposes [25]. All bovine primers are displayed in Table 1.