VITAMIN E ANALOGS EXHIBIT ANTIOXIDATIVE FUNCTIONS AND INHIBIT PRODUCTION OF A CYTOCHROME P450 DERIVED OXYLIPID

By

Matthew Joseph Kuhn

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ABSTRACT

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Preventing and controlling disease during the transition period in cattle is the most significant health challenge facing veterinarians and farm managers. In initial studies, it was found that cattle have increased biomarkers of oxidative stress around the time of calving in addition to significant shifts in the abundance of pro-inflammatory lipid inflammatory mediators, known as oxylipids, compared to other stages of lactation. These factors, brought upon by the intense physiological shifts around the time of calving, can become detrimental and contribute to disease predisposition during the transition period in animals that do not properly adapt to such physiological changes. There is a significant need to find means to reduce oxidative stress and the production of pro-inflammatory lipid mediators at this time. One potential way is by augmenting the well-known effects of α-tocopherol with other analogs of vitamin E which have been shown in other species to have antioxidant functions and share a metabolic pathway with the pro-inflammatory oxylipid 20-HETE. Little is known about this shared metabolic pathway, specially the cytochrome P450 family 4 sub-family F member 2 enzyme, in dairy cattle. Additionally, many other cytochrome P450 enzymes which are involved in the production of other oxylipids and metabolism of fat-soluble vitamins essential to appropriate immune regulation remain mostly unexplored in cattle. A range of such cytochrome P450 enzymes was explored in both bovine tissues and common cell culture models used for oxidative stress and inflammation modeling. These data revealed which models may best represent specific cytochrome P450 metabolic pathways in vivo. A bovine mammary endothelial cell model of oxidative stress was utilized to

determine the antioxidative effects of vitamin E analogs in vitro. Gamma-tocopherol was found to not only reduce the accumulation of reactive metabolites but protected cellular health by reducing apoptosis and protecting the endothelial barrier integrity. Further, γ-tocopherol reduced the production of 20-HETE produced from human and bovine-kidney cytochrome P450 microsomes, likely by competitive inhibition. These data provide credence to further explore the functions of γ-tocopherol and other non-α-tocopherol analogs of vitamin E in cattle. Overall, this dissertation underscores the challenges faced by dairy cattle during the transition period and highlights gaps in knowledge left to be explored regarding the production of inflammatory mediators, especially those from the cytochrome P450 pathway. Given the relative safety of feeding mixed tocopherols to dairy cattle, further efforts should be undertaken to confirm these initial safety assessments and aim to understand their potential contributions to the prevention of oxidative stress and dysfunctional inflammation during the transition period.

This dissertation is dedicated to my wife, who has never faltered in her support of my passions and dreams, no matter their obscurity.

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KEY TO ABBREVIATIONS

1,25-$(OH)_2D_3$	1,25-dihydroxyvitamin D_3
2-AG	2-Arachidonylglycerol
20-HETE	20-hydroxyeicosatetraenoic acid
25-$(OH)D_3$	25-hydroxyvitamin D_3
αT	α-tocopherol
αTTP	α-tocopherol transport protein
AA	Arachidonic acid
AEA	Anandamide (arachidonoylethanolamine)
AOP	Antioxidant potential
AOR	Antioxidant reducing agent
AT-RA	All-trans retinoic acid
AUC	Area under the curve
BAEC	Bovine aortic endothelial cells
BHT	Butylated hydroxytoluene
BMEC	Bovine mammary endothelial cells
CDMD$(en)_2$HC	Carboxydimethyldecadienylhydroxychromanol
CDMHenHC	Carboxymethylhexenylhydroxychromanol
CDMOenHC	Carbodimethyloctenylhydroxychromanol
CDMO$(en)_2$HC	Carbodimethyloctdienylhydroxychromanol
CDMOHC	Carboxymethyloctylhydroxychromanol
CEHC	Carboxyethylhydroxychromanol
CMBHC	Carboxymethylbutylhydroxychromanol

CMBenHC	Carboxymethylbutadienylhydroxychromanol
COOH	Carboxychromanol
COX	Cyclooxygenase
CT	Cycles to threshold
CYP2C	Cytochrome P450 family 2 sub-family C
CYP26	Cytochrome P450 family 26
CYP4	Cytochrome P450 family 4
CYP450	Cytochrome P450
DHA	Docosahexaenoic
DHET	Dihydroxyeicosatrienoic acid
DetaNO	(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
DiHOME	Dihydroxyoctadecenoic acid
DIM	Days in milk
DMEM	Dulbecco's Modified Eagle Media
ECIS	Electrical Cell-substrate Impedance Sensing
EDTA	Ethylenediaminetetraacetic acid
EET	Epoxyeicosatrienoic acid
EPA	Eicosapentaenoic acid
EPHX2	Soluble epoxide hydrolase
EpOME	Epoxyoctadecenoic acid
FBS	Fetal bovine serum
γT	γ-tocopherol

γT3	γ-tocotrienol
GC/MS	Gas chromatography mass spectrometry
H_2O_2	Hydrogen peroxide
HBSS	Hank's balanced salt solution
HETE	Hydroxyeicosatetraenoic acid
HODE	Hydroxyoctadecadienoic acid
HpETE	Hydroperoxyeicosatetraenoic acid
HPLC	High performance liquid chromatography
HpODE	Hydroperoxyoctadecadienoic acid
IACUC	Institutional animal care and use committee
IL	Interleukin
LA	Linoleic acid
LC/MS/MS	Liquid chromatography tandem mass spectrometry
LDH	Lactate dehydrogenase
LL	Late-lactation
LTB4	Leukotriene B4
LOX	Lipoxygenase
LXA4	Lipoxin A4
MAC-T	Immortalized bovine mammary epithelial cells
MDBK	Madin-Darby bovine kidney epithelial cells
MeOH	Methanol
ML	Mid-lactation
MRM	Multiple reaction monitoring

NAD	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified fatty acid
NIFA	National Institute of Food and Agriculture
NRC	National Research Council
OH	Hydroxychromanol
OSi	Oxidant stress index
OxoETE	Oxoeicosatetraenoic acid
OxoODE	Oxooctadecadienoic acid
PGE2	Prostaglandin E2
PMN	Polymorphonuclear neutrophil
PP	Periparturient
PUFA	Polyunsaturated fatty acid
RA	Retinoic acid
RFU	Relative fluorescence units
RNS	Reactive nitrogen species
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
sEH	Soluble epoxide hydrolase
SEM	Standard error of the mean
SOD2	Superoxide dismutase 2
STOH	Sub-terminal ω-hydroxylase
TCOH	Terminal carbon ω-hydroxylase

TPP	Tetraphenylporphyrin
UV	Ultraviolet

CHAPTER 1

REVIEW: CYTOCHROME P450 ENZYME INVOLVEMENT IN HEALTH AND INFLAMMATORY-BASED DISEASES OF DAIRY CATTLE

Matthew J. Kuhn,[1] and Lorraine M. Sordillo*[1]

[1]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing 48824, United States

***Corresponding author**

Dr. Lorraine M. Sordillo,

Department of Large Animal Clinical Sciences

College of Veterinary Medicine

Michigan State University

sordillo@msu.edu

Tel: (517) 432-8821

Fax: (517) 432-8822

ABSTRACT

Compromised immune responses and oxidative stress predispose some dairy cattle to disease around the time of calving. Previous studies have shown that potent lipid inflammatory mediators, or oxylipids, and fat-soluble vitamins with antioxidant functions can alter the effectiveness of the immune system during the transition period. The cytochrome P450 (CYP450) family of enzymes is involved in both the production of oxylipids and regulation of fat-soluble vitamin concentrations. For example, CYP450 pathways can metabolize fatty acids to form a group of pro- and anti-inflammation oxylipids that influence the overall immune homeostasis on a local and systemic scale. Additionally, CYP450 participate in the activation of vitamin D and degradation of vitamins A, D, and E, which influence antioxidant defense mechanisms. This review will detail the known contributions of bovine CYP450 to the regulation of oxylipids and the fat soluble vitamins A, D, and E. Although the activity of specific CYP450 is generally conserved amongst mammals, important differences exist in cattle, such as the isoforms primarily responsible for activation of vitamin D. Additionally, a CYP450 driven inflammatory positive feedback loop is proposed, which may contribute to the dysfunctional inflammatory responses commonly found during the transition period. Establishing the individual contributions of CYP450 to the biosynthesis of oxylipids and the regulation of vitamins A, D, and E may reveal how this family of enzymes can impact inflammatory responses during times of increased susceptibility to disease. Determining the potential impact of each CYP450 on disease or disease predisposition allows for informed development of targeted CYP450 inhibitors or inducers. By manipulating specific CYP450 isoforms, nutrition of dairy cattle can be more precisely tailored to the oxidative and immune status of cows at greatest risk for health disorders during the transition period.

Key words*:* cytochrome p450, dairy cow, oxylipid, hydroxylase, transition period

INTRODUCTION

In dairy cattle, the transition period is marked by an increase in the occurrence of inflammatory-based diseases such as mastitis and metritis that cost farmers over $426 and $262 per case, respectively (Liang et al., 2017). The increase in disease incidence during the transition period is partly due to metabolic stress, which consists of a triad of factors including oxidative stress, dysfunctional inflammatory responses, and altered nutrient metabolism (Sordillo and Raphael, 2013). Oxidative stress results from inadequate antioxidant defenses to compensate for the increased production of pro-oxidants during the transition period that results in damage to macromolecules (Sordillo and Raphael, 2013). The phenomenon of aberrant or dysfunctional inflammatory responses around the time of calving also have been attributed to imbalances in the production of lipid inflammatory mediators known as oxylipids (Sordillo, 2018). Although oxylipids regulate all aspects of inflammation, it is the overall profile and timing of their expression during tissue insult that will determine the effectiveness of the inflammatory response (Gabbs et al., 2015). Indeed, there is ample evidence to suggest that it is an imbalance in the production of pro-inflammatory and anti-inflammatory oxylipids that contributes to dysfunctional inflammatory responses around the time of calving and during mastitis (Sordillo, 2018). Such aberrant inflammation not only reduces the immune system's barrier to infection, but eventually responds to pathogen challenge in an overly-robust manner, causing tissue damage beyond that of the infection (Mavangira and Sordillo, 2018). Both oxidative stress and dysfunctional inflammation can form positive feed-back loops to exacerbate the incidence and severity of dairy cattle diseases. For example, oxidative stress not only causes apoptosis of cells essential to an effective inflammatory response but also increases the production of pro-inflammatory oxylipids through redox sensitive enzymes (Zamamiri-Davis et al., 2002, Ryman et al., 2016). Conversely,

dysregulated inflammation can result in an over-accumulation of PMN and macrophages in tissues which, if unable to cope with the intense production of pro-oxidants by these cells, can cause oxidant damage (Mavangira and Sordillo, 2018).

Fat-soluble vitamins with antioxidant functions play an important role in the antioxidant defenses needed to prevent oxidative stress. Through their antioxidant functions, vitamins A, D, and E can mitigate damage to the immune system from oxidative stress, such as weakening of the endothelial barrier, which regulates leukocyte migration to sites of infection during inflammatory responses (Ryman et al., 2016). The production of oxylipids is partly dictated by PUFA substrate availability, however, much of the regulation of their production is carried out by changes in enzyme activity from the cyclooxygenase (COX), lipoxygenase (LOX), or cytochrome P450 (CYP450) enzymatic pathways, or due to alteration in non-enzymatic production from interaction with reactive oxygen species (ROS) (Gabbs et al., 2015, Sordillo, 2018). Oxidative stress or the relative oxidant balance as a consequence of increased ROS accumulation plays an important role in determining the overall pool of oxylipids that are produced. Vitamins that reduce pro-oxidant production may limit the number of non-enzymatically produced oxylipids, including 9- or 11-hydroxyeicosatetraenoic acid (HETE), that are thought to have pro-inflammatory activities (Mavangira and Sordillo, 2018). Additionally, both COX and LOX enzymes are induced by pro-oxidants (Zamamiri-Davis et al., 2002). Although these pathways do not exclusively produce pro-inflammatory oxylipids, many of their metabolites promote inflammation (Gabbs et al., 2015). Through these mechanisms, vitamins A, D, and E have integral roles in limiting oxidative stress and the general pro-inflammatory shift of the immune balance during the transition period.

Interestingly, the CYP450 family of enzymes directly contribute to the creation or prevention of oxidative stress and dysfunctional inflammation. Specific CYP450 are involved in

the production of and degradation of the bioactive forms of certain vitamins with antioxidant functions. Additionally, CYP450 are involved in PUFA metabolism, the most well studied of which are arachidonic acid (AA) and linoleic acid (LA). Cytochrome P450 mediated metabolism of PUFA contributes to the oxylipid pool by producing both pro-inflammatory and anti-inflammatory oxylipids determined by substrate and the specific CYP450 (Gabbs et al., 2015). These roles in the regulation of oxidative stress and dysfunctional inflammation make CYP450 essential to discerning, and potentially intervening in, the development of metabolic stress in transition cows.

The origins of CYP450 predate pre-aerobic life allowing time for substantial diversification of individual isoforms across all clades of life (Hrycay and Bandiera, 2015). Diversification has allowed CYP450 to become integral in the metabolism of a plethora of compounds ingested or synthesized by animals. Early research into the role of CYP450 in cattle focused on their ability to either activate or break down xenobiotics, steroids, and pollutants (Balk et al., 1984, Waterman and Simpson, 1985). More recently, literature has turned its attention to other roles of CYP450 including regulation of the immune system. Cytochrome P450 enzymes are typically associated with the degradation and inactivation of fat-soluble vitamins as is the case with vitamins A, D, and E (Figure 1.1). Uniquely, the vitamin D activation cascade is a multi-step process involving 3 distinct steps of CYP450 metabolism, 2 in the form of activation and 1 of inactivation.

Cytochrome P450 enzymes also have substantial substrate promiscuity, allowing some CYP450 involved in vitamin metabolism to additionally biosynthesize oxylipids from PUFA substrate. For example, CYP450 family 4 subfamily F member 2 (CYP4F2) degrades vitamin E and oxidizes AA to form 20-HETE. For the remainder of this review, specific CYP450 will be abbreviated in this manner- CYP followed by the family number, the subfamily letter, and lastly

the member number. The substrate promiscuity of CYP450 creates a complicated network of substrates, enzymes, and metabolites. In this network, several CYP450 may metabolize a given substrate, a single CYP450 may metabolize many substrates, and a single CYP450 may produce differing products from a single substrate. For example, CYP2J2 can carry out the first step of vitamin D activation by metabolizing cholecalciferol. This enzyme additionally metabolizes linoleic acid and arachidonic acid into typically pro-inflammatory or anti-inflammatory oxylipids substrate dependently. During the transition period, many of the factors within this network considered to be associated with immune regulation are found at significantly different concentrations than other stages of lactation. For decades, studies profiling CYP450 substrates such as plasma vitamin A and E have found systemic concentrations to reach a nadir shortly after calving (Goff and Stabel, 1990). More recently, we have come to learn that vitamin D does as well (Holcombe et al., 2018). Poly-unsaturated fatty acid substrates and oxylipid products also can fluctuate significantly, both increasing and decreasing around calving as does the gene expression of many CYP450 themselves (Table 1.1) (Haga et al., 2015, Kuhn et al., 2017).

The depth of knowledge surrounding the metabolic activities and contribution to disease states of specific CYP450 in humans and murine species far outpaces our grasp of CYP450 in cattle. These gaps in knowledge have led to the design of bovine studies in which the contribution of CYP450 to health or disease is based upon extrapolation of other species rather than known activities in cattle. For example, without a consensus as to which CYP450 are the major vitamin D 25-hydroxylases in cattle, some studies have not assessed important contributors, instead focusing on prominent CYP450 in humans (Tellez-Perez et al., 2012). Further, nutritional interventions are limited by CYP450 mediated metabolism of certain substrates. Manipulating enzymatic activity in concurrence with dietary changes may offer a more targeted and efficacious

approach to disease prevention. This review aims to detail the known contributions of CYP450 to the regulation of health and disease in cattle with a focus on what has been specifically shown in cattle rather than extrapolated from other species. With a strong appreciation of what is known and remains unknown, further research can uncover the pieces needed to use CYP450 manipulation as a viable prevention and treatment strategy for diseases of dairy cattle.

. VITAMIN METABOLISM BY CYP450

Vitamin A

Vitamin A is as an umbrella term for several metabolites derived from the diet, including β-carotene and retinol, and their subsequent metabolic products that serve several key purposes in cattle including supporting immune system maturation, immune cell function, and reducing pro-oxidant load (Weiss, 1998, Nonnecke et al., 1999, Shi et al., 2018). Multiple studies have associated greater plasma concentrations of vitamin A at calving or early lactation with reduced incidence of disease underscoring the importance of vitamin A at this life stage (Johnston and Chew, 1984, LeBlanc et al., 2004). Additionally, regulation of vitamin A during pregnancy is just as delicate a balance in cattle as in humans (Ross and Zolfaghari, 2011). Adequate vitamin A during pregnancy promotes embryogenesis yet vitamin A becomes a teratogen at excessive concentrations (Pennimpede et al., 2010). The involvement of CYP450 in the regulation of vitamin A metabolism is well established within human literature, however, apart from confirming that indeed CYP450 degrade retinoic acids (RA), the bioactive metabolite of vitamin A, little is known about their relative role in controlling circulating concentrations of vitamin A in cattle (Figure 1.1) (Leo et al., 1989, Ross and Zolfaghari, 2011). The fact that such a gap in knowledge exists is surprising given the relationships that are known to exist between dietary vitamin A, circulating concentrations of vitamin A, and correlations with disease. As mentioned previously, greater

concentrations of plasma retinol are associated with reduced risk of mastitis, however, others have found feeding greater concentrations of vitamin A had no effect on incidence of mastitis (Oldham et al., 1991, Weiss, 1998). Although there are many enzymatic steps between intake of vitamin A and excretion, CYP450 likely play an important role in regulating concentrations of active vitamin A metabolites including RA. As research into vitamin A supplementation advances, so too should investigations into the CYP450 enzymes that degrade their active metabolites.

In humans, CYP26A1, CYP26B1, and CYP26C1 are implicated as the major CYP450 RA-hydroxylases, degrading the functional metabolites of vitamin A. Gene expression of cytochrome P450 family 26 (CYP26) enzymes, like most CYP450, are robustly responsive to substrate abundance and widely distributed in the organs and tissues of dairy cattle (Table 1.1) (Ross and Zolfaghari, 2011, Kuhn et al., 2020).

Kruger et al. (2005) found that when feeding calves colostrum or milk replacer with or without vitamin A (retinyl esters), transcript expression of several CYP450 was induced by vitamin A. Liver samples from calves fed vitamin A supplemented milk replacer or colostrum had *CYP26A1* mRNA abundance 32-fold and 8-fold greater, respectively, than those fed un-supplemented milk replacer (Kruger et al., 2005). The increase in *CYP26A1* in colostrum fed calves may have been due to the greater amount of β-carotene found in colostrum compared to commercial milk replacers (Calderon et al., 2007). This study confirms that CYP26 enzymes in cattle are indeed induced by vitamin A substrate as in other mammals.

In a liver transcriptomic analysis, hepatic *CYP26A1* mRNA expression was significantly decreased after parturition compared to 3 wk prior to calving (Ha et al., 2017). In other species, dietary vitamin A has a significant positive influence on liver transcript expression of *CYP26A1*, a relationship also measured in calves by Kruger et al. (2005). Reduced transcript expression after

parturition coincides with decreases in transition period feed intake, including reduced dietary vitamin A, at least partially explaining a reduction in *CYP26A1* mRNA expression (Goff and Stabel, 1990). Goff et al. (2002), however, showed that in mastectomized cows, plasma retinol and 9,13-di-cis RA increase after calving compared to declines in these metabolites in intact cows. An increase in vitamin A metabolites in mastectomized cows suggests that even though a reduction in dietary intake may reduce transcript expression, sequestration of vitamin A in colostrum and milk likely plays a greater role in regulating CYP26 gene expression and plasma vitamin A concentration than changes in feed intake (Goff et al., 2002).

Nonetheless, although serum vitamin A does not reflect tissue stores, concentrations found in blood reach a nadir in cows at calving. Several studies have associated low serum vitamin A with inflammatory disease at the transition period suggesting it as an opportune time to target interventions aimed at increasing circulating concentrations of vitamin A (Johnston and Chew, 1984, LeBlanc et al., 2004). In vitro studies using several bovine cell types have shown significant changes in inflammatory cascades after increased vitamin A supplementation, which may reveal some of the mechanisms by which increased circulating vitamin A is beneficial during the transition period. In bovine adipocytes, all-*trans* RA significantly reduced the gene expression of pro-inflammatory cytokines, both basally and after induction with LPS, including tumor necrosis factor-α, IL-1β, IL-6 or IL-17, while increasing the production of anti-inflammatory IL-10 and transforming growth factor-β (Xu et al., 2019). Pro-inflammatory cytokines, as with all components of the initial inflammatory response, are essential to mounting an adequate defense to pathogen challenge. During the transition period, however, inflammatory responses can become dysregulated and overly robust, becoming detrimental and causing excessive tissue damage (Sordillo and Raphael, 2013). Reducing pro-inflammatory cytokines at this life stage may reduce

the inflammatory imbalances of the transition period, which contribute to dysregulated inflammatory responses. In addition to an inflammatory imbalance, transition cows concurrently face an oxidant imbalance which can result in oxidative stress if antioxidant defenses are overcome by pro-oxidants. Shi et al., (2018) found that in primary bovine mammary epithelial cells treated with nitric oxide, all-*trans* RA pre-treatment significantly reduced the gene expression of pro-inflammatory cytokines and increased the activity of antioxidant enzymes. This work by Shi et al. suggests that the benefits of vitamin A may reach beyond mitigating the inflammatory response to antioxidant functions.

In whole animal studies, acute increases in vitamin A supplementation result in the induction of CYP26 enzymes limiting the efficacy of short term vitamin A supplementation (Kruger et al., 2005). Rather, it may take months of supplementation to alter concentrations of stored vitamin A. Not only does this length of time prevent focused supplementation of vitamin A, but it limits how much vitamin A can be safely supplemented. Because vitamin A can be teratogenic during fetal development, sustained increases in supplementation during pregnancy would be contraindicated. Rather, down-regulating CYP26 activity may be integral to maximizing the benefits of vitamin A supplementation by limiting the degradation of its active form during the transition period. Such inhibitory interventions may allow for more targeted supplementation and reduced risk for adverse effects during pregnancy. Although such inhibitors have been assessed in other species, their use has not been applied to cattle as of yet (Nelson et al., 2013).

A final consideration in the regulation of vitamin A is the potential for involvement of CYP450 outside of the CYP26 family in RA metabolism. Due to the substrate promiscuity of CYP450, it is unlikely that CYP26 enzymes are the sole contributors to the breakdown of Vitamin A. In humans, many CYP450 (CYP2A6, CYP2B6, CYP3A4, CYP3A5, CYP3A7, CYP2C8,

CYP2C9, CYP2C18, CYP2C22, CYP2C39) may be involved in the metabolism of all-*trans* RA (Leo et al., 1989, Marill et al., 2002, Ross and Zolfaghari, 2011). None of these CYP450 have been specifically evaluated for their ability to metabolize RA in cattle. Most CYP450 that metabolize vitamin A in other species are transcriptionally positively regulated by RA concentration, similar to *CYP26A1* in cattle. Transcript expression of *CYP2A6, CYP2E1, CYP2C8,* and *CYP3A4* in dairy calves were unaltered by changes in concentrations of vitamin A, suggesting that vitamin A is not a significant substrate for these isoforms (Kruger et al., 2005). Nonetheless, the potential for unknown RA-hydroxylases to exist remains. Until such non-CYP26 RA-hydroxylases are identified, unintended impacts on vitamin A metabolism should be considered when manipulating expression or activity of CYP450 for other purposes. For example antifungal treatments such as ketoconazole are known for their CYP450 inhibiting activity on enzymes such as CYP3A4 or CYP2C9 (Marill et al., 2002). If these CYP450 were to ultimately have a significant role in metabolizing vitamin A, such treatments could disrupt vitamin A regulation.

Vitamin D

Research into the physiological activities of vitamin D in cattle has been ongoing for nearly a century with a primary focus on calcium homeostasis (Chick and Roscoe, 1926). A concentration on calcium has resulted in NRC supplementation standards which ensure adequate circulating 25-hydroxyvitamin D_3 (25-[OH]D_3), the precursor for active vitamin D, to maintain calcium homeostasis and met by nearly all dairy cattle in the United States (National Research Council, 2001). More recent work in both humans and veterinary species, however, suggests that greater circulating concentrations of 25-(OH)D_3 (>70 ng / mL in cattle) are required for optimum immune performance (Nelson et al., 2012, Chin et al., 2017, Wisnieski et al., 2020). Beyond the systemic circulation, a new focus has additionally been placed on the unknown contribution of vitamin D

activation in local compartments, such as in the mammary gland, which may have important implications for mastitis prevention and treatment.

25-hydroxylases. Interest in vitamin D's role in immune health has increased research focusing on the CYP450 pathway. Vitamin D_3 (cholecalciferol) is either initially consumed through the diet or synthesized in the skin from 7-dehydrocholesterol upon UV light exposure (Pacheco et al., 2018). Cholecalciferol is further metabolized to 25-(OH)D_3 by potentially 5 CYP450 cattle, namely CYP2J2, CYP2R1, CYP3A4, CYP11A1, and CYP27A1 (Schuster, 2011, Tellez-Perez et al., 2012, Slominski et al., 2015). The activity of 25-hydroxylases occurs predominantly in the liver and to a lesser extent in other organs and circulating cells (Tellez-Perez et al., 2012)**.** The dominant 25-hydroxylase in humans and mice is CYP2R1, yet the relative contribution of specific CYP450 to 25-hydroxylase activity in cattle remains speculative (Schuster, 2011).

High density genotyping by Casas et al. (2013) revealed a strong correlation between SNP within *CYP2J2* and 25-(OH)D concentrations in beef calves suggesting that indeed CYP2J2 plays an important role in the activation of vitamin D (Table 1.1). Supporting this finding, a whole-genome sequencing scan by Pacheco et al. (2018) found only 2 CYP450 genes to be associated with milk fever incidence: *CYP27A1* and *CYP2J2*. These studies suggest that cattle, unlike other mammals, may not have a single dominant 25-hydroxylase enzyme. Rather, CYP2J2 and CYP27A1 may each contribute significantly to the production of 25-(OH)D_3 (Figure 1.1). On a transcription expression basis, *CYP27A1* is exceedingly greater than *CYP2J2* or any other proposed 25-hydroxylase throughout all tissues of cattle (Kuhn et al., 2020). Although mRNA expression of *CYP2R1* in cattle increases dose dependently with supplementation of cholecalciferol, indicating that cholecalciferol is likely a substrate, there is not yet strong evidence

that CYP2R1 plays a prominent role as a systemic 25-hydroxylase as in other species (Tellez-Perez et al., 2012). Interestingly, Naderi et al. (2018) found that a SNP within a region of the genome containing *CYP2R1* had a significant association with clinical mastitis. Potential activity of CYP2R1 as a 25-hydroxylase may certainly associate it with mastitis incidence, however, it has other roles aside from vitamin D metabolism, such as cholesterol, steroid, and lipid synthesis. Any of these activities may contribute to such an association as well. The connections found by Pacheco et al. (2018) and Naderi et al. (2018) between SNPs of *CYP2J2, CYP27A1,* and *CYP2R1* and clinical disease additionally provide evidence contrary to the believe that CYP27B1 1α-hydroxylation is the rate limiting step in activation of vitamin D as in other species.

1α-hydroxylase and 24-hydroxylase. After initial formation of 25-(OH)D_3, CYP27B1 metabolizes this compound into bioactive 1,25-dihydroxyvitamin D_3 (1,25-$[OH]_2D_3$). The 1α-hydroxylase activity of CYP27B1 is concentrated within the kidney and is tightly regulated to maintain adequate circulating concentrations of 1,25-$(OH)_2D_3$ (Nelson et al., 2010b). For example, arctic reindeer have evolved to maintain adequate calcium concentrations despite living in a high latitude low-light environment by having CYP27B1 activity significantly greater than other ruminants without differences in activity of other CYP450 (Lin et al., 2019). Activity of CYP27B1 in the kidney is primarily driven by parathyroid hormone and 25-(OH)D_3 to meet calcium demands (Horst, 1986). Evidence is mounting, however, that activation by other co-factors may regulate local concentrations of vitamin D (Nelson et al., 2010b). The last step in Vitamin D metabolism is its degradation of 25-(OH)D_3 and 1,25-$(OH)_2D_3$ to calcitroic acid and other minor metabolites by CYP24A1. Transcript expression and activity of CYP24A1 is controlled primarily by concentrations of 1,25-$(OH)_2D_3$ as a negative feedback loop.

In cattle, *CYP27B1* transcript expression robustly increases in response to exogenous challenges, such as in milk somatic cells after LPS or *Streptococcus uberis* challenge. Such changes in gene expression result in increased transcript expression of *NOS2* and *CCL5*, contributors to microbial killing and chemoattraction of other leukocytes (Nelson et al., 2010b, Merriman et al., 2018). This cytokine production enhances the antimicrobial capabilities of the mammary gland revealing a potential mechanism of pathogen defense by CYP27B1. Emphasizing the importance of vitamin D regulation at the local level, although *CYP27B1* was upregulated in mammary somatic cells by mastitis challenge, plasma leukocytes or those from contralateral unchallenged glands were unaffected. In this regard, the value of understanding vitamin D regulation within localized tissues, rather than by systemic concentrations, is made clear. Interestingly, the typically strong induction of *CYP24A1* transcript expression by 1,25-$(OH)_2D_3$ is inhibited in phagocytes and other mammary cells in the presence of LPS providing an additional mechanism by which vitamin D regulation may contribute to the immune defenses of the mammary gland (Nelson et al., 2011, Yue et al., 2017). Together, the changes in expression of the differing CYP450 involved in vitamin D regulation show a protective response to pathogen challenge representing an important host defense system.

The selectiveness of CYP27B1 and CYP24A1 to only metabolize substrate within the vitamin D pathway, to our knowledge, also makes them viable targets for intervention with little chance of off-target effects on other metabolic pathways. In this manner, increasing the activity of CYP27B1 or reducing the activity of CYP24A1 may be intriguing methods to increase concentrations of 25-$(OH)_2D_3$ without the caveats generally associated with CYP450 intervention.

Vitamin E

In addition to vitamins A and D, metabolism of vitamin E is additionally dependent upon a CYP450 enzyme. As a potent antioxidant, Vitamin E is an essential vitamin to prevent or limit the cellular damage of pro-oxidants. Unlike vitamins A and D, a single enzyme, CYP4F2, is responsible for the degradation of vitamin E analogs, beginning a β-oxidation cascade of water-soluble molecules (Figure 1.1). The term vitamin E refers to a group of 8 vitamers with similar structure, including a chromanol ring and phytyl-like tail. The most biologically active and well-studied of the 8 analogs is α-tocopherol (αT). In addition, there are 3 tocopherols, β-, γ-(γT), and δ-tocopherol and 4 tocotrienols, α-, β-, γ-, and δ-tocotrienol. Despite human diets consisting of primarily γT, αT circulates and is found in tissues at greater concentrations due to increased metabolism of non-αT analogs, a characteristic shared amongst mammals (Parker et al., 2004). Although the mechanisms behind this phenomenon of αT preservation are not fully understood, 2 factors have stood out including the presence of a hepatic αT transfer protein (αTTP) and a preference of CYP4F2 to metabolize non-αT analogs at a faster rate than αT.

Despite the focus on αT, there is mounting evidence that other analogs may indeed have important biological activity including reducing the production of pro-inflammatory oxylipids, acting as direct antioxidants, and having a considerable ability to reduce reactive nitrogen species (Jiang et al., 2000, Mazlan et al., 2006). In order to elicit the benefits of non-αT analogs, however, interventions likely need to be taken to increase the physiological retention time of such vitamers. A reduction in the activity of CYP4F2 may be a viable means to increase circulating concentrations of all tocopherols and tocotrienols.

In cattle, despite significant research into the association between αT and disease, investigations into αT's metabolism and excretion have been limited. Increases in αT

supplementation over the last 4 decades have overcome overt clinical deficiencies, yet oxidative stress remains prevalent in modern dairy cattle (Kuhn et al., 2018). Oxidative stress during the transition period is due, in part, to a nadir of plasma αT at parturition and the ever increasing energy demands of the modern dairy cow (Goff and Stabel, 1990, Haga et al., 2018). Determining the cause of reduced αT after calving has been elusive. Although the suppressed feed intake of transition cattle, including dietary vitamins, contributes to diminished circulating concentrations of αT, the correlation between feed intake and circulating αT suggests other factors contribute as well (Haga et al., 2018). Such factors may include sequestration of αT in colostrum or reduced liver function. The transcript expression of *CYP4F2* is also increased shortly after calving, potentially increasing the breakdown of vitamin E analogs at the time they are needed the most (Table 1.1) (Haga et al., 2018). Further complicating matters, the depression of serum αT at calving is a trend not noted for all vitamin E analogs. Whereas serum αT, γT, and β-tocotrienol are significantly lower in dairy cattle prior to calving, α-, γ-, and δ-tocotrienol were unchanged across the transition period (Sadri et al., 2015). The differences in relative analog concentration across the transition period would suggest that other unknown factors are at play, such as novel transport proteins like supernatant protein factor, which have been suggested in humans but remain unstudied in cattle (Jiang, 2014).

Transcriptional expression of *CYP4F2* is found widely, yet variably, in tissues of dairy cattle (Haga et al., 2015, Kuhn et al., 2020). Our conception of the factors influencing *CYP4F2* expression and activity is still in its infancy in cattle. Unlike most CYP450 whose transcript expression is highly substrate driven, it appears that *CYP4F2* is not strongly, if at all, influenced by concentrations αT (Haga et al., 2015). Although *CYP4F2* mRNA in the liver is significantly greater 1 wk postpartum than a month before or after calving, *CYP4F2* in the mammary gland does

not increase in the same manner. In mammary tissue, *CYP4F2* mRNA is lowest 1 wk prior to calving and increases 3-fold to its peak by the sixth week of lactation (Sadri et al., 2015, Haga et al., 2018). The increase of *CYP4F2* mRNA in the mammary gland over the first few weeks of lactation may then be a contributing factor to the decline of milk αT seen over the same period (Calderon et al., 2007). Unfortunately, there is no current data profiling kidney expression of *CYP4F2* through the transition period despite having the greatest organ transcript expression in cattle (Kuhn et al., 2020).

Greater αT supplementation alone has not proven to be a viable solution to the oxidative stress faced at parturition by some dairy cows. One study supplementing αT just 3-fold greater than NRC recommendations counterintuitively reported increased oxidative stress and disease incidence (Bouwstra et al., 2010a, Bouwstra et al., 2010b). Together, these factors implore the need for new approaches to mitigating oxidative stress that do not involve merely increased αT supplementation. Instead, a different approach may include inhibition of *CYP4F2* to reduce breakdown of non-αT vitamin E analogs. Such approaches will be discussed in the later section discussing ω-hydroxylases.

PUFA METABOLISM BY CYP450

A hallmark of the transition period in cattle is a state of dysfunctional inflammation that underlies marked predisposition to disease (Sordillo and Raphael, 2013). One of the reasons for this inflammatory dysfunction is a shift in the production of oxylipids which play crucial roles in the regulation of inflammation during health and disease. Oxylipids may be produced either non-enzymatically through interactions between PUFA and ROS or enzymatically through COX, LOX, and CYP450 pathways. Whereas COX and LOX pathways have been extensively studied in relation to oxylipid production, more recent research in dairy cattle has provided evidence that the

CYP450 pathway may actually be a greater contributor to inflammatory dysregulation during the transition period and in clinically diseased animals (Mavangira et al., 2015, Kuhn et al., 2017).

Oxylipids of CYP450 PUFA oxidation are primarily from ω-6 PUFA due to their relative abundance in the diet of North American dairy cattle despite a preference of many CYP450 to metabolize ω-3 PUFA (Spector and Kim, 2015). As individual oxylipids may have either pro-inflammatory or anti-inflammatory characteristics, the relative abundance of each individual oxylipid can have significant impacts on inflammation. As such, it is important to recognize the specific roles each individual CYP450 enzyme plays in this convoluted web of substrates as differing CYP450 preferentially produce specific oxylipids.

Epoxygenases of Arachidonic Acid

Several CYP450 enzymes act upon PUFA as epoxygenases, oxidizing a mid-chain double bond to form an epoxide group. As PUFA have several mid-chain double bonds, each substrate can be metabolized into an equal number of isomers albeit at unequal ratios. Arachidonic acid having 4 double bonds is metabolized to 5,6-, 8,9-, 11,12-, and 14,15-eicosatrienoic acid (EET). Importantly, each of these isomers are further metabolized into respective dihydroxyeicosatrienoic acids (DHET) by soluble epoxide hydrolase (sEH). The secondary oxylipid metabolites derived from AA tend to be less bioactive than their precursors, making sEH an important enzyme to the CYP450 oxylipid cascade. Although many CYP450 have epoxygenase activity, in cattle there is little information evaluating the relative contribution of specific CYP450 to the overall pool of EET aside from CYP2J2 and CYP2C11.

Epoxyeicosatrienoic acids have received the bulk of attention amongst ω-6 fatty acid CYP450 products in human research due to roles in cardiovascular control (Anwar-mohamed et al., 2010). The use of bovine in vitro models to study the vasodilatory effects of such oxylipids

dates back nearly 3 decades, yet such models have rarely been used to probe potential activities of EET in cattle (Rosolowsky and Campbell, 1993). Whereas cardiovascular disease is not a significant concern for dairy cattle, EET have other roles in combating disease. Epoxyeicosatrienoic acids protect bovine endothelial cells from hypoxic injury, inflammation-induced apoptosis, and oxidative stress (Yang et al., 2001, Yang et al., 2007). This cellular protection has been shown with exogenous addition of EET and through cellular overexpression of *CYP2J2* (Yang et al., 2001, Yang et al., 2007). Many etiologies of mastitis, such as *Streptococcus uberis* or coliform species, cause significant tissue injury and are known for their ability to damage the endothelial barrier within the mammary gland (Zhao and Lacasse, 2008). This endothelial damage allows localized and treatable infections to become systemic, life-threatening, and unresponsive to intervention by disrupting vascular endothelial cells and the inflammatory response. In this context, increasing mammary or systemic concentrations of EET, which may protect endothelial cells from hypoxic or oxidative damage, potentially provides an additional defense against several forms of mastitis. Despite these studies showing impressive protection of bovine cells by upregulating CYP450 epoxygenases, this knowledge has been all but overlooked by bovine researchers.

In addition to the role of epoxygenases in tissue protection, EET may also help to control the typically over-robust inflammatory response to infection noted in many transition cattle. Both exogenous 11,12-EET and *CYP2J2* overexpression in bovine endothelial cells abolish the up-regulation of VCAM-1 after inflammatory cytokine stimulation and inhibit adherence of PMN to endothelium (Node et al., 1999, Pratt et al., 2002). By reducing the adherence of leukocytes to endothelium, EET may act to slow the progression of inflammation and associated production of ROS, limiting further tissue injury.

Knowing the specific roles CYP450 epoxygenases may have in mediating inflammation offers an insight into what changes in expression and activity practically mean to dairy cattle health. In general, the expression and activity of CYP450 epoxygenases is reduced under conditions of stress and disease. For example, epoxygenases *CYP2C19* and *CYP2E1* are each downregulated in bovine liver samples after challenge with both *E. coli* and LPS (Table 1.1) (Jorgensen et al., 2012). Noninfectious conditions of heat stress and oxidative stress downregulate *CYP2J2* and differing cytochrome P450 family 2 subfamily C enzymes (CYP2C) as well (Larsen et al., 2008, McCracken et al., 2015). Additionally, unidentified physiological changes during the transition period cause a reduction in *CYP2C19* (Ha et al., 2017). Given what we know of the roles these CYP450 play in reducing the inflammatory response and protecting cell viability during infection, their downregulation during stress, disease, and the transition period may be contributing to the progression and damage of infectious disease.

Epoxygenases of Linoleic Acid

Like AA, LA is oxidized by CYP450 at mid-chain double bonds forming 9,10-epoxyoctadecenoic acid (EpOME) and 12,13-EpOME and that are further metabolized to respective dihydroxyoctadecenoic acids (DiHOME) by sEH. Unlike EET derived DHET, however, DiHOME are more biologically active than their precursor metabolite (Zheng et al., 2001). Whereas EET and DHET have been extensively studied, little is known about the activity of LA derived oxylipid epoxides and dihydroxides in cattle despite being more abundant in plasma and milk (Mavangira et al., 2015).

Epoxyoctadecenoic acids and DiHOME have been implicated in other species as positive regulators of pain and inflammation in contrast to the generally anti-inflammatory portrayal of EET and DHET (Moran et al., 2000). Supporting this assertion is the positive outcomes noted

across various species by blocking sEH and preventing the production of DiHOME (Guedes et al., 2017). The lack of knowledge regarding EpOME and DiHOME activity and production in cattle is a clear impediment to discerning their involvement in inflammation. Given the significant shifts towards the production of LA derived CYP450 oxylipids during inflammatory events, a greater research emphasis should be placed on the CYP450 mediated production of these oxylipids to reveal their contribution to disease.

ω-Hydroxylases of Arachidonic Acid

In addition to epoxide formation, several CYP450 metabolize AA by ω-hydroxylation, either at the terminal carbon or 1 of several sub-terminal carbons. These reactions yield the terminal carbon hydroxylated 20-HETE or isomers of sub-terminal hydroxylation, 15-, 16-, 17-, 18-, or 19-HETE. This distinction, however minor, has important impacts on the relative activity of the oxylipid produced, being generally pro-inflammatory in the case of 20-HETE and anti-inflammatory regarding 15-19-HETE.

Two primary terminal carbon ω-hydroxylases (TCOH) likely exist in cattle as in humans, CYP4A11 and CYP4F2. In humans, CYP4F2 is the primary enzyme responsible for the production of 20-HETE in the kidney and liver, found in greater amounts and having a smaller K_m value of the 2 enzymes (Powell et al., 1998). Interestingly, in cattle, *CYP4A11* mRNA is expressed to a greater amount in all organs in which the 2 enzymes are found, although as of yet, no information is available as to relative pharmacokinetics of CYP4F2 and CYP4A11 in cattle (Kuhn et al., 2020). If, as in humans, the transcript expression of each of these CYP450 correlated with their activity, this would suggest that CYP4A11 is dominant in cattle.

As mentioned previously, Haga et al. (2018) noted an increase in *CYP4F2* mRNA expression in the liver and mammary tissue after calving (Figure 1.2). This increase in *CYP4F2*

expression fits with observations of increased 20-HETE in plasma and milk during this same period (Kuhn et al., 2017). Understanding the primary driver of 20-HETE production is essential to reducing 20-HETE concentrations, which can increase over 70-fold in the mammary gland during cases of mastitis (Mavangira et al., 2015). The cause of such a robust increase in 20-HETE during mastitis is currently unknown. One explanation for this phenomenon is that mastitis activates phospholipases, releasing AA substrate stored in cellular membranes (Corl et al., 2008). As the major driver oxylipid production is commonly substrate availability, such an influx of AA may significantly increase the production of 20-HETE. Another explanation, however, is a change in the activity of CYP4F2 and CYP4A11. Whereas a small increase in mRNA expression of *CYP4F2* has been profiled just after calving, no studies have yet assessed potential changes in expression of *CYP4A11* by lactation stage. In other species, however, evidence does show that *CYP4A11* is upregulated by LPS, suggesting an additional contributing factor to increased 20-HETE production during coliform mastitis (Figure 1.2) (Anwar-mohamed et al., 2010).

The search for practical inhibitors of CYP450 hydroxylases has been fraught with a lack of specificity for CYP4 enzymes although certain compounds have made a successful transition from the lab to clinical trials. For example, one of the most promising inhibitors is HET0016, which inhibits the production of 20-HETE through inhibition of CYP4F2 and to a lesser extent CYP4A11. Use of HET0016 in vivo reduces oxidative stress and inflammation in rodent models without apparent signs of off-target effects, yet its use has yet only been for toxicological research in cattle (Parkinson et al., 2012, Toth et al., 2013).

A more natural inhibitory compound yet to be explored in cattle involves the use of sesame as an inhibitor of cytochrome P450 family 4 (CYP4) enzymes. Particularly, the predominant plant lignan in sesame, sesamin, has shown a strong ability to reduce 20-HETE production in human

liver and kidney microsomes through inhibition of CYP4 enzymes with little impact on the production of other oxylipids (Wu et al., 2009). Despite not reaching as great of an inhibitory maximum as HET0016, partial inhibition by sesamin may nonetheless be a practical outcome as 20-HETE remains an important signaling molecule in specific organs. Promisingly, a reduction of 20-HETE production was found in humans supplemented with sesamin without noted adverse effects attesting to its safety and efficacy (Wu et al., 2009). As CYP4F2 is primarily inhibited by sesamin, several human studies have shown an ability to increase αT and γT concentrations in subjects who increase dietary consumption of sesame infused bars or a paste of sesame oil as well (Wu et al., 2009, Barbosa et al., 2017). The potential for a concurrent increase in tocopherols and reduction in 20-HETE should be a serious focus for transition cow researchers.

In addition to TCOH enzymes, several CYP450 with epoxygenase activity also carry out sub-terminal ω-hydroxylase (STOH) activity in other mammals, notably, CYP1A1, CYP1A2, CYP2E1, and CYP2U1 (Powell et al., 1998). Such sub-terminal hydroxylation activity has not yet been shown in cattle. Knowledge of 15-, 16-, 17-, and 18-HETE activities are limited in all species, likely due to the difficulties associated with detection of oxylipids produced at such limited quantities. Instead, the stereoisomer 19-HETE has been the nexus of research surrounding this class of oxylipids. This focus stems from the ability of 19-HETE to directly antagonize the activity of 20-HETE (Shoieb et al., 2019). Although no current research has studied the impacts of 19-HETE in cattle, in other species, it has shown to be beneficial in several disease conditions as reviewed by Shoieb et al. (2019).

In cattle, *CYP1A1* has widespread and robust transcript expression in tissues and circulating leukocytes compared to *CYP1A2* or *CYP2E1* making it the most likely CYP450 to contribute to systemic 19-HETE production (Darwish et al., 2010, Girolami et al., 2011, Kuhn et al., 2020). In

support of its positive potential, in vitro overexpression of *CYP1A1* in bovine mammary epithelial cells suppressed pro-inflammatory effects of LPS, such as restoring the normal proliferative ability of cells by ameliorating increases inflammatory cytokine stimulation (Zhang et al., 2018b). One explanation for this activity is the antagonistic activity of 19-HETE against 20-HETE, which otherwise increases production of TNF-α and NFκB (Ishizuka et al., 2008). Another explanation may be the increase in EET production from *CYP1A1* which would likely have similar effects on proliferation.

Unfortunately, expression of *CYP1A1* and other STOH are reduced by both viral and bacterial pathogens and during clinical mastitis (Jorgensen et al., 2012, Zhang et al., 2018b, Tian et al., 2019, Toka et al., 2019). Additionally, liver *CYP2U1* expression was found by Ha et al. (2018) to be reduced after calving. Altogether, with evidence in other species that *CYP4A11* is stimulated by LPS, transition cows or those with mastitis may have reduced 19-HETE and increased 20-HETE production, shifting the inflammatory balance further towards a pro-inflammatory and dysfunctional phenotype (Figure 1.2) (Anwar-mohamed et al., 2010).

CONCLUSIONS AND FUTURE DIRECTIONS

Advances in research of vitamin metabolism and oxylipid production have begun to uncover the complexity and importance of CYP450 enzymes to the maintenance of dairy cattle health. Despite this, the description used in literature of CYP450 activities in cattle are largely extrapolated from human medicine with limited research devoted to discerning species-specific differences. Although CYP450 activity is generally conserved among species, exceptions certainly exist. Importantly, evidence in cattle suggests that the physiologically relevant enzymes to produce 25-(OH)D_3 are CYP2J2 and CYP27A1 rather than CYP2R1 in other species. Additionally, the expression of *CYP4A11* mRNA in cattle is more robust than that of *CYP4F2* in all organs. If the

relative transcript expression of *CYP4F2* and *CYP4A11* is indicative of their activity as in other species, this would suggest that CYP4A11 is the major TCOH responsible for the majority of 20-HETE in cattle. As many ω-hydroxylase inhibitors focus on CYP4F2 with lesser impact on CYP4A11, their translation to bovine research should be undertaken with this important caveat.

Further research into the specific roles CYP450 play in cattle and potential to manipulate their activities are likely to work alongside nutritional research. Supplementation of fat-soluble vitamins alone may be limited by increased in vivo degradation due to substrate induction of CYP450. Reducing the activity of specific CYP450, such as CYP4F2 (vitamin E), CYP26A1 (vitamin A), or CYP24A1 (vitamin D), may lessen the need for ever greater increases in supplementation. Further, the ω-6 PUFA diet fed to most modern dairy cattle tends to produce a pro-inflammatory oxylipid profile, especially at times of disease predisposition such as the transition period. Rather than dramatically shifting the diet of cattle away from ω-6 PUFA, altering the activity of specific CYP450 may reduce the pro-inflammatory nature of oxylipid production. Increasing the activity of CYP450 such as CYP2J2 which produces primarily EET or CYP1A1 producing 19-HETE while reducing the activity of 20-HETE producing CYP4F2 and CYP4A11 could dramatically tip the inflammatory scales towards a more balanced phenotype.

Despite the many potential uses for inhibitors of specific CYP450 to prevent or treat disease, research into their use has been fraught with unintended off-target effects due to the complexity of CYP450 metabolism. Many inhibitors that potently inhibit a single CYP450 still exert partial inhibition of others. Additionally, the substrate multiplicity of CYP450 means that inhibiting a CYP450 isoform with multiple substrates may impact several metabolic pathways. Although the use of specific inhibitors may pose challenges, partial inhibitors of targeted CYP450 have great potential for increasing the health and productivity of our dairy cattle. Such targeted

inhibition of CYP26, CYP24A1, CYP4F2, or CYP4A11 could still considerably increase the antioxidant capacity of cattle and reduce the shift towards a dysregulated and pro-inflammatory state found in some transition cattle. As our comprehension of the specific mechanisms of bovine disease grows and our use of disease biomarkers expands there will be an ever-increasing need for targeted care in animal agriculture. Inhibition of specific CYP450 in those animals most at risk for disease allows for focused interventions on specific animals or cohorts of animals to reduce the overall incidence of disease.

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CHAPTER 2

REVIEW: THE POTENTIAL ROLE OF VITAMIN E ANALOGS AS ADJUNCTIVE ANTIOXIDANT SUPPLEMENTATION FOR TRANSITION COWS

Matthew J. Kuhn,[1] and Lorraine M. Sordillo*[1]

[1]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing 48824, United States

***Corresponding author**

Dr. Lorraine M. Sordillo,

Department of Large Animal Clinical Sciences

College of Veterinary Medicine

Michigan State University

sordillo@msu.edu

Tel: (517) 432-8821

Fax: (517) 432-8822

ABSTRACT

Dairy cattle face a greater risk for disease during the transition period due, in part, to oxidative stress. Despite routine supplementation of vitamins with antioxidant functions to dairy cattle, such as α-tocopherol, the high energy demand of the transition period nonetheless creates a pro-oxidant state that can overcome antioxidant defenses and damage macromolecules. Oxidant damage of macromolecules, or oxidative stress, impairs normal cellular functions and host immune defenses, predisposing cattle to disease. Additionally, oxidative stress positively feeds into a state of dysfunctional inflammation that some cattle undergo if unable to properly cope with the significant physiological changes of the transition period. Dysfunctional inflammation involves an uncoordinated production of lipid inflammatory mediators, known as oxylipids, that coordinate all aspects of inflammation. In animals that do not properly adapt to the physiological shifts of the transition period, inflammatory responses can become aberrant and cause tissue damage when excessive or unresolving. In search of interventions to limit oxidative stress and the development of dysfunctional inflammation in transition cows, non-α-tocopherol analogs of vitamin E have shown functions in other species that may benefit cattle in this manner but have largely remained unstudied in cattle. Although research into the supplementation of α-tocopherol has been well studied in dairy cattle, evidence suggests that further dietary supplementation of α-tocopherol alone, merely 3 times National Research Council recommendations, may paradoxically increase oxidative stress and sub-clinical disease. As non-α-tocopherol analogs of vitamin E have functions similar to α-tocopherol, yet are more rapidly metabolized, they may provide further antioxidative functions with a reduced risk for adverse effects. Several of these analogs, most notably γ-tocopherol and δ-tocopherol, have been successfully used in human clinical trials as supplements for several inflammatory disorders, generally showing little to no adverse effects. Given that non-

α-tocopherol analogs of vitamin E reduce the production of pro-inflammatory mediators and act as antioxidants, their safety and efficacy for these purposes should be further evaluated in cattle.

Key words: Vitamin E, dairy cattle, oxidative stress, tocopherol, tocotrienol

INTRODUCTION

Intense physiological and physical changes of the transition period in dairy cattle can lead to a disturbance in the delicate immune balance in animals unable to properly cope with such changes, predisposing them to disease. This predisposing condition, known as metabolic stress, develops from a triad of factors including oxidative stress, dysfunctional inflammatory responses, and altered nutrient metabolism that can positively feed back into one another to disrupt inflammatory regulation in animals that do not properly cope with the physiological shifts of the transition period (Sordillo and Mavangira, 2014). Ultimately, metabolic stress can result in the development of aberrant inflammatory responses that lead to an increase in disease predisposition and severity. As a key component of the metabolic stress triad, oxidative stress, or damage to macromolecules from pro-oxidants, has been a focus of research aimed at reducing the incidence of disease around the time of calving. Although not all transition cows will undergo oxidative stress severe enough to disrupt normal cellular functions, managing the accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), the causative agents of macromolecule damage, has become an important consideration of dairy cattle nutrition.

The supplementation of cattle with vitamins that have antioxidant functions has reduced the prevalence of overt clinical deficiencies of such vitamins over the last 3 decades. Unfortunately, due in part to a significant drop in feed intake during the transition period, fat-soluble vitamins such as α-tocopherol are significantly lower in blood and liver tissue at calving and into early lactation compared to other stages of the lactation cycle (Goff and Stabel, 1990, Sadri et al., 2015). It is for this reason that several groups have studied the efficacy of parenteral administration of α-tocopherol rather than relying solely on dietary supplementation (Erskine et al., 1997, LeBlanc et al., 2002, Jensen et al., 2020). Vitamin E has been focused of the dairy

industry and human medicine for its antioxidant function in breaking lipid peroxidation chain-reactions which can ultimately lead to cell death (Galli et al., 2017). Although supplementation of vitamin E to dairy cattle has increased robustly over the past few decades, some cattle still undergo oxidative stress severe enough to hinder normal cellular function during the transition period (Goff and Stabel, 1990, Haga et al., 2018, Kuhn et al., 2018).

In cattle, α-tocopherol has been considered to be one of the least toxic vitamins according to the National Research Council (NRC) despite few studies specifically focusing on its safety when supplemented to cattle (2001). Even in human medical literature debate persists as to whether chronic high dose supplementation of vitamin E can be detrimental to health (Schmolz et al., 2016). Despite being regarded as safe for cattle, feeding α-tocopherol at only 3-times NRC recommended concentrations, still well within the a stated toxicity concentration of 75 IU / kg, raised concerns over further increases in its supplementation (Council, 2001, Bouwstra et al., 2010a, Bouwstra et al., 2010b). At this higher supplementation plane, Bouwstra et al. (2010a, 2010b) found increased ROS production in certain groups of animals and an increase in transition cow disease. Although speculation, these counterintuitive findings may be due to the antioxidant phenomenon of "inversion of activity," when antioxidants conversely act as pro-oxidants under certain conditions (Kamal-Eldin and Appelqvist, 1996). Specific to vitamin E, the inversion of activity can occur either when vitamin E is found at a considerably high concentration or when there is inadequate ascorbic acid to regenerate reduced vitamin E (Kamal-Eldin and Appelqvist, 1996). With evidence that supplementing α-tocopherol at amounts only 3-times greater than NRC recommendations may increase ROS accumulation or incidence of disease, a new approach to antioxidant supplementation is necessary.

VITAMIN E ANALOGS

Although nearly all vitamin E research in cattle has focused on α-tocopherol, a burgeoning field in other mammals has begun to uncover beneficial effects of non-α-tocopherol analogs (Wiser et al., 2008, Galli et al., 2017, Burbank et al., 2018). These 8 analogs are split into 2 sub-groups, the tocopherols with a saturated phytyl tail and tocotrienols with unsaturated phytyl tails (Figure 2.1). Each of these sub-groups is comprised of an α, β, γ, and δ analog based upon methylation of the chromanol ring. In addition, each of these analogs are broken down into a cascade of water-soluble metabolites prior to excretion by initial CYP450 mediated ω-hydroxylation and subsequent steps of β-oxidation. Despite α-tocopherol receiving the greatest attention from research, γ-tocopherol is the major form of vitamin E found in North American human diets (Jiang et al., 2001). This focus on α-tocopherol is thus due to its far greater retention and bioavailability compared to non-α-tocopherol analogs in both humans and other mammals (Schmolz et al., 2016). Although the mechanisms are not fully understood, 2 primary and independent processes play a role in preserving hepatic concentrations of α-tocopherol over other analogs. First, the rate limiting and initial step in vitamin E metabolism, undertaken by cytochrome P450 family 4 sub-family F member 2 (CYP4F2) preferentially metabolizes non-α-tocopherol analogs of vitamin E over α-tocopherol in microsomal reaction studies (Sontag and Parker, 2007). Secondly, the α-tocopherol transport protein (αTTP), imperative for transport of vitamin E out of the liver and protection from metabolism, if considered to have 100% affinity for α-tocopherol, has only 38% affinity for β-tocopherol and 12% or fewer for all other analogs (Hosomi et al., 1997).

In the basal diet of cattle, analogs of vitamin E can be found primarily in forages or concentrated in oils (Council, 2001, Jiang, 2014, Adeyemi et al., 2016). Supplementation of vitamin E almost exclusively comes from synthetic mixes of α-tocopherol added to TMR.

Interestingly, such exclusive supplementation of α-tocopherol in TMR may actively increase the ratio of α-tocopherol to non-α-tocopherol analogs by a third mechanism. Increased concentrations of α-tocopherol in CYP450 microsomal reaction systems increase the metabolism of non-α-tocopherol analogs through heterotropic cooperativity (Sontag and Parker, 2007). Supplementing α-tocopherol alone may not only increase the intake of α-tocopherol but additionally increase the breakdown of non-α-tocopherol analogs found basally in the diet. This mechanism may additionally contribute to the 10- to 1000-times greater concentration of α-tocopherol compared to non-α-tocopherol analogs found in serum and liver of cattle (Sadri et al., 2015).

Despite being used clinically for the treatment of human inflammatory conditions, such as asthma or nicotine replacement therapy, few studies have supplemented mixed tocopherols or specific non-α-tocopherol analogs in cattle and none, to the authors' knowledge, have purposefully supplemented tocotrienols (Mah et al., 2015, Burbank et al., 2018). The Elsasser group was foundational for such research, establishing how non-α-tocopherol analogs may accumulate in bovine tissues, blood, and milk after short-term mixed tocopherol supplementation (Qu, 2017). After feeding a tocopherol mix (α: 9%, β: 1%, δ: 24%, γ: 62%) to mid-lactation dairy cattle for 9 d, concentrations of α-tocopherol were reduced in hepatic tissue as concentrations of γ-tocopherol increased in liver, muscle, and mammary tissue. Reduced liver accumulation of α-tocopherol may be due to the αTTP being expressed almost solely in the liver. Since αTTP has affinity, however low, for γ-tocopherol, there is potential that increased concentrations of γ-tocopherol may displace some α-tocopherol and allow for greater metabolism. Interestingly, both α-tocopherol and γ-tocopherol were increased in the blood, with peak concentrations after 5 d of feeding. After noting that mixed tocopherol supplementation indeed increased concentrations of γ-tocopherol in tissue and plasma, Qu et al. (2018) fed mid-lactation multiparous dairy cattle a mixture of tocopherol

analogs and measured a variety of health parameters. This study concluded that feeding such a vitamin mix would not negatively affect either circulating concentrations of leukocytes or impair their ability to mount an inflammatory response to challenge. This review will coalesce evidence from other species, with an emphasis on ruminants and in vitro studies, that offer insights into the potential benefits that supplementation of mixed vitamin E analogs may offer in dairy cattle. Using mixed tocopherol supplementation dates back to the 1940's in cattle, yet little evidence exists in modern research investigating the functions of non-α-tocopherol analogs of vitamin E (Phillips et al., 1948).

ANTIOXIDANT CAPACITY

Analog Antioxidant Functions

Primarily known for its antioxidative capacity, α-tocopherol, and all other vitamin E analogs, directly scavenge reactive metabolites by donating a hydrogen atom from the phenolic group of their chromanol ring. The advantage of this antioxidant function is its inclination to break the chain reaction of lipid peroxidation in cellular membranes, limiting reactive metabolite damage (Figure 2.2A) (Jiang, 2014). Although this suggests that all analogs of vitamin E have antioxidant functions, the extent of these functions varies. The most widely studied of the 3 other tocopherol analogs, γ-tocopherol, shows a proclivity for scavenging of RNS due to its unsubstituted group at the 5 position of the chromanol ring, a characteristic also found in δ-tocopherol, γ-tocotrienol, and δ-tocotrienol, opposed to α-tocopherol's, or other α and β analogs', methyl group at this position (Figure 2.2B) (Wiser et al., 2008). One of the few studies focused on vitamin E analogs in cattle used the characteristic of γ- and δ-tocopherol to reduce RNS as a major outcome variable for their study. Elsasser et al. (2013) fed a diet to beef calves supplemented with γ-tocopherol and δ-tocopherol, measuring protein damage due to nitrosative stress created intravenous by LPS

administration. Calves supplemented with either α-tocopherol or a γ-tocopherol / δ-tocopherol mix indeed had reduced cellular protein damage as measured by tyrosine nitration after LPS challenge compared to the control calves (Elsasser et al., 2013). An important consideration for this study is that supplementation of the γ-tocopherol / δ-tocopherol mix was greater than that of α-tocopherol to compensate for relative increased metabolism. The outcomes of this study are promising for the use of non-α-tocopherol analogs to reduce cellular damage due to the accumulation of RNS, which are generally cytotoxic and disrupt many cell signaling pathways (Pacher et al., 2007). Although RNS have beneficial roles in the initiation of inflammation and phagocyte microbial killing, excessive or chronic accumulation has been linked to many human health disorders and likely contributes to the excessive tissue damage of metabolically stressed animals undergoing disease (Bouchard et al., 1999, Pacher et al., 2007).

Aside from cattle, tocopherol supplementation to reduce reactive metabolite mediated cellular damage has been shown in other ruminants as well. In sheep, those fed either α-tocopherol or γ-tocopherol in late gestation had reduced serum concentrations of 8-isoprostane as measured by ELISA compared to control ewes starting 7 d after supplementation until the final sample collection point 2 wk prior to lambing (Kasimanickam and Kasimanickam, 2011). As lipids are the most susceptible macromolecule to oxidant damage, 8-isoprostane quantification, markers of such lipid damage, are the gold standard for measuring oxidative stress when quantified by liquid chromatography tandem mass spectrometry. Quantification of 8-isoprostane by ELISA, however, is less reliable due to cross-reactivity with similar metabolites that are not indicators of oxidative stress (Mavangira et al., 2016). Nonetheless, akin to results noted by Elsasser, et al. (2013), those ewes fed γ-tocopherol supplement had lower 8-isoprostane concentrations than those fed α-tocopherol (Kasimanickam and Kasimanickam, 2011). A greater ability of γ-tocopherol to reduce

ELISA quantified 8-isoprostane compared to α-tocopherol has also been noted in rats fed supplements for merely 3 d (Jiang and Ames, 2003). Similarly, mice fed either γ-tocopherol or γ-tocotrienol had reductions in serum 8-isoprostane as measured by gas chromatography mass spectrometry (Yoshida et al., 2005).

Interestingly, a human study was conducted with a supplement equivalent to the tocopherol mixture used by Qu et al. (2018), which showed significant reduction the accumulation of 5-nitro-γ-tocopherol, a byproduct of the reaction of γ-tocopherol and RNS (Figure 2.2B) (Wiser et al., 2008). Reductions in 5-nitro-γ-tocopherol should only occur if either supplementation of γ-tocopherol is reduced, supplementation of α-tocopherol is increased, or there is a reduction of RNS for γ-tocopherol to react with. In studies that supplement γ-tocopherol, reduction in 5-nitro-γ-tocopherol indirectly quantifies the presence of RNS. Similar reductions in 5-nitro-γ-tocopherol have also been noted in γ-tocopherol supplemented rats (Hernandez et al., 2013). Similar to results in cattle, Wiser et al. (2008) conducted a safety assessment in humans and found no changes in complete blood counts, liver enzymes, and reported no adverse health events in patients. Unfortunately, assessing redox balance or oxidative stress was not an aim of the feeding trail in dairy cattle by Qu et al. (2018).

Analog Concentrations in Cattle

A potential contributor to oxidative stress around the time of calving, in addition to increased reactive metabolite production from increased metabolism, is the significant drop in DMI, including supplemented vitamins such as α-tocopherol. Such a reduction in vitamin intake contributes to reduced serum and liver α-tocopherol at this time in addition to reduced liver function around the time of calving, limiting the exportation of vitamin E from the liver (Haga et al., 2018). Although liver samples for tocopherol quantification are the most accurate assessment

of vitamin E status, serum concentrations are believed to give an accurate representation of total body status and may help to put changes in liver status into perspective. To account for changes in liver function around the time of calving, concentrations of vitamin E are commonly reported as a ratio with cholesterol to account for alterations in liver function. Unfortunately, no ratios for non-α-tocopherols of vitamin E in plasma or serum have yet been reported.

Interestingly, not all vitamin E analogs fluctuate similarly across the transition period and into lactation. In serum, α-tocopherol, γ-tocopherol, and β-tocotrienol follow a similar pattern, increasing from a lesser amount at days -21 to 1 relative to calving to a greater plateau from 21-70 DIM (Sadri et al., 2015). Alternatively, α-tocotrienol, γ-tocotrienol, and δ-tocotrienol remain unchanged across this time period. These differences in concentration fluctuations may reflect the preference of αTTP in the liver and its capacity to export greater amounts of tocopherols as the liver begins to function more efficiently as lactation progresses. Alternatively, as diets may change by lactation stage, changes in diet ingredients may alter the relative availability of individual dietary analogs. Given the proven benefits of non-α-tocopherol analogs in other species for reducing oxidative stress and potential safety when supplemented at greater concentrations, further studies should confirm their potential for causing adverse effects and focus study outcomes their potential to reduce oxidative stress biomarkers during the transition period when blood and liver α-tocopherol concentrations are reduced (Kamal-Eldin and Appelqvist, 1996, Sadri et al., 2015).

Parenteral injections of α-tocopherol have been studied previously as an intervention to overcome the reduction in DMI and liver function of the transition period (Erskine et al., 1997, LeBlanc et al., 2002, Pontes et al., 2015). Their use has been shown to have diminishing returns in animals that are adequately supplemented with α-tocopherol in the dry period, however, on farms with lower dietary supplementation, even a single parenteral injection of α-tocopherol can have

significant health benefits (Erskine et al., 1997, LeBlanc et al., 2002). Given the potential benefits of non-α-tocopherol analogs of vitamin E, parenteral administration is an unstudied but intriguing intervention. The increased labor of administering injections is an obvious draw back to the use of parenteral vitamins compared to dietary supplementation, however, the ability to use them in a targeted manner for rapid supplementation fits in well with potential applications of mixed tocopherols, especially for use as a potential therapeutic for animals with an overly robust or chronic inflammatory condition or who are harmfully oxidatively stressed.

INFLAMMATORY REGULATION

Pro-Inflammatory Signaling

Inflammation is a carefully orchestrated balance between pro-inflammatory and anti-inflammatory mediators that must meet pathogen challenge with an adequate response to contain infection yet controlled to prevent excessive tissue damage (Sordillo and Mavangira, 2014). During the transition period, inflammation in dairy cattle can become aberrant in some animals and result in excessive inflammation or inflammation that fails to resolve in an appropriate amount of time (Sordillo and Mavangira, 2014). In either case, the excessive or delayed resolution of inflammation can result in tissue damage due to increased exposure to pro-oxidants. Although inflammation should not be hindered in carrying out normal and necessary inflammatory responses, anti-inflammatory interventions can play an important part is limiting excessive damage to tissues from inflammation that has become dysregulated and damaging, as can be the case during transition inflammatory disease.

Studies have provided a wide range of anti-inflammatory functions of vitamin E analogs amongst many mammalian species (Qureshi et al., 2010, Wagner et al., 2014, Muid et al., 2016). In RAW 264.7 murine macrophages stimulated with endotoxin, supplementation with α-

tocotrienol, γ-tocotrienol, or δ-tocotrienol reduced expression of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) (Qureshi et al., 2010). Interestingly, this response was not elicited in α-tocopherol supplemented cells. When repeated in vivo with BALB/c mice fed individual tocotrienol supplements, serum TNF-α was again reduced in supplemented animals after LPS challenge (Qureshi et al., 2010). Inflammatory cytokines such as TNF-α are an essential component of an effective inflammatory response to infection, however, metabolically stressed cattle have compromised the inflammatory responses that can be excessive and destructive. In such cases, the anti-inflammatory nature of vitamin E analogs may limit further tissue injury.

An important control point for inflammatory responses is the vascular endothelium. Regulated leukocyte recruitment and migration to sites of infection are crucial for fighting infection, however excessive or prolonged leukocyte infiltration in response to infection can result in exacerbated tissue damage beyond that of infection alone. Vitamin E analogs have shown in human models of endotoxin induced inflammation a capacity to reduce the expression of adhesion molecules which would otherwise contribute to the recruitment of leukocytes to underlying tissue. In human umbilical vein endothelial cells, supplementation with δ-tocotrienol and γ-tocotrienol significantly reduced expression of genes necessary for the adhesion of leukocytes to the endothelium, namely *ICAM1*, *VCAM1*, and *SELE* (E-Selectin) (Muid et al., 2016). The practical outcome of reducing expression of these adhesion molecules was further shown in rats supplemented with γ-tocopherol. When challenged with nebulized LPS, γ-tocopherol supplemented rats had both reduced expression of pro-recruitment genes such as *MIP-2*, *CINC-1*, and *MCP-1* and reduced migration of neutrophils and total leukocytes (Hernandez et al., 2013, Wagner et al., 2014). These examples demonstrate anti-inflammatory functions of vitamin E analogs that reduce recruitment of leukocytes in several species. Although necessary for the initial

responses to infection and instances of life-stage dependent tissue remodeling, such as dry off, reducing leukocyte recruitment to resolve prolonged or excessive inflammation may be a potential use for analogs of vitamin E. The use of vitamin E analogs in a treatment capacity, rather than prophylactic, may allow for targeted resolution of inflammation when deemed appropriate without compromising the inflammatory responses necessary to prevent or eliminate disease.

Lipid Mediator Aberration

A significant driver of inflammation, especially in the transition period, is the alteration production a class of inflammatory lipid mediators known as oxylipids (Sordillo and Mavangira, 2014). Although several PUFA may act as a precursor for oxylipid production, arachidonic acid metabolites, known as eicosanoids, have been the most widely profiled of such oxylipids. These metabolites are produced both enzymatically, through the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450) pathways, and non-enzymatically through interaction with a reactive metabolite.

The precise inflammatory activities for oxylipids in cattle are an active area of study with significant gaps in knowledge remaining. The majority of research focused on the relationship between vitamin E analogs and oxylipds has focused on arachidonic acid derived oxylipids, however, a small number of studies have pointed to an apparent effect of non-α-tocopherol analogs on linoleic acid derived hydroxyoctadecadienoic acid (HODE) production. Specifically, 9-and 13-HODE, downstream metabolites of linoleic acid hydroperoxides, hydroperoxyoctadecadienoic acids (HPODE), produced by either COX, LOX, or non-enzymatic reactions. The HODEs have been found in lower circulating and tissue concentrations in mice fed α-tocopherol, γ-tocopherol, and α-tocotrienol (Yoshida et al., 2005, Yoshida et al., 2007, Gabbs et al., 2015). Certainly, Ryman et al. (2015) demonstrated in vitro that 13-HPODE can be a destructive metabolite, causing cellular

apoptosis and reducing bovine endothelial barrier integrity, however, 13-HODE did not negatively impact cellular health or function. No studies have yet explored a potential effect of vitamin E analogs specifically on HPODE. As HPODE and HODE concentrations are not always correlated and our understanding of the functions of HODE in cattle remain undeveloped, the findings that vitamin E analogs may reduce the production of HODE are difficult to presently put in context yet should not be overlooked as research into oxylipid function continues to advance.

In addition to linoleic acid derived HODE, production of the leukotriene group of oxylipids is reduced by analogs of vitamin E. Several studies have now presented evidence that non-α-tocopherol analogs of vitamin E can reduce the activity of LOX enzymes and subsequently production of arachidonic acid derived leukotrienes such as leukotriene B4 (LTB4) and leukotriene C4 after inflammatory stimulus in vitro and in vivo (Jiang and Ames, 2003, Jiang et al., 2011). Predominately produced by neutrophils, leukotrienes are important chemotactic factors and increased significantly in cattle with acute and chronic mastitis (Boutet et al., 2003, Mavangira et al., 2015). Increased concentration of leukotrienes during inflammatory events underscores the importance of these oxylipids in orchestrating an effective inflammatory response to infection. Persistently increased concentrations of leukotrienes, however, may lead to delayed resolution of inflammation and subsequent tissue damage. In such cases, reducing the production of leukotrienes can contribute to the resolution of inflammation and allow for tissue to heal. In a rodent model of carrageenan-stimulated inflammation, Jiang and Ames (2003) reported significant reductions in the production of LTB4 in animals dietarily supplemented with γ-tocopherol, but not those supplemented with α-tocopherol after subcutaneous inflammatory stimulation.

Both the COX and CYP450 pathways have been implicated as being regulated by vitamin E as well (Farley et al., 2013). The activity of vitamin E analogs on the production of oxylipids by

the COX pathway is mixed in the literature dependent on cell type and the vitamin E analog of focus. Many groups have shown reductions in the production of prostaglandins, typically pro-inflammatory oxylipids, produced from COX1 and COX2 enzymes (*PTGS1* and *PTGS2*) (Jiang and Ames, 2003, Yam et al., 2009, Kim et al., 2018). The mechanism by which the effects of vitamin E analogs influence COX derived oxylipid production remains unclear, potentially being mediated by changes in gene expression, post-transcriptional regulation, alterations in PUFA availability, or direct competition between arachidonic acid and vitamin E analogs.

In RAW 264.7 murine macrophages, α-tocotrienol and δ-tocotrienol both showed an ability to reduce the production of prostaglandin E_2 (PGE2) after stimulation with LPS as α-tocopherol significantly increased production of PGE2 (Yam et al., 2009). As expected, *COX1* gene expression was not impacted by LPS or any analog. The robust stimulation of *COX2* gene expression by LPS, however, was ameliorated not by α-tocopherol but by α-tocotrienol, δ-tocotrienol, and γ-tocotrienol (Yam et al., 2009). This suppression of *COX2* induction was noted in LPS challenged primary murine macrophages supplemented with γ-tocotrienol as well (Kim et al., 2018). Protein expression of COX2 was unchanged by any analog of vitamin E in RAW 264.7 cells stimulated by LPS (Yam et al., 2009). Shirode and Sylvester (2010), however, found a reduction in COX2 protein expression after γ-tocotrienol treatment in murine mammary epithelial cells. These studies have uncovered several mechanisms in which analogs of vitamin E may lessen the inflammatory response to LPS. Given that in many transition cows with coliform mastitis an overwhelming inflammatory response can cause excessive tissue damage, the ability of vitamin E analogs to reduce the proinflammatory effects of LPS may be a beneficial function.

Beyond mRNA or protein expression, γ-tocopherol and δ-tocopherol, but not α-tocopherol, reduced COX2 activity in a dose dependent manner in human A549 lung epithelial cells, albeit

through an unexpected mechanism. In this study, blocking metabolism of tocopherols and tocotrienols with an inhibitor of CYP4F2 significantly reduced the inhibitory activity of vitamin E analogs on PGE2 production from COX (Jiang et al., 2008). This suggests that, in regard to inhibition of COX2 activity, post-CYP4F2 metabolites of vitamin E analogs have greater bioactivity than their precursor molecules. Certainly, it is possible that had a more complete inhibitor of CYP4F2 been used, preventing all metabolism of vitamin E analogs, there may have been no inhibition of COX2 at all. Rather, sesamin is an incomplete inhibitor of CYP4F2 at the concentration used (1 μM) and therefore would reduce the metabolism of vitamin E analogs but not prevented it fully (Wu et al., 2009). Jiang et al. (2008) showed that specifically 9'-COOH and 13'-COOH, early metabolites of analog metabolism, were responsible for the inhibition of COX activity by directly competing with arachidonic acid for COX metabolism (Figure 2.1). Surprisingly, computer simulations suggested that 13'-COOH binds to COX1 with a greater affinity than arachidonic acid whereas 9'-COOH binds with less affinity.

Considering the potential activities of vitamin E analog downstream metabolites will be important if research is to further understand the potential roles of CYP4F2 inhibitors that may reduce their production. Supplementing a CYP4F2 inhibitor, such as sesamin, alongside an analog mix would help to reduce the amount of non-α-tocopherol analogs necessary to supplement to overcome their preferential metabolism (Ikeda et al., 2001). The aforementioned research on COX inhibition, however, shows that inhibiting CYP4F2 may reduce some anti-inflammatory properties of vitamin E analogs. The competing properties of pre- and post-CYP4F2 metabolites means that practical supplementation in cattle would need to determine which specific balance of analog function is most beneficial to animals and will add a layer of complexity to initial research.

Evidence of significant activity due to post-CYP4F2 vitamin E metabolites goes well beyond the production of PGE2, however. The initial breakdown product of δ-tocopherol, δ-13'-COOH, reduced the production of LTB4 in human HL-60 cells, an activity which was additionally validated for both δ-tocopherol and δ-13'-COOH in human primary neutrophils (Jiang et al., 2011). Specifically, this decrease in production was due to a reduction in the activity of the 5LOX enzyme. Further research has additionally shown significant activity of downstream metabolites of vitamin E. In human peripheral blood mononuclear cells stimulated with a COX inducer, both α-3'-COOH (α-CEHC) and γ-3'-COOH (γ-CEHC) significantly reduced ROS production (Wiser et al., 2008). A similar phenomenon was noted for human neutrophils stimulated with a nuclear factor kappa B inducer in which γ-3'-COOH (γ-CEHC) and δ-3'-COOH (δ-CEHC) reduced superoxide production to a greater degree than α-3'-COOH (α-CEHC) (Varga et al., 2008). Of all of the metabolites produced from tocopherols, it seems many have some form of anti-inflammatory or antioxidative activities. Overall, 3'-COOH (CEHC) appears to be the most active compound in the vitamin E analog cascade (Wagner et al., 2014).

SAFETY OF IN VIVO SUPPLEMENTATION

Non-α-tocopherol analogs of vitamin E are used to treat, ameliorate, and prevent inflammatory conditions in non-bovine species yet limited research has sought to understand such applications in dairy cattle (Jiang, 2014). One of the reasons for this is a justified concern for creating adverse effects by supplementing vitamin E analogs, especially the tocotrienols (Galli et al., 2017). In mice injected subcutaneously with δ-tocotrienol and γ-tocotrienol, a severe skin irritation was noted at doses above 200 mg / kg, an amount 4-20 times greater than that used for most in vivo studies. Aside from this cutaneous reaction, however, a 200 mg / kg dose was tolerated with no measurable or visible impact on internal organs (Swift et al., 2014). A human profiling

study further showed that individuals with greater plasma γ-tocopherol had reduced plasma α-tocopherol and β-carotene concentrations in addition to increased plasma F_2-isoprostane as measured by gas chromatography mass spectrometry (Abdulla et al., 2018). This study, however, was not a clinical trial but rather a profile of general population. Due to this, it is difficult to be certain that increasing concentrations of γ-tocopherol were indeed a causative factor for increasing oxidative stress or reduced vitamin concentrations rather an unknown confounding variable, such as patient diet. Certainly, many studies have shown that the dose at which non-α-tocopherols cause cytotoxic effects are much lower in various cells types than that of α-tocopherol in vitro; the concentrations at which non-α-tocopherol analogs have bioactivity, however, has also routinely been shown to be significantly lower than that of α-tocopherol meaning doses cannot be directly compared (McCormick and Parker, 2004, Mazlan et al., 2006, Yam et al., 2009).

Although cytotoxicity of all vitamin E analogs should be assessed, there has not yet been an indication of detrimental effects of non-α-tocopherols supplemented dietarily in ruminants (Kasimanickam and Kasimanickam, 2011, Adeyemi et al., 2016, Qu et al., 2018). Supplementation of tocopherol mixes in goats, sheep, and cattle have all showed that at up to at least 18 mg / kg of total tocopherol no adverse outcomes were noted, nor were any increases in indicators of oxidative stress or immune suppression (Table 2.1) (Kasimanickam and Kasimanickam, 2011, Elsasser et al., 2013, Adeyemi et al., 2016). Dietary vitamin mixes containing significant amounts of tocotrienols on the other hand have not been evaluated in ruminants. Additionally, parenteral administration of non-α-tocopherol analogs has not been reported in ruminants. Studies of parenteral injection of α-tocopherol, however, provide no evidence of injection site hypersensitivity reactions or other adverse effects (LeBlanc et al., 2002, Pontes et al., 2015).

As of yet, the limited number of feeding trials in cattle using mixed tocopherols have not garnered the same positive results as those seen in humans. Although feeding mixed tocopherols to beef calves slightly reduced LPS induced liver inflammation and oxidant damage, supplementing mid-lactation dairy cows with a tocopherol mix did not elicit as beneficial of results (Elsasser et al., 2013, Qu et al., 2018). Mid-lactation cows were studied by Qu et al. (2018) to avoid the dramatic shifts of transition period inflammatory responses. When supplemented with a tocopherol mix at a very similar dose to that of a human supplementation trial (~10 mg / kg), neutrophils of mid-lactations cows challenged with LPS did not show a difference in inflammatory gene expression between those from supplemented and un-supplemented cows. This differs from the results of the related human trial in which LPS stimulation of peripheral blood mononuclear cells had reduced IL-1β, IL-6, and TNFα expression when derived from supplemented humans compared to un-supplemented (Wiser et al., 2008). A lack of differential response to LPS may be due to the difference in response to LPS by neutrophils compared to peripheral blood mononuclear cells. Additionally, given the proclivity for transition cattle to face dysfunctional inflammation compared to mix-lactation cows, a more practical study may focus on animals in the transition period when inflammatory responses to LPS may indeed be aberrant and potentially excessive. Alternatively, the species different responses may be explained blood γ-tocopherol concentrations being about 1/3 less in supplemented cattle than that of humans, despite similar supplementation concentrations, suggesting absorption, metabolism, or storage of vitamin E may differ significantly between the 2 species. Several reasons may explain why cattle have less of an increase in circulating tocopherols despite similar supplementation to humans. One such explanation is the vehicle used being a capsule in the human trial whereas cattle received an oil form of vitamin mix resulting in differential uptake up the supplement. Additionally, as analogs of vitamin E are

packaged into lipoproteins, differential liver activity, likely lower in cattle compared to humans, would result in lower release from bovine liver compared to humans. Because of this, it may be necessary to supplement these vitamin E analog mixes either in higher concentrations to cattle or to use parenteral administration, avoiding any reductions in bioavailability that may be cause by the rumen.

CONCLUSIONS

The use of vitamin E analogs in other mammalian species has revealed that not all vitamin E analogs have equivalent biological activities. Each has unique antioxidant and anti-inflammatory activities that vary by species, cell type, and other physiological conditions. Rather than trying to identify a single best analog to supplement alongside α-tocopherol, taking a broader approach to supplementation with a mixture of analogs likely offers the greatest benefits. Based upon this and the disproportionate prevalence of oxidative stress and dysfunctional inflammation around the time of calving rather than other stages of lactation, the use of a mixed analog supplement should be evaluated for targeted supplementation. As mentioned previously, peak plasma concentrations tocopherols are reached after only 5 d of feeding suggesting that long-term supplementation is likely not necessary (Qu, 2017). Alternatively, peak concentrations of blood α-tocopherol have been reported at similarly short periods after parenteral administration, notably after 7 d when injected subcutaneously or only 1-2 d if given intramuscularly, although a time course for liver accumulation was not undertaken in these studies (LeBlanc et al., 2002, Jensen et al., 2020). Although not a tocopherol mix, these studies suggest that such mixes would likely reach peak blood concentrations in a short period of time, however, the time to peak liver accumulation of mixed tocopherols supplemented either dietarily or parenterally remains unknown. Focusing on those animals at greatest risk for disease and at life stages where concentrations of α-tocopherol

typically lower than average in blood and tissues, such as cattle at calving and into early lactation, may provide the greatest benefit with lowest risk of negative effects. There is ample reason to move forward with greater use of vitamin E analogs in disease models to begin to discern what benefits they may afford to dairy cattle, or potentially what harm could be caused if inflammatory responses are overly constrained. With reported safety at specific doses there is also the potential to begin supplementation in vivo, monitoring for unknown adverse effects, to determine what impacts such tocopherol or tocotrienol mixes may have on the redox balance or regulation of inflammation.

The most significant obstacle that this research will face is the preferential metabolism of non-α-tocopherol analogs. Without interventions to reduce this metabolism, benefits to supplementation will be limited by the rapid breakdown of such vitamers. The previously mentioned inhibitor of CYP4F2, sesamin, has been used in human clinical trials and shown to indeed increase the circulating concentrations of non-α-tocopherols (Wu et al., 2006). Given that the relative contribution of the post-CYP4F2 metabolites of vitamin E analogs is only beginning to be uncovered, utilizing an incomplete inhibitor, such as sesamin, may reduce the chances of fully inhibiting the production of these downstream metabolites and preventing their potentially beneficial activities. For these reasons, supplementation of sesamin or sesame oils, or other partial CYP4F2 inhibitors, alongside a vitamin E analog mix is likely to provide additive beneficial effects.

The use of vitamin E analogs as dietary supplements in cattle remains relatively unexplored despite evidence that such analogs may be able to contribute to the antioxidant potential of an individual. Mixed tocopherols have been supplemented safely in both cows, calves, and other ruminant species justifying further research into their safety and biological activities in dairy cattle.

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CHAPTER 3

DIFFERENCES IN THE OXYLIPID PROFILES OF BOVINE MILK AND PLASMA AT DIFFERENT STAGES OF LACTATION

Matthew J. Kuhn,[1] Vengai Mavangira,[1] Jeffery C. Gandy,[1] Chen Zhang,[2] A. Daniel Jones,[2,3] and Lorraine M. Sordillo*[1]

[1]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing 48824, United States, [2]Department of Chemistry, and [3]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing 48824, United States

***Corresponding author**

Dr. Lorraine M. Sordillo,

Department of Large Animal Clinical Sciences

College of Veterinary Medicine

Michigan State University

sordillo@msu.edu

Tel: (517) 432-8821

Fax: (517) 432-8822

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ABSTRACT

Mastitis is caused by a bacterial infection of the mammary gland, which reduces both milk quality and quantity produced for human consumption. The incidence and severity of bovine mastitis are greatest during the periparturient period that results from dysfunctional inflammatory responses and causes damage to milk synthesizing tissues. Oxylipids are potent fatty acid-derived mediators that control the onset and resolution of the inflammatory response. The purpose of this study was to investigate how oxylipid profiles change in bovine milk at different stages of the lactation cycle. Results showed significantly lower concentrations of both milk polyunsaturated fatty acid content and total oxylipid biosynthesis during early lactation when compared to mid- or late-lactation. The only oxylipid that was higher during early lactation was 20-hydroxyeicosatetraenoic acid (HETE), which is often associated with inflammatory-based diseases. Milk oxylipid profiles during the different stages of lactation differed from plasma profiles. As such, plasma fatty acid and oxylipid concentrations are not a proxy for local changes in the mammary gland during the lactation cycle.

Key words: bovine milk, eicosanoids, fatty acids, lipid mediators, mammary gland, oxylipids, oxylipins

INTRODUCTION

Bovine milk is an important food source in the human diet that is rich in carbohydrates, proteins, and lipids (Haug et al., 2007). A major factor affecting the quality and nutrient content of milk is mastitis, which is caused by intramammary bacterial infections and results in an uncontrolled inflammatory response (Aitken et al., 2011). There are specific times in the lactation cycle when dairy cows are most susceptible to mastitis. The incidence and severity of mastitis is most pronounced during the periparturient period when metabolic stress is at its peak and inflammatory responses are dysfunctional (Sordillo et al., 2009). Dysfunctional inflammatory responses in the mammary glands of periparturient dairy cattle may become acute or chronic, resulting in excessive tissue damage, and cause lifetime decreases in milk production (Archer et al., 2013). There is a need to identify host factors that are differentially expressed during lactation that impact the onset and resolution of inflammation thereby influencing the susceptibility of cows to new intramammary infections.

Optimal inflammatory responses are tightly regulated such that the initial reaction must be robust enough to limit and eliminate infection, followed immediately by healing and repair of damaged tissues after the resolution of infection. A balance between proinflammatory and anti-inflammatory metabolites must be maintained in order to achieve an effective inflammatory response (Serhan, 2008). Polyunsaturated fatty acid (PUFA) derived lipid mediators known as oxylipids coordinate a balanced inflammatory response. Each oxylipid possesses proinflammatory and/or anti-inflammatory functions (Gabbs et al., 2015). The relative abundance of different oxylipids creates a profile that can shift the inflammatory balance, either maintaining proper control for successful elimination of the pathogen or undergoing loss of regulatory control leading to chronic infection or injurious inflammation.

Oxylipids are derived from PUFA including linoleic acid (C18:2n-6, LA) (Ramsden et al., 2012), arachidonic acid (C20:4n-6, AA) (Rosenthal et al., 1995), eicosapentaenoic acid (C20:5n-3, EPA) (Serhan, 2008), and docosahexaenoic acid (C22:6n-3, DHA) (Wittwer and Hersberger, 2007). These PUFA are mainly hydrolyzed from the cellular plasma membrane by phospholipase-A2 and metabolized by the cyclooxygenase (COX), lipoxygenase (LOX), or cytochrome P450 (CYP) enzymatic pathways or can be nonenzymatically converted to oxylipids by interaction with reactive oxygen species (ROS) (Buczynski et al., 2009, Murakami et al., 2011). Accurate measurements of oxylipids have many inherent challenges. Many oxylipids are thermally unstable and readily autoxidize. As such, there is a need for proper sample collection, handling, and preparation in order to ensure accurate identification and quantification of these lipid-derived metabolites. Several measurement modalities have been utilized to quantify oxylipids, including colorimetric (Jessup et al., 1994) and spectrophotometric assays (Nourooz-Zadeh, 1999), ELISA, gas chromatography mass spectrometry (GC/MS), and liquid chromatography mass spectrometry (LC/MS) (Niki, 2014); however, liquid chromatography tandem mass spectrometry (LC/MS/MS) has become the gold standard for sensitive and specific oxylipid determination.

A previous study in dairy cattle addressing changes in oxylipids during severe coliform mastitis found that 15 of the 63 oxylipids measured were increased in milk from clinically ill animals compared to healthy controls (Mavangira et al., 2015). Oxylipids with proinflammatory and/or anti-inflammatory functions were altered to various degrees. For example, the proinflammatory oxylipid 20-hydroxyeicosatetraenoic acid (HETE) was increased over 700-fold over control while anti-inflammatory lipoxin A4, derived from the same PUFA substrate, increased only 9-fold over control. Whereas all oxylipids were increased during mastitis, proinflammatory oxylipids were increased to a considerably greater degree than anti-inflammatory

oxylipids (Mavangira et al., 2015). Previous studies associated increased proinflammatory oxylipid profiles in both plasma and milk of mastitic cows with the dysfunctional inflammatory responses of the mammary gland that contributes to disease severity (Aitken et al., 2011, Mavangira et al., 2015, Ryman et al., 2015b). However, there are no studies documenting oxylipid profiles in the plasma and milk of periparturient cows that are more susceptible to mastitis when compared with later stages of lactation. This information would be essential to determine if baseline changes in oxylipid biosynthesis may be a contributing factor leading to dysfunctional inflammatory responses and increased disease susceptibility during the periparturient period. Using LC/MS/MS, we report changes in both plasma and milk oxylipid expression in periparturient cows and compare these profiles to those obtained during later stages of lactation when cows are less susceptible to disease.

METHODS

Safety

All chemicals used for extraction and processing of metabolites were disposed of according to Michigan State University Environmental Health and Safety regulations and guidelines. Proper personal protective equipment was worn by all individuals involved in the processing of samples to limit any exposure to chemicals.

Animals

The use of animals in this study was approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC, reference number 03/14-059-00). The study was conducted at a 3,300 lactating cow commercial dairy operation in Michigan with consent from the owner. The herd milking average was approximately 12,250 kg, milking twice daily, and cows were housed in free stall barns, grouped according to lactation number, days in milk (DIM),

and milk yield. Diets were formulated based on production energy requirements (Table 3.1), and feed was delivered 2 times / d as total mixed rations.

Study Design

Thirty-six multiparous dairy cows of at least third lactation (average age of 5.29 yr old) were enrolled and divided by stage of lactation into periparturient (PP) (n = 13), mid-lactation (ML) (n = 13), and late-lactation (LL) (n = 10) groups. All animals in the periparturient group were 1–2 DIM. The mid-lactation group animals were between 80 and 95 DIM. The late-lactation animals were 184 to 207 DIM. Negative bacterial cultures on milk, absence of overt clinical signs, and a somatic cell count of <250,000 cells / mL on last test day were required for each group. Bacterial milk cultures were performed on blood agar and selective media for coliform bacteria according to the National Mastitis Council guidelines such that individual mammary gland quarter milk was streaked onto sheep blood agar and selective media and incubated at 37 °C for up to 48 h (Council, 1999). Samples were considered positive for bacterial culture based on the presence of 3 or more colonies on both media.

Chemicals

Acetonitrile, methanol, and formic acid of high performance liquid chromatography (HPLC) grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deuterated and nondeuterated oxylipid standards were purchased from Cayman Chemical (Ann Arbor, MI, USA). Butylated hydroxytoluene (BHT) was purchased from ACROS (New Jersey, USA), Ethylenediaminetetraacetic acid (EDTA) and triphenylphosphine (TPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and indomethacin was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Sample Collection

Milk samples were aseptically collected and flash frozen. Blood was drawn by coccygeal venipuncture into evacuated tubes with EDTA and immediately placed on ice for transport to the laboratory. Blood tubes were subsequently centrifuged at 2000*g* for 18 min at 4 °C, and the plasma was harvested, aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C pending analysis within 2 months of sample collection.

Lipidomics

Targeted PUFA, monounsaturated fatty acid, saturated fatty acids, and oxylipids were quantified using LC/MS/MS. A full list of metabolites can be found in Supplemental Table 1. Blood and milk samples were extracted and analyzed using methods published previously by Mavangira et al (Mavangira et al., 2015). Briefly, milk (4 mL) and plasma (2 mL) samples were mixed with an antioxidant reducing agent mixture (4 μL of antioxidant reducing agent / 1 mL of sample) to prevent degradation of preformed oxylipids and prevent ex vivo lipid peroxidation as described previously (O'Donnell et al., 2009). The antioxidant reducing agent mixture consisted of 50% methanol, 25% ethanol, and 25% water with 0.9 mM of BHT, 0.54 mM EDTA, 3.2 mM TPP, and 5.6 mM indomethacin. Samples were flash frozen and stored at −80 °C until analyses. For processing, samples were thawed on ice and combined with a mixture of internal standards containing 5(S)-HETE-d_8 (0.25 μM), 15(S)-HETE-d_8 (0.25 μM), 8(9)-EET-d_{11} (0.5 μM), PGE_2-d_9 (0.5 μM), 8,9-DHET-d_{11} (0.25 μM), AA-d_8 (50 μM), 2-AG-d_8 (2 μM), and AEA-d_8 (0.25 μM). After addition of acetonitrile, methanol, and formic acid, samples were vortexed for 2 min, incubated at room temperature for 15 min, and centrifuged at 4816*g* for 20 min at 4 °C. Supernatant was diluted with HPLC water containing 0.1% formic acid. Solid phase extraction was carried out with Oasis HLB 12CC LP extraction columns (Waters, Milford, MA, USA) preconditioned with

methanol and HPLC water. Supernatants were loaded into the columns, washed with methanol, and eluted with a 50:50 mixture of methanol and acetonitrile. Volatile solvents were removed using a Savant SpeedVac, and residues were reconstituted in methanol, mixed at a 1.5:1 ratio with HPLC water, and stored in glass chromatography vials with glass inserts at −20 °C until analysis. Data analysis was performed by generating 7 point linear curves with standards purchased from Cayman Chemical, Ann Arbor, MI. The curves for oxylipids and endocannabinoids were 5-fold dilution ranging from 100 nM to 0.01 nM. The curve for PUFA, monosaturated fatty acids, and saturated fatty acids was a 5-fold dilution ranging from 500 μM to 0.001 μM. The linear curves generated produced R^2 values of 0.99 with percent deviations of less than 100%.

Liquid Chromatography Tandem Mass Spectrometry Analyses

Details of LC/MS/MS analysis are described in Mavangira et al (2015). In short, the quantification of metabolites was accomplished on a Waters Xevo-TQ-S tandem quadrupole mass spectrometer using multiple reaction monitoring (MRM) as summarized in Supplemental Table 1. Chromatography separation was performed with an Ascentis Express C18 HPLC column (10 cm × 2.1 mm; 2.7 μM particles, Sigma-Aldrich, St. Louis, MO) held at 50 °C, and the autosampler was held at 10 °C. Mobile phase A was water containing 0.1% formic acid, and mobile phase B was acetonitrile. Flow rate was fixed at 0.3 mL / min. Liquid chromatography separation took 15 min per sample. All oxylipids and fatty acids were detected using electrospray ionization in negative-ion mode. Endocannabinoids were detected using positive-ion mode. MRM parameters including cone voltage, collision voltage, precursor ion, product ion, and dwell time were optimized based on Waters QuanOptimize software by flow injection of pure standard for each individual compound.

Statistical Analysis

Statistical analyses were performed using the SAS software (version SAS 9.4; SAS Institute Inc., Cary, NC). Data were expressed as means ± standard error of the mean (SEM) and tested for normality. Where Gaussian distribution was satisfied, a one-way ANOVA was performed with contrasts specified as follows: PP vs ML, PP vs LL, and PP vs LL. Data deviating from the Gaussian distribution were either square root or log transformed prior to analyses. An ANOVA and Tukey test for comparison between means were performed to determine differences among fatty acid concentrations in the same sample type (milk or plasma). Pearson correlations were calculated for multiple relationships among fatty acid substrates; fatty acid substrates and their oxylipid metabolites; related upstream and downstream oxylipid metabolites and concentrations of the same substrate / metabolite in milk vs plasma. Bivariable linear regression analyses were performed to determine trends between ω-6 : ω-3 ratios and lactation stage using Stata software (version Stata 14.2). In each model, pairwise comparisons (Bonferroni adjusted) were used to compare ratios at different lactation stages. The levels of significance for correlation analyses were adjusted for multiple comparisons. Statistical significance was set at 5%.

RESULTS AND DISCUSSION

The oxygenation of fatty acids results in a vast, interconnected, and highly regulated network of lipid mediators known as oxylipids. In the present study, changes in plasma oxylipids at different lactation stages support the concept that oxylipid biosynthesis is complex. Figure 3.1 shows that both pro- and anti-inflammatory metabolites were higher and lower in the PP compared to other stages of lactation. For example, previous studies documented the proinflammatory functions of 20-HETE, 9,10-DiHOME (Moran et al., 1997, Edin et al., 2011), and 5-oxoETE (Ryman et al., 2015b). In the current study, while proinflammatory 20-

HETE was higher, other oxylipids with known proinflammatory functions (9,10-DiHOME and 5-oxoETE) were lower during the PP. There are also several oxylipids with known anti-inflammatory properties including 5(*S*),6(*R*),15(*S*)-lipoxin A4 (LXA4) (Lee et al., 1989, Chinthamani et al., 2012) and 13-oxooctadecadienoic acid (oxoODE) (Altmann et al., 2007). Whereas LXA4 was higher during the PP, there also was a concomitant reduction in 13-oxoODE at this stage of lactation. Thus, the observed changes in plasma oxylipid biosynthesis during the PP may represent several potentially antagonistic mechanisms of regulation that could impact the severity and duration of the inflammatory response.

Each oxylipid has its own roles in physiology including the maintenance of reproductive efficiency (Fortier et al., 2008) to regulating the inflammatory cascade (Chinthamani et al., 2012, Raphael and Sordillo, 2013, Raphael et al., 2014). The proinflammatory or anti-inflammatory properties of oxylipids may depend on the timing of their expression during an inflammatory response. For example, while 13-hydroxyoctadecadienoic acid (HODE) responds quickly during the initiation of inflammation (Nieman et al., 2016) with increased adhesion molecule expression (Friedrichs et al., 1999), epoxyeicosatrienoic acid (EET) isoforms have proresolving and angiogenic effects to reduce inflammation and aid in tissue repair (Bystrom et al., 2011, Panigrahy et al., 2013). The timing and relative abundance of oxylipids with either proinflammatory or proresolving functions is essential for optimal inflammatory response that is characterized by an initially robust response during tissue injury yet resolves promptly once the source of tissue injury is eliminated. Maintaining a proper balance that shifts at appropriate times can dictate the difference between return of tissues to homeostasis or establishment of chronic infection. The findings from the present study indicate that oxylipid profiles do change as a function of lactation

stage. Additional studies are required to determine how shifts in oxylipid profiles may contribute to aberrant inflammatory responses associated with the periparturient dairy cattle.

Substrate Availability

The biosynthesis of specific oxylipids is dependent upon the availability of PUFA substrates that can be oxidized through enzymatic or nonenzymatic pathways. In agreement with other studies (Contreras et al., 2013), LA was the most abundant PUFA quantified for all stages of lactation, followed by another ω-6 fatty acid, AA. The ω-3 fatty acids EPA and DHA were found at much lower concentrations than the ω-6 fatty acids (Figure 3.2A). While not unexpected due to the influence of LA-enriched corn in the modern dairy cattle diet, the difference between concentrations of ω-6 and ω-3 fatty acid substrates can account for the oxylipid profiles reported in this study. Generally, oxylipids derived from ω-6 fatty acids have proinflammatory tendencies. A notable exception to this is the many CYP derived oxylipids from AA (Bystrom et al., 2011). On the other hand, ω-3 fatty acids are generally substrates that form anti-inflammatory acting oxylipids (Gabbs et al., 2015). As the production of oxylipids is partly dependent on substrate availability, a greater proportion of ω-6 fatty acids equates to a greater production of proinflammatory characterized oxylipids (Poulsen et al., 2008, Contreras et al., 2012a). However, because the enzymatic pathways have substrate preferences, many times preferentially oxidizing ω-3 PUFA, increases in ω-3 PUFA not only increase the production of anti-inflammatory oxylipids but promote competition with ω-6 PUFA for enzymatic oxidation. This can lead to an increase in ω-3 oxylipids as well as a decrease in those produced from ω-6 PUFA (Westphal et al., 2015).

In plasma, while no statistical change across lactation was noted in concentrations of EPA, other PUFA were increased significantly during the PP compared to ML. LA and AA remained unchanged between ML and LL while DHA had a significant increase in LL. These results support

the current literature that shows an increase in LA and AA concentration from 15 days before parturition up to 10 DIM (Raphael et al., 2014). The observed shift in PUFA concentrations around the time of calving was in line with the physiological changes occurring in dairy cows during the periparturient period. As cows are near calving, they fall into a state of negative energy balance due to decreased feed intake and the onset of milk production. This draw for more energy leads to the mobilization of adipose tissue and a significant increase in non-esterified fatty acids (NEFA), of which PUFAs are a constituent together with saturated and monounsaturated fatty acids (Bertoni et al., 2010, Contreras et al., 2010). As cattle return to energy homeostasis in ML, NEFA decrease (Table 3.2), as did the PUFAs. Currently, the impact of high NEFA on oxylipid production is unclear. Certainly, it has been shown that increased NEFA is correlated with a proinflammatory cellular phenotype (Contreras et al., 2012b) and severity of disease (Burvenich et al., 2003); however this is likely due to increased saturated fatty acids, such as palmitic acid, which was increased significantly during the PP and has known proinflammatory activities (Harvey et al., 2010). Our data did not provide evidence that increased PUFA in plasma during the PP had a clear impact on oxylipid biosynthesis.

Milk PUFA quantification revealed that the ω-6 fatty acids LA and AA predominated over the ω-3 fatty acids EPA and DHA; however, quite unexpectedly, the amount of total PUFA abundance was decreased significantly in the PP and ML compared to LL for all PUFA except DHA ($P < 0.1$) as seen in Figure 3.2B. Previously, due to several studies correlating human breast milk PUFA with plasma PUFA, we had expected that PUFA in bovine milk would mimic that available in systemic circulation (Cherian and Sim, 1996, Marangoni et al., 2002, Torres et al., 2006). We now hypothesize that this observed trend is due to the significant synthesis of milk fat during lactation as the observed concentrations of milk PUFA follow a typical lactation curve

(Silvestre et al., 2009). In order to evaluate if there was selective transport and/or metabolism of particular fatty acids during milk synthesis, we compared the relative values of the ω-3 and ω-6 PUFA across lactation. The ratio of ω-3 to ω-6 fatty acids in milk and plasma was similar at all stages of lactation (Figure 3.3). This suggests that PUFA were utilized for lactation at nearly equal proportions. As with plasma PUFA, the direct impact of changes in substrate availability on mammary gland oxylipid production is not fully understood, and changes seen in milk could have been due to several regulatory mechanisms.

Oxylipid Production

The existence of several pathways of oxylipid production including enzymatic and nonenzymatic systems makes it difficult to determine their relative contributions to the formation of oxylipids. For example, 11-HETE is a predominantly nonenzymatically derived oxylipid whose production from AA is primarily determined by the amount of ROS (Zein et al., 2012, Puri et al., 2016). In the present study, the amount of milk 11-HETE was significantly correlated with milk AA substrate concentration (r = 0.603, P < 0.0001); however, this relationship suggests that the changes in 11-HETE production are only partially attributable to substrate availability. Based on changes observed in other oxylipids, it is likely that the changes in 11-HETE production not only are attributable to substrate availability but also can be due to the oxidative environment of the mammary gland (Table 3.3). One way to determine the relative contribution of oxidative status on oxylipid production is by using a ratio of (9-HETE + 11-HETE) / 15-HETE. In addition to 11-HETE, 9-HETE is also produced through nonenzymatic reactions with ROS (Shishehbor et al., 2006) while 15-HETE is enzymatically (LOX) derived (Dobrian et al., 2011). As all three oxylipids are biosynthesized from AA, changes in this ratio may reflect shifts in AA metabolism between enzymatic and nonenzymatic oxidation. Our data reveal a change in this ratio in milk,

having significantly lower relative nonenzymatic oxylipid production during the PP compared to ML or LL (Figure 3.4A). Like 9-HETE and 11-HETE derived from AA, a LA metabolite of nonenzymatic oxidation, 9-HODE, was additionally found to be significantly decreased during the PP compared to ML or LL (Figure 3.4B). These findings were unexpected as periparturient period dairy cattle often suffer from oxidative stress and would be expected to produce more nonenzymatically derived oxylipids.

Although ROS-generated oxylipids were low in milk during the PP, changes in oxidant status may affect oxylipid biosynthesis indirectly by regulating different enzymatic oxidation pathways. For example, a pro-oxidant environment can either enhance LOX-mediated enzymatic metabolism of PUFA or decrease production of oxylipids through CYP-dependent pathways (Shishehbor et al., 2006, Larsen et al., 2008). In the present study, 13-HODE biosynthesis through the LOX pathways was significantly decreased in milk from cattle in the PP (Figure 3.5). Thus, based on the reduced biosynthesis of both nonenzymatic and LOX-derived oxylipids, one may speculate that the PP mammary gland may represent a decreased oxidative environment when compared to other stages of lactation. Oxylipid profiles also may be a reflection of the degree of downstream metabolism following biosynthesis of the initial oxidation product (Gabbs et al., 2015). For example, one of the most influential enzymatic reactions is the hydration of specific oxylipids by soluble epoxide hydrolase (sEH). sEH is positively regulated by ROS status, increasing in activity and increasing the metabolism of oxylipids to downstream metabolites as ROS levels increase (Abdelhamid and El-Kadi, 2015). In milk, the ratios of upstream 9,10-epoxyoctadecenoic acid (EpOME) and 12,13-EpOME to downstream 9,10-dihydroxyoctadecenoic acid (DiHOME) and 12,13-DiHOME, respectively, were significantly higher in the periparturient period than ML and LL in both isomers (Figure 3.6). An increased ratio

during the PP suggests a decrease in sEH activity at this period compared to other stages of lactation as would be expected during a stage with low levels of ROS. The change in activity of sEH and decrease of 13-HODE production during the PP conforms with the data from nonenzymatically derived oxylipids suggesting that ROS levels in the mammary gland during the PP may not impact oxylipid production in the manner once previously thought. Additionally, the lack of a change in corresponding plasma oxylipids suggests that the oxidant state of the mammary gland may not reflect the systemic state.

Correlations with Plasma Oxylipids

A total of 29 oxylipids were detected in milk, but only 15 were significantly different during at least one period of lactation compared to other stages. In comparison, only five plasma oxylipids changed over the course of lactation where only 2 of these plasma oxylipids changed in a similar manner in milk (Table 3.4). This substantial difference between the number of oxylipids changing systemically and in the mammary gland was interesting to observe in healthy animals. Of note in plasma, LXA4 was increased during the periparturient period without a concurrent increase in other similarly produced oxylipids. LXA4 is derived through more than one enzymatic pathway, each stemming from initial oxygenation by different LOX enzymes (Dobrian et al., 2011, Tam, 2013, Gabbs et al., 2015). Lipoxin A4 is classically thought of as being produced from the original oxygenation products of 5-LOX and 15-LOX, 5-hydroperoxyeicosatetraenoic acid (HPETE) and 15-HPETE, respectively. The increase in LXA4 without concurrent increase in either of 5-HETE or 15-HETE, direct downstream metabolites of 5-HPETE and 15-HPETE, raises the proposition of preferential production of LXA4 over 5-HPETE or 15-HPETE. This is of interest as LXA4 is a potent anti-inflammatory oxylipid with some antioxidant properties (Lee et al., 1989, Chinthamani et al., 2012). Increasing the production of LXA4 through interventions has

been studied previously (Serhan, 1997) and the increase of LXA4 over proinflammatory oxylipids during the periparturient period in both milk and plasma supports the justification to explore LXA4 further as a means to combat the low grade inflammatory state found systemically in periparturient dairy cattle (Turk et al., 2008, Abuelo et al., 2016).

Interestingly, as periparturient dairy cattle had an increase in milk LXA4, a similar increase in LXA4 was observed in cattle with severe coliform mastitis when compared to healthy controls (Mavangira et al., 2015). In fact, a number of similarities were found between cattle experiencing severe mastitis and those in the periparturient period. Most striking was the similarities of the milk oxylipids influenced by the animal's oxidant status. While abundance of 9-HETE and 11-HETE was found to be increased in coliform cattle compared to controls, unlike the decrease seen in the PP compared to ML or LL, the ratio of (9-HETE + 11-HETE) / 15-HETE was decreased, albeit not significantly, in coliform cattle, partly due to a significant increase in enzymatic production of 15-HETE. Further, the ratios of 9,10-EpOME/9,10-DiHOME and 12,13-EpOME/12,13-DiHOME were both significantly higher in coliform cows as was seen in cattle during the PP. Similar also was the change in 20-HETE during mastitis and the PP, both increasing in these animals compared to controls or other stages in lactation (Mavangira et al., 2015). As 20-HETE is a potent proinflammatory mediator, this is an important relationship to observe. Raphael et al. found that 20-HETE was increased from 15 days prior to calving and steadily decreased to ML in healthy animals, suggesting that the increase in 20-HETE is related to physiological changes occurring during the transition period (Raphael et al., 2014). The similarities between cattle in the PP and those with severe coliform mastitis indicate that the PP itself creates an environment in the mammary gland that in ways reflects that which is seen in severe disease. Although speculative,

the increase in 20-HETE during the PP may contribute to the predisposition to disease and uncontrolled inflammatory responses that occur during this time.

In conclusion, these data illustrate that the physiological changes occurring during the periparturient period in healthy dairy cattle have a significant impact on the production of specific oxylipids by several regulatory mechanisms. Further, the changes occurring during the periparturient period are consistent with changes found during cattle suffering from severe coliform mastitis reflecting a profile of oxylipids favoring greater proinflammatory characteristics than found during other stages of lactation. These changes could significantly contribute to the increased incidence and severity of disease that occurs during the periparturient period. Additional studies that define how changes in specific oxylipids may impact inflammatory responses are warranted and could identify potential therapeutic targets for the control of inflammatory based diseases in dairy cows.

CHAPTER 4

PRODUCTION OF 15-F2t-ISOPROSTANE AS AN ASSESSMENT OF OXIDATIVE STRESS IN DAIRY COWS AT DIFFERENT STAGES OF LACTATION

Matthew J. Kuhn[1], Vengai Mavangira[1], Jeffery C. Gandy[1], and Lorraine M. Sordillo*[1]

[1]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing 48824

***Corresponding author**

Dr. Lorraine M. Sordillo,

Department of Large Animal Clinical Sciences

College of Veterinary Medicine

Michigan State University

sordillo@msu.edu

Tel: (517) 432-8821

Fax: (517) 432-8822

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ABSTRACT

Oxidative stress contributes to dysfunctional immune responses and predisposes dairy cattle to several metabolic and inflammatory-based diseases. Although the negative effects of oxidative stress on transition cattle are well established, biomarkers that accurately measure oxidative damage to cellular macromolecules are not well defined in veterinary medicine. Measuring 15-F_{2t}-isoprostane, a lipid peroxidation product, is the gold standard biomarker for quantifying oxidative stress in human medicine. The aim of our study was to determine whether changes in 15-F_{2t}-isoprostane concentrations in plasma and milk could accurately reflect changes in oxidant status during different stages of lactation. Using liquid chromatography-tandem mass spectrometry, 15-F_{2t}-isoprostane concentrations were quantified in milk and plasma of 12 multiparous Holstein-Friesian cows that were assigned to 3 different sampling periods, including the periparturient period (1-2 d in milk; n = 4), mid lactation (80-84 d in milk; n = 4), and late lactation (183-215 d in milk; n = 4). Blood samples also were analyzed for indicators of oxidant status, inflammation, and negative energy balance. Our data revealed that 15-F_{2t}-isoprostane concentrations changed at different stages of lactation and coincided with changes in other gauges of oxidant status in both plasma and milk. Interestingly, milk 15-F_{2t}-isoprostane concentrations and other indices of oxidant status did not follow the same trends as plasma values at each stage of lactation. Indeed, during the periparturient period, systemic 15-F_{2t}-isoprostane increased significantly accompanied by an increase in the systemic oxidant status index. Milk 15-F_{2t}-isoprostane was significantly decreased during the periparturient period compared with other lactation stages in conjunction with a milk oxidant status index that trended lower during this period. The results from this study indicate that changes in 15-F_{2t}-isoprostane concentrations in

both milk and plasma may be strong indicators of an alteration in redox status both systemically and within the mammary gland.

Key words: isoprostane; oxidative stress; oxylipid; reactive oxygen species.

INTRODUCTION

Drastic shifts in feed consumption and energy demands before and after calving pointedly alter the metabolic environment of the modern dairy cow, setting the stage for predispositions to health disorders during the periparturient period (Sordillo and Mavangira, 2014). For example, mastitis may occur at any time during a production cycle but presents with a significantly higher incidence and severity during the periparturient period (Hogeveen et al., 2011). Several other infectious and metabolic disorders such as metritis, ketosis, and milk fever occur most frequently around the time of calving and early lactation (Pinedo et al., 2010). In dairy cattle, the underlying cause of increased incidence of disease during the periparturient period is metabolic stress. Metabolic stress ultimately compromises the immune status of the animal through a triad of risk factors including altered nutrient metabolism, dysfunctional inflammation, and oxidative stress (Sordillo and Mavangira, 2014). Altered nutrient metabolism can be triggered around the time of calving as the demand for energy increases due to fetal growth and the onset of lactation resulting in negative energy balance. Increased lipid mobilization as a consequence of negative energy balance may increase inflammatory responses and the generation of reactive oxygen species (ROS) (Sordillo and Raphael, 2013). An imbalance between the production of ROS coupled with the decreased intake of dietary antioxidants due to decreased overall feed intake can lead to a pro-oxidant shift in the redox balance (Sordillo and Aitken, 2009). When excessive, ROS can damage cellular macromolecules such as lipids, protein, and DNA. The severity of oxidative stress can be determined by measuring any macromolecule damage, but lipid peroxidation events are often used to assess oxidative stress in dairy cattle (Castillo et al., 2005, Celi, 2011).

An alteration in the redox balance toward a greater pro-oxidant redox status can compromise dairy cattle immune competence by influencing the production of oxylipids, which

are potent lipid mediators produced from oxidized PUFA that can control essentially every aspect of the inflammatory response (Sordillo, 2018). The role of oxylipids in regulating inflammation is convoluted because they may produce both pro- and anti-inflammatory effects (Mancuso et al., 1998, Cheng et al., 2008). In periparturient cows, for example, oxylipids can significantly affect the susceptibility and duration of diseases such as mastitis by overrecruiting leukocytes to the site of infection and increasing damage to the mammary gland (Mavangira et al., 2015, Sordillo, 2018). Oxylipids are generally produced enzymatically but can be nonenzymatically formed through direct oxidation of lipids by ROS. Because lipids are the most sensitive macromolecule to oxidative damage, nonenzymatic production yields quantifiable oxylipids, including the isoprostanes, that can be used as specific and sensitive markers for oxidative stress. Specifically, the 15-F2t-isoprostane, also referred to as 8-isoprostane, has emerged in research as the gold standard for the measurement of oxidative stress in human and veterinary medicine (Niki, 2014, Mavangira et al., 2016).

The aim of this study was to quantify 15-F2t-isoprostane in milk as a direct measure of oxidative stress in the mammary gland. Because the mammary gland is physiologically isolated to a certain extent from the systemic circulation by the blood–milk barrier, there is the potential that the oxidative environment of the mammary gland does not mimic that of the systemic circulation. Accurately quantifying the local oxidative environment of the mammary gland provides a new resource to redox balance research, allowing for direct quantification of oxidative damage rather than extrapolation from the systemic circulation.

MATERIALS AND METHODS

Chemicals

Acetonitrile, methanol, and formic acid of liquid chromatography–MS grade were purchased from Sigma-Aldrich (St. Louis, MO). Deuterated and nondeuterated oxylipid standards were purchased from Cayman Chemical (Ann Arbor, MI). Butylated hydroxy toluene was purchased from Acros (Waltham, MA), 156 EDTA and triphenylphosphine were purchased from Sigma-Aldrich, and indomethacin was purchased from Cayman Chemical.

Animals

This study was approved by the Michigan State University Institutional Animal Care and Use Committee (reference number 03/14-059-00), and cows were enrolled with client consent. The study was conducted at a commercial dairy operation in Michigan with an average of 3,300 lactating cows with an approximate rolling herd-average milk production of 12,250 kg. Cows were housed in free-stall barns and grouped per lactation number, DIM, and milk yield. Cows were milked 2 times daily. Two diets, one for periparturient cows and a second for mid-lactation and late-lactation cows (Table 4.1), were formulated to meet the energy requirements based on production, and feed was delivered 2 times per day as a TMR.

Study Design

Twelve multiparous dairy cows of at least third lactation with an average age of 5.2 yr were enrolled and divided evenly according to their current stage of lactation into periparturient (PP; n = 4), mid-lactation (ML; n = 4), and late-lactation (LL; n = 4) groups. All animals in the periparturient group were 1 to 2 DIM. The mid-lactation animals were between 80 and 84 DIM. The late-lactation animals were 183 to 215 DIM. Negative milk bacterial cultures and an absence of overt clinical signs at the time of collection as well as SCC of <250,000 cells / mL on the most

recent Dairy Herd Improvement Association test day were required for inclusion in each group. Bacterial milk cultures were performed on blood agar and selective medium for coliform bacteria according to the National Mastitis Council guidelines (Council, 1999). Briefly, individual mammary gland quarter milk (10 μL) was streaked onto sheep blood agar and selective medium using a sterile loop inoculator. Cultures were incubated at 37°C, read at 24 and 48 h, and considered positive for bacterial growth based on the presence of 3 or more colonies on blood or selective medium.

Sample Collection and Analyses

Blood samples were collected via coccygeal venipuncture into serum-separator or 156 EDTA evacuated tubes and placed on ice with added antioxidant reducing agent (AOR) within 15 min of collection to be transported to the laboratory. Milk samples were aseptically collected before morning milking and flash frozen within 15 min of collection for transportation to the laboratory. Urine samples were collected via free catch in plain 15-mL conical tubes and flash frozen within 15 min of collection for transportation to the laboratory. At the laboratory, blood tubes were centrifuged at 2,000 × g for 18 min at 4°C and plasma and serum were harvested, aliquoted, and snap frozen in liquid nitrogen within 2 h of initial collection for subsequent storage at −80°C and analyses within 2 mo. Plasma, urine, and milk 15-F2t-isoprostane quantification samples were mixed with an AOR at 4 μL / mL as described previously (Mavangira et al., 2015). Reactive metabolites including both reactive oxygen and nitrogen species (RONS) were analyzed with plasma and whole milk not containing AOR using the Cell Biolabs (San Diego, CA) OxiSelect in vitro ROS/RNS assay, which involves sample reaction with dichlorodihydrofluorescin DiOxyQ and fluorometric quantification as previously reported (Mavangira et al., 2016). Plasma and whole milk without added AOR were also used to determine

antioxidant potential (AOP) as described previously (Re et al., 1999). In short, AOP was quantified by sample reaction with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) solution. The ability of the sample to reduce 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) was standardized to Trolox used in a standard curve. Serum amyloid A and haptoglobin were measured in serum samples using the commercial assays from Tridelta Development Ltd. (Maynooth, Ireland). Plasma albumin, cholesterol, and non-esterified fatty acids (NEFA) were analyzed at the Michigan State University Veterinary Diagnostic Laboratory (Lansing, MI). The oxidant status index (OSi) was created by dividing the RONS value by AOP (Abuelo et al., 2013).

Quantification of 15-F2t-Isoprostane

15-F2t-isoprostanes were determined using liquid chromatography–tandem MS. Plasma (2 mL) and milk (4 mL) samples were mixed with an AOR mixture to prevent degradation of preformed oxylipids and prevent ex vivo lipid peroxidation as described previously (O'Donnell et al., 2009). The AOR mixture consisted of 50% methanol, 25% ethanol, and 25% water with 0.9 mM butylated hydroxy toluene, 0.54 mM EDTA, 3.2 mM triphenylphosphine, and 5.6 mM indomethacin. Samples were flash frozen in liquid nitrogen and stored at −80°C until analyses. A mixture of internal standards containing 5(S)-HETE_d8, 15(S)-HETE_d8, 8(9)-EET_d11, PGE2_d9, 8,9-DHET_d11, AA_d8, 2-AG_d8, and AEA_d8 was prepared to final concentrations of 0.25, 0.25, 0.5, 0.5, 0.25, 50, 2, and 0.25 µM, respectively. The internal pure standards mixture was added to each sample and used to generate a 6-point standard curve ranging from 500 to 0.001 µM in concentration of unlabeled fatty acid and oxylipid standards.

Sample Processing

Frozen plasma and whole milk samples were thawed on ice and processed as follows: 4 mL of milk was combined with 9.15 mL of 4°C acetonitrile, and 2 mL of plasma was combined

with 5 mL of 4°C methanol and 2 µL of 88% liquid chromatography MS-grade formic acid. For urine, 4 mL of sample was initially mixed with 24 µL of formic acid. To each sample mixture, 15 µL of internal standard cocktail was added, and the sample was vortexed for 2 min, incubated at room temperature for 15 min, and centrifuged at 4,816 × g for 20 min at 4°C. For plasma and urine, the supernatant was diluted with 95 mL of HPLC water and 95 µL of formic acid, and supernatant in milk samples was diluted with 170 mL of HPLC-grade water and 170 µL of formic acid.

Solid Phase Extraction

Solid phase extraction was performed for each sample with Oasis HLB 12cc (500 mg) LP extraction columns (Waters, Medford, MA). Columns were conditioned with 6 mL of methanol followed by 6 mL of HPLC water. Extracts were loaded onto the columns, which were then washed with 6 mL of 20% methanol and then dried under full vacuum for 15 min. Analytes were eluted with 6 mL of methanol and acetonitrile (50:50 vol/vol). The volatile solvents were removed under vacuum using a Savant SpeedVac Concentrator (ThermoFisher Scientific, Asheville, NC). The residues were reconstituted in 150 µL of methanol, mixed at a 1.4:1 ratio with HPLC water, and stored in glass chromatography vials with glass inserts at −20°C until analysis. The curve for 15-F2t-isoprostane was a 5-fold dilution ranging from 100 to 0.001 µM. The deuterated internal standard concentrations mentioned above were added to each point on the curves. The linear curves generated produced coefficient of determination (R2) values of 0.99 with percentage deviations of less than 100%.

Liquid Chromatography–Tandem MS Analyses

Details of liquid chromatography–tandem MS procedures are described in Mavangira et al. (2015). Briefly, the quantification of 15-F2t-isoprostane was accomplished on a Waters Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer using multiple

reaction monitoring. Chromatography separation was performed with an Ascentis Express C18 HPLC column (10 cm × 2.1 mm, 2.7 µm; Supelco, Bellefonte, PA) held at 50°C. The autosampler was held at 10°C. Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile. Flow rate was fixed at 0.3 mL / min. Liquid chromatography separation took 15 min with linear gradient steps programmed as follows (A/B ratio): time 0 to 0.5 min (99/1), to (60/40) at 2.0 min; to (20/80) at 8.0 min; to (1/99) at 9.0 min; 0.5 min held at (1/99) until min 13.0; then return to (99/1) at 13.01 min, and held at this condition until 15.0 min. 15-Ft2-isoprostane was detected using electrospray ionization in negative-ion mode. Cone voltages and collision voltages were optimized using Waters QuanOptimize software. All samples were processed, extracted, measured, and analyzed as a single batch, minimizing intra-assay variation; however, a specific intra-assay variation coefficient is not available. Because a single batch of samples was assayed, an inter-assay variation coefficient does not apply.

Statistical Analyses

Statistical comparisons were performed using the MIXED procedure in SAS (SAS Institute Inc., Cary, NC). Data were checked for normality in SAS and analyzed by 1-way ANOVA with Tukey's adjustment for multiple comparisons. Data failing to satisfy the Gaussian (normal) distribution were transformed (log or square root) and rechecked for normality before comparisons were performed. For presentation (tables or figures), transformed data were back transformed to the observed scale and reported as the geometric means. Where data satisfied the Gaussian distribution requirements but with unequal variances, the Kenward-Roger adjustment for multiple comparisons was performed (this was done only for Haptoglobin data). Pearson correlation coefficients were determined both within specific lactation groups and across all sample periods

for all data in which both milk and plasma samples were available for correlation within specific lactations and across all sample periods. All comparisons were considered different at $\alpha \leq 0.05$.

RESULTS

Indices of inflammation and liver function included measuring haptoglobin, serum amyloid A, and plasma albumin, whereas altered nutrient metabolism was quantified through NEFA and cholesterol concentrations (Table 4.2). Abundance of RONS was measured fluorometrically. In plasma, RONS levels were higher in PP and significantly lower ($P < 0.01$) in ML and LL ($P < 0.05$; Figure 4.1). In addition to RONS, the AOP was quantified by the ability of the sample to reduce 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) against a standard of Trolox (Figure 4.1). In plasma, AOP was lower in the PP period compared with other lactation periods but only significantly lower when compared with late lactation ($P < 0.01$). High levels of RONS and a low AOP during the PP created a significantly ($P < 0.01$) higher OSi during the PP period compared with ML and LL (Figure 4.1). These measurements allude to a systemic pro-oxidant environment in the cow during the PP compared with other stages, as would be expected in animals facing a negative energy balance.

In addition to measuring RONS, AOP, and the OSi in plasma, these values can be obtained in milk as has been shown previously (Albenzio et al., 2016, Mavangira et al., 2016). Rather than providing systemic values, milk provides a measure of oxidant status locally in the mammary gland. Results obtained from milk were overall inconsistent with those found systemically. Although a significant correlation ($P < 0.001$) between plasma and milk RONS was found across all lactation groups ($r = 0.95$), AOP levels in milk did not correlate with plasma AOP at any lactation stage. Specifically, AOP values in milk were significantly ($P < 0.01$) higher during the PP stage compared with the ML and LL stages (Figure 4.1). This combination of increased RONS

in the presence of high AOP suggests that although an abundance of RONS exists, a likely compensatory response of local antioxidant mechanisms has been triggered to combat the pro-oxidant load and maintain a stable redox environment. When the OSi is applied, it reveals a relatively low index value during the PP stage compared with the ML and LL stages, albeit not significant, concurring with this assessment.

Although these observations offer valuable insights, quantifying RONS or AOP only indirectly determines redox status. Measurement of isoprostanes, specifically 15-F2t-isoprostane, has become the gold standard of direct oxidative stress quantification. When measured across lactation in plasma, 15-F2t-isoprostane concentrations were significantly higher during the PP and LL stages compared with the ML stage ($P < 0.05$; Figure 4.2). Interestingly, in milk, 15-F2t-isoprostane concentrations in the PP stage were significantly lower than those measured in the ML or LL stages ($P < 0.05$; Figure 4.2). A correlation ($P < 0.05$) between milk and plasma 15-F2t-isoprostane concentrations was noted only during the ML stage (r = 0.98). 15-F2t-isoprostanes were additionally quantified in urine across lactation (presented as mean ± SE mg / mg of creatinine). In urine, the PP stage (5.49 ± 3.02 mg / mg of creatinine) and LL stage (29.02 ± 15.03 mg / mg of creatinine) were found to have significantly higher concentrations than the ML stage (0.25 ± 0.21 mg / mg of creatinine; $P < 0.05$). Although these trends are similar to those seen in plasma, no correlation was found between plasma and urine.

DISCUSSION

Oxidative stress in dairy cattle can cause increased disease susceptibility, longer durations of clinical disease, and slower return to peak production (Bertoni et al., 2008, Jozwik et al., 2012, Sordillo and Raphael, 2013). Although a plethora of studies have outlined potential causes and consequences of systemic oxidative stress in dairy cattle (Sordillo and Aitken, 2009), few have

addressed the oxidative environment of the mammary gland. The detrimental effects of oxidative stress on immune defenses are due, in part, to the peroxidation of cellular lipids resulting in the generation of several oxidized lipid metabolites, including the family of isoprostanes (Sordillo, 2018). Indeed, previous works in cattle have validated the use of measuring 15-F2t-isoprostane in the plasma and milk of cows with mastitis and during early lactation as an assessment of oxidative stress (Vernunft et al., 2014, Mavangira et al., 2016). A systemic pro-oxidant shift of the redox balance in dairy cattle is integral to the development of metabolic stress in the periparturient period. In this study, increased plasma 15-F2t-isoprostane concentrations paralleled the higher plasma OSi, a more comprehensive measure of oxidative balance that takes into account both RONS and AOP (Abuelo et al., 2013). Additionally, 15-F2t-isoprostane reflected biomarkers of inflammation (acute phase proteins) and altered nutrient metabolism (NEFA, cholesterol) characteristic of metabolic stress (Bertoni et al., 2008, Sordillo and Raphael, 2013, Sordillo and Mavangira, 2014). Thus, the findings of this study support the contention that plasma 15-F2t-isoprostane concentrations are a valid way to assess oxidative stress at different stages of the lactation cycle.

During acute coliform mastitis, milk 15-F2t-isoprostane concentrations were reported to resemble changes observed in plasma and urine (Mavangira et al., 2016). Similarly, changes in plasma and milk 15-F2t-isoprostane concentrations have been shown to be correlated in healthy cows during the second month of lactation (Vernunft et al., 2014). Interestingly, it was during this ML stage in the current study that 15-F2t-isoprostane concentrations were significantly correlated between milk and plasma. Whereas 15-F2t-isoprostane concentrations in plasma and milk were correlated strictly during the ML period, values of RONS for plasma and milk were correlated throughout all 3 lactation periods, even though milk RONS amounts were 100 times those found in plasma. Nevertheless, these values are consistent with those reported previously using the same

assay methodology (Abuelo et al., 2016, Mavangira et al., 2016). The difference in RONS abundance is most likely due to the concentration of the metabolites in milk while they are relatively diluted throughout the body in plasma. In our study, the concentrations of 15-F2t-isoprostane in milk and plasma did not consistently trend in similar directions across all lactation stages. Indeed, 15-F2t-isoprostane concentrations were greatest in plasma but lowest in milk around the time of calving. Vernunft et al. (2014) also noted a comparable trend, reporting an increase in milk isoprostanes between d 38 and 71 postpartum, whereas plasma isoprostane concentrations decreased during this period.

Although several trends exist between our results and those published previously with respect to oxidant status in plasma and milk, the absolute concentration of isoprostanes varies in the literature. Several reasons may explain the conflict between the findings in our study and results reported previously. The health status of a dairy cow can clearly affect lipid peroxidation, as indicated previously with the influence of coliform mastitis on isoprostane concentration in urine, milk, and plasma (Mavangira et al., 2016). All cows in the present study were clinically healthy, as was shown by haptoglobin, serum amyloid A, and plasma albumin concentrations residing within published reference limits at all stages of lactation. The significant differences noted in the PP compared with other stages of lactation are consistent with previous studies and represent a normal physiologic variation found during this time (Cozzi et al., 2011). Additionally, concentrations of NEFA and cholesterol were consistent with previously described values for the 3 different stages of lactation (Bell, 1995, Adewuyi et al., 2005, Contreras et al., 2010). A previous study in healthy cows also reported concomitant increases in both plasma and milk concentrations of 15-F2t-isoprostane as related to ovulation (Vernunft et al., 2014). An important distinction between the previous and present study is the analytical methods used to determine 15-F2t-

isoprostane concentrations. Vernunft et al. (2014) used commercial ELISA-based assays that are known to be susceptible to cross-reactivity problems due to the structural similarities across the entire prostanoid series of molecules. As such, ELISA-based assays are considered only as semiquantitative estimations that often yield significantly higher 15-F2t-isoprostane concentrations than corresponding chromatography and MS methods that were used in the present study (Klawitter et al., 2011, Sordillo, 2018). Thus, the methods used to measure isoprostanes should be carefully considered when comparing studies in the scientific literature and interpreting the information. Additional differences, such as those noted between Contreras et al. (Contreras et al., 2017)and the presented results, may stem from differences in the basal redox status of cattle that live under different management conditions and are fed different rations (Hanschke et al., 2016, Khatti et al., 2017). Sampling time may also contribute to published differences because small changes in the physiological state of a cow may significantly affect isoprostane production. As such, it is difficult to directly compare absolute values of cattle in their dry period or shortly after the periparturient period with those of cattle at parturition or near peak lactation. The differences in 15-F2t-isoprostane concentrations reported in the literature emphasize the need to establish more consistent sampling times and analytical techniques before this metabolite of lipid peroxidation can be used as a standardized biomarker of oxidative stress and disease susceptibility.

Although milk and plasma 15-F2t-isoprostane concentrations did not follow the same trends across lactation stage, the lower milk concentration is consistent with the trends found in a recent study addressing shifting quantities of biosynthesized oxylipids across lactation (Kuhn et al., 2017). For example, the ratio of arachidonic acid–derived oxylipids in milk, including 9-hydroxyeicosatetraenoic acid (HETE) and 11-HETE to 15-HETE, was significantly lower during the PP stage compared with the ML and LL stages (Kuhn et al., 2017). Although these HETE are

derived from the same fatty acid substrate, 9-HETE and 11-HETE are primarily nonenzymatically produced through oxidation by ROS, whereas 15-HETE is primarily enzymatically biosynthesized by the 15-lipoxygenase-1 enzyme (Kuhn et al., 2017). Expression of 15-lipoxygenase-1 is increased significantly in mammary tissue around the time of calving (Aitken et al., 2009); however, our data and others do not reflect a significant increase in 15-HETE during this period (Raphael et al., 2014, Kuhn et al., 2017). Rather, a decrease in nonenzymatically derived 9-HETE and 11-HETE was responsible for the change in the ratio of enzymatic and nonenzymatic pathways of oxidation (Kuhn et al., 2017). Lower milk concentrations of 9-HETE and 11-HETE during the PP stage may suggest that the pro-oxidant environment in the mammary gland was not sufficient to drive non-enzymatic oxylipid biosynthesis.

The potential role of negative redox balance in the mammary gland around calving and into early lactation was previously characterized (Hodgkinson et al., 2007, Aitken et al., 2009). Changes in antioxidant defenses (e.g., glutathione peroxidase) and measures of inflammation (including vascular cell adhesion molecule 1 or tumor necrosis factor α) were not significantly increased in mammary tissues during the periparturient period when compared with other physiological stages of the production cycle (Hodgkinson et al., 2007, Aitken et al., 2009). Although the periparturient period is associated with an increased production of systemic and milk ROS values, the mammary gland antioxidant defense mechanisms may be enhanced to combat local ROS accumulation and tissue damage. In support of this contention, previous research showed increased activity in the selenoproteins glutathione peroxidase-1 and glutathione peroxidase-4, which play key roles in the protection of cells against ROS-induced peroxidation of membrane phospholipids (Aitken et al., 2009, Sordillo and Aitken, 2009). Thus, the lower milk concentrations of 15-F2t-isoprostane reported in the present study during the onset of lactation

may be explained by increased AOP associated with mammary tissues during a time of increased metabolic demands. Whole milk was used in this study to include all considerations within milk that may affect redox status. Unpublished work in our laboratory evaluated the difference between centrifugally separated milk samples and whole milk samples, revealing that whole milk offered a higher AOP. Additionally, work by Albenzio et al. (2016) showed that whole milk contained higher levels of RONS than individual milk protein fractions. Indeed, different milk fractions have been shown to harbor specific antioxidant abilities (Chen et al., 2000, Castillo et al., 2013).

Systemic oxidative stress may still play a significant role in the onset of mastitis as well regardless of the local oxidant environment. At the onset of infection, when a robust yet measured inflammatory response is necessary to contain and eliminate foreign pathogens, oxidative stress dysregulates the control over this response. Reactive oxygen species increase the permeability of the endothelial barrier between the circulation and the mammary gland, allowing for increased extravasation of leukocytes (Boueiz and Hassoun, 2009). Additionally, neutrophils in an increased oxidant environment produce less superoxide dismutase than those supplemented with antioxidants, decreasing their killing abilities (Politis et al., 2004). In turn, this dysregulated response leads to the onset of infection, and the severity of mastitis during this period is heightened by a systemic shift toward a pro-oxidant redox balance. Without proper control of the inflammatory response, an overreaction to the initial stimulus can occur, causing further tissue damage from inflammatory cells as well as chronic inflammation (Aitken et al., 2011).

Current research in cattle is only beginning to uncover the abilities of local compartments, such as the urinary tract, ovaries, or mammary gland, to regulate their local oxidant environment independently from the systemic circulation (De Bie et al., 2016). Although the focus of this study was on the mammary gland, data generated from urine 15-F2t-isoprostane were in line with

previous research in our laboratory, which has also shown no correlation between plasma and urine 15-F2t-isoprostane (Mavangira et al., 2016). Both human and murine models have shown a direct correlation between 15-F2t-isoprostane excretion in urine and plasma levels, making our findings noteworthy (Takahashi et al., 1992, Ware et al., 2011). The lack of a significant correlation between these fluids in cattle suggests that there may be another variable affecting the excretion of 15-F2t-isoprostane in cattle, such as differential phospholipase activity (Stafforini et al., 2006).

Although not the first to quantify 15-F2t-isoprostane in milk, this study validates the use of measuring 15-F2t-isoprostane in milk to quantify oxidative stress in the mammary gland across different stages of the lactation cycle. The methodology used in the present study is arguably more accurate than ELISA-based assays (Klawitter et al., 2011), and we showed for the first time that milk 15-F2t-isoprostane is not merely a reflection of plasma concentration but rather reflects lipid peroxidation events of the mammary gland. Additionally, this study supports previously reported data suggesting that the mammary gland may maintain its oxidative balance in the face of systemic oxidative stress during the periparturient period. The current study focused on comparing stages of lactation that represent drastically different physiological environments and, more specifically, significant differences in oxidant status; however, it is recognized that dramatic physiological changes still occur between each of the stages studied herein. Specifically focusing on changes that occur between calving and the immediate postpartum period may better characterize the oxidative changes occurring in the periparturient period. Due to the small sample size used, although significant differences were found in 15-F2t-isoprostane concentration and other indicators of redox status both amid different lactation stages and between milk and plasma, a more robust study following a greater number of individuals across their entire lactation would be beneficial to sustain these conclusions. The deductions expressed in this paper are supported by

the modest power of the post hoc analyses of 0.69 for plasma 15-F2t-isoprostane concentrations; however, despite the low numbers, the analyses for milk 15-F2t-isoprostane concentrations had a high power of 0.87. The ability of the mammary gland to regulate oxidant status independent of systemic measures may direct future studies and interventions toward the most effective strategies to prevent and control oxidative stress.

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CHAPTER 5

WIDESPREAD BASAL CYTOCHROME P450 EXPRESSION IN EXTRAHEPATIC BOVINE TISSUES AND ISOLATED CELLS

Matthew J. Kuhn[1], Ashley K. Putman[1], and Lorraine M. Sordillo*[1]

[1]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing 48824

***Corresponding author**

Dr. Lorraine M. Sordillo,

Department of Large Animal Clinical Sciences

College of Veterinary Medicine

Michigan State University

sordillo@msu.edu

Tel: (517) 432-8821

Fax: (517) 432-8822

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ABSTRACT

Periparturient cattle face increased risk of both metabolic and infectious diseases. Factors contributing to this predisposition include oxidized polyunsaturated fatty acids, also known as oxylipids, whose production is altered during the periparturient period and in diseased cattle. Alterations in the production of oxylipids derived from cytochrome P450 (CYP450) enzymes are over-represented during times of increased disease risk and clinical disease, such as mastitis. Many of these same CYP450 enzymes additionally regulate metabolism of fat-soluble vitamins, such as A, D, and E. These vitamins are essential to maintaining immune health, yet circulating concentrations are diminished near calving. Despite this, a relatively small amount of research has focused on the roles of CYP450 enzymes outside of the liver. The aim of this paper is to describe the relative gene expression of 11 CYP450 in bovine tissues and common in vitro bovine cell models. Eight tissue samples were collected from 3 healthy dairy cows after euthanasia. In vitro samples included primary bovine aortic and mammary endothelial cells and immortalized bovine kidney and mammary epithelial cells. Quantitative real-time-PCR was carried out to assess basal transcript expression of CYP450 enzymes. Surprisingly, CYP450 mRNA was widely expressed in all tissue samples, with predominance in the liver. In vitro CYP450 expression was less robust, with several cell types lacking expression of specific CYP450 enzymes altogether. Overall, cell culture models did not reflect expression of tissue CYP450. However, when CYP450 were organized by activity, certain cell types consistently expressed specific functional groups. These data reveal the widespread expression of CYP450 in individual organs of healthy dairy cows. Widespread expression helps to explain previous evidence of significant changes in CYP450-mediated oxylipid production and fat-soluble vitamin metabolism in organ microenvironments during periods of oxidative stress or disease. As such, these data provide a foundation for targeted

functional experiments aimed at understanding the activities of specific CYP450 and associated therapeutic potential during times of increased disease risk.

Key words: cytochrome p450, dairy cow, oxylipid, vitamin D

INTRODUCTION

During the periparturient period, inflammation in dairy cattle becomes dysregulated, causing impaired immune health. Dysregulated inflammation predisposes cattle to disease and excessive inflammatory tissue damage, resulting in reduced productivity (Sordillo and Raphael, 2013). Many factors contribute to the dysregulation of inflammation during the periparturient period, including the altered generation of oxidized PUFA inflammatory mediators, collectively termed oxylipids, and an inadequate amount of fat-soluble vitamins necessary for abrogating oxidative stress (Sordillo, 2016).

Oxylipids are derived non-enzymatically from exposure to reactive oxygen species and enzymatically through cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450)-mediated pathways (Gabbs et al., 2015, Sordillo, 2018). Despite the historical focus placed on the COX and LOX pathways, recent work among cattle has suggested that the CYP450 pathway has a significant role in influencing health and disease. In studies profiling oxylipids in periparturient cows compared with those in other lactation stages, and cows with coliform mastitis compared with healthy controls, changes in the production of CYP450-derived oxylipids outnumbered those from either COX or LOX pathways in plasma and milk (Mavangira et al., 2015, Kuhn et al., 2017). Surprisingly, the differences noted in milk outnumbered those found in plasma, suggesting substantial local regulation of CYP450-derived oxylipids in the mammary gland.

Despite previous work showing significant production of CYP450-derived oxylipids in the mammary gland, little information is available regarding extrahepatic expression of CYP450 enzymes that may offer insight into the mechanisms behind local regulation of these lipid mediators (Mavangira et al., 2015, Kuhn et al., 2017). Given the diverse activities of CYP450

enzymes (Figure 5.1), functional studies are necessary to understand the specific CYP450 enzymes contributing to oxylipid production. Establishing a transcript expression profile of CYP450 enzymes builds a foundation for such studies, given that extrahepatic expression for many CYP450 has not yet been shown.

The multiple activities of individual CYP450 and redundant activities between them create a complex network of oxylipid and vitamin regulation with multiple instances of substrate and function crossover. Understanding the unique activities of individual CYP450 and overlapping activities between them is key to understanding their roles in health and disease. For example, several CYP450 may metabolize arachidonic acid into oxylipids, 7 of which were included in this study (Figure 5.1A). Currently, no study in cattle has established the relative contribution of differing CYP450 to the overall oxylipid pool. Due to this, CYP450 that metabolize PUFA chosen for this study were selected based on their significant contribution to the oxylipid pool in other species, such as CYP2C19, CYP2E1, CYP3A4, CYP4A11, and CYP4F2, or previous research in cattle that has explored the activity of a specific CYP450, such as CYP1A1 or CYP2J2 (Lasker et al., 2000, Yang et al., 2001, Fer et al., 2008, Zhang et al., 2018b).

A subset of these PUFA-metabolizing CYP450, the CYP450 family 4 (CYP4) class, including CYP4A11 and CYP4F2, have predominantly terminal carbon ω-hydroxylase activity (TCOH), producing the highly pro-inflammatory 20-hydroxyeicosatetraenoic acid (20-HETE) as their primary product (Powell et al., 1998). In contrast, CYP1A1, CYP2C19, CYP2E1, CYP2J2, and CYP3A4 generally act as epoxygenases, creating 4 isomers of epoxyeicosatrienoic acids (EET) as a primary product. These EET are further metabolized by the non-CYP450 enzyme soluble epoxide hydrolase (EPHX2) to secondary and presumably less anti-inflammatory oxylipid metabolites. As such, EPHX2 is an enzymatic step that has been targeted in other veterinary

species for therapeutic intervention for the reduction of pain due to inflammation (Bylund et al., 1998, Guedes et al., 2017). Secondarily, and to a lesser extent, these latter 5 epoxygenases metabolize arachidonic acid by hydroxylating carbons 16–19, thus acting as subterminal ω-hydroxylases (STOH), a class of oxylipids whose activities are only beginning to be understood in veterinary research.

Whereas oxylipids are direct mediators of inflammation, fat-soluble vitamins A, D, and E act upon inflammation both directly, through regulation of inflammatory signaling, and indirectly, as antioxidants curtailing oxidative stress (Sordillo, 2016). Although the mechanisms by which fat-soluble vitamins can influence inflammation differ from oxylipids, their regulation shares many of the same CYP450 enzymes that regulate oxylipid production. For example, epoxygenases CYP2J2 and CYP3A4 also act as a 25-hydroxylase of vitamin D, along with CYP27A1, to form 25-hydroxyvitamin D_3 [25-(OH)D_3; Figure 5.1B]. Although several CYP450 have been identified as 25-hydroxylases in other species, no studies have conclusively identified the relative contribution of each of these enzymes to the overall production of 25-(OH)D_3 in cattle. Polymorphisms in CYP2J2 and CYP27A1, however, lead to reduced serum 25-(OH)D_3 and ultimately are associated with incidence of milk fever (Casas et al., 2013, Pacheco et al., 2018). Although these studies do not provide direct evidence of activity or discount the potential activity of other CYP450 25-hydroxylases, they nonetheless provide evidence of specific CYP450 that have clinical effects on the health of cattle. Metabolism of vitamin D continues with the activation of 25-(OH)D_3 to 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the active form of vitamin D, solely by CYP27B1. Last in this cascade, CYP24A1 alone is responsible for the ultimate degradation of 25-(OH)D_3 and 1,25-$(OH)_2D_3$ to inactive compounds (Christakos et al., 2016).

Vitamins A and E also can be degraded by CYP450. In addition to its TCOH activity, CYP4F2 is the single CYP450 responsible for the degradation of analogs of vitamin E to their inactive forms (Figure 5.1C) (Sontag and Parker, 2002). Further, the cytochrome P450 family 26 (CYP26) class of enzymes degrades active forms of vitamin A to less bioactive metabolites (Figure 5.1D) (Ross and Zolfaghari, 2011). As with CYP450-derived oxylipids, the regulation of these vitamins is disturbed during times of greatest need (LeBlanc et al., 2004, Holcombe et al., 2018). Regulation of vitamin metabolism is another essential aspect of understanding how CYP450 enzymes affect cow health and disease.

Modeling the function of CYP450 enzymes will require an understanding of their transcriptional expression in cell culture systems that are relevant to bovine health and production. Bovine mammary alveolar epithelial cells (MAC-T) and Madin-Darby bovine kidney cells (MDBK) are commonly sought for their immortalized nature, providing reproducible results through many passages (Madin and Darby, 1958); Huynh et al., 1991). On the other hand, primary cells, such as bovine aortic endothelial cells (BAEC) and bovine mammary endothelial cells (BMEC), are valued for their phenotypic similarities to in vivo counterparts (Yang et al., 2001, Ryman et al., 2016).

Although CYP450 have the capacity to significantly affect inflammation by regulating vitamin metabolism and contributing to the oxylipid pool, their relative expression in cattle and potential for study using in vitro models is largely unknown. The aim of this study was to describe the relative basal transcript expression of CYP450 enzymes relevant to the regulation of inflammation in healthy cattle. Additionally, with the need for in vitro models for functional studies of CYP450 enzymes, expression of these CYP450 was evaluated in both primary and immortalized cell lines of endothelial and epithelial cells, respectively. Many studies have

evaluated a single CYP450 or a small subset of such enzymes; however, none have explored such a wide range of enzymes among the many tissues of cattle nor presented data in a manner that allows for comparison between CYP450, as described herein. Furthermore, no studies as of yet have evaluated relative expression of CYP450 between commonly used in vitro bovine models. This report expands our current understanding of CYP450 expression in cattle, showing novel tissue expression of several enzymes and offering a foundation for future functional assays to build from.

MATERIALS AND METHODS

Chemicals and Reagents

QIAzol lysis regent and RLT buffer were purchased from Qiagen Sciences (Germantown, MD). Chloroform was purchased from Thermo Fisher Scientific (Fair Lawn, NJ) and Dulbecco's modified Eagle's medium was obtained from Gibco (Thermo Fisher Scientific). Collagenase and Hanks' balanced salt solution (HBSS) were purchased from Sigma-Aldrich (St. Louis, MO). Ham's F-12K medium was purchased from Irvine Scientific (Santa Ana, CA).

Animals and Tissue Sampling

Three multiparous Holstein cows were culled from the Michigan State University Dairy Teaching and Research Center (East Lansing) for non-disease-related reasons. Parity ranged from lactation 3 to 7, with all cows between 74 and 163 DIM. Production in the previous lactation ranged from 8,095 to 13,641 kg of milk on a 305-d milking average. Two cows were open, and 1 was pregnant at the time of culling. The cows were milked twice daily, kept in a free-stall barn, offered water ad libitum, and fed a TMR twice daily.

Cows were euthanized by captive bolt and exsanguination, and tissues were collected within 15 min of euthanasia. Samples of liver, kidney, heart, skeletal muscle, spleen, mammary gland, lung, and uterus were taken aseptically by removing roughly 0.5-cm pieces of organ tissue from areas devoid of serosal surfaces or large vasculature. Samples were placed in 2-mL Eppendorf tubes, flash frozen in liquid nitrogen, and stored at −80°C for less than 3 mo before RNA extraction.

Primary Cell Isolation

Bovine mammary endothelial cells and BAEC were collected as described previously (Aherne et al., 1995). Briefly, for BAEC isolation, a 10-cm full-circumference section of descending aorta, just distal to the branch of the subclavian artery, was collected at a commercial abattoir and immediately submerged in HBSS with 0.05 mg / mL gentamicin on ice. Pudendal artery for BMEC isolation was collected by taking a 4-cm full-circumference sample of pudendal artery near the cut edge of the mammary gland, submerged immediately in HBSS with 0.05 mg / mL gentamicin, and placed on ice. Once transported to the laboratory for processing, aorta samples were cut lengthwise and laid flat in a solution of 2 mg / mL collagenase in Krebs-Ringer bicarbonate with 4% BSA and allowed to incubate at 37°C for 10 min. Pudendal artery samples were rinsed with HBSS and clamped at one end, filled with a solution of 2 mg / mL collagenase in Krebs-Ringer bicarbonate with 4% BSA, clamped at the remaining open end, and incubated for 10 min at 37°C. After each incubation, the collagenase solution was collected and the luminal sides of tissues were rinsed with HBSS, collecting the rinse solution. The rinse solution was added to collected collagenase and samples were centrifuged at 160 × g for 10 min at 15°C. Resulting cellular pellets were plated in T25 cell culture flasks until confluent in Ham's F12K medium containing 10% fetal bovine serum, 10 mM HEPES buffer, 0.25% sodium bicarbonate, 2 mM l-

glutamine, 1% 1 : 1 antibiotic : antimycotic, 100 μg / mL heparin, 10 μg / mL insulin, 5 μg / mL transferrin, and 40 ng / mL sodium selenite.

Cells were detached via incubation with 0.05% trypsin, diluted serially, and plated in 96-well plates. To exclude fibroblasts and other cell types, wells containing only colonies derived from a single cell were selected for propagation. Wells were selected by typical endothelial cobblestone morphology for further propagation and confirmed by von Willebrand factor staining. Cells were frozen at passage 3 and grown to passage 6 for RNA isolation, representing the earliest passage at which cells could realistically be used for assays.

Cell Line Culture

Madin-Darby bovine kidney cells were obtained from the American Type Culture Collection (Manassas, VA), and MAC-T cells were obtained from the USDA Agricultural Research Service as a line of primary mammary alveolar cells transfected with SV-40 large T-antigen for immortalization (Huynh et al., 1991). Both MDBK and MAC-T cells were cultured in Dulbecco's modified Eagle's medium (+ 4.5 g / L d-glucose) devoid of phenol red, sodium pyruvate, or l-glutamine. Added to this medium were the following: 10% fetal bovine serum, 10 mM HEPES buffer, 0.25% sodium bicarbonate, 2 mM l-glutamine, 1% 1:1 antibiotic : antimycotic, 100 μg / mL heparin, 10 μg / mL insulin, 5 μg / mL transferrin, and 40 ng / mL sodium selenite. Cell were incubated at 5% CO2 and 37°C.

RNA Extraction

Tissues were cut into 30- to 40-mg pieces with a sterile scalpel while still frozen and added to 450 μL of QIAzol lysis reagent (Qiagen). Samples of muscle tissue were cut into 80-mg pieces for homogenization. A TissueRuptor II tissue homogenizer (Qiagen) was run at maximum allowable speed for 1 min for tissue homogenization. An additional 450 μL of QIAzol was added

to samples, which were then incubated at room temperature for 5 min. After incubation, 180 uL of chloroform was added to each tube, and tubes were vigorously shaken for 15 s and incubated at room temperature for 3 min. Samples were centrifuged at 12,000 × g, 4°C, for 15 min, and the upper aqueous layer was removed and added to a new 2-mL tube for RNA extraction.

Cultured cells were grown on 100-mm cell culture treated dishes. Once confluent, dishes were washed twice with HBSS and 600 μL of buffer RLT (Qiagen) was added for cell lysis. Buffer RLT was collected, added to QIAShredders nested in 2-mL Eppendorf tubes, and spun at 16,000 × g for 2 min. Flow-through was stored at −20°C.

Once all tissue and cell culture samples were obtained, RNA was extracted according to the Qiagen RNeasy protocol with DNase digest, using a QIAcube (Qiagen). RNA quantity and quality were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher).

Reverse Transcription

For cDNA generation, RNA was diluted with nuclease-free water to standardize all samples. A master mix of 10× reverse-transcription buffer, 25× dNTP, 10× random primers, Multiscribe reverse transcriptase, RNase inhibitor, and RNase nuclease-free water from a high-capacity cDNA reverse-transcription kit with RNase inhibitor (all kit components from Applied Biosystems, Vilnius, Lithuania), was added at an equal volume to diluted RNA. Samples were placed in a PTC-200 Peltier Thermo Cycler (MJ Research, Waltham, MA), which ran as follows: stage 1, 25°C for 10 min; stage 2, 37°C for 2 h; stage 3, 852ºC for 5 min; stage 4, hold at 4°C.

Real-Time PCR

Real-time PCR was carried out with predesigned TaqMan primers and FAM-MGB probes (Applied Biosystems, Pleasanton, CA) for those genes listed in Table 5.1. Two genes were created as custom TaqMan primers, listed in Table 5.2. All primers and probes were used in a 7500 Fast

Real-time PCR system (Applied Biosystems). Genes were evaluated in triplicate with 2× TaqMan Gene Expression Master Mix (Applied Biosystems), 20× Custom TaqMan Gene Expression Assay Mix (Applied Biosystems), sample cDNA (50 ng / well for tissues, 100 ng / well for cells), and nuclease-free water for a total of 10 μL per reaction well. A 20× pre-designed TaqMan Gene Expression Assay for RPS9 was used as endogenous control (Applied Biosystems). Thermal cycling conditions for the Fast 2-step PCR system were as follows: stage 1, 95°C for 20 s; stage 2, 95°C for 3 s; stage 3, 60°C for 30 s, with 40 cycles of stages 2 and 3. Data were recorded and compiled using ExpressionSuite Software version 1.0.4 (Applied Biosystems) and analyzed using DataAssist Software version 3.01 (Applied Biosystems, Foster City, CA). Data from tissues and in vitro cell samples, having no standard cell type for comparison, are expressed as $2^{-\Delta CT}$.

RESULTS

Tissue Expression

Gene expression of CYP450 enzymes in tissues of major organs of dairy cattle was surprisingly widespread, with every CYP450 evaluated found to be expressed in at least 1 sample of every tissue type (Figure 5.2). As expected, the liver, in general, expressed all CYP450 to the highest degree, with the only exceptions being CYP2J2, CYP24A1, and CYP27B1.

Oxylipid Metabolism. The CYP450 that metabolize oxylipids from PUFA substrate in this study include CYP1A1, CYP2C19, CYP2E1, CYP2J2, CYP3A4, CYP4A11, and CYP4F2. Of the CYP450 with significant epoxygenase and STOH activity, including CYP1A1, CYP2C19, CYP2J2, CYP2E1, and CYP3A4, all were found to have greatest expression in the liver except for CYP2J2. Instead, CYP2J2 was expressed in splenic and lung tissue 6.06-fold and 1.42-fold above liver expression, respectively ($2^{-\Delta\Delta CT}$, reference gene RPS9). Additionally, CYP1A1 and CYP3A4 each had their second-greatest expression in lung tissue, whereas CYP2E1 and CYP2C19

had the lowest tissue expression in the lung. Although there are inherent differences between the isolation of specific CYP450 and reverse transcriptase efficiency, samples were processed as a single batch, ensuring appropriate RNA quality, and used a single lot of reverse transcriptase to reduce these effects. Comparing CYP450, we found CYP2C19 to be expressed to a considerably greater amount in the liver than the next closest epoxygenase (CYP2C19 $2^{-\Delta CT}$ of 87.69, compared with 28.09 of CYP2E1 or 5.19 of CYP1A1; reference gene RPS9) and was the predominate epoxygenase in the mammary gland, heart, and uterus, in each case followed in expression by CYP1A1 and CYP2E1 sequentially. In the lung, CYP2C19 dominated epoxygenase expression with little comparable expression by any other epoxygenase. Interestingly, the spleen was comparatively devoid of epoxygenase expression apart from CYP2J2.

Of the 2 TCOH enzymes, CYP4A11 was most highly expressed in the liver and secondarily in the kidney, whereas CYP4F2 had a significantly greater expression in the kidney than any other tissue. Additionally, CYP4F2 was also the only CYP450 not reliably expressed in each tissue type, with samples of skeletal muscle, spleen, and uterus devoid of expression of this enzyme. Despite increased expression of CYP4F2 in the kidney compared with the liver, overall transcription of CYP4F2 paled in comparison to CYP4A11 in all tissues.

Although not a CYP450 enzyme, the EPHX2 enzyme is instrumental in secondary metabolism of CYP450-derived epoxides, such as metabolizing EET to DHET. We found that EPHX2 was expressed to the greatest amount in the liver compared with reference gene RPS9 ($2^{-\Delta CT}$ of 1.3), and to a lesser amount in other tissues, having its second highest expression in the kidney ($2^{-\Delta CT}$ of 0.31), followed by other organs, which were expressed at similar amounts ($2^{-\Delta CT}$ ranging from 0.012 to 0.12).

Vitamin D Metabolism. Of the 3 25-hydroxylases evaluated in this study, CYP27A1 and CYP3A4 were both expressed to the highest extent in liver tissue, whereas CYP2J2 was expressed most highly in the spleen. Between CYP450, CYP27A1 was found to be the most highly expressed 25-hydroxylase in every tissue type and had the most consistent expression profile between tissues.

We found CYP27B1 expressed to a considerably greater amount in the kidney than in any other tissue, with the next highest expression in heart and uterine tissues. The 24-hydroxylase CYP24A1 was also relatively highly expressed in the kidney compared with all other tissues, apart from the uterus. Interestingly, 1 sample of uterine tissue was found to have expression of CYP24A1 comparable to those found in the kidney, whereas the remaining 2 uterine tissue samples had levels near that of liver.

Vitamin A Metabolism. We found CYP26A1 to be transcribed in all tissue types, with the highest expression in the liver, followed closely by uterine tissue. Moderate expression was noted in heart, kidney, and mammary tissue, with low expression in lung, skeletal muscle, and spleen.

Cell culture Expression

Oxylipid Metabolism. Gene expression of oxylipid-producing CYP450 in cultured bovine cells was far less robust than noted in tissues. We found that CYP2C19 and CYP4F2 were not reliably expressed by any cell type evaluated, CYP4A11 only consistently expressed in BAEC, and CYP2E1 only consistently expressed in BAEC and MAC-T (Table 5.3). Additionally, CYP2J2 was not expressed by a clone of the MDBK cell line. The MAC-T cell line expressed both CYP1A1 and CYP2J2 to considerably greater amounts, over 10-fold, compared with the next highest-expressing cell type (Figure 5.3). Expression of CYP3A4 varied between cell types but was nonetheless reliably expressed in each sample. We found that EPHX2 was similarly expressed among cell types but considerably lower in MDBK cells.

Vitamin D Metabolism. Of all 25-hydroxylases evaluated, CYP27A1 was expressed to the highest amount and was consistently expressed among cell types, aside from MAC-T cells, for which CYP27A1 had measurable yet low expression (Figure 5.3). The final 2 steps of vitamin D metabolism, CYP27B1 and CYP24A1, were both reliably expressed in all cell types. Although both CYP27B1 and CYP24A1 had greater expression in MAC-T cells compared with either endothelial cell type, MDBK expression was converse, representing the lowest expressing cell type of CYP27B1 yet highest expressing cell type of CYP24A1.

Vitamin A Metabolism. In cell culture models, CYP26A1 was consistently expressed among all cell types, with greatest expression in MAC-T and lowest in MDBK cells. Compared with other CYP450, however, CYP26A1 was expressed to a comparatively lesser amount (Figure 5.3).

DISCUSSION

Circulating concentrations of oxylipids and fat-soluble vitamins A, D, and E fluctuate during the periparturient period in cattle, contributing to a state of oxidative stress and dysfunctional inflammation at this time (Sordillo, 2016, 2018). Despite influencing inflammation through differing mechanisms, these metabolites share regulation by the CYP450 enzymatic pathway. Nevertheless, before this study, little was known about the extrahepatic expression of specific CYP450 in dairy cattle. Surprisingly, every CYP450 evaluated herein, apart from CYP4F2, was transcriptionally expressed in every tissue sampled. Although extrahepatic expression was relatively low for many CYP450, local effects of CYP450 should not be overlooked. For example, infusion of LPS into the mammary gland results in isolated activation of CYP450 involved in the regulation of vitamin D without changes in activation of CYP450 noted outside of the mammary gland (Merriman et al., 2018).

Conclusions drawn from these data are limited by the understanding that gene expression does not directly relate to activity of an enzyme. Post-transcriptional and translational modification through phosphorylation, glycosylation, and protein degradation are all known to modify the activity of CYP450 (Aguiar et al., 2005). In general, however, CYP450 activity is influenced most by substrate availability, such as the induction of CYP24A1 by 1,25-$(OH)_2D_3$ or CYP26A1 by vitamin A (Kruger et al., 2005, Merriman et al., 2017). As such, detailing mRNA expression to understand the breadth of CYP450 distribution in various organs is needed as a first step for functionality research.

Samples with cycles to threshold (CT) values of greater than 35 cycles were not included in Figure 5.3, as quantitative accuracy diminishes beyond this point (Lorenz, 2012). Such samples, included in Table 5.3, cannot be compared directly to those with reliable expression. Tissue samples included bovine liver, kidney, mammary gland parenchyma, lung, spleen, heart, skeletal muscle, and full-thickness uterus from healthy, multiparous, lactating cows.

Epoxygenases

The epoxygenases examined in this study were CYP1A1, CYP2C19, CYP2E1, CYP2J2, and CYP3A4 (Fer et al., 2008). Unlike previous reports, we found that CYP1A1 was not distributed evenly among tissues but had a preponderance in the liver similar to other CYP450 (Darwish et al., 2010). Prominent lung expression, compared with other CYP450, however, is in line with a previous report in human tissue profiling. Such lung expression may underscore the importance of STOH activity also noted from epoxygenases such as CYP1A1. Relatively high lung expression of CYP1A1 may support the necessity of 19(R)-HETE production in the local pulmonary environment, where 20-HETE is potently vasoactive (Bieche et al., 2007, Fan et al., 2016). Robust hepatic CYP2C19 expression is unsurprising, given its role in xenobiotic

metabolism; however, the greater hepatic expression relative to other xenobiotic-metabolizing CYP450 is not conserved in other species (Bieche et al., 2007, Hirota et al., 2013). Additionally, the extrahepatic expression of CYP2E1 and CYP3A4 was unexpected and novel, as previously published findings in cattle found little to no extrahepatic expression (Kruger et al., 2005, Darwish et al., 2010).

Interestingly, we found robust splenic expression of CYP2J2 compared with its expression in other tissues, as opposed to the relatively pronounced cardiac expression seen in other ruminants (Messina et al., 2010). In this regard, among CYP450 evaluated, CYP2J2 was the highest-expressing CYP450 in splenic tissue as well. This relatively greater splenic expression may be due to the preference of CYP2J2 for producing EET rather than linoleic acid–derived oxylipids or subterminal HETE. Because EET are a vasodilatory class of oxylipids, such increased expression relative to other tissues may facilitate at least partial control over splenic and hepatic blood flow (Fer et al., 2008, Di Pascoli et al., 2016).

When measuring oxylipids derived from the CYP450 enzymatic pathway, in addition to absolute values, it has proven beneficial to put outcomes in a context comparing the primary produced oxylipid to the metabolite formed from EPHX2. For example, in dairy cattle, the ratio of primary to secondary metabolites of the ω-6 PUFA linoleic acid (epoxyoctadecenoic acid to dihydroxyoctadecenoic acid), is increased significantly in the milk and plasma of cows with coliform mastitis despite not all oxylipid concentrations significantly differing between sick and healthy animals (Mavangira et al., 2015). Differences in oxylipid abundance such as this have led others to focus on EPHX2 as a therapeutic target with clinically beneficial results, such as the amelioration of inflammation and pain from laminitis in horses (Guedes et al., 2017). Interestingly, despite its close-knit activity to CYP450 epoxygenases, EPHX2 expression was not distributed

similarly to any epoxygenase enzyme. Nevertheless, EPHX2 had widespread expression throughout all tissues. As such, EPHX2 may indeed significantly contribute to oxylipid metabolism not only systemically but at the local level in tissues throughout the body.

Expression of epoxygenases in differing bovine in vitro cell types was overall less robust than expression in tissues, likely due to the cellular heterogeneity of tissues and known reduction of CYP450 expression soon after removal from parent tissues (Xiang et al., 2019). In general, no in vitro cell type expressed a profile of epoxygenases similar to the profile of any tissue. The low expression of CYP3A4 and CYP2E1 in cultured bovine cells, compared with the organ types from which they were originally derived, has indeed been noted previously (Talbot et al., 2016).

ω-Hydroxylases

In addition to epoxide formation of mid-chain double bonds, CYP450 hydroxylate PUFA at the terminal carbon. Terminal carbon ω-hydroxylases CYP4A11 and CYP4F2 produce the highly pro-inflammatory oxylipid 20-HETE from arachidonic acid (Johnson et al., 2015). Although not the only 2 enzymes to produce 20-HETE, CYP4F2 and CYP4A11 are the major contributors to 20-HETE production in other species (Lasker et al., 2000). Although the relative input of each CYP4 to 20-HETE production is unknown in cattle, these data suggest that on a transcription basis, CYP4A11 is the greater of the 2, with production predominantly in the liver and kidney.

Aside from arachidonic acid, CYP4F2 has several substrates, including the oxylipid leukotriene B4 (LTB4) and vitamin E (Zhang et al., 2000, McDonald et al., 2009, Schmolz et al., 2016). Interestingly, despite reported widespread expression of CYP4F2 in cattle, this was the only CYP450 enzyme not found consistently in all tissues (Haga et al., 2015). Nonetheless, bovine CYP4 enzymes have proven to participate in mediating immune functions in organ

microenvironments. Indeed, 20-HETE production in the mammary gland appears to be locally regulated (Mavangira et al., 2015, Kuhn et al., 2017). Cattle with lower expression of cutaneous CYP4F3, another CYP450 which metabolizes LTB4, also show resistance to ectoparasites by maintaining greater amounts of LTB4 (Tabor et al., 2017). Differential expression of CYP4F2 may also alter the rate of degradation of vitamin E. Certainly in other species, reduced expression of CYP4F2 slows the breakdown of vitamin E; however, the contribution of CYP4F2 to circulating vitamin E concentrations in cattle is unknown (Bartolini et al., 2017). The lack of consistent expression of TCOH CYP4F2 and CYP4A11 cell culture models underscores why such research is so difficult to undertake. Although the CYP4 family of enzymes shows indications of important functions in organ microenvironments, a lack of cell culture models, as supported by these data, has prevented the study of such functions without transfection.

Vitamin D Metabolism

Initial vitamin D from the diet or UV light exposure of the skin is metabolized to 25-(OH)D_3 by several CYP450, including CYP2J2, CYP3A4, and CYP27A1, evaluated in this study. Of bovine 25-hydroxylases, thus far only CYP2J2 and CYP27A1 have been associated with measurable contributions to calcium homeostasis. Such findings suggest that although other 25-hydroxylases may significantly produce 25-(OH)D_3, at a minimum CYP2J2 and CYP27A1 play a role in vitamin D metabolism important enough to influence clinical disease (Casas et al., 2013, Pacheco et al., 2018). In other species, CYP2R1 appears to be the primary 25-hydroxylase, and in cattle, gene expression of CYP2R1 in mammary epithelial cells stimulated with cholecalciferol certainly increases in a dose-dependent manner, as would be expected with substrate supplementation (Tellez-Perez et al., 2012, Christakos et al., 2016). Additionally, CYP2R1 has been associated with incidence of mastitis; however, it is unclear whether this is due to 25-

hydroxylase activity or to a separate mechanism (Naderi et al., 2018). Despite these understandings, no direct evidence of the relative contribution of CYP2R1 to 25-(OH)D_3 has been shown in cattle.

The importance of 25-hydroxylases in health is beginning to draw greater attention, as our understanding of how clinical and subclinical hypocalcemia influence the predisposition to other diseases improves. Given that hypocalcemia is implicated as a contributing factor to several periparturient cow disorders, the importance of 25-hydroxylases goes beyond milk fever alone (Rodriguez et al., 2017). The relatively high expression of CYP27A1 compared with other 25-hydroxylases bolsters the proposition by Pacheco et al. (2018) that it is indeed one of the primary drivers of 25-(OH)D_3 production in cattle.

The active form of vitamin D_3, 1,25-$(OH)_2D_3$, is formed solely by CYP27B1 (Christakos et al., 2016). Previously thought to occur primarily in the kidney, these data and recent research in cattle show that expression is found widely throughout the body in many tissues and circulating leukocytes (Nelson et al., 2010a, Nelson et al., 2011). The activity of CYP27B1 outside of the kidney may be critical for the immune-modulating activities of 1,25-$(OH)_2D_3$. Specifically in the mammary gland, several studies have noted the antibacterial and anti-inflammatory properties of vitamin D, as well as the importance of CYP27B1 upregulation in response to pathogen insult (Merriman et al., 2017, Merriman et al., 2018). With relatively high expression in lung and uterine tissues, which commonly face pathogen challenge, CYP27B1 may contribute to the antibacterial defenses of these organs as well.

The final step in the vitamin D pathway is carried out by CYP24A1, which is generally induced by the metabolites it ultimately degrades to maintain appropriate 1,25-$(OH)_2D_3$ amounts (Christakos et al., 2016). The induction of CYP24A1 is inhibited by LPS, however, potentially

acting as a defense mechanism against pathogen insult to prolong the activity of 1,25-$(OH)_2D_3$ (Nelson et al., 2010b).

Vitamin A Degradation

Three CYP450 of the CYP26 family of enzymes (CYP26A1, CYP26B1, CYP26C1) are primarily responsible for the degradation of the active forms of vitamin A, namely all-trans retinoic acid (AT-RA), 9-cis retinoic acid, and 13-cis retinoic acid. Of the 3 CYP26 enzymes, CYP26A1 was included in this profile due to its widespread tissue expression in humans, which was recapitulated in the current study despite previous research in cattle showing little extrahepatic expression (Kruger et al., 2005, Ross and Zolfaghari, 2011). Prominent expression in liver samples is conserved with other mammals, whereas robust expression in uterine tissue is not yet may represent a means for the tight regulation of vitamin A necessary during pregnancy in cattle (Topletz et al., 2012).

Similarly, CYP26A1 is also likely a key regulator in the role played by active forms of vitamin A in contributing to the inflammatory response to bacterial insult. Like CYP24A1, the induction of CYP26A1 is significantly reduced by LPS (Zolfaghari et al., 2007). Given the ability of AT-RA to protect bovine cells from oxidative stress and to potentiate the LPS-induced inflammatory cascade, preventing the induction of CYP26A1 may serve as a protective mechanism by prolonging the activity of AT-RA during infection (Shi et al., 2018, Xu et al., 2019).

CONCLUSIONS

This study provides evidence that CYP450 enzymes are widely transcriptionally expressed throughout all tissues of dairy cattle, although to varying amounts. Such widespread expression in distinct cell types aids in explaining how CYP450-derived oxylipids are differentially produced in localized compartments such as the mammary gland. Furthermore, profiling of commonly utilized

in vitro cells revealed significantly lower overall CYP450 expression in cultured cells compared with isolated organ tissues. Nonetheless, these data provide a valuable foundation for future studies aiming to understand the metabolism of vitamins A, D, and E in cattle as well as the production of CYP450-derived oxylipids.

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CHAPTER 6

VITAMIN E ANALOGS LIMIT IN VITRO OXIDANT DAMAGE TO BOVINE MAMMARY ENDOTHELIAL CELLS

Matthew J. Kuhn,[1] and Lorraine M. Sordillo*[1]

[1]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing 48824, United States

***Corresponding author**

Dr. Lorraine M. Sordillo,

Department of Large Animal Clinical Sciences

College of Veterinary Medicine

Michigan State University

sordillo@msu.edu

Tel: (517) 432-8821

Fax: (517) 432-8822

ABSTRACT

Disease in transition dairy cattle is exacerbated in individuals unable to cope with an increased pro-oxidant load that results in oxidative stress. The supplementation of the vitamin E analog α-tocopherol has increased over the last few decades to bolster the antioxidant capacity of dairy cattle, yet some remain unable to manage an appropriate oxidant balance during the transition period. Research suggests supplementation of α-tocopherol to 3-times National Research Council recommendations can counterintuitively increase reactive metabolite production and disease incidence. Therefore, a new approach to supplementing vitamin E is needed. Alpha-tocopherol is only 1 of 8 analogs of vitamin E, all of which have varying antioxidant properties in other mammals and a shorter physiological half-life compared to α-tocopherol, potentially reducing their risk for causing adverse effects. A primary bovine mammary endothelial cell oxidant challenge model was utilized to determine functions of vitamin E analogs. The aim of this study is to determine if other analogs, namely γ-tocopherol or γ-tocotrienol, have antioxidative functions in bovine cells and if these functions may protect cellular viability and endothelial function from oxidant damage. Non-α-tocopherol analogs at physiological concentrations reduced the accumulation of reactive oxygen species more potently than α-tocopherol. Further, γ-tocotrienol decreased cell cytotoxicity to a greater amount than other analogs whereas γ-tocopherol reduced lipid peroxidation and apoptosis more effectively than other analogs. Lastly, α-tocopherol and γ-tocopherol significantly slowed pro-oxidant induced loss of endothelial cell barrier integrity using an electrical cell-substrate impedance sensing system. Concerningly, γ-tocotrienol drastically reduced the endothelial barrier integrity despite no apparent effect on cellular viability at like concentrations. Our results suggest that γ-tocopherol has antioxidant activities and reduces cellular damage and loss of function due to oxidant challenge as well as α-tocopherol. This research sets

the foundation for further investigation into the antioxidant properties of vitamin E analogs in other bovine cells types or whole animal models.

Key Words: Vitamin E, dairy cattle, oxidative stress, tocopherol, tocotrienol

INTRODUCTION

Around the time of calving, dairy cattle that fail to properly adapt to physiological shifts occurring during the transition period may undergo metabolic stress. Caused by the combined factors of oxidative stress, dysfunctional inflammation, and altered nutrient metabolism, which can form a positive and destructive feedback loop if not properly controlled, metabolic stress is a predisposing condition for transition cow health disorders (Sordillo and Mavangira, 2014). A hallmark of the transition period is a significant increase in metabolic activity due to lactogenesis and fetal calf growth that results in increased reactive oxygen species (ROS) production. In some animals, such an increase in ROS production may overcome antioxidant defenses of the animal resulting in damage to macromolecules (Celi, 2011, Mavangira and Sordillo, 2018). Macromolecule damage, such as that of lipids, proteins, and DNA, is known as oxidative stress and one of the contributing factors to metabolic stress. By causing such damage to macromolecules and contributing to the other factors of metabolic stress, oxidative stress is a major factor in the predisposition to disease that some transition cows face (Sordillo and Mavangira, 2014).

Reactive oxygen species are mediators of normal cellular signaling, however, overproduction during the transition period can pose a danger to animals with insufficient compensatory antioxidant responses. Vitamins with antioxidant functions are utilized widely by the dairy industry to increase the antioxidant capacity of animals and promote a balanced oxidant environment. Vitamin E is one such vitamin with antioxidant functions and has been increasingly supplemented over the past 30 yr in its most bioactive form, α-tocopherol (Goff and Stabel, 1990, Haga et al., 2018). Despite doubling circulating concentrations of α-tocopherol over the last 3 decades, some dairy cattle still face oxidative stress around the time of calving (Kuhn et al., 2018). Although it may be intuitive to supplement greater concentrations of α-tocopherol, further

increases in supplementation above current industry standards have yielded concerning results. In 1 study, supplementing α-tocopherol at 3-times National Research Council recommendations resulted in increased incidence of disease and an increased production of reactive oxygen metabolites in certain groups of animals (Council, 2001, Bouwstra et al., 2010a, Bouwstra et al., 2010b). With a need for increased antioxidant capacity and reluctance to supplement greater amounts of α-tocopherol, there is a considerable need for a new approach to antioxidant supplementation in transition dairy cattle.

Although α-tocopherol is found at the greatest concentrations of any analog in mammals, it is only 1 of 8 analogs, all of which retain certain functions similar to α-tocopherol such as antioxidant activities (Jiang, 2014). Analogs differ based upon the methylation of their chromanol ring, resulting in α, β, γ, and δ configurations, and their phytyl tail being saturated (tocopherols) or un-saturated (tocotrienols) (Figure 6.1). These slight differences in configuration result in significantly different rates of metabolism, generally following a pattern of γ > δ > β > α and tocotrienol > tocopherol (Sontag and Parker, 2007). Additionally, analogs without a methyl group at the 5 position of the chromanol ring, such as those designated with γ and δ configurations, have a greater capacity to reduce reactive nitrogen species (Jiang, 2014). For these reasons, there is the potential that non-α-tocopherol analogs of vitamin E may contribute to the overall antioxidant capacity of an animal with a reduced risk of adverse effects due to faster metabolism.

Despite examples of antioxidative function in other species, research into non-α-tocopherol analog bioactivity and potential use in dairy cattle is sparse. Mixed tocopherol supplements have been fed to calves and mid-lactation cattle without adverse effects, however, tocotrienols are yet to be used in a similar manner leaving their safety and efficacy in cattle unknown (Elsasser et al., 2013, Qu et al., 2018). Promisingly, calves fed a tocopherol mix had reduced nitration of tyrosine,

a marker of reactive nitrogen species production, in liver biopsy samples after intravenous challenge with LPS compared to control calves. The reduction in tyrosine nitration conferred by γ-tocopherol was comparable to those fed strictly α-tocopherol suggesting that a non-α-tocopherol mix can protect tissue from damage equally well as α-tocopherol (Elsasser et al., 2013). This study in calves and the remainder of studies which have fed mixed tocopherols to cattle lack further details on the potential oxidant balance of animals or provide a direct measure of oxidative stress (Borher et al., 2002, Elsasser et al., 2013, Qu et al., 2018).

The aim of this study was to assess the capacity of vitamin E analogs γ-tocopherol and γ-tocotrienol in comparison to α-tocopherol to limit cellular damage, death, and loss of function in an in vitro primary bovine mammary endothelial cell (BMEC) oxidant challenge model. The outcomes focus on establishing if non-α-tocopherol analogs of vitamin E have antioxidant functions in cultured bovine cells and if such functions are robust enough to limit damage to endothelial call viability and function from a pro-oxidant environment. Because oxidative stress can influence disease predisposition by damaging cells and altering cellular signaling, understanding the effects of pro-oxidants on cellular viability and function places antioxidant characteristics in a physiological context.

MATERIALS AND METHODS

Chemicals and Reagents

Vitamin E analogs were obtained from either Millipore Sigma (α-tocopherol and γ-tocopherol) (Cerillant Co., Round Rock, TX) or Cayman Chemical (γ-tocotrienol) (Ann Arbor, MI). YoPro and propidium iodide (PI) dyes were purchased from Invitrogen (Eugene OR) and (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-iuM-1,2-diolate (DetaNO) was purchased from Cayman Chemical Co. (Ann Arbor, MI). Antibiotic : antimycotic, trypsin (0.05%),

glutamine, and bovine collagen were obtained from Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) was produced by Hyclone Laboratories Inc. (Logan, UT) and HEPES buffer and HAM's F12K by Corning Inc. (Corning, NY). Hanks Balanced Salt Solution was purchased from Sigma (St. Louis, MO) and gentamycin and collagenase were purchased from Gibco (Grand Island, NY).

Primary Cell Isolation and Culture

Primary BMEC were isolated as described previously (Aherne et al., 1995). In short, pudendal arteries were obtained from an abattoir and placed in ice cold HBSS with 0.1% gentamycin for transportation to the laboratory. Once at the laboratory, vessels were rinsed with HBSS, clamped at one end, and filled with collagenase solution (100 mg collagenase in 48 mL Kreb's ringer bicarbonate and 2 mL 7.5% bovine serum albumin). Once filled, the open end was clamped shut and vessels are incubated at 37ºC for 10 min. After incubation, collagenase solution was collected, and vessels were rinsed with rinse liquid collected into the same tube as collagenase solution. Cells were pelleted, resuspended in growth media, and transferred to a T25 culture flask. After reaching confluency, cells were trypsinized for collection and serially diluted for plating on 96-well plates for single cell isolation. Wells growing colonies from single endothelial cells were propagated before freezing in liquid nitrogen and eventual use. Cells were thawed and used at passages 6-10 for assays.

Cell Viability

Cell viability was measured using the Cell Titer-Glo (Promega, Madison, WI) assay according to manufacturer instructions. The Cell Titer-Glo assay utilizes cellular ATP released after cell lysis to drive a luciferase reaction that results in a quantifiable luminescent compound. The amount of ATP available for this reaction is reflective of the number of viable cells in each

sample. Luminescence was measured on a BioTek Synergy H1 microplate reader (Winooski, VT).

Analog cytotoxicity was quantified by lactate dehydrogenase (LDH) release with a CyQUANT LDH release assay (Invitrogen, Eugene, OR) according to manufacturer instructions. This assay utilizes LDH released from cells due to cellular damage or death as a catalyst for a lactate to pyruvate reaction that, in turn, reduces NAD+ to NADH. Subsequently, NADH is used to produce a quantifiable color shift by reducing tetrazolium salt to red formazan that is proportional to cell cytotoxicity. Colorimetric readings were taken on a BioTek Synergy H1 microplate reader.

Reactive Metabolite Quantification

Reactive oxygen species were quantified with the OxiSelect intracellular ROS assay (Cell Biolabs, San Diego, CA) by reaction of 7'-dichlorodihydrofluorescin dye with ROS creating fluorescent dichlorodihydrofluorescein. After incubation overnight, cells were washed twice with warm HBSS and loaded with a 1:200 dilution of the cell-permeable fluorogenic probe 2',7'-dichlorodihydrofluorescin diacetate. Plates were then incubated for 20 min at 37ºC. After incubation, dye solution was removed, and cells were again washed twice with warm HBSS and treated with 100 uL of media with or without treatment. Fluorescence was measured at appropriate timepoints on a BioTek Synergy H1 microplate reader.

Cellular Damage

Quantification of lipid membrane peroxidation was conducted using the Abcam Lipid Peroxidation Assay Kit (Cell-based) (Branford, CT) according to manufacturer's instructions. In short, cells were plated in a 12-well plate and incubated for 18 h after which they were treated with appropriate analogs or media alone for 6 h. After incubation, cells were again treated with analogs and 250 μM hydrogen peroxide (H_2O_2) for 30 min in 0% FBS F12K media. Media was removed

and a HBSS solution containing assay dye at 1X was added for incubation at 37ºC 5% CO_2 for an additional 30 min. The solution was removed, and cells were washed twice with HBSS, trypsinized, pelleted, and resuspended in 200 uL HBSS for analysis by flow cytometry. Lipid peroxidation was determined by shift of fluorescence from red to green represented by the red : green ratio as measured on an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ). Cytotoxicity of cells was created by treating cultures with 2mM of DetaNO for 24h. Cytotoxicity was quantified by LDH release with a CyQUANT LDH release assay according to manufacturer instructions as discussed above. Colorimetric readings were taken on a BioTek Synergy H1 microplate reader.

Apoptosis was measured by flow cytometry using YoPro dye as an indicator for early apoptotic cells and PI to differentiate necrotic cells. Cells were plated in 6-well tissue-culture plates and allowed to incubate overnight. After 16 h, media was removed and replaced with or without vitamin E analogs at 5 μM or 10 μM each. After 6 h, media was again removed and replaced with or without vitamin E analogs at 5 μM or 10 μM and 0.5 μM DetaNO and incubated at 37ºC 5% CO_2 for 24 h. After incubation, media was removed and collected. Cells were collected by trypsinization and added to previously collected media. Cells were pelleted, resuspended in 1 mL ice cold PBS and transferred to round-bottom polystyrene tubes on ice. A 1 μL amount of YoPro dye was added to appropriate tubes which were incubated for 20 min on ice. After incubation, 1 μL of PI was added to each tube 2 min prior to analysis. Cells were analyzed for percentage of cells fluorescing with YoPro and PI on an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ). Cells fluorescing both YoPro and PI dyes were considered to be necrotic whereas those expressing just YoPro were classified as apoptotic.

Endothelial Barrier Integrity

Analysis of endothelial cell barrier integrity was carried out using the Electrical Cell-Substrate Impedance Sensing system (ECIS) (Applied Biophysics, Inc., Troy, NY). Cells were plated in a 96-well collagen coated plate (96W10idf PET, Applied Biophysics, Inc., Troy, NY) with each well containing an electrode compromising 2.09 mm^2 of the well surface area. After incubating for approximately 14 h and resistance having plateaued, media was replaced with 10% FBS F12K media with or without vitamin E analogs. After 6 h, media was replaced with 1% FBS F12K with or without vitamin E analogs and DetaNO. Cells were analyzed continuously for 48 h. Parameters for analysis were as follows: single frequency, 4000 hz, 180 s interval time. Resistance was normalized for individual wells to the timepoints prior to addition of the DetaNO treatment. Resistance curves were used to derive area under the curve (AUC) values.

Statistics

Statistical analysis was carried out using JMP Pro 14.3.0 (SAS Institute Inc.). Statistics represented an asterisk were compared to a single treatment as specified using parametric pairwise comparison with Dunnett's correction for multiple comparisons or non-parametric Steel test. Differences outlined by differing letters were determined using pairwise Tukey-Kramer HSD with multiple comparisons. Single timepoint analysis for assessing endothelial barrier integrity was carried out using a repeated-measures 2-way ANOVA Dunnett's multiple comparison test ($P < 0.05$).

RESULTS

Cell Viability

Effects on cell viability of vitamin E analogs varied according to specific analog, dose, and incubation time. Cell viability assessed by cellular ATP abundance showed no differences among

treatments after 6 h of incubation ($P < 0.05$) (Figure 6.2A). After 24 h of incubation, α-tocopherol increased cell viability 15.7% ± 1.9% above control whereas γ-tocopherol did not impact viability of cells ($P < 0.05$). We found viability of cells treated with a 50μM dose of γ-tocopherol to be reduced to 41.5% ± 12.9% ($P < 0.05$) (Figure 6.2B).

Cytotoxicity of analogs was assessed by LDH release after 6 h and 24 h of incubation (Figure 6.2C/D). All 3 analogs showed an increase in cytotoxicity with increasing dosage at both 6 and 24 h yet increases due to α-tocopherol and γ-tocopherol, while significant, never increased to > 5% ($P < 0.05$). Unlike the tocopherols, γ-tocotrienol caused more severe cytotoxicity at 25 μM (10.1% ± 2.1%) and 50 μM (21.2% ± 0.2%) doses, greater than that of α-tocopherol or γ-tocopherol ($P < 0.05$).

Reactive Oxygen Species

Reactive oxygen species production was evaluated in BMEC after 30 min, 1 h, and 6 h of treatment with 1 mM DetaNO, a nitric oxide donor (Figure 6.3). At a 1 mM concentration, DetaNO was found to reliably stimulate ROS accumulation without negatively impacting cell viability (Data not shown). Concentrations of 10 μM and 50 μM of vitamin E analogs were utilized to represent the high end of the physiologic range of γ-tocopherol and α-tocopherol, respectively, in blood of dairy cattle supplemented with either analog. When using 10 μM of analogs, both γ-tocopherol and γ-tocotrienol were found to reduce the production of ROS compared to DetaNO antagonist alone ($P < 0.05$; Figure 6.3). When treated with 50 μM, all 3 analogs reduced the production of ROS by DetaNO to a similar amount ($P < 0.05$).

Cellular Damage and Death

The antagonist DetaNO alone did not produce lipid peroxides as measured by the utilized protocol (Data not shown). Instead, H_2O_2 was used as a reactive metabolite to quantify potential

reductions in lipid peroxide production by vitamin E analogs (Figure 6.4). Although H_2O_2 did not increase lipid peroxidation statistically, the difference trended towards a difference ($P = 0.06$) as did that between H_2O_2 and 10 μM α-tocopherol. The only analog to reduce the formation of lipid peroxides after a 30 min H_2O_2 challenge was 10 μM γ-tocopherol ($P < 0.05$).

Vitamin E analogs were evaluated for their ability to limit cell cytotoxicity after 2 mM DetaNO treatment, a dose determined to cause cellular cytotoxicity. After treatment of 24 h, γ-tocopherol and γ-tocotrienol reduced DetaNO derived cytotoxicity and γ-tocotrienol did so to a greater amount than γ-tocopherol ($P < 0.05$) (Figure 6.5A). Additionally, when 30 μM of α-tocopherol alone was compared to 10μM of each analog mixed, the analog mixture was found to offer greater protection from cellular damage or death than α-tocopherol alone ($P < 0.05$; Figure 6.5B).

In addition to measuring cytotoxicity, early stage apoptosis was evaluated. A 0.5 mM treatment of DetaNO for 24 h resulted in a 1.6 fold increase in apoptosis in cells. Unlike the effects of analogs on cellular cytotoxicity, γ-tocopherol alone reduced the number of cells undergoing apoptosis and did so at both 5 μM and 10 μM concentrations ($P < 0.05$). A treatment of 5 μM γ-tocopherol nearly reduced apoptosis to values equal to control samples, reducing apoptosis to only 1.04 ± 0.41 greater fold-change of control cells (Figure 6.6).

Electrical Cell-Substrate Impedance Sensing

The endothelial barrier integrity of BMEC was disrupted by 0.5 mM of DetaNO. At individual timepoints over 48 h, 5 μM of γ-tocopherol was the only dose-analog combination to limit the damage to barrier integrity inflicted by DetaNO, specifically at 29-41 h timepoints ($P < 0.05$; Figure 6.7A/B). Although α-tocopherol did not have an impact at individual timepoints, AUC analysis showed that for both 5 μM and 10μM treatments, α-tocopherol increased the AUC

compared to DetaNO alone, representing a greater sustained barrier integrity over the 48 h time course ($P < 0.05$; Figure 6.7C/D). Similarly, the AUC of γ-tocopherol was greater than DetaNO alone for both 5 μM and 10 μM doses and did not differ from that of α-tocopherol ($P < 0.05$). Surprisingly, γ-tocotrienol dose dependently reduced the endothelial cell barrier more severely and at a faster rate than 0.5 mM DetaNO treatment alone, differing from the DetaNO treatment at timepoints 14-48 h and 12-48 h for 5 μM and 10 μM analog concentrations, respectively. Such a reduction in barrier integrity by γ-tocotrienol reduced the AUC for both treatment doses compared to DetaNO treatment alone as well ($P < 0.05$).

DISCUSSION

Through proper management and nutrition, the prevalence of oxidative stress can be reduced, yet there remains a need for further interventions to reduce its contribution to disease predisposition in transition dairy cows. One such intervention may be the use of non-α-tocopherol analogs of vitamin E. Non-α-tocopherol analogs of vitamin E have considerably shorter physiological half-lives compared to α-tocopherol which may reduce the risk for adverse effects potentially associated with greater supplementation of α-tocopherol (Bouwstra et al., 2010a, Bouwstra et al., 2010b, Schmolz et al., 2016). In this study, γ-tocopherol was found to have antioxidative functions at physiological concentrations in an in vitro primary BMEC oxidant challenge model without evidence of adverse effects on cellular viability. Intriguingly, these effects were found to be more potent than α-tocopherol.

Primary BMEC play a unique role in modeling the interaction between oxidants and vitamin E analogs. Endothelial cells act as a physical barrier to circulating cells and as regulators of cellular signaling between circulation and underlying tissue (Ryman et al., 2015a). Not only are they impacted by the oxidant environments of both tissues and systemic circulation, but they

directly and indirectly control the passage of inflammatory cells from vasculature to sites of infection (Ryman et al., 2015a). Controlling migration of leukocytes as a physical barrier and through inflammatory signaling places endothelial cells at a nexus between oxidative stress and its ultimate negative effects on inflammatory responses. For this reason, maintaining viable and functional endothelial cells is an essential part of effective immune responses.

An additional unique characteristic of BMEC is a lack of *CYP4F2* transcript expression (Kuhn et al., 2020). The cytochrome P450 family 4 sub-family F member 2 enzyme (CYP4F2), is thought to be the sole enzyme responsible for the breakdown of vitamin E analogs (Sontag and Parker, 2002). Without C*YP4F2*, BMEC are not believed to readily metabolize α-tocopherol, γ-tocopherol, or γ-tocotrienol which serves 2 purposes in our model. First, without metabolism all analogs are evaluated at equal concentrations, removing differential metabolism as a reason for differences in outcome measures. Secondly, downstream metabolites of vitamin E analogs are known to have biological effects in other species, such as reducing the activity of cyclooxygenase (COX) enzymes (Jiang et al., 2008). Altering the activity of an enzyme such as COX-2, which induces ROS production and several pro-inflammatory lipid mediators, could have measurable impacts on ROS production or cell health independent from functions of the primary vitamin E analogs (Hernanz et al., 2014). Without transcript expression of *CYP4F2*, these factors are removed from consideration in the interpretation of our results. A final consideration for cells lacking *CYP4F2* are potential impacts on cell viability. Tocotrienols are metabolized faster than any tocopherol in other mammals (Sontag and Parker, 2007). Although their relative metabolism is currently unknown in cattle, tocotrienols are indeed found in cattle at substantially lower concentrations than tocopherols (Sadri et al., 2015). Without active enzymatic metabolism, analogs may be maintained at greater concentrations in our model than would be found in bovine tissues

where *CYP4F2* is present and analogs may be broken down. Although transcript expression of *CYP4F2* has been found widely amongst cattle tissues, its relative activity in different organs has not been evaluated in any specific bovine tissues (Kuhn et al., 2020). The absence of metabolism of vitamin E analogs may account, in part, for the cytotoxicity noted for γ-tocotrienol. A relatively greater cytotoxicity of γ-tocotrienol is consistent with non-bovine studies which show that although typically found at lower concentrations, tocotrienols are generally more cytotoxic compared to tocopherols when utilized at similar concentrations (McCormick and Parker, 2004, Parker et al., 2004, Yam et al., 2009).

When focusing on the antioxidative capacity of vitamin E analogs, we were surprised to find that γ-tocopherol and γ-tocotrienol more potently reduced the accumulation of ROS in BMEC compared to α-tocopherol. Even more, the reduction in ROS production by γ-tocopherol translated into a reduction in lipid peroxidation, yet γ-tocotrienol did not reduce lipid peroxidation, suggesting potentially differing antioxidant functions. Finding antioxidative functions at the lower concentration of 10 μM, and a reduction in apoptotic cells at 5 μM of γ-tocopherol, was important as γ-tocopherol ranges from 300 nM – 1 μM in serum of un-supplemented cattle but has been shown to reach 7.2 μM when dietarily supplemented suggesting the concentrations presented herein could indeed be reached in vivo (Sadri et al., 2015, Qu et al., 2018).

Serum or plasma α-tocopherol concentrations are typically found between 5 and 50 μM depending on a farm's specific supplementation scheme (Bouwstra et al., 2010b, Sadri et al., 2015, Qu et al., 2018). Effects of feeding γ-tocotrienol or mixed tocotrienols on circulating or tissue concentrations are currently unknown as such supplementation has not been reported. Basal concentrations, however, have been found to be 3-5 nM in serum of dairy cattle (Sadri et al., 2015). Our data suggest that although non-α-tocopherol analogs of vitamin E are found at lesser

concentrations than α-tocopherol, they have antioxidant potential at these lower concentrations and may still contribute to antioxidant defenses.

Although α-tocopherol and γ-tocopherol reduced ROS accumulation when supplemented at 50 μM, it is difficult to interpret these results in comparison to the equivalent γ-tocotrienol treatment when ROS results are put into the context of cell viability. Cytotoxicity from α-tocopherol and γ-tocopherol showed dose dependent and statistically significant increases, however, values never reached greater than 5% and there was no effect of analog concentration noted on ATP-based viability measurement. Such minimal effects on cell viability make it unlikely that effects of tocopherol analogs influenced the production of ROS through changes in viability. Alternatively, γ-tocotrienol reduced viability and increased cytotoxicity at higher concentrations after 24 h of incubation. Although 6 h treatments did not reveal any concerns with cellular viability, it is difficult to rule out the potential contribution of cell death to the reduction of ROS accumulation in cells treated with 50 μM of γ-tocotrienol.

The importance of the antioxidant capacity afforded by γ-tocopherol and γ-tocotrienol becomes apparent when cellular viability in a pro-oxidant environment is assessed. An important result of oxidative stress is damage to lipids in cell membranes that create peroxidation chain reactions, ultimately leading to cellular death (Kamal-Eldin and Appelqvist, 1996). Breaking these chain reactions is a primary antioxidative function of α-tocopherol (Kamal-Eldin and Appelqvist, 1996). For the antioxidative functions of γ-tocopherol and γ-tocotrienol to be meaningful in a physiological context, any reduction in ROS accumulation by vitamin E analogs should reduce oxidant damage to cellular macromolecules in some manner. Indeed, γ-tocopherol did reduce the formation of lipid peroxides in cells as α-tocopherol trended towards a significant reduction. A reduction in cellular cytotoxicity caused by DetaNO shows that non-α-tocopherol analogs of

vitamin E can limit oxidant damage and cell death. Promisingly, utilizing analogs as a mixture, as is more likely to be fed in vivo provided greater protection against oxidant cytotoxicity than α-tocopherol alone at an equivalent concentration (Figure 6.5B). Although in vivo non-α-tocopherol analogs would be metabolized faster than α-tocopherol, this difference in cytotoxicity underscores the potential benefits to including non-α-tocopherol analogs in vitamin E supplements.

The apparent greater potency of γ-tocopherol and γ-tocotrienol in both reducing ROS accumulation and protecting cellular cytotoxicity compared to α-tocopherol raises an additional research question. Although these analogs could simply be provided in addition to current α-tocopherol supplements, there is evidence in other species that reducing some supplementation of α-tocopherol when supplementing analog mixes may enhance the effectiveness of such mixed supplements. Microsomal studies assessing the interactions between vitamin E analogs show that increasing concentrations of α-tocopherol hastens the metabolism of non-α-tocopherol analogs through heterotropic cooperativity, reducing their physiological half-life (Sontag and Parker, 2007). Additionally, the α-tocopherol transport protein (αTTP), a protein which confers protection from metabolism to any analog bound to it, prefers binding of α-tocopherol, reducing its relative metabolism. With relative binding affinity for α-tocopherol set at 100%, αTTP binds for γ-tocotrienol and γ-tocopherol at only 12% and 9% respectively (Hosomi et al., 1997). As concentration of α-tocopherol increases so too does the displacement of non-α-tocopherol analogs from αTTP. For these reasons, knowing that γ-tocopherol and γ-tocotrienol may be more potent than α-tocopherol, reducing α-tocopherol to a certain extent may slow the metabolism of non-α-tocopherol analogs and in turn increase their antioxidant efficacy.

A specific outcome from the development of oxidative stress are lipid peroxidation chain reactions in cellular plasma membranes that ultimately result in apoptosis (Catala, 2009).

Apoptosis is one mechanisms by which oxidative stress contributes to dysfunctional inflammatory responses (Ryman et al., 2015a). Reducing apoptosis is a functional measure of the capacity of vitamin E analogs to limit lipid peroxidation chain reactions. Gamma-tocopherol, unlike α-tocopherol or γ-tocotrienol, reduced the percentage of cells undergoing oxidant induced apoptosis. The capacity of γ-tocopherol to also reduce the production of lipid peroxides produced by hydrogen peroxide is therefore likely not a coincidence. Although cause and effect are not directly shown, reducing lipid peroxidation is certainly one explanation for how γ-tocopherol may reduce cellular apoptosis in a pro-oxidant environment.

Apoptosis and other causes of cellular death are one of several reasons that an endothelial barrier may break down, compromising the ability of the vascular endothelium to regulate inflammatory responses. Although the endothelial barrier may become slightly less cohesive to facilitate migration of leukocytes during inflammatory events, this is a tightly regulated process that does not result in severe reductions in barrier integrity. Sustained or severe weakening of the endothelial barrier disrupts its ability to orchestrate inflammatory responses and is a detrimental outcome of pathogen or oxidative damage (Ryman et al., 2016). The ability of α-tocopherol and γ-tocopherol to slow the deterioration of the endothelial barrier due to oxidative damage is a very promising and applicable outcome from this study. Although the mechanism by which these tocopherols reduced oxidant damage of the endothelial barrier function was not directly determined, γ-tocopherol's suppression of oxidant induced apoptosis represent one possible mechanism. These data suggest that in vivo, increased endothelial concentrations of γ-tocopherol may indeed reduce or slow damage to vascular endothelium from oxidative stress faced by some animals during transition or infection (Mavangira et al., 2016, Kuhn et al., 2017).

We found the rapid deterioration in barrier integrity of cells treated with γ-tocotrienol surprising and inconsistent with cellular viability and cytotoxicity data. A lack of cell death or cytotoxicity from 10 μM γ-tocopherol after 24 h suggests that the ECIS system is sensitive to other causes of cellular stress which reduce barrier integrity by an undetermined mechanism. In other species, γ-tocotrienol has been shown to be potently anti-angiogenic and induces apoptosis (Li et al., 2011b). Gamma-tocotrienol was not found to induce apoptosis after 24 h treatment as quantified by YoPro dye inclusion, a timepoint after barrier integrity began to diminish, however, in this assay γ-tocotrienol was supplemented alongside DetaNO, which may have masked pro-apoptotic effects. Additionally, as the ECIS system relies on cell to cell junctions to provide barrier resistance, γ-tocotrienol in conjunction with DetaNO may have further stressed cells and caused significant changes in the formation and maintenance of cellular junctions (Gonzalez-Mariscal et al., 2011).

Given the results of this study and the relative toxicity of γ-tocotrienol noted in other cellular and animal models, caution should be taken when supplementing a vitamin E mix containing tocotrienols (Swift et al., 2014). Certainly, tocotrienols have been used in other species in vivo without adverse effects, however, cattle should be monitored closely until safe baseline values are found (Ikeda et al., 2001, Zhao et al., 2015). Alternatively, tocopherol mixes including oils rich in γ-tocopherol and δ-tocopherol have already been studied in cattle without measured adverse effects (Elsasser et al., 2013, Qu et al., 2018). Unfortunately, these prior studies did not include well rounded analyses of redox balance or any direct measure of oxidative stress, such as quantification of lipid peroxidation. The results presented in our study provide evidence that assessing markers of redox status and oxidative stress when supplementing γ-tocopherol to oxidatively challenged cattle is warranted.

Although these data provide credence for further evaluation of γ-tocopherol use in dairy cattle, evidence that γ-tocotrienol can damage cells provides a warning of the work left to be done. The optimal or potentially toxic concentrations in blood or tissue for analogs of vitamin E in cattle remains essentially unknown. Measuring both tocopherols and tocotrienols in such oils in future studies will help to establish safe or unsafe baselines for supplementation. Nonetheless, the use of analog mixes in dairy cattle deserves greater attention. Specifically, the potential of non-α-tocopherol analogs to increase the antioxidant capacity and reduce oxidative stress in oxidatively challenged animals should be assessed. Dairy cattle that experience oxidative stress during the transition period face an increased predisposition to disease, which can result in lost productivity, increased veterinary costs, and reduced animal welfare. The potential to increase antioxidant capacity and reduce a predisposition to disease during the transition period makes non-α-tocopherol analogs of vitamin E, and specifically γ-tocopherol, important vitamers for further study.

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CHAPTER 7

INHIBITION OF 20-HYDROXYEICOSATETRAENOIC ACID BIOSYNTHESIS BY VITAMIN E ANALOGS IN HUMAN AND BOVINE CYTOCHROME P450 MICROSOMES

Matthew J. Kuhn,[1] and Lorraine M. Sordillo*[1]

[1]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing 48824, United States

***Corresponding author**

Dr. Lorraine M. Sordillo,

Department of Large Animal Clinical Sciences

College of Veterinary Medicine

Michigan State University

sordillo@msu.edu

Tel: (517) 432-8821

Fax: (517) 432-8822

ABSTRACT

Dairy cattle can be predisposed to disease around the time of calving due to an improper alteration in the production of lipid inflammatory mediators, or oxylipids, which regulate all aspect of the inflammatory response. Shifts in the production of oxylipids due to an inability to properly cope with the physiological changes of the transition period can result in aberrant inflammatory responses that reduce immune defenses against infection and exacerbate tissue damage from overly-robust inflammation. For example, 20-hydroxyeicosatetraenoic acid (HETE) is an oxylipid derived from cytochrome P450 enzymes (CYP450) that is found at significantly greater concentration around calving and during clinical disease. Biosynthesis of 20-HETE occurs almost exclusively from 2 specific cytochrome enzymes, CYP450 family 4 sub-family F member 2 (CYP4F2) and CYP450 family 4 sub-family A member 11 (CYP4A11) of which CYP4F2 is the predominant ω-hydroxylase of arachidonic acid in humans. To further study the activities of 20-HETE and potentially reduce its production in vivo, methods to mitigate the production of 20-HETE must be explored. Additional substrates of CYP4F2, such as vitamin E, are known to both increase and decrease the metabolism of other CYP4F2 substrates. The objective of this study was to determine if vitamin E analogs may reduce the production of 20-HETE through competition for CYP4F2 activity. In this study, interactions between arachidonic acid and analogs of vitamin E were assessed in human CYP4F2, bovine-kidney, and bovine-mammary microsomes. Gamma-tocopherol reduced production of 20-HETE from human and bovine-kidney microsomes (35.3% and 27.5%, respectively) whereas γ-tocotrienol only reduced 20-HETE production from human microsomes (40.1%). Finally, bovine-mammary microsomes were not found to produce a quantifiable amount of 20-HETE suggesting this local compartment may not be a significant contributor to the production of 20-HETE during an unstimulated inflammatory state. Together

these data show that analogs of vitamin E can reduce the production of 20-HETE though competition with arachidonic acid for metabolism by CYP4F2. For further study of the activities of 20-HETE, vitamin E analogs, especially γ-tocopherol, should be further assessed for its capacity to reduce 20-HETE production.

Key words: Vitamin E, microsome, 20-HETE, tocopherol, tocotrienol, dairy cow

INTRODUCTION

Dairy cows in the transition period face a heightened risk of disease if they fail to properly adapt to the physiological changes associated with calving and lactogenesis. Known as metabolic stress, cattle can be negatively impacted by a triad of factors that form a destructive feedback loop if not properly managed by individuals (Sordillo and Mavangira, 2014). One component of this triad, dysfunctional inflammation, involves an inappropriate shift in the production of inflammatory lipid mediators, known as oxylipids, in cattle unable to properly regulate their production. Oxylipids control all aspects of inflammation from onset through resolution (Mavangira and Sordillo, 2018). Although changes in oxylipid production are necessary to respond to specific physiological changes, such as inflammation necessary for dry period remodeling or as a response to disease, unregulated changes in production can lead to aberrant inflammatory responses (Mavangira and Sordillo, 2018). Such aberrant responses may be slow to respond to pathogen challenge yet overly-robust and prolonged after infection onset. Overall, the inflammatory environment of these cattle predisposes them to disease and causes excessive tissue damage due to the overaccumulation of leukocytes and associated oxidative damage

Oxylipids are derived from poly-unsaturated fatty acids by either enzymatic oxidation pathways or by interaction with reactive oxygen species. Cytochrome P450 enzymes (CYP450) represent 1 of the 3 enzymatic pathways of oxidation, along with cyclooxygenase and lipoxygenase enzymes. Recent studies aimed at understanding how the production of oxylipids may be different during disease or across lactation found shifts in CYP450 produced oxylipids outnumbered those from other pathways (Mavangira et al., 2015, Kuhn et al., 2017). This suggests that the CYP450 pathway of oxylipid metabolism may be an important, yet currently less studied, contributor to regulating inflammation. Additionally, 1 oxylipid from the CYP450 pathway in

particular, 20-hydroxyeicosatetraenoic acid (HETE), has repeatedly been found to be at a greater concentration in cattle undergoing inflammatory shifts, such as during clinical mastitis or the transition period compared to other stages of lactation (Mavangira et al., 2015, Kuhn et al., 2017). These findings underscore the potential importance of 20-HETE to the inflammatory response in cattle and the need to better understand its inflammatory activities.

Metabolized by CYP450 with ω-hydroxylase activity, 20-HETE is created with the addition of a hydroxyl group to the terminal carbon of arachidonic acid (Imig, 2016). Primarily studied for its activity as a vasoconstrictor, 20-HETE is implicated as a causative agent of human hypertension yet may prevent ischemic stroke as well (Ward et al., 2008, Shekhar et al., 2019). The duality of activities shown by 20-HETE in this regard emphasizes the need for a balance in its production. In humans, 20-HETE is primarily produced from either CYP450 family 4 sub-family A member 11 (CYP4A11) or CYP450 family 4 sub-family F member 2 (CYP4F2) (Imig, 2016). Like most CYP450, CYP4F2 metabolizes additional substrates including vitamin K, vitamin E, and leukotriene B4 (LTB4), an eicosanoid derived from the lipoxygenase pathway (Figure 7.1) (Jin et al., 1998, Farley et al., 2013). Unique amongst these substrates is vitamin E, which is only broken down by CYP4F2 and no other CYP450 (Parker et al., 2004).

Vitamin E is a collective term for a constellation of 8 analogs consisting of α, β, γ, and δ isoforms, determined by methylation of the chromanol ring. Each isoform class is further split into the families of tocopherols and tocotrienols, differentiated by either an unsaturated or saturated phytyl tail, respectively (Jiang, 2014). Interestingly, individual analogs of vitamin E appear to interact differently with CYP4F2. A structural understanding of the human or bovine CYP4F2 isoform is undeveloped and without a reported crystal structure, explanations for interactions between substrates of CYP4F2 are largely speculative based upon characteristics of other CYP450

conformations. Sontag and Parker (2007) exemplified potential heterotropic cooperativity of CYP4F2 substrates using human CYP4F2 transfected insect-cell microsomes. This work revealed that although non-α-tocopherol analogs of vitamin E did not alter the metabolism of α-tocopherol, increasing concentrations of α-tocopherol increased the rate of metabolism of non-α-tocopherol analogs (Sontag and Parker, 2007). Although all CYP450 isoforms have a single heme-based active site, some have also shown to contain multiple binding sites within the active site, such as bovine CYP21A2 (Peng et al., 2015). Multiple binding sites may allow for simultaneous binding of substrates if size and conformation of the active site allows. A potential for multiple binding sites may explain the phenomenon reported by Sontag and Parker (2007). In general, the single active site of CYP450 allows for binding of only a single substrate at any given time, making heterotropic cooperativity an exception to the rule. Other substrates of CYP4F2 compete for metabolism as is more common amongst substrates of specific CYP450 (Figure 7.1). In addition to its acceleration of non-α-tocopherol metabolism, α-tocopherol conversely slows the V_{max} of vitamin K metabolism (Farley et al., 2013). Additionally, arachidonic acid reduces LTB4 ω-hydroxylation by 30% in human liver microsomes (Jin et al., 1998).

Despite the evidence of interactions between substrates of CYP4F2, vitamin E's capacity to increase or decrease the production of 20-HETE by competing or cooperating with arachidonic acid for CYP4F2 activity remains unknown. The aim of the current study is to determine if analogs of vitamin E may have an impact on the production of 20-HETE in CYP450 microsomes.

MATERIALS AND METHODS

Chemicals

Nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide (NAD), arachidonic acid, and γ-tocotrienol were obtained from Cayman Chemical

Co. (Ann Arbor, MI). Collagenase type I, glacial acetic acid, and Dulbecco's modified eagle medium (DMEM) were obtained from ThermoFisher Scientific (Waltham, MA) and fetal bovine serum (FBS) from Hyclone Laboratories Inc. (Logan, UT). Hanks Balanced Salt Solution (HBSS) hyaluronidase from bovine testes, α-tocopherol (Cerrilant Co.) and γ-tocopherol (Cerrilant Co.) were purchased from Millipore Sigma (St. Louis, MO). Acetonitrile was obtained from VWR Chemicals (Radnor, PA).

Tissue Microsome Isolation

All tissues were obtained within 15 min of slaughter from cull dairy cows without gross appearance of pathology and immediately placed in ice cold Krebs Ringer solution for transportation to the laboratory. Once at the laboratory, kidney and mammary tissues were rinsed thoroughly with ice cold wash buffer (50 mM Tris-base, 150 mM KCl, 1 mM EDTA), cut into approximately 1 g pieces, and rinsed again. Tissues from 3 cows were combined into a single conical tube containing 5mL of homogenization buffer (50 mM monobasic K_3PO_4, 125 mM KCl 50 mM, 1 mM EDTA, 250 mM sucrose) and homogenized with a TissueRuptor II (Qiagen Sciences, Germantown, MD). Additionally, a secondary procedure involving enzymatic tissue dissociation based upon Mackenzie et al. (1982) was used for mammary tissue prior to mechanical disruption to ensure enzymes had not been mechanically inactivated by the TissueRuptor II (Mackenzie et al., 1982). In short, minced mammary tissue was slowly mixed in DMEM with 10% FBS, 400 units / mL hyaluronidase, and 300 units / mL collagenase at 37ºC for 8 h. Large pieces of tissue were strained out and cells were homogenized with a Dounce homogenizer with 15 strokes.

Disrupted cell samples were spun at 9,000 x g for 20 min at 4º C and supernatant was transferred to a new tube. Samples were then spun at 108,000 x g for 70 min at 4º C, supernatant

was discarded, and the pellet was resuspended in resuspension buffer (50 mM monobasic K_3PO_4, 150 mM KCl) and spun a second time at 108,000 x g for 70 min at 4º C. Supernatant was discarded and samples were resuspended a final time in homogenization buffer. An aliquot was taken for protein quantification and samples were flash frozen in LNO_2 and stored at -80º C until use. Protein was quantified according to manufacturer instructions of the Pierce BCA protein assay kit (ThermoFisher Scientific, Waltham, MA).

Microsome Enzymatic Reactions

Microsomal reactions were based, in part, upon Sontag and Parker (2007). Microsomal reactions were carried out with either 10 pM of human CYP4F2 microsomes derived from transfected insect cells (Corning Supersomes, Woburn, MA) or 500 μg of tissue-microsome protein. Reaction mixtures contained 50 mM phosphate buffer (Corning, Woburn, MA), 25 μM arachidonic acid, H_2O, and vitamin E analogs or vehicle methanol (MeOH). Vitamin E analogs were incubated at 37ºC with buffer and microsomes for 60 min prior to initiation of the reaction. Initiation was carried out with 1 mM NADPH and 1 mM NAD. A single reaction for each replicate received H_2O as a negative control in place of NADPH + NAD. Reactions were carried out for either 15 or 60 min at which point 100 uL of reaction mix was removed and added to a tube containing 25 μL of ice cold 94% acetonitrile and 6% glacial acetic acid. A 60 min reaction was not undertaken in with bovine microsomes due to limited sample quantity. Samples were centrifuged at 10,000 x g for 3 min and 100 μL was transferred to a chromatography vial containing 10 μL of internal standard containing 5(S)-HETE-d_8 (0.25 μM), 15(S)-HETE-d_8 (0.25 μM), 8-9-EET-d_{11} (0.5 μM), prostaglandin E_2-d_9 (0.5 μM), 8,9-dihydroxyeicosatrienoic acid-d_{11} (0.25 μM).

Liquid Chromatography Tandem Mass Spectrometry

Details of liquid chromatography tandem mass spectrometry analysis are described in detail in Mavangira et al. (2015). Quantification of metabolites was accomplished on a Waters Xevo-TQ-S tandem quadrupole mass spectrometer using multiple reaction monitoring.

Statistical Analysis

Statistical analysis was carried out using a one-way ANOVA with Dunnett's correction with statistical significance set at $P < 0.05$ using JMP Pro 14.3.0 (SAS Institute Inc). Data are presented as mean ± SEM.

RESULTS

Human CYP4F2 Microsomes

Microsomes from human CYP4F2 were utilized to determine the effect of vitamin E analogs on CYP4F2 without other CYP450 present. After 15 or 60 min of reaction time, control reactions triggered with H_2O rather than NADPH + NAD had little to no production of 20-HETE. Production of 20-HETE in reactions triggered with NADPH + NAD, however, was greater than 200 nM of 20-HETE after 15 min and greater than 400 nM of 20-HETE after 60 min showing there was time dependent production. Although 20-HETE production did not differ from control by any treatment in 15 min reactions containing 10 μM of vitamin E analog (Figure 7.2A), 20-HETE production was reduced in 15 min reactions containing 25 μM of both γ-tocopherol and γ-tocotrienol ($P < 0.001$; Figure 7.2B). The reduction in 20-HETE formation by 25 μM of γ-tocopherol and γ-tocotrienol reached 35.3% and 40.1% respectively. After 60 min of reaction time, 20-HETE concentration was not reduced by 10 μM of any vitamin E analog, however, MeOH vehicle control significantly reduced 20-HETE production ($P < 0.01$; Figure 7.2C). All 3 analog treatments ($P < 0.001$) and MeOH vehicle control ($P < 0.01$) reduced 20-HETE at a 25 μM dose

after 60 min of reaction (Figure 7.2D). In these reactions, γ-tocopherol ($P < 0.01$) and γ-tocotrienol ($P < 0.001$), however, significantly reduced 20-HETE production when compared to MeOH vehicle control as well.

Bovine Organ Microsomes

Of the 50 bovine-mammary microsome reactions undertaken, only 3 produced quantities of 20-HETE greater than negative control samples and 9 reactions did not reach the limit of detection for 20-HETE (0.01 nM) regardless of isolation method. Kidney microsomes activated by negative control H_2O did not produce any measurable quantity of 20-HETE. Similar to human CYP4F2 microsomes, after a 15 min reaction, bovine-kidney microsomes treated with 25 μM of γ-tocopherol produced 27.5% less 20-HETE ($P < 0.05$) as compared to a 35.3% reduction in human CYP4F2 microsomes of like treatment (Figure 7.2E/F).

DISCUSSION

Greater concentrations of 20-HETE in transition cows compared to those in other stages of lactation and those with clinical mastitis compared to healthy controls suggests that 20-HETE is either has pro-inflammatory activities or is produced as a compensatory response to pro-inflammatory shifts (Mavangira et al., 2015, Kuhn et al., 2017). Evidence in humans and murine species in addition to bovine in vitro work supports the pro-inflammatory nature of 20-HETE (Toth et al., 2013, Hoopes et al., 2015, Mavangira et al., 2020). The work done with a bovine in vitro model of oxidative stress, however, observes the effects of 20-HETE at concentrations orders of magnitude greater than have been quantified in cattle making extrapolation of results difficult (Mavangira et al., 2020). Without further evidence, the nature in 20-HETE in cattle is based upon informed speculation. Nonetheless, developing interventions to limit the production of 20-HETE in vitro or in vivo will allow for further research into the specific activities of 20-HETE in cattle.

This study shows that vitamin E analogs, and most potently γ-tocopherol, have the capacity to reduce 20-HETE production by competing with arachidonic acid for CYP4F2 activity. These data lay a foundation for further studies into the potential of vitamin E analogs to mitigate 20-HETE production.

Assessing 20-HETE production with CYP450 microsomes limits the factors that contribute to differential metabolite production, such as CYP450 gene expression, cellular viability, or substrate availability. Instead, only interactions between substrate, enzyme, and potentially other reaction components contribute to metabolite production. The significant inhibition of 20-HETE production from human CYP4F2 conferred by γ-tocopherol and γ-tocotrienol reveals that these substrates interact in an inhibitory rather than cooperative manner with arachidonic acid.

These data may have been confounded by a reduction in activity of CYP4F2 by MeOH, specifically 60 min reactions with α-tocopherol or γ-tocopherol. Methanol is known to inactivate certain CYP450 at roughly ≥ 2% total reaction volume (Vuppugalla et al., 2007, Sakalli et al., 2015). The breakpoint for MeOH's inactivation of CYP4F2 is unknown and in this study, MeOH accounted for 0.43% and 1.08% of the total reaction volume for 10 μM and 25 μM doses, respectively, for α-tocopherol and γ-tocopherol treatments. The γ-tocotrienol treatment was prepared in a more concentrated form such that MeOH made up a maximum of only 0.1% of total reaction volume. Although MeOH reduced CYP4F2 activity (Figure 7.2C/D), the extent to which for γ-tocopherol and γ-tocotrienol reduced 20-HETE production was significantly greater than that of MeOH alone.

Cytochrome P450 enzymes are generally conserved amongst species, yet differences certainly can exist. For this reason, experiments were repeated with bovine isolated microsomes from either kidney or mammary tissues. Bovine renal tissue has the greatest CYP4F2 expression

on an mRNA basis of any organ making it potentially an important contributor to systemic 20-HETE production (Kuhn et al., 2020). Unlike the human CYP4F2 microsome preparations, whole organ bovine microsomes include all CYP450 present within a given tissue type, aside from a select few CYP450 which are not endoplasmic reticulum associated. With all CYP450 present, CYP4F2 is likely no longer the sole enzyme producing 20-HETE. Rather, CYP4A11 would be expected to produce considerable amounts of 20-HETE, potentially diluting the inhibitory effects of vitamin E analogs on CYP4F2 (Imig, 2016). The potential or additional production of 20-HETE by CYP4A11 in these samples made finding a 27.5% reduction in 20-HETE production by γ-tocopherol a surprising amount, likely representing an even greater inhibition of CYP4F2 individually.

Isolation of bovine-mammary microsomes was undertaken in an attempt to contrast the activity of CYP450 in renal tissue with mammary tissue. Studies profiling differences in 20-HETE concentrations between plasma and milk samples have found milk 20-HETE concentrations shift to a greater amount than in plasma in cows with clinical mastitis compared to healthy controls and in the transition period compared to other stages in lactation (Mavangira et al., 2015, Kuhn et al., 2017). The explanation for greater changes in milk 20-HETE concentration compared to plasma remains unknown. One justification suggests that mammary tissue may produce a significant amount of 20-HETE during times of increased inflammation, such as during mastitis. An inability to consistently quantify 20-HETE produced from bovine-mammary microsomes suggests mammary tissue man not significantly contribute to the 20-HETE found in milk, a finding supported by the relatively low transcript expression of both CYP4F2 and CYP4A11 in mammary tissue compared to other organs (Kuhn et al., 2020). The potential for an increase in CYP4F2 or CYP4A11 expression and activity by an inflammatory stimulus in order to produce copious

amounts of 20-HETE still remains as the samples for this study were obtained from cows without signs of clinical disease. Given the relatively small changes in expression and activity of these enzymes in cattle or other species by stimuli or different stages of lactation, however, this remains an unlikely scenario (Edson and Rettie, 2013, Sadri et al., 2015, Haga et al., 2018). More realistically, significant changes in milk 20-HETE concentrations are due to either sequestration of plasma 20-HETE in milk or production from locally infiltrating leukocytes (Tsai et al., 2011).

The precise activities of 20-HETE in dairy cattle remain elusive. If the changes in 20-HETE production during the transition period or clinical disease are to be placed into proper physiological context, further research into the role 20-HETE may play in exacerbating or mitigating inflammation must be undertaken. Analogs of vitamin E, and specifically γ-tocopherol, are potentially useful compounds to mitigate the production of 20-HETE for the further study of its activities in dairy cattle or in vitro models. Other factors may contribute to 20-HETE formation in a cellular model or living animal, including arachidonic acid availability, activity of phospholipase A2, or changes in CYP450 expression and activity. These data nonetheless provide foundational evidence to further assess the relationship between vitamin E, arachidonic acid, and the production of 20-HETE in dairy cattle.

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CHAPTER 8

SUMMARY AND CONCLUSIONS

Profiling Transition Physiology

Raphael et al. (2014) first described significant shifts in transition period oxylipid production beyond only eicosanoids. This work, however, was limited by gas chromatography technology and the scope of oxylipids profiled. Thereafter, Mavangira, et al. (2015, 2016) described differences in oxylipid concentration between healthy animals and those with severe coliform mastitis through the more sensitive and specific liquid chromatography tandem mass spectrometry (LC/MS/MS) quantitative method. Mavangira, et al. (2016) included the human-medicine gold standard for quantification of oxidative stress, the 8-isoprostane class of oxylipids measured by LC/MS/MS. These works galvanized the use of LC/MS/MS in dairy cattle to quantify oxidative stress and broadened our understanding of the role oxylipids may play in mediating inflammatory responses. With methods validated, an opportunity arose to build on these foundational works to fill gaps in knowledge of the transition period in dairy cattle.

For decades, dairy cattle have been suspected to undergo substantial shifts in oxidant balance during the transition period due to increased energy demands. Many previous studies that focused on the oxidative status of transition dairy cattle lacked measures of both pro- and anti-oxidants making it difficult to draw strong conclusions about the overall redox balance (Turk et al., 2013, Zahrazadeh et al., 2018). Additionally, the use of non-specific biomarkers of oxidative stress that can vary in concentration due to slight differences sample processing, such as malondialdehyde or thiobarbituric acid-reactive substances, resulted in conflicting findings regarding the nature of oxidative stress during transition (Castillo et al., 2005, Turk et al., 2008, Hanschke et al., 2016, Tsikas, 2017). Our research in Chapter 3 was the first to use LC/MS/MS

quantification of 8-isoprostanes for transition period samples and show that indeed, systemic oxidative stress is significantly more severe during the transition period compared to other stages of lactation. Importantly, this research revealed that stark differences exist between plasma and milk, representative of the systemic circulation and mammary gland, respectively suggesting that the mammary gland may respond differently to the oxidative challenges of transition than other parts of the body. Further evaluation of tissue specific oxidative stress biomarkers is required to confirm this assertion. Many modalities for quantifying oxidative stress exist, however, focusing on tissue biopsies rather than further evaluation of representative fluids may be the most direct method. Although fluids such as urine, blood, or milk may provide generalized indications of oxidative stress by potentially pooling biomarkers from whole organs or several tissues, histological analysis of tissue sections may allow for a more localized assessment of specific tissues. Immunohistochemical staining of mammary tissue for 4-hydroxynonenal (lipid damage), 8-hydroxy-2'-deoxyguanosine (nucleic acid damage), and 3-nitrotyrosine (protein damage) have all been carried out in cattle previously and could provide more well-rounded analysis of the severity and tissue specificity of oxidative stress at transition if compared to other tissues, such as the liver (Si et al., 2018, Strickland et al., 2019).

If local body compartments indeed respond uniquely to the oxidative challenge of transition, these results would change the paradigm of how we view transition oxidative stress and pursue preventive measures and treatments. For example, if seeking non-invasive means to screen cattle for systemic oxidative stress, milk would not be an appropriate sample given that it is representative of the mammary gland's oxidative status rather than that of the whole animal. The validity of the compartmentalization theory was bolstered by evidence in Chapter 4 and more recent work by Contreras, et al. (2017) showing changes in the accumulation of specific oxylipids

by lactation stage differs by the body compartment being assessed. Substrate availability plays a significant role in the production of classes of oxylipids and may explain, to an extent, the differences found between the systemic circulation and the mammary gland. Likely not a coincidence, all but one oxylipid, 20-HETE, were reduced in milk during the transition period compared to other stages of lactation. Fatty acid utilization for milk-fat synthesis may reduce the availability of PUFA for oxylipid production, a reduction which may be enhanced during transition compared to other stages of lactation as cattle adapt to the onset of lactogenesis. Transcript expression of enzymes that contribute to oxylipid biosynthesis also varies widely between differing cells and tissues in cattle (Pfaffl et al., 2003, Kuhn et al., 2020). This alone may contribute to differences between compartments as well as potential changes in enzyme activity across lactation.

Conclusions drawn from Chapters 3 and 4 are limited by their cross-sectional study design, comparing different animals from various life stages, rather than a longitudinal design, following individual animals through lactation. Confounding variables including varying diets or management differences between groups may have influenced these results. As diets change through lactation, so does the relative abundance of PUFA available for oxylipid formation. Additionally, this dissertation has raised questions as to the interaction between vitamin E analogs and the production of 20-HETE. Although almost wholly unstudied, several other potential interactions could occur such as between vitamin D and epoxyeicosatrienoic acid production through CYP2J2. These unknown interactions could contribute to differences in oxylipid production created by changes in diet as vitamin supplementation varies with diets formulated for specific lactation groups. A more precise study would include samples from individual cows prior to calving and throughout lactation on a single, standardized diet to determine how oxylipid

production and measures of oxidant balance fluctuate within individuals. Conclusions from these chapters also emphasizes the need for more localized measures of oxidative stress and oxylipid production. Although milk may be utilized to represent the mammary gland as a whole, the impact of the systemic circulation on the oxylipid composition of milk remains elusive. A more accurate assessment of the locally produced oxylipid profile could involve *ex vivo* culture of mammary tissue (Chen et al., 2019). *Ex vivo* culture and assessment of oxylipid production may remove the contribution of oxylipids produced by other tissues from analysis, however, this method would not consider how systemic inflammatory signaling may influence the relative production of oxylipids.

It is unfortunate that milk may not appropriately reflect systemic oxidative stress as collecting milk is less invasive and typically more convenient than obtaining blood samples. Alternative methods for oxidative stress assessment that do not require blood collection have been utilized in humans including urine isoprostanes or other biomarkers in breath condensate, yet such alternative methods remain mostly unstudied in cattle (Pelclova et al., 2016). The use of breath condensate was attempted in cattle by Ranade et al. (2014), however only indirect measures of redox balance were measured. Without follow-up research on the utility of breath condensate, a significant gap in non-invasive oxidative stress testing capabilities remains. If the utility of oxidative stress quantification is to become useful for cow-side diagnostics, not only will reference ranges for biomarkers need to be set, but methods of sample collection and analysis need to be developed. Whereas LC/MS/MS quantification of isoprostanes is an essential part of oxidative stress research, less time consuming and technical methods of analysis are necessary for on-farm applications.

Focusing on 20-Hydroxyeicosatetraenoic Acid

Profiling differences in oxylipid concentrations across lactation exposed a similar increase in 20-HETE concentration between cattle undergoing clinical mastitis and those in the transition period (Mavangira et al., 2015, Ryman et al., 2015b, Kuhn et al., 2017). At this time, observations of 20-HETE have been mostly descriptive and do not resolve whether 20-HETE is perpetuating inflammation or a physiologic response to inflammation. Although an in vitro report of its effects on bovine aortic endothelial cells reveals pro-oxidant and pro-inflammatory activity, the dose used was orders of magnitude greater than physiological concentrations (Mavangira et al., 2020). Nonetheless, given the potential pro-inflammatory nature of 20-HETE, there is a significant need to understand what may be causing an increase in 20-HETE during transition and disease states. Cytochrome P450 enzymes (CYP450) involved in the metabolism of drugs and pollutants have been the focus of bovine CYP450 research for decades, however, those involved with pathways directly influencing metabolic stress remain only partially understood (Nebbia, 2001). To create an appropriate model to evaluate factors influencing 20-HETE production, it was paramount to understand how 20-HETE may be produced in vivo and modeled in vitro.

Our profiling of CYP450 transcript expression in Chapter 5 aimed to determine if any commonly utilized bovine in vitro models of inflammation or oxidative stress may be appropriate to model in vivo 20-HETE production or fat-soluble vitamin metabolism. Chapter 5 revealed widespread expression of CYP450 in bovine tissues including the liver, kidney, lung, mammary, gland, heart, skeletal muscle, spleen, and uterus. Although few similarities were found between in vitro cell culture models and organ expression profiles, these findings set the foundation for further studies into the expression and activity of CYP450 in cattle. These data, however, are only representative of a basal inflammatory state and lack insight into how expression may change

during disease or across lactation. The occurrence of dramatic shifts in oxylipid production and circulating vitamin concentrations during times of disease or physiological changes certainly support the potential for significant changes in CYP450 activity to occur.

An unexpected outcome from Chapter 5 was the comparison between transcript expression of CYP4F2 and CYP4A11 which account for nearly all 20-HETE production in most mammalian species (Edson and Rettie, 2013). Interestingly, CYP4A11 was found to be transcriptionally expressed at a considerably greater amount than CYP4F2 in all organs, proposing the inverse relationship from what has been detailed in other mammals (Powell et al., 1998, Lasker et al., 2000). This finding highlighted a second shortcoming of Chapter 5, that conclusions were drawn from transcript expression rather than enzymatic activity. If CYP4A11 was to be a greater contributor to 20-HETE production than CYP4F2, it would complicate finding interventions to reduce 20-HETE production in cattle as most inhibitors of 20-HETE production currently studied have a greater inhibitory effect upon CYP4F2 than CYP4A11 (Wu et al., 2009). Chapter 7, however, provided evidence to the contrary.

Inhibition of 20-HETE Production

When assessing the potential inhibitory effects of vitamin E analogs on 20-HETE production, Chapter 7 showed for the first time that analogs of vitamin E, especially γ-tocopherol, can significantly inhibit the production of 20-HETE, likely in a competitive manner. These data were exciting as they represent a novel interaction between substrates of CYP4F2 in any species. The reduction of 20-HETE production in bovine kidney microsomes by γ-tocopherol also emphasized the potentially considerable contribution of CYP4F2 to 20-HETE production in cattle. When compared to human CYP4F2 microsomes, where CYP4F2 is the lone CYP450 present, γ-tocopherol inhibition of bovine kidney 20-HETE was only 7.8% lower (35.3% inhibition in human

microsomes vs 27.5% in bovine). Given that both CYP4F2 and CYP4A11 are present in bovine microsomes, there should have been stark differences between inhibition of human microsomes compared to bovine microsomes had CYP4A11 been the major contributor to 20-HETE production. The relatively robust inhibition of 20-HETE biosynthesis by γ-tocopherol suggests that similar to other mammals, CYP4F2 is likely the prominent arachidonic acid ω-hydroxylase in cattle. As no specific and complete inhibitors of either CYP4F2 or CYP4A11 are available aside from specific antibodies, to truly test this theory, individual bovine microsomes would need to be isolated. Similar to the human CYP4F2 microsomes utilized in Chapter 7, this would involve the transfection of the bovine CYP4F2 or CYP4A11 gene into a cell line, such as the BTI-Tn-5B1 line used for human CYP4F2 microsomes, for specific isolation of a single bovine enzyme.

Chapter 7 further highlighted the importance of studying CYP450 activity, rather than transcription, when focusing on mammary microsomes. Although CYP4F2 and CYP4A11 were found to be transcriptionally expressed in mammary tissue, 20-HETE could not be measured from mammary microsomal reactions. The absence of 20-HETE biosynthesis may be due to error in isolation of microsomes. Several methods which were successful in isolating such enzymes in other tissues, however, were assessed to address this concern. An inability to quantify 20-HETE from mammary microsomes questions whether changes in milk 20-HETE are due to the accumulation of 20-HETE from systemic circulation, local production from mammary tissue, or infiltrating leukocytes. Ascertaining the true reason for differences in 20-HETE production could involve assessing changes in mRNA, protein expression, and activity of CYP4F2 and CYP4A11 in transition cows compared to other stages of lactation in the liver and kidney, their sites of highest expression, and the mammary gland. Further, somatic cells could be collected during the transition period and assessed for their ability to produce 20-HETE through microsome isolation and

reaction. Vitamin E and 20-HETE's potential importance around the time of calving and during disease make assessing changes in expression and activity of CYP4F2 and CYP4A11 during these times a prudent endeavor.

The significance in a more robust cellular or in vivo model of vitamin E analogs inhibiting the ω-hydroxylation of arachidonic acid remains to be determined. Many other factors contribute to the production of 20-HETE, potentially including the redox environment, the activity of phospholipase A2 enzymes, or the cellular uptake of substrate. Additional studies should assess what impact vitamin E analogs may have on 20-HETE production in a cellular model that would account for these factors. Potential adverse effects caused by CYP4F2 inhibition in dairy cattle remain unknown as well. In humans, polymorphisms associated with a reduction in CYP4F2 and CYP4A11 activity mainly result in predisposition to cardiovascular diseases not typically found in cattle (Sirotina et al., 2018, Zhang et al., 2018a). Unintended consequences should still be considered, however. As 20-HETE is a highly vasoactive metabolite, reduced systemic blood pressure may result in increased blood flow to the mammary gland altering milk production (Cai et al., 2018). Alternatively, 20-HETE has inverse pulmonary activities, causing vasodilation rather than constriction. In this case, reductions in 20-HETE could exacerbate conditions such as pulmonary hypertension in susceptible cattle or those at higher altitudes (Birks et al., 1997).

Antioxidant Capacity of Vitamin E Analogs

The potential to use analogs of vitamin E to compete with arachidonic acid for activity of CYP4F2 is especially intriguing as mixed tocopherols have already been used in dairy cattle without adverse effects (Elsasser et al., 2013, Qu et al., 2018). A preliminary study by Elsasser et al. (2013) also promisingly presented evidence in calves that short term γ-tocopherol can reduce oxidative injury to the liver, revealing antioxidative functions similar to those of α-tocopherol.

Unfortunately, although Qu et al. (2018) fed a similar vitamin E analog supplement to adult cows, outcome measures did not evaluate oxidative stress.

There is reason to suspect that supplementation of some non-α-tocopherol analogs in dairy cattle may not bring about the adverse effects found when supplementing α-tocopherol at three-fold greater than National Research Council recommendations (Council, 2001, Bouwstra et al., 2010b). These adverse effects from α-tocopherol supra-supplementation may be due a phenomenon of antioxidants known as the "inversion of activity" where activities switch from anti- to pro-oxidant in specific situations (Kamal-Eldin and Appelqvist, 1996). For vitamin E, this occurs at either high concentrations or without adequate ascorbic acid available for regeneration of reduced vitamin E (Kamal-Eldin and Appelqvist, 1996). Fortunately, using non-α-tocopherol analogs may avoid such concerns. Due to a faster metabolic rate, non-α-tocopherol analogs do not concentrate in blood or tissue to the extent of α-tocopherol (Sadri et al., 2015). Further, the concentration necessary to reach a point of inversion of activity is analog-dependent and greater for γ-tocopherol and δ-tocopherol than α-tocopherol (Kamal-Eldin and Appelqvist, 1996).

Nonetheless**,** increasing the tissue and circulating concentration of vitamin E analogs to bolster antioxidant capacity has been a focus of several groups in human medicine (Wu et al., 2006, Wiser et al., 2008). In bovine mammary endothelial cells (BMEC), γ-tocopherol provided clear benefits to cellular viability when supplemented prophylactically prior to oxidant challenge. Importantly, these properties of γ-tocopherol were not found when vitamin E was utilized as a treatment following oxidant challenge. Gamma-tocotrienol's rapid and severe breakdown in endothelial barrier integrity was surprising as it did not appear to be caused by cellular death. Rather, the ECIS system may have been sensitive to cellular changes, such as cells beginning to undergo apoptosis or alterations in cellular junctions. Previous studies show that γ-tocotrienol

specifically, rather than other analogs, induces apoptosis in various non-bovine cell types supporting this conclusion (Shah and Sylvester, 2004, Tang et al., 2019). These studies, like Chapter 6, use supraphysiological doses of γ-tocotrienol to determine its effects. Baseline values of circulating γ-tocotrienol have been measured in dairy cattle, yet it remains unknown how dietary supplementation may affect circulating concentrations. Although Chapter 6 supplemented γ-tocotrienol as low as 5 μM, further in vitro studies should continue to lower this dose to determine at what concentration adverse effects are no longer found and if γ-tocotrienol may still have antioxidant capacity or other physiological effects at lesser concentrations.

Any application of these results to in vitro models or live animals is limited by a lack of metabolism of vitamin E analogs by BMEC. Whereas this made BMEC an opportunistic model for assessing vitamin E analogs, the role of differing metabolic rates among analogs is an additional gap in knowledge that should be explored. An absence of CYP4F2 expression also prevented BMEC from being utilized to evaluate potential inhibitors. Other species have shown that concentrations of γ-tocopherol can be increased with natural supplements, such as sesamin, which reduce CYP4F2 activity. If reducing 20-HETE is considered favorable, CYP4F2 inhibitors that increase tocopherol concentrations may also beneficially reduce 20-HETE biosynthesis. Just as an inappropriately intense inflammatory response can be damaging to animals, a lack of response to challenge can allow for the onset and increased severity of disease. We lack an understanding of this balance, especially when it comes to the role of specific oxylipids. Although reducing 20-HETE production at some periods of lactation may be beneficial to limiting excessive inflammatory damage, its reduction at disease onset may prove harmful. Because non-α-tocopherol analogs are metabolized much more rapidly than α-tocopherol, in vivo supplementation of a partial CYP4F2 inhibitor may be helpful to maximize potential benefits of mixed tocopherol

supplements. If this is undertaken, changes to the production of 20-HETE and associated effects, either positive or negative, should not be overlooked.

Altogether, this dissertation highlights the intense challenges dairy cattle potentially face around the time of calving. These data underscore the need for further studies into transition oxidative stress and associated body compartment specific responses and the potential dramatic changes in inflammation-mediating oxylipids. Analogs of vitamin E, especially γ-tocopherol, represent a potentially practical intervention for the mitigation of both oxidative stress and dysfunctional inflammation. The greatest benefits of mixed tocopherols are likely to be afforded from prophylactic systemic supplementation rather than targeted treatment of disease such as intramammary infusion. As scrutiny of modern agriculture from the public continues to increase, methods to prevent and decrease disease severity become ever more pressing. The use of mixed tocopherols in cattle represents a compelling method of improving antioxidant capacity while reducing production of potentially harmful oxylipids, such as 20-HETE.

APPENDICES

APPENDIX A: Tables

Table 1.1. Factors effecting transcript expression of cytochrome P450 enzymes involved in health and disease of cattle

CYP450	Substrates	Gene expression change	Reference
CYP1A1	Xenobiotics, PUFA	Increased in cultured bovine hepatocytes with supplementation of fish oil extracts	(Guruge et al., 2009)
		Decreased in bovine macrophage cell line by bacterial and viral PAMP	(Toka et al., 2019)
		Increased in rumen papillae of calves with greater tretinoin (vitamin A)	(Nishihara et al., 2018)
		Decreased in bovine hoof dermal cells by LPS	(Tian et al., 2019)
CYP1B1	Xenobiotics	Decreased in bovine macrophage cell line by bacterial PAMP	(Toka et al., 2019)
CYP2B6	Xenobiotics, (vitamin A)	Increased colon of calves fed colostrum and vitamin A supplemented milk replacer compared to standard milk replacer	(Kruger et al., 2005)
CYP2C8	Xenobiotics, (vitamin A)	Increased liver of calves fed colostrum rather than standard milk replacer	(Kruger et al., 2005)
CYP2C19	Xenobiotics, PUFA	Decreased in liver of cows after intramammary challenge with E. coli or LPS	(Jorgensen et al., 2012)
		Decreased in liver of cows fed lipolysis reducing rumen protected niacin at parturition	(Ringseis et al., 2019)
		Decreased in liver post-calving compared to pre-calving	(Ha et al., 2017)
		Decreased in liver of cows after intramammary challenge of E. coli and LPS	(Jorgensen et al., 2012)
		Decreased in mid-lactation and late-lactation cows undergoing heat stress	(McCracken et al., 2015)
CYP2C31	Xenobiotics	Increased in liver of cows post-calving compared to pre-calving	(Ha et al., 2017)
CYP2C42	Unknown	Decreased in liver of cows fed lipolysis reducing rumen protected niacin at parturition	(Ringseis et al., 2019)

Table 1.1. (Cont'd)

CYP2C87	Endogenous hormones	Decreased in liver of cows fed lipolysis reducing rumen protected niacin at parturition	(Ringseis et al., 2019)
		Increased in liver of cows post-calving compared to pre-calving	(Ha et al., 2017)
CYP2E1	Xenobiotics, PUFA, (Vitamin A)	Increased in liver of calves fed colostrum compared to those fed standard milk replacer	(Kruger et al., 2005)
		Increased in whole blood in transgenic cattle with a FAT-1 gene insertion	(Guo et al., 2011)
		Decreased in liver of cows by intramammary challenge of E. coli and LPS	(Jorgensen et al., 2012)
CYP2J2	PUFA, Cholecalciferol	Negative correlation with milk fever	(Pacheco et al., 2018)
		Positive correlation between adipose expression and both circulating free fatty acids and β-hydroxybutyrate	(Contreras et al., 2017)
		Increased in adipose tissue after calving compared to pre-calving	(Contreras et al., 2017)
CYP2S1	Xenobiotics, (Vitamin A)	Increased in whole blood of transgenic cattle with FAT-1 gene insertion	(Guo et al., 2011)
CYP2U1	Xenobiotics, PUFA	Decreased in liver post-calving compared to pre-calving	(Ha et al., 2017)
		Decreased in bovine macrophage cell line by viral PAMP	(Toka et al., 2019)
CYP2R1	Lipids, Cholecalciferol	Decreased in bovine primary mammary epithelial cells after treatment with Staphylococcus aureus	(Tellez-Perez et al., 2012)
		Increased dose dependently in bovine primary mammary epithelial cells treated with cholecalciferol	(Tellez-Perez et al., 2012)
		Small nucleotide polymorphism association with clinical mastitis	(Naderi et al., 2018)
CYP3A4	Xenobiotics, PUFA, (Vitamin A, Cholecalciferol)	Increased in whole blood of transgenic cattle with FAT-1 gene insertion	(Guo et al., 2011)
		Increased in the abomasum of cattle resistant to nematode infestation compare to susceptible animals	(Li et al., 2011a)
		Decreased in mid-lactation cows undergoing heat stress	(McCracken et al., 2015)

Table 1.1. (Cont'd)

		Decreased in bovine hoof dermal cells by LPS	(Tian et al., 2019)
CYP4A11	PUFA	Increase in mRNA expression and copy number results in increased lipogenesis in beef cattle	(Yang et al., 2017)
		Increased at 1 and 2 wk post-calving in liver of cows fed a high energy diet during the dry period compared to low energy diet	(Khan et al., 2015)
CYP4B1	Xenobiotics	Increased in the abomasum of cattle resistant to nematode infestation compare to susceptible animals	(Li et al., 2011a)
CYP4F2	PUFA, Vitamin E, Vitamin K, LTB4	Increased in liver of cows 1 wk post-calving compared to 1 wk pre-calving and 1 month post-calving	(Haga et al., 2018)
		Increased in mammary tissue 6 wk post-calving compared to parturition with a nadir 1 wk prior to calving	(Haga et al., 2018)
		No change in any organ after supplementation with α-tocopherol	(Haga et al., 2015)
		Increased in rumen papillae of calves with greater tretinoin (vitamin A)	(Nishihara et al., 2018)
CYP4F3	LTB4 (PUFA)	Decreased in cutaneous tissue of breeds resistant to ectoparasites infestation when challenged with ticks	(Piper et al., 2010)
		Increased in cutaneous tissue of cattle susceptible to tick infestation compared to resistant animals	(Carvalho et al., 2014)
		Increased in whole blood of transgenic cattle with FAT-1 gene insertion	(Guo et al., 2011)
		Increased in rumen papillae of calves with greater tretinoin (vitamin A)	(Nishihara et al., 2018)
CYP11A1	Cholesterol	Decreased in cutaneous tissue of breeds resistant to ectoparasites infestation when challenged with ticks	(Piper et al., 2010)
CYP24A1	25-(OH)D_3, 1,25-$(OH)_2D_3$	Decreased in a bovine mammary epithelial cell line treated with Staphylococcus aureus	(Yue et al., 2017)
		Increased in a bovine mammary epithelial cell line supplemented with either 25-(OH)D_2 or 25-(OH)D_3	(Yue et al., 2017)
		Increased in cultured PBMCs treated with 1,25-$(OH)_2D_3$ or concurrently 25-(OH)D_3 and Mycobacterium bovis purified protein derivative	(Nelson et al., 2011)

Table 1.1. (Cont'd)

		Increased in bovine macrophages dose dependently by 1,25-$(OH)_2D_3$	(Garcia-Barragan et al., 2018)
		Increased in bovine monocytes by 1,25-$(OH)_2D_3$ although only increased 1/5th as much when concurrently treated with both 1,25-$(OH)_2D_3$ and LPS	(Nelson et al., 2010b)
		Increased in mammary tissue and milk CD14- somatic cells after intramammary Streptococcus uberis challenge	(Nelson et al., 2010a)
		Increased in peripheral blood monocytes after supplementation with 1,25-$(OH)_2D_3$ although only increased 1/20th as high when concurrently treated with both 1,25-$(OH)_2D_3$ and LPS	(Merriman et al., 2015)
		Increased in CD14+ and CD14- milk somatic cells after intramammary infusion of 1,25-$(OH)_2D_3$	(Merriman et al., 2015)
		Increased in milk somatic cells after multiple intramammary infusions of 25-$(OH)D_3$	(Merriman et al., 2018)
CYP26A1	Vitamin A	Increased in liver of calves fed colostrum and milk replacer supplemented with vitamin A compared to those fed standard milk replacer	(Kruger et al., 2005)
		Decreased in liver of cows post-calving compared to pre-calving	(Ha et al., 2017)
CYP26B1	Vitamin A	Decreased in bovine macrophage cell line by viral PAMP	(Toka et al., 2019)
CYP27A1	Cholecalciferol, Cholesterol	Negative correlation with milk fever	(Pacheco et al., 2018)
CYP27B1	25-$(OH)D_3$	Decreased in a bovine mammary epithelial cell line treated with Staphylococcus aureus	(Yue et al., 2017)
		Increased in bovine PBMC culture by LPS and Mycobacterium bovis purified protein derivative	(Nelson et al., 2011)
		Increased in bovine macrophages after treatment with LPS or infection with Mycobacterium bovis	(Garcia-Barragan et al., 2018)
		Increased in bovine monocytes by multiple toll like receptor ligands	(Nelson et al., 2010b)
		Increased in peripheral blood monocytes by LPS and decreased by 1,25-$(OH)_2D_3$ with no impact of 25-$(OH)D_3$	(Nelson et al., 2010b)

Table 1.1. (Cont'd)

	Increased in bovine primary mammary epithelial cells after treatment with Staphylococcus aureus or cholecalciferol	(Tellez-Perez et al., 2012)
	Increased in mammary tissue and CD14+ milk somatic cells after intramammary infusion with Streptococcus uberis	(Nelson et al., 2010a)
	Increased in milk macrophages and neutrophils after intramammary infusion of LPS	(Merriman et al., 2018)

Table 2.1 Ruminant feeding trials using tocopherol mixes as dietary supplements, supplement parameters, and major outcomes[1]

Species	Dose	Mixture	Duration	Outcome	Source
Bovine (Dairy)	13.16 mg / kg (598 kg cow)	αT: 9.4% βT: 1.0% γT: 64.6% δT: 25.0%	7-9 d	Reduction of liver and muscle α-tocopherol as γ-tocopherol increased in liver, muscle, and mammary tissue. α-tocopherol and γ-tocopherol peaked in blood after 5 d	(Qu, 2017)
Bovine (Dairy)	15.47 mg / kg (598 kg cow)	αT: 23.6% βT: 0.9% γT: 58.7% δT: 16.8%	7 d	No negative effects on health, leukocyte populations, or immune health	(Qu et al., 2018)
Bovine (Beef)	D1: 5.9 mg / kg, D2: 18.2 mg / kg (211 kg calf)	D1: αT: 100.0% D2: γT: 77.7% δT: 22.3%	5 d	D2 increased plasma and liver α-tocopherol, D1 and D2 reduced oxidative damage after LPS treatment.	(Elsasser et al., 2013)
Bovine (Beef)	D1: 111.5 mg / kg D2: 193.1 mg / kg D3: 227.2 mg / kg (375 kg steer)	αT: 13.5% γT: 58.2% βT+δT: 28.3%	Single dose	Peak γ-tocopherol at 38 h followed by drop. α-tocopherol sustained after 38 h. D2 showed greatest plasma increase	(Borher et al., 2002)
Ovine	D1: 7.7 mg / kg D2: 15.4 mg / kg (65 kg ewe)	D1: αT 100% D2: γT 100%	37 d	Serum isoprostanes were reduced by γ-tocopherol to a greater degree than α-tocopherol	(Kasimanickam and Kasimanickam, 2011)

[1]αT: α-tocopherol, βT: β-tocopherol, γT: γ-tocopherol, δT: δ-tocopherol, D1: 1st diet in trial, D2: 2nd diet in trial, D3: 3rd diet in trial, SAA: serum amyloid A

Table 3.1 Nutrient composition of the diet for the experimental animals

item	nutrient analysis (%DM)	
	PP group	ML/LL group
dry matter	46.14	47.64
forage DM	47.00	40.87
crude protein	17.09	17.23
MP	12.48	11.97
ME (Mcal/kg)	3.05	2.98
NEL (Mcal/kg)	1.81	1.79
ADF	17.51	16.23
NDF	27.48	29.56
Forage ADF	12.44	10.92
Forage NDF	17.19	17.46
NFC	44.33	42.36
starch (non-sugar)	28.55	29.85
fat	4.70	4.34

Abbreviations: PP, periparturient; ML, mid-lactation; LL, late lactation; DM, Dry Matter; MP, Metabolizable Protein; ME, Metabolizable Energy; NEL, Net-Energy for Lactation; ADF, Acid Detergent Fiber; NDF, Neutral Detergent Fiber; NFC, Non-fiber Carbohydrates. For the PP group the following ingredients were added to the base ration: Vitamins: Vit A 8328 IU/kg, Vit D 1456 IU/kg and Vit E 35 IU/kg; Minerals (% DM): 0.80 Ca, 0.37 P, 1.32 K, 0.29 Mg, 0.25 S, 0.34 Na, 0.48 Cl, 0.47 Salt; Ca:P ratio = 2.16; trace minerals (ppm): 61.97 Zn, 3.66 Fe, 14.08 Cu, 46.20 Mn, 0.79 Co, 0.79 I, 0.28 Se; DCAB = 19.48 mEq / 100g; Rumensin 13.14 g / ton. For the ML and LL groups, the following ingredients were added to the base ration: Vitamins: Vit A 8369 IU / kg, Vit D 1455 IU / kg and Vit E 33 IU / kg; Minerals (% DM): 1.04 Ca, 0.48 P, 1.10 K, 0.34 Mg, 0.26 S, 0.56 Na, 0.32 Cl, 0.35 Salt; Ca:P ratio = 2.17; trace minerals (ppm): 51.8 Zn, 7.6 Fe, 11.5 Cu, 37.0 Mn, 0.6 Co, 0.6 I, 0.37 Se; DCAB = 27.6 mEq / 100g; Rumensin 9.5 g / ton.

Table 3.2 Non-esterified fatty acids quantified in plasma and milk samples compared between lactation stages[1]

oxylipid	fatty acid form	plasma			milk		
		peripartorient period	mid-lactation	late-lactation	peripartorient period	mid-lactation	late-lactation
LA	Ω-6 PUFA	11210 ± 2070 [a]	3698 ± 838 [b]	5466 ± 301 [b]	1608 ± 464 [b]	2054 ± 464 [ab]	3599 ± 529 [a]
AA[3]	Ω-6 PUFA	2099 ± 416 [a]	976 ± 251 [b]	1692 ± 62 [ab]	304 ± 94 [b]	387 ± 106 [b]	1539 ± 240 [a]
EPA[2,3]	Ω-3 PUFA	153 ±1.7	95.8 ± 1.8	171 ± 2	27.4 ± 10.7 [b]	59.5 ± 15.8 [b]	160 ± 30 [a]
DHA[2,3]	Ω-3 PUFA	792 ± 214 [a]	141 ± 50 [b]	388 ± 88 [a]	56.7 ± 2.0	59.2 ± 2.0	154 ± 3
oleic acid[2]	monounsaturated	39220 ± 9550 [a]	8778 ± 649 [b]	6987 ± 1381 [b]	7700 ± 1520.	7780 ± 1520	3260 ± 2280
palmitic acid[2]	saturated	15700 ± 3950 [a]	6340 ± 2010 [ab]	3440 ± 1460 [b]	548 ± 1460	7260 ± 1460	1580 ± 2190
stearic acid[2,3]	saturated	6460 ± 139	3880 ± 218	7180 ± 382	n/a[c]	n/a[c]	n/a[c]

[1]Geometric means ± SEM. [2]Plasma data transformed for statistical purposes. [3]Milk data transformed for statistical purposes. Values expressed as μM. Abbreviations: LA, Linoleic Acid; AA, Arachidonic Acid; EPA, Eicosapentaenoic Acid; DHA, Docosahexaenoic Acid; PUFA, Polyunsaturated Fatty Acid. [ab] Values with different letters in a row, within a single sample type, are significantly different ($P < 0.05$). [c] Fatty acids that are found in less than half of samples and omitted from the final analyses.

Table 3.3 Oxylipids measured in milk exhibiting significant changes in concentration between lactation stages[1]

oxylipid	precursor	periparturient period	mid-lactation	late-lactation
9-HODE[2]	LA	0.575 ± 0.158 [b]	1.830 ± 0.482 [a]	1.881 ± 0.567 [a]
13-HODE[2]	LA	1.758 ± 0.364 [b]	5.139 ± 1.064 [a]	7.064 ± 1.671 [a]
12,13-DiHOME[2]	LA	0.246 ± 0.141 [b]	4.935 ± 2.704 [a]	2.966 ± 1.878 [a]
9,10-EpOME[2]	LA	0.213 ± 0.049 [b]	0.289 ± 0.064 [ab]	0.489 ± 0.125 [a]
9,10-DiHOME[2]	LA	0.138 ± 0.032 [b]	6.645 ± 1.562 [a]	7.703 ± 2.071 [a]
LXA4[2]	AA	0.005 ± 0.002 [ab]	0.010 ± 0.002 [a]	0.002 ± 0.001 [b]
20-HETE[2]	AA	0.018 ± 0.004 [a]	0.007 ± 0.002 [b]	0.014 ± 0.004 [ab]
14,15-DHET[2]	AA	0.0039 ± 0.0011 [ab]	0.0005 ± 0.0004 [b]	0.0018 ± 0.0008 [a]
AEA[2]	AA	0.040 ± 0.013 [b]	0.045 ± 0.011 [b]	0.121 ± 0.025 [a]
2-AG[2]	AA	4.137 ± 2.026 [b]	16.953 ± 3.413 [a]	15.212 ± 4.406 [a]
9-HETE	AA	0.010 ± 0.006 [b]	0.021 ± 0.006 [ab]	0.035 ± 0.007 [a]
11-HETE[2]	AA	0.0214 ± 0.007 [b]	0.044 ± 0.010 [ab]	0.085 ± 0.015 [a]
Resolvin D2[2]	DHA	0.008 ± 0.001 [ab]	0.014 ± 0.002 [a]	0.005 ± 0.001 [b]
19,20-EpDPE	DHA	0.132 ± 0.039 [b]	0.222 ± 0.038 [ab]	0.294 ± 0.043 [a]

[1]Geometric means ± SEM. [2]Data transformed for statistical purposes. Values expressed as μM. Abbreviations: HODE, Hydroxyoctadecadienoic Acid; DiHOME, Dihydroxy-12Z-octadecenoic Acid; EpOME, Epoxy-12Z-octadecenoic Acid; LXA4, 5(S),6(R),15(S)-lipoxin A4; HETE, Hydroxyeicosatetraenoic Acid; DHET, Dihydroxyeicosatrienoic Acids; AEA, Arachidonyl Ethanolamide; 2-AG, 2-Arachidonyl Glycerol ; EpDPE, Epoxydocosapentaenoic Acid; DiHETE, Dihydroxyeicosatrienoic Acid. [ab] Values with different letters differ ($P < 0.05$).

Table 3.4 Oxylipids measured in plasma exhibiting significant changes in concentration between lactation stages[1]

oxylipid	substrate	periparturient period	mid-lactation	late-lactation
13-oxoODE[2]	LA	0.311 ± 0.119 [a]	0.195 ± 0.078 [a]	0.039 ± 0.017 [b]
9,10-DiHOME[2]	LA	8.757 ± 1.480 [b]	19.813 ± 2.318 [a]	20.256 ± 2.567 [a]
LXA4[2]	AA	0.020 ± 0.006 [a]	0.035 ± 0.018 [a]	0.003 ± 0.001 [b]
20-HETE[2]	AA	0.888 ± 0.205 [a]	0.267 ± 0.041 [b]	0.218 ± 0.062 [b]
5-oxoETE[2]	AA	0.006 ± 0.001 [b]	0.015 ± 0.004 [a]	0.009 ± 0.002 [ab]

[1]Geometric means ± SEM. [2]Data transformed for statistical purposes. Values expressed as μM. Abbreviations: oxoODE, Oxooxtadecadienoic Acid; DiHOME, Dihydroxy-12Z-octadecenoic Acid; LXA4, 5(S),6(R),15(S)-lipoxin A4; HETE, Hydroxyeicosatetraenoic Acid; oxoETE, Oxoeicosatetraenoic Acid. [ab] Values with different letters differ ($P < 0.05$).

Table 4.1. Nutrient composition of the diets for the experimental animals

Item	Nutrient analysis (%DM)[1]	
	PP group[2]	ML/LL group[3]
DM	46.14	47.64
Forage DM	47.00	40.87
CP	17.09	17.23
MP	12.48	11.97
ME (Mcal / kg)	3.05	2.98
NE_L (Mcal / kg)	1.81	1.79
ADF	17.51	16.23
NDF	27.48	29.56
Forage ADF	12.44	10.92
Forage NDF	17.19	17.46
NFC	44.33	42.36
Starch (non-sugar)	28.55	29.85
Fat	4.70	4.34

[1] Groups: PP = Periparturient; ML = Mid-lactation; LL = Late lactation.
[2]Additions to base ration: Vitamins: Vit A 8328 IU / kg, Vit D 1456 IU / kg and Vit E 35 IU / kg; Minerals (% DM): 0.80 Ca, 0.37 P, 1.32 K, 0.29 Mg, 0.25 S, 0.34 Na, 0.48 Cl, 0.47 Salt; Ca:P ratio = 2.16; trace minerals (ppm): 61.97 Zn, 3.66 Fe, 14.08 Cu, 46.20 Mn, 0.79 Co, 0.79 I, 0.28 Se; DCAB = 19.48 mEq / 100 g; Rumensin 13.14 g / ton.
[3]Additions to base ration: Vitamins: Vit A 8369 IU / kg, Vit D 1455 IU / kg and Vit E 33 IU / kg; Minerals (% DM): 1.04 Ca, 0.48 P, 1.10 K, 0.34 Mg, 0.26 S, 0.56 Na, 0.32 Cl, 0.35 Salt; Ca:P ratio = 2.17; trace minerals (ppm): 51.8 Zn, 7.6 Fe, 11.5 Cu, 37.0 Mn, 0.6 Co, 0.6 I, 0.37 Se; DCAB = 27.6 mEq / 100g; Rumensin 9.5 g / ton.

Table 4.2. Serum and plasma metabolites from periparturient (n = 4), mid-lactation (n = 4), and late-lactation (n = 4) cows (mean ± SEM)

	Periparturient period		Mid-lactation		Late-lactation	
Metabolite	Mean	SEM	Mean	SEM	Mean	SEM
Serum Amyloid-A[1] (μg/mL)	135.71	33.91	91.54	33.91	28.56	33.91
Haptoglobin[1] (mg/mL)	1.378	0.051	1.064	0.010	0.003	0.009
Albumin[2] (g/dL)	2.98b	0.05	3.03b	0.06	3.57a	0.06
Cholesterol[2] (mg/dL)	54.75b	2.14	205.25a	169.40	265.00a	11.17
Non-Esterified Fatty Acids[2] (mEq/L)	0.64a	0.11	0.09b	0.11	0.10b	0.11

[a,b]Means within a row with different superscripts differ ($P < 0.05$)
[1]Serum sample
[2]Plasma sample

Table 5.1. Proprietary TaqMan primer/probe reference information and gene activity

Gene	NCBI reference sequence	TaqMan assay ID	Activity
CYP1A1	N/A	Custom Assay[2]	Epoxygenase, STOH
CYP2C19	NM_001109792.2	Bt03268476_m1	Epoxygenase, STOH
CYP2E1	NM_174530.2	Bt03237496_m1	Epoxygenase, STOH
CYP2J2	N/A	Custom Assay[2]	Epoxygenase, STOH, 25-hydroxylase
CYP3A4	NM_001099367.1	Bt03293209_sH	Epoxygenase, STOH, 25-hydroxylase
CYP4A11	NM_001077908.1	Bt03243984_m1	TCOH
CYP4F2	NM_001035042.1	Bt03221777_m1	TCOH, vitamin E, and LTB4 metabolism
CYP24A1	NM_001191417.1	Bt04306544_m1	24-hydroxylase
CYP26A1	NM_001168662.1	Bt04280827_m1	Vitamin A metabolism
CYP27A1	NM_001083413.2	Bt03255966_m1	25-hydroxylase
CYP27B1	NM_001192284.1	Bt04311113_g1	1α-hydroxylase
EPHX2	NM_001075534.1	Bt03241449_m1	Epoxide hydrolase
RPS9	NM_001101152.2	Bt03272017_m1	Reference gene

[1]National Center for Biotechnology Information reference sequence found in the nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore/).
[2]Information regarding the creation of custom TaqMan primer/probe sets can be found in Table 2.
STOH = sub-terminal ω-hydroxylase, TCOH = terminal-carbon ω-hydroxylase, LTB4 = leukotriene B4

Table 5.2. Primer sequences for custom TaqMan primers

Gene		Primer sequence (5'-3')	bp	Accession no.[1]
CYP1A1	forward	TTGTGAACCAGTGGCAGATCAA	64	XM_005222018.4
	reverse	GGCCGGAACTCAGATGGAT		
CYP2J2	forward	TCCTACCTCATCCAGACGATAAGAG	78	XM_002707809.6
	reverse	GGAAACGGCATTGTTGATTGTCA		

[1]Accesion numbers from National Center for Biotechnology Information gene bank database (https://www.ncbi.nlm.nih.gov/gene).

Table 5.3. Cell samples with cycles to threshold (CT) values > 35 cycles or undetected (n = 3)

	Cell type[1]			
Gene	BAEC	BMEC	MAC-T	MDBK
CYP2C19	30.05	37.33	35.54[2]	27.03[2]
CYP2E1	28.86	35.94[2]	34.27	35.56[2]
CYP4A11	33.62	29.07[2]	37.09[2]	36.99[2]
CYP4F2	--[3]	--[3]	33.31[2]	36.95[2]

[1]BAEC = bovine aortic endothelial cell; BMEC = bovine mammary endothelial cell; MAC-T = bovine immortalized mammary epithelial cells; MDBK = Madin-Darby bovine kidney epithelial cell.

[2]Excludes sample(s) with no expression

[3]No expression found in any sample (100ng RNA)

APPENDIX B: Figures

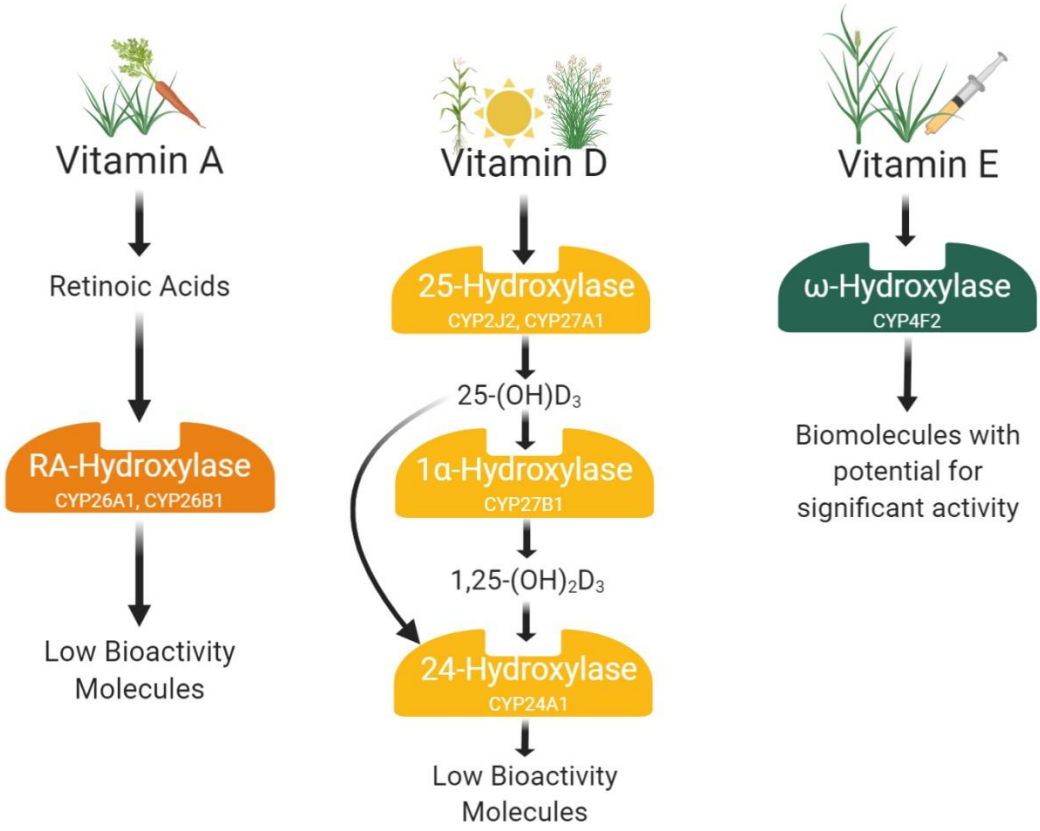

Figure 1.1. Several fat-soluble vitamins are metabolized by cytochrome P450 enzymes (CYP450). Vitamin A, once activated into a retinoic acid (RA), is likely degraded by several CYP450, however, only CYP26A1 and CYP26B1 have been evaluated in cattle. Vitamin D goes through first step activation to 25-hdyroxyvitamin D_3 (25-[OH]D_3) by potentially several CYP450, however, there is only ample evidence for this activity with CYP2J2 and CYP27A1 in cattle. The vitamin D metabolic cascade continues with activation of 25-(OH)D_3 to 1,25-dihydroxyvitmain D_3 (1,25-[OH]$_2D_3$) by CYP27B1 and ultimate inactivation by CYP24A1. Vitamin E analogs are degraded exclusively by CYP4F2 to biomolecules with undetermined activity in cattle. Figure created at BioRender.com.

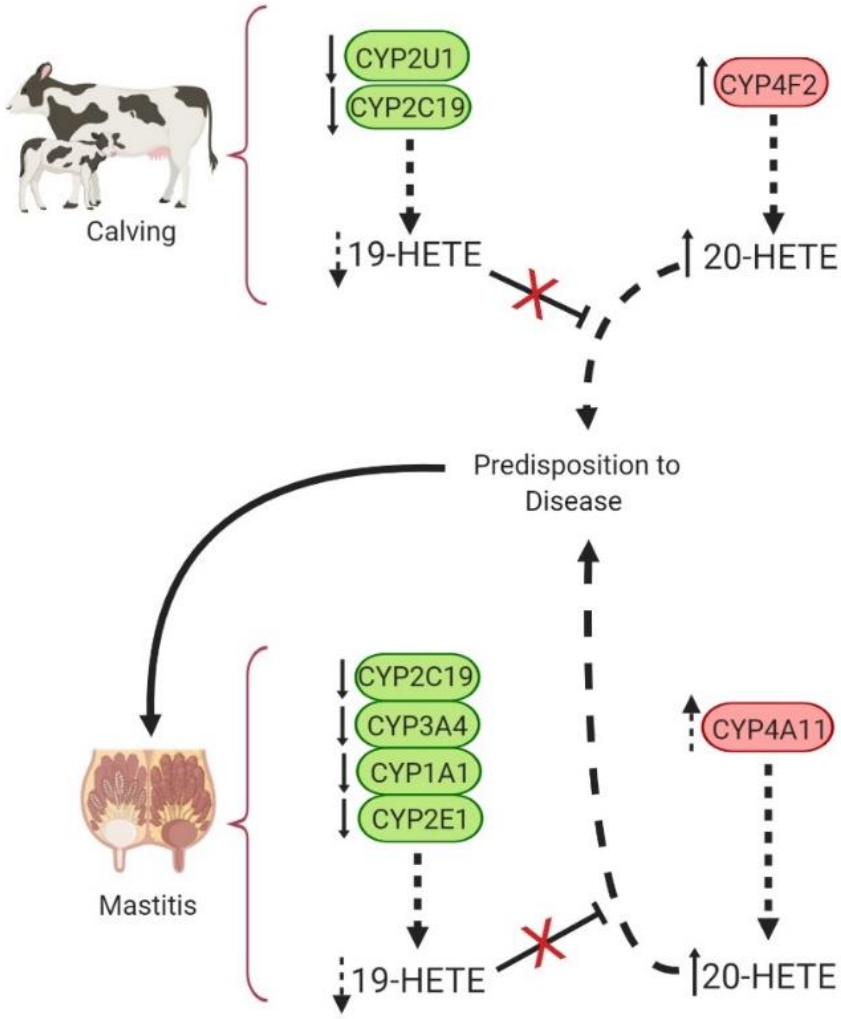

Figure 2.1. Around the time of calving, a down regulation of cytochrome P450 enzymes (CYP450) that produce 19-hydroxyeicosatetraenoic acid (HETE) may result in a reduction in its production. An increased expression of 20-HETE-producing CYP4F2 at this same time period may contribute to a state of dysfunctional inflammation during the transition period as 20-HETE has been shown to increase reactive oxygen species and breakdown endothelial barrier integrity. Dysfunctional inflammation predisposes animals to disease and results in an increased prevalence of mastitis during the transition period. Mastitis, in turn, further reduces mRNA expression of 19-HETE producing CYP450 and increases expression of CYP4A11 resulting in additional production of 20-HETE. This imbalance of plasma 19-HETE and 20-HETE, creates a negative feedback loop potentially furthering inflammation. Solid lines are known changes or associations in cattle whereas dashed lines are proposed activities or associations extrapolated from other mammalian species or in vitro data. Figure created at BioRender.com.

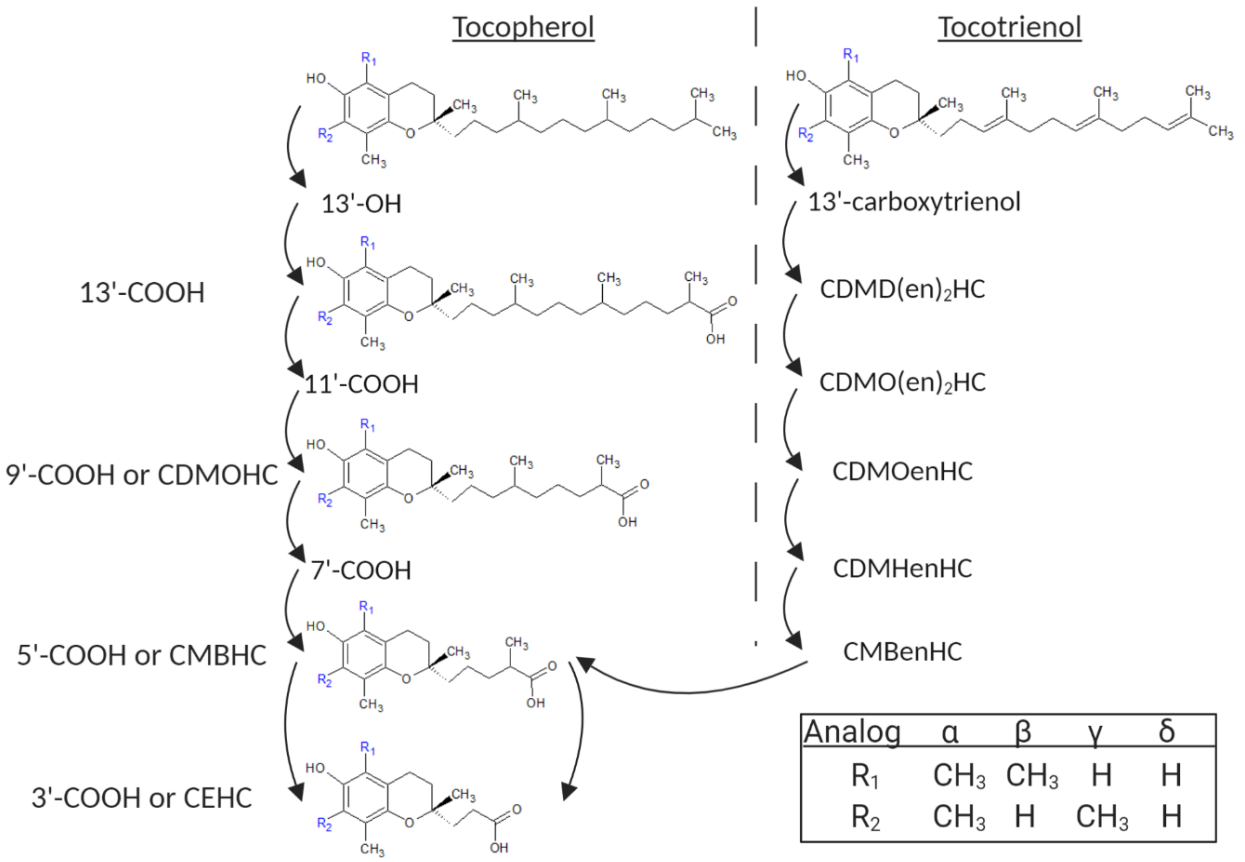

Figure 1.1. Metabolic cascade of vitamin E tocopherol and tocotrienol compounds. Metabolites with significant bioactivity and referenced within the body of this review are represented structurally whereas those not referenced are listed stepwise as their generally accepted abbreviation. Carboxychromanol (COOH) Hydroxychromanol (OH); Carboxymethyloctylhydroxychromanol (CDMOHC); Carboxymethylbutylhydroxychromanol (CMBHC); Carboxyethylhydroxychromanol (CEHC); Carboxydimethyldecadienylhydroxychromanol (CDMD(en)$_2$HC); Carbodimethyloctdienylhydroxychromanol (CDMO(en)$_2$HC); Carbodimethyloctenylhydroxychromanol (CDMOenHC); Carboxymethylhexenylhydroxychromanol (CDMHenHC); Carboxymethylbutadienylhydroxychromanol (CMBenHC). Created with Biorender.com and ChemSketch from ACD Labs.

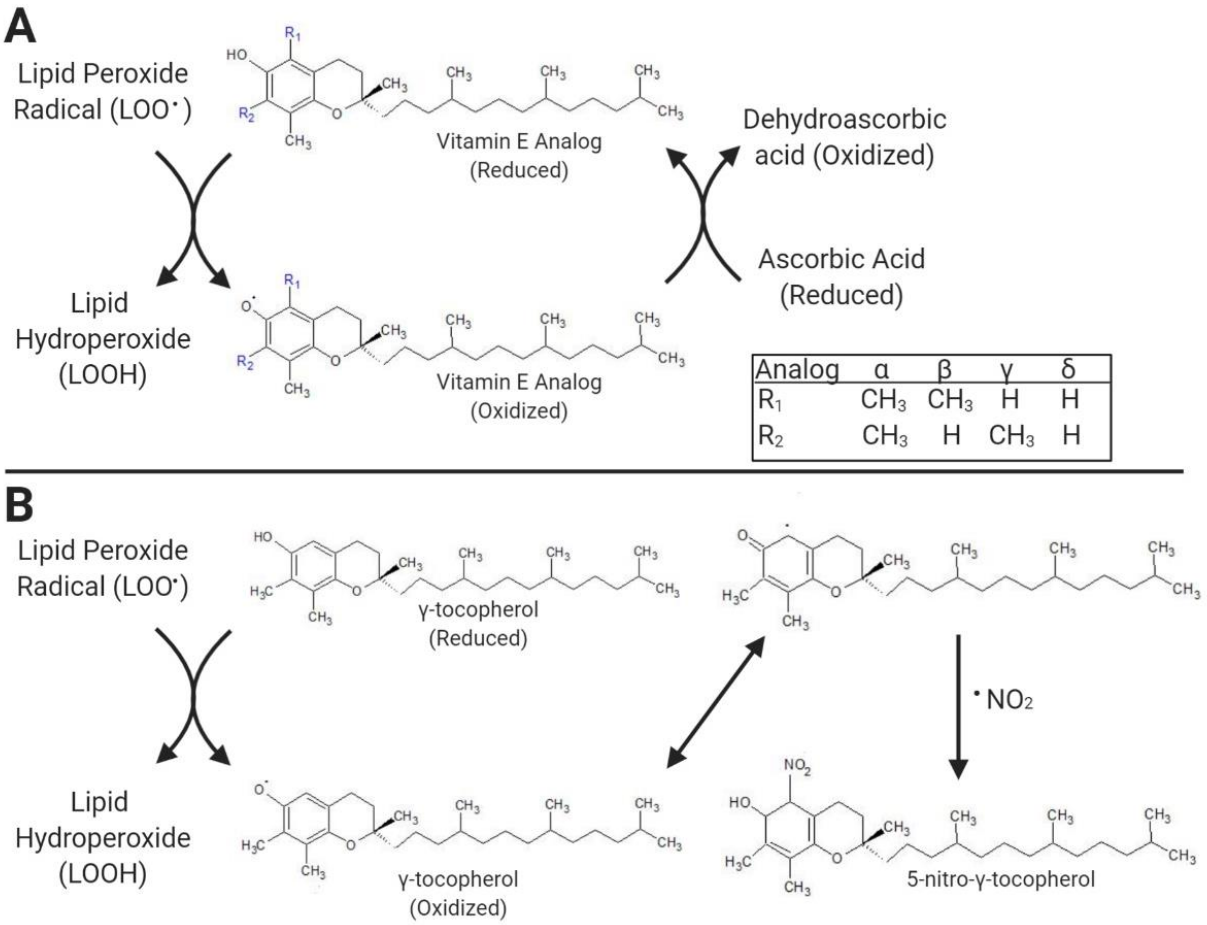

Figure 2.2. As antioxidants, the primary known function of vitamin E analogs (represented by a tocopherol structure in [A]) is to break lipid peroxidation chain reactions by reducing lipid peroxides to lipid hydroperoxides (A) by forming an analog radical. Vitamin E analog radicals are then reduced by ascorbic acid to dehydroascorbic acid regenerating reduced vitamin E analogs. Dehydroascorbic acid is subsequently is reduced to ascorbic acid by reduced glutathione. Vitamin E analogs with an unsubstituted 5' position of the chromanol ring, as designated by R1 in (A) and represented by γ-tocopherol in (B), can further reduce reactive nitrogen species, for example nitrogen dioxide ($\bullet NO_2$), to form 5-nitro-γ-tocophoerol, a marker of redox balance.

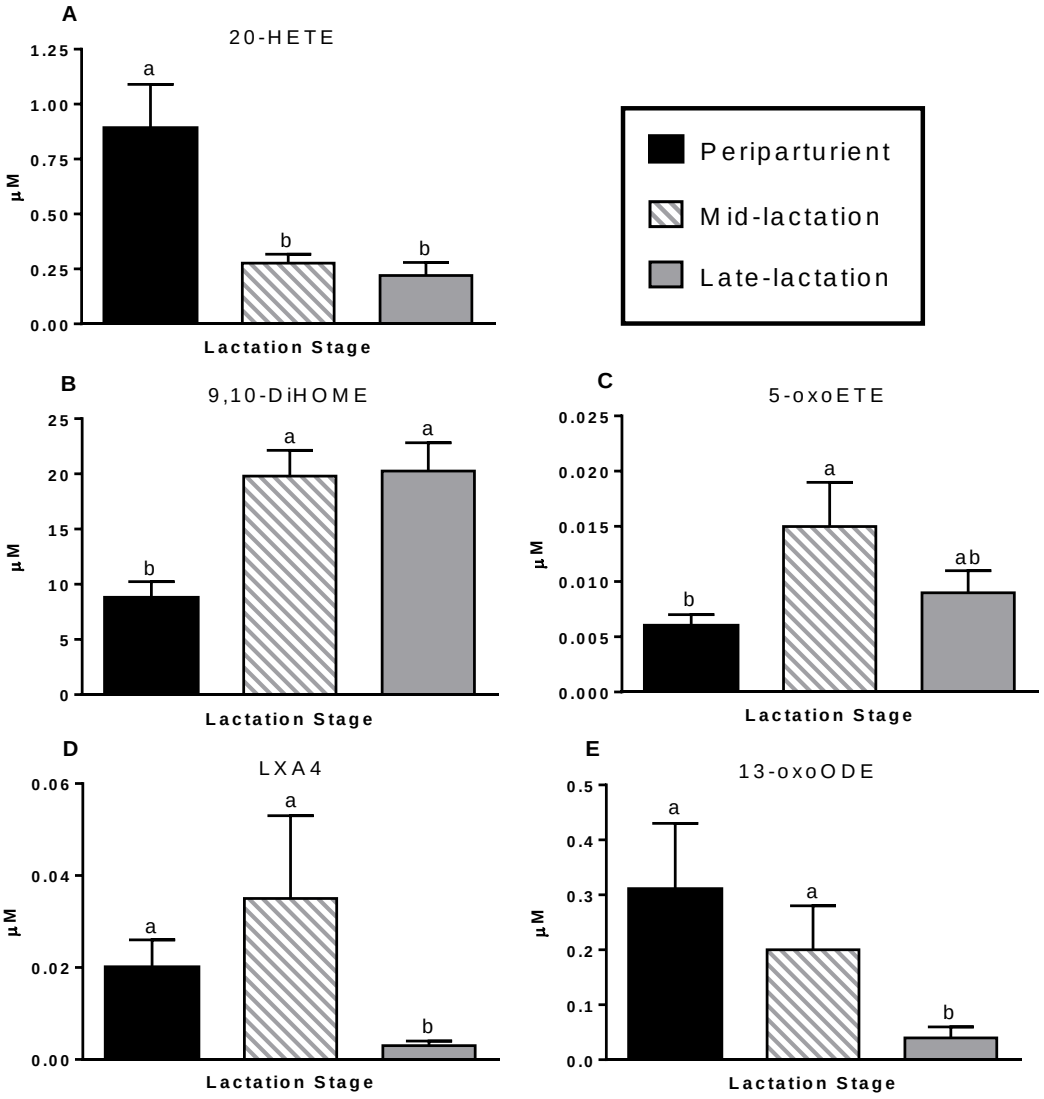

Figure 3.1: Plasma concentrations of oxylipids 20-hydroxyeicosatetraenoic acid (HETE) (A), 9,10-dihydroxyoctadecenoic acid (DiHOME) (B), 5-oxoeicosatetraenoic acid (oxoETE) (C), Lipoxin-A4 (LXA4) (D), and 13-oxooctadecadienoic acid (oxoODE) (E) in µM as geometric mean ± SEM. Periparturient n = 13, Mid-lactation n = 13, Late-lactation n = 10. [ab] Values with different letters differ ($P < 0.05$).

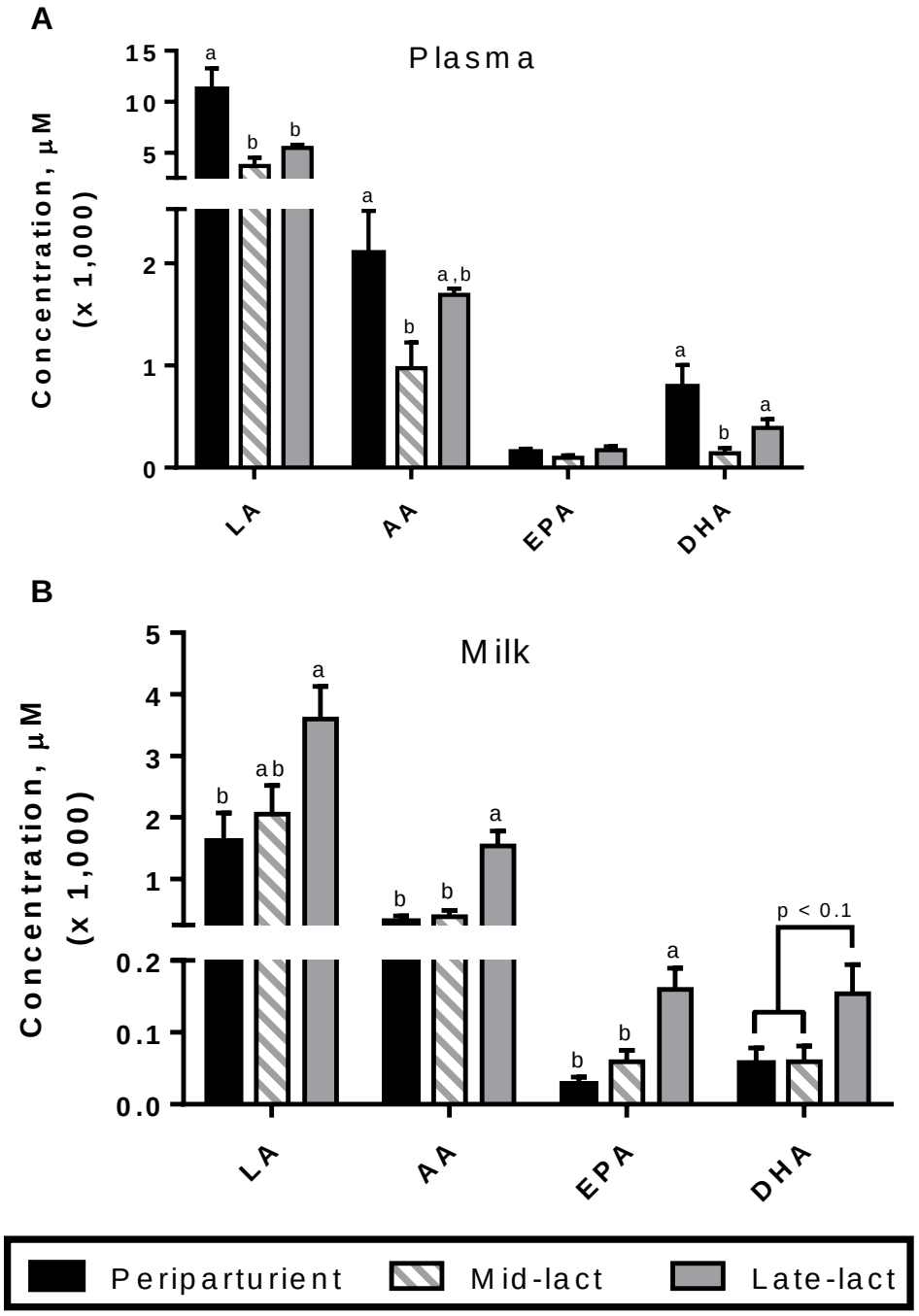

Figure 3.2: Non-esterified polyunsaturated fatty acid concentrations (x1000 µM) in plasma (A) and milk (B) samples as geometric mean ± SEM. Periparturient n = 13, Mid-lactation n = 13, Late-lactation n = 10. Abbreviations: Linoleic acid, LA; Arachidonic acid, AA; Eicosapentaenoic acid, EPA; Docosahexaenoic acid, DHA [ab] Values with different letters differ ($P < 0.05$).

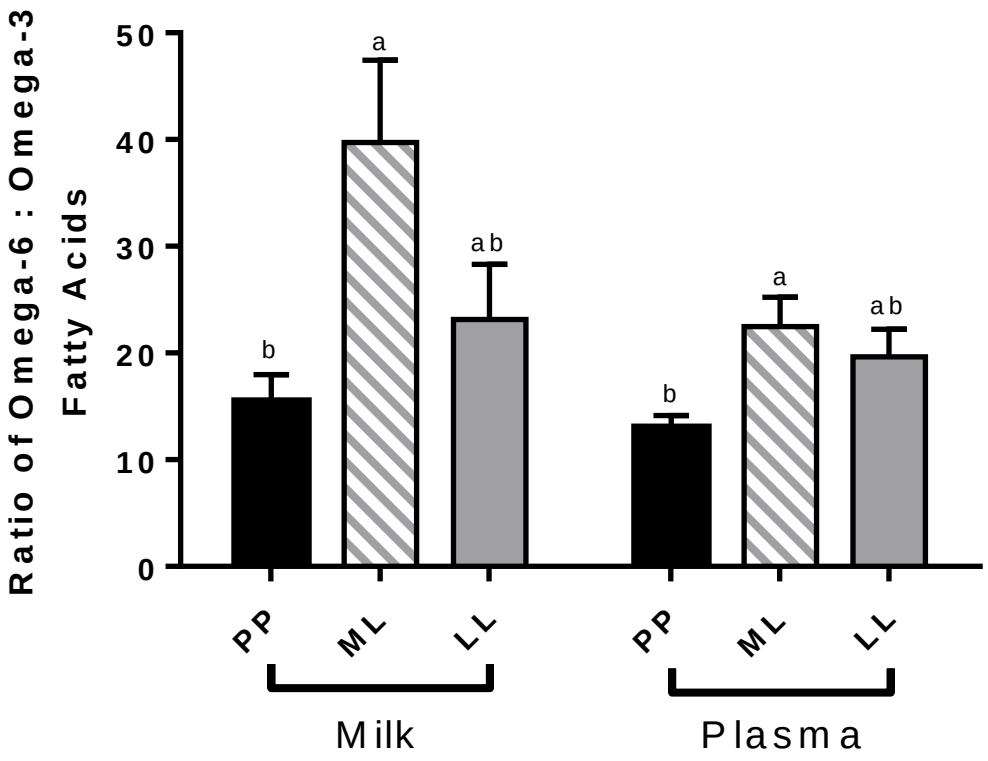

Figure 3.3: Ratio of non-esterified ω-6 to ω-3 fatty acids in milk and plasma as geometric mean ± SEM. Periparturient (PP) n = 13, Mid-lactation (ML) n = 13, Late-lactation (LL) n = 10.

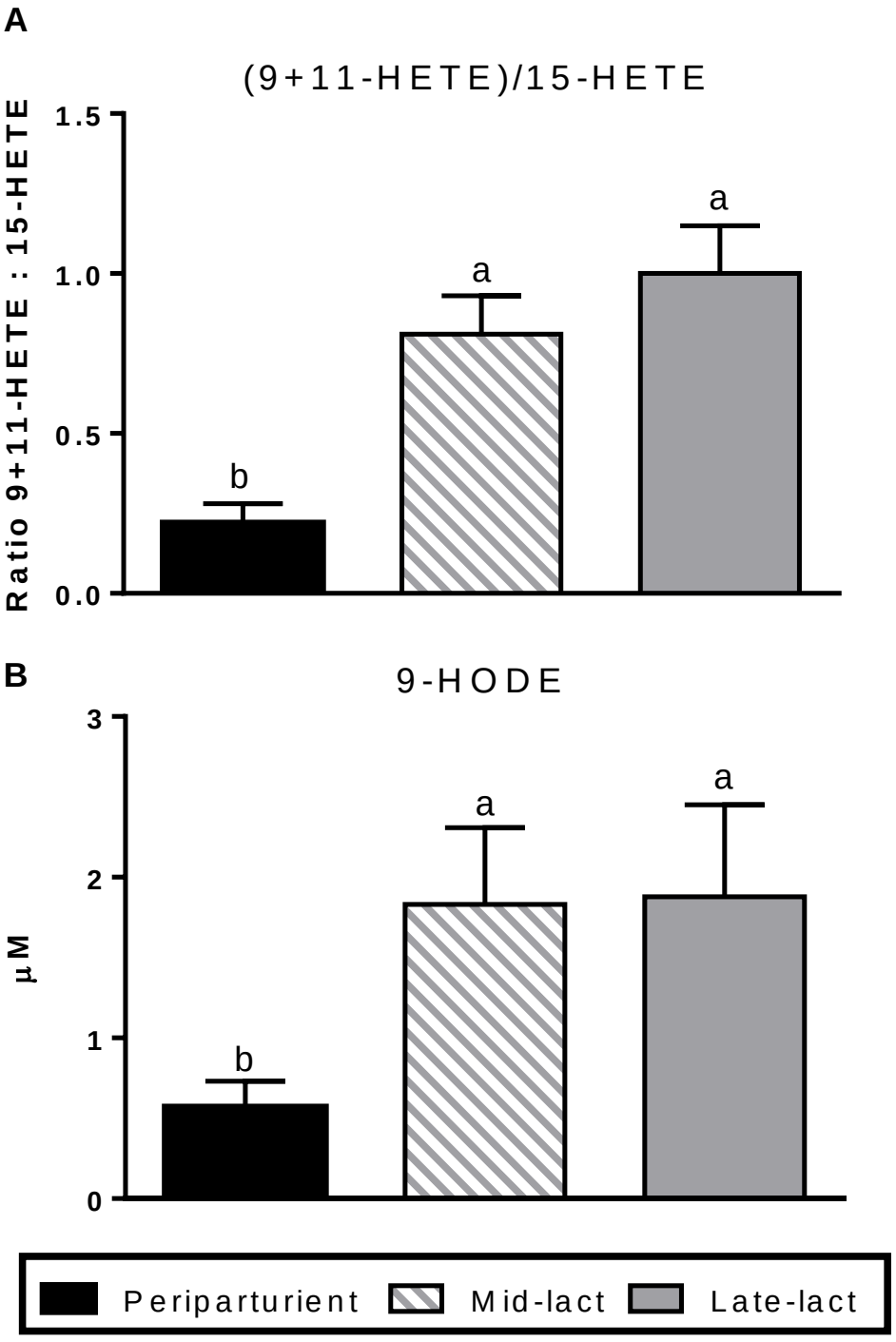

Figure 3.4: Ratio of non-enzymatically produced 9-hydroxyeicosatetraenoic acid (HETE) and 11-HETE to enzymatically derived 15-HETE (A) and concentration of non-enzymatically produced 9-hydroxyoctadecadienoic acid (HODE) in µM in milk and plasma as geometric mean ± SEM. Periparturient n = 13, Mid-lactation n = 13, Late-lactation n = 10. [ab] Values with different letters differ ($P < 0.05$).

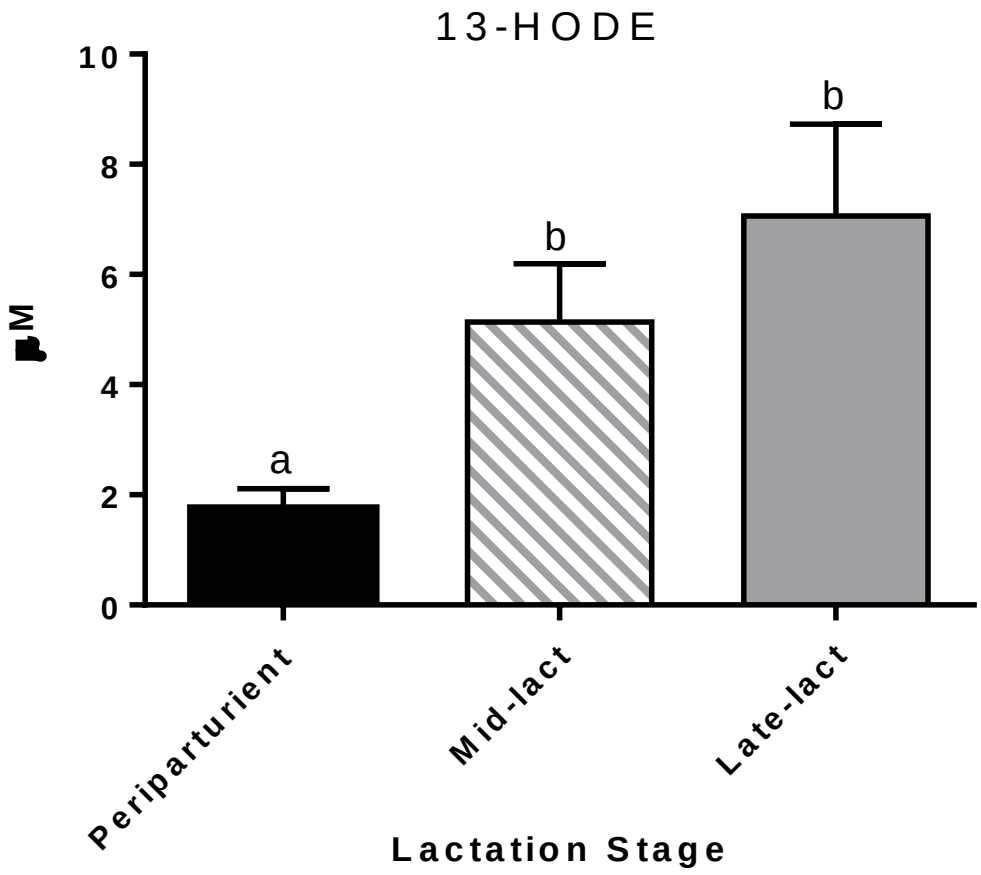

Figure 3.5: Concentration of milk oxylipid 13-hydroxyoctadecadienoic acid (HODE) in μM as geometric mean ± SEM. Periparturient n = 13, Mid-lactation n = 13, Late-lactation n = 10. [ab] Values with different letters differ ($P < 0.05$).

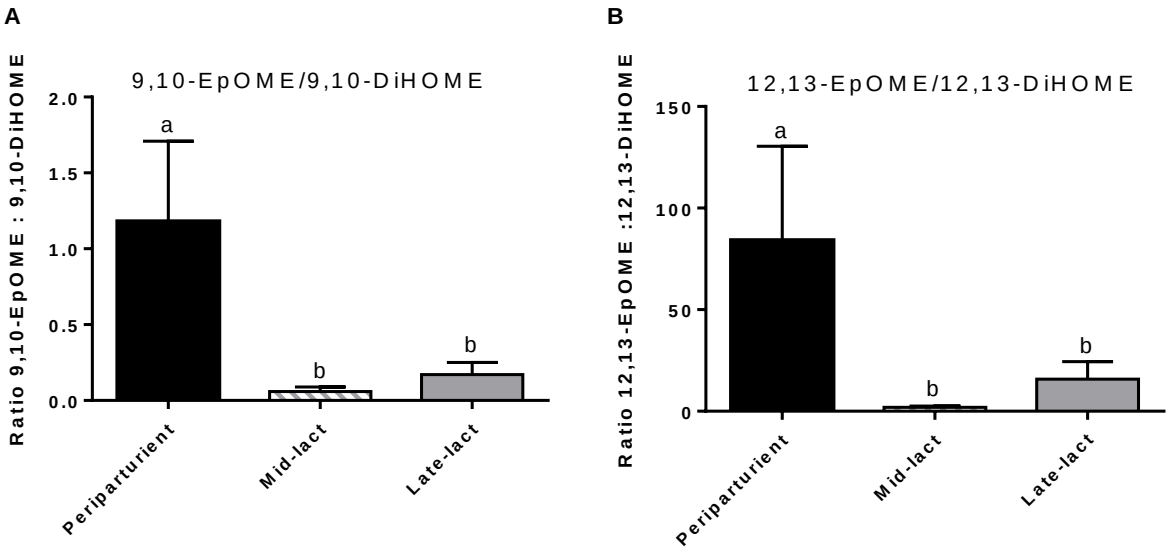

Figure 3.6: Ratio of 9,10-epoxyoctadecenoic acid (EpOME) to 9,10-dihydroxyoctadecenoic acid (DiHOME) (A) and 12,13-EpOME to 12,13-DiHOME (B) as geometric mean ± SEM. Periparturient n = 13, Mid-lactation n = 13, Late-lactation n = 10. [ab] Values with different letters differ ($P < 0.05$).

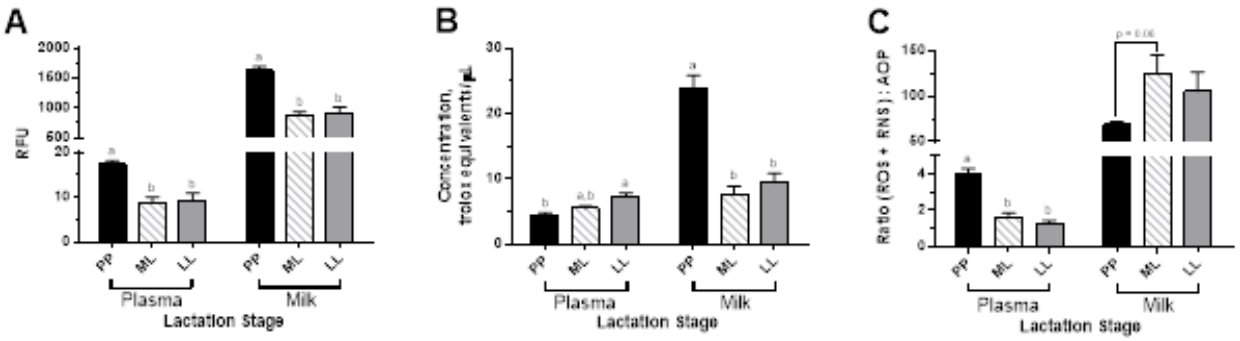

Figure 4.1. Plasma and milk concentrations (mean ± SEM) of reactive oxygen and reactive nitrogen species in fluorescence units (RFU) / μL (A), antioxidant potential, in Trolox equivalents / μL (B), and oxidant status index (OSi), ratio of reactive oxygen species (ROS) and reactive nitrogen species (RNS) to antioxidant potential (AOP) (C) were inconsistently higher or lower between the 2 fluids in cows from the periparturient (PP), mid-lactation (ML), and late lactation (LL) stages ultimately revealing opposite trends in the OSi. Bars with different letters (a or b) differ ($P < 0.05$) within a single graph unless otherwise noted by a specific P-value pertaining to what the specific bars its bracket signifies.

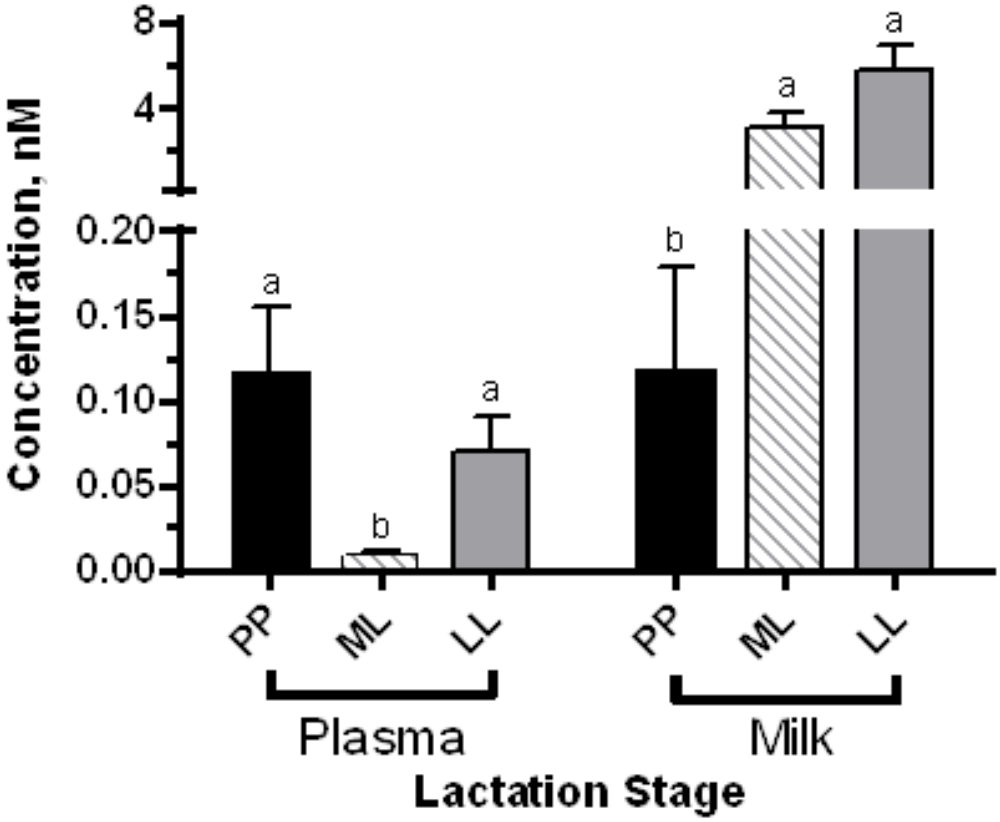

Figure 4.2. Plasma and milk concentrations (mean ± SEM) of 15-F2t-isoprostanes were elevated in periparturient (PP) cows in plasma while milk was elevated in mid-lactation (ML) and late lactation (LL) cows. Bars with different letters (a or b) differ ($P < 0.05$) within a single graph.

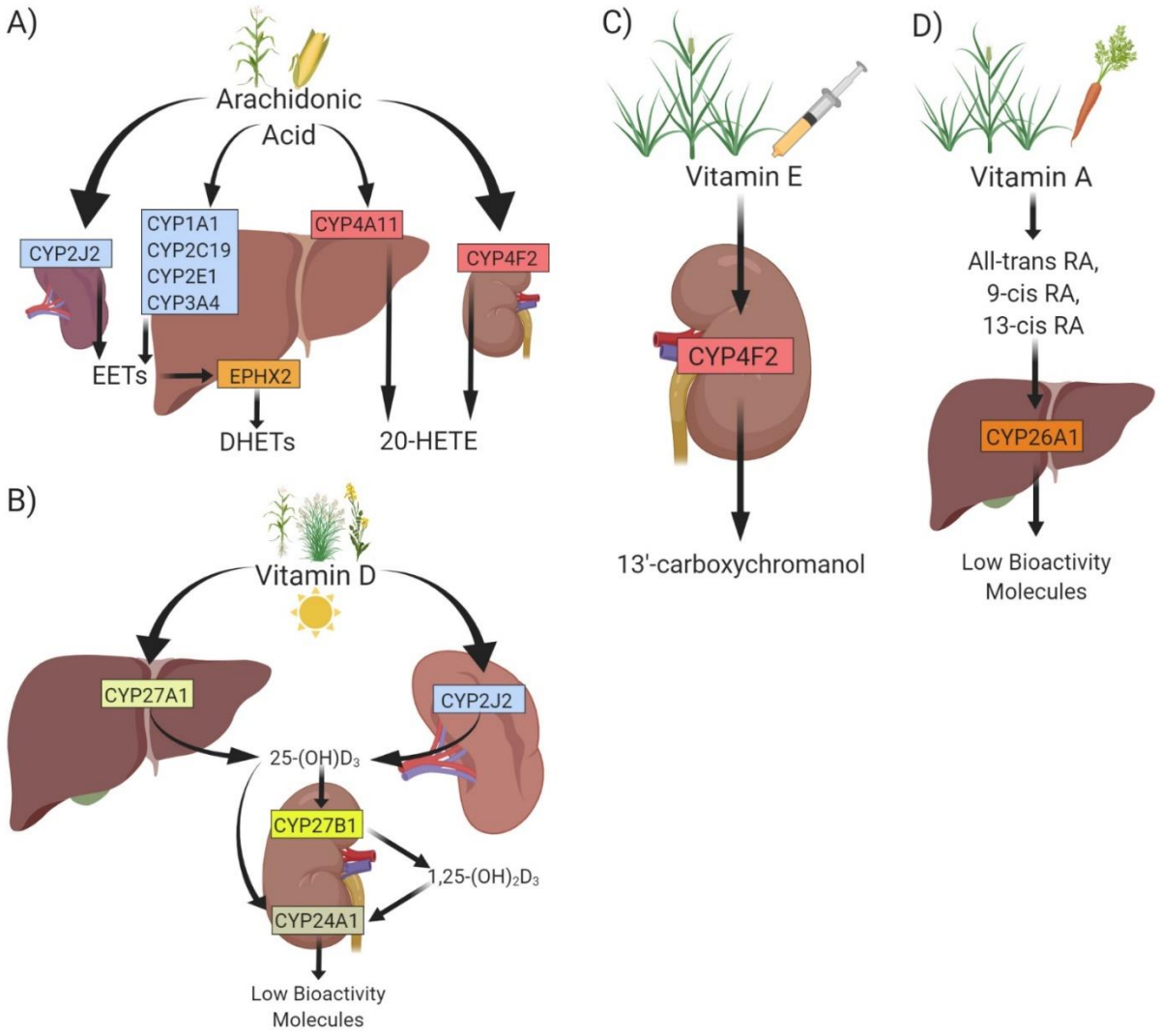

Figure 5.1: Cytochrome P450 enzymes are placed upon their organ of highest mRNA expression as follows: A) from left to right, spleen, liver, kidney; B) clockwise from upper left, liver, spleen, kidney; C) kidney; D) liver. A) Several cytochrome P450 enzymes are capable of metabolizing poly-unsaturated fatty acids; shown here are pathways of arachidonic acid metabolism. Cytochrome P450 enzymes backed by blue and on the left act as epoxygenases producing epoxyeicosatrienoic acids (EET) that can be further metabolized by the enzyme *EPHX2* to dihydroxyeicosatrienoic acids (DHET). Cytochrome P450 enzymes backed in red, on the right, have predominantly terminal carbon ω-hydroxylase activity producing 20-hydroxyeicosatetraenoic acid (20-HETE). B) Cytochrome P450 enzymes involved in the activation and inactivation of vitamin D. Vitamin D is obtained from either exposure of the skin to ultraviolet light or directly from diet as either vitamin D_2 or vitamin D_3. Shown is the cascade for vitamin D_3 as the initial compound is metabolized to 25-hydroxyvitamin D_3 [25-(OH)D_3] by at least 2 cytochrome p450 enzymes in cattle, *CYP27A1* and *CYP2J2*. 25-hydroxyvitamin D_3 is then metabolized by the 1α-hydroxylase *CYP27B1* to produce active vitamin D, 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]. Ultimately, both 25-(OH)D_3 and 1,25-$(OH)_2D_3$ are inactivated by *CYP24A1*

to molecules of low bioactivity such as 24,25-dihydroxycholecalciferol or calcitroic acid. C) All 8 analogs of vitamin E are hydroxylated by *CYP4F2* to form analog specific forms of 13'-carboxychromanol. 13'-carboxychromanol is readily metabolized by other means to water-soluble metabolites, some of which have significant bioactivity. D) Vitamin A is a group of compounds including carotenoids and retinyl esters ultimately metabolized to forms of retinoic acids, including all-trans retinoic acid (RA), 9-cis RA, and 13-cis RA. Such retinoic acids are metabolized by cytochrome P450 family 26 enzymes, specifically *CYP26A1* evaluated in this study, to metabolites with little bioactivity. Created with BioRender.com.

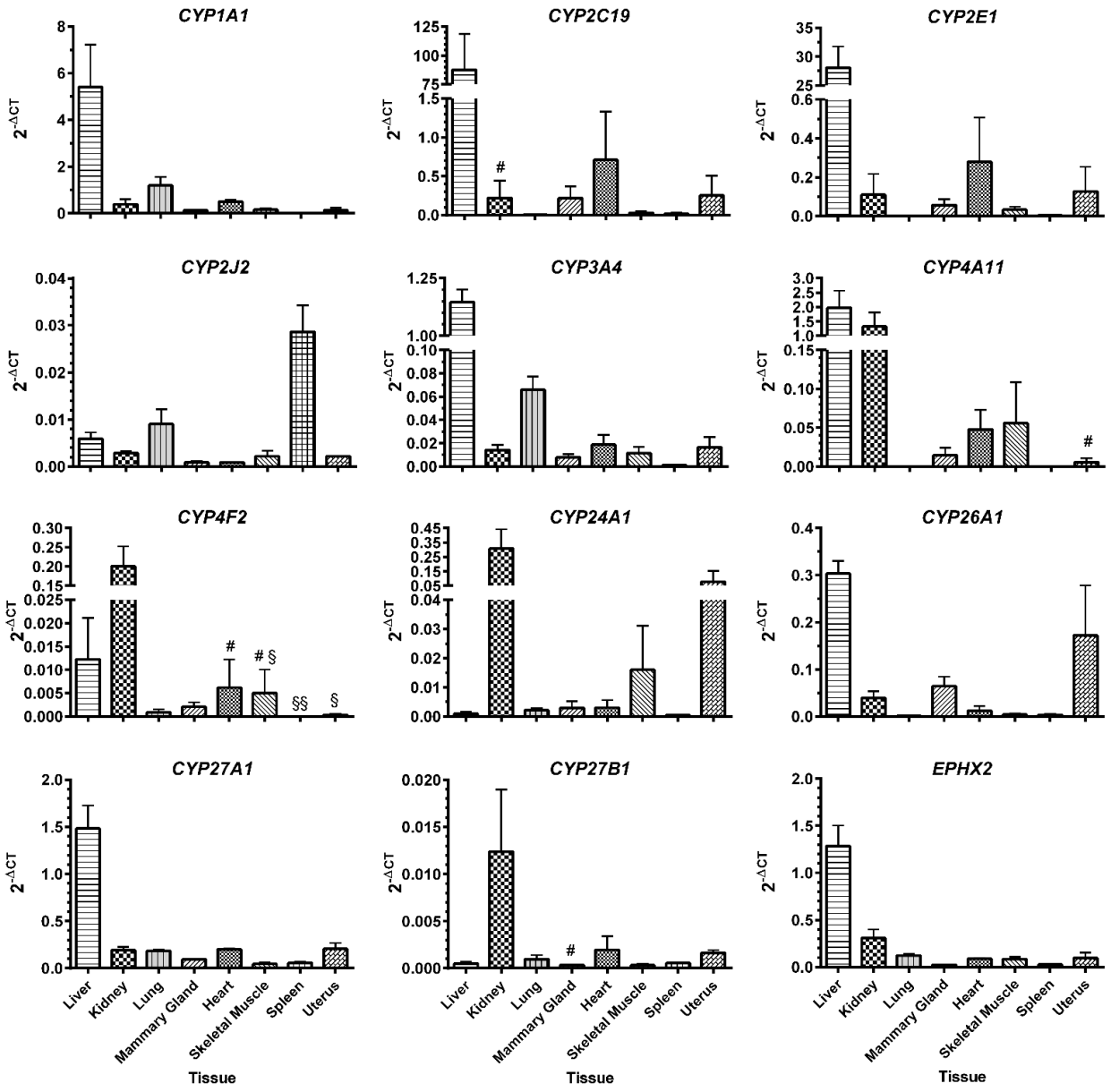

Figure 5.2: Relative abundance (mean ± SEM) of gene transcripts for *CYP1A1*, *CYP2C19*, *CYP2E1*, *CYP2J2*, *CYP3A4*, *CYP4A11*, *CYP4F2*, *CYP24A1*, *CYP26A1*, *CYP27A1*, *CYP27B1*, and *EPHX2* found in liver, kidney, lung, mammary gland, heart, skeletal muscle, spleen, and uterine tissues from healthy, lactating dairy cows (n = 3). § Presented value excludes sample not found during PCR cycling. # Presented value contains sample(s) with cycle to threshold value > 35 cycles. Values are presented as transcript abundance ($2^{-\Delta CT}$) relative to endogenous control gene *RPS9.*

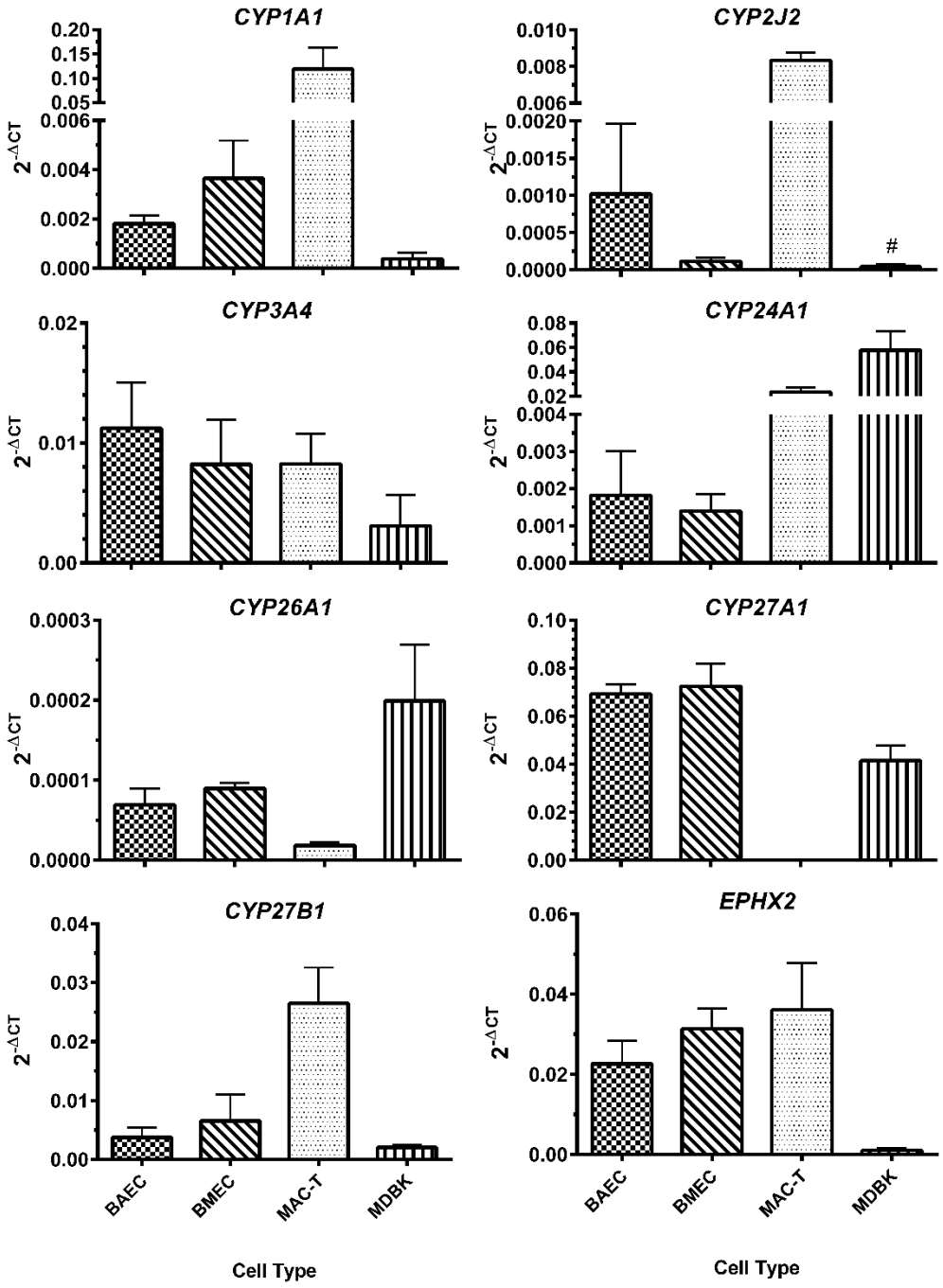

Figure 5.3: Relative abundance (mean ± SEM) of gene transcripts for *CYP1A1*, *CYP2J2*, *CYP3A4*, *CYP24A1*, *CYP26A1*, *CYP27A1*, *CYP27B1*, and *EPHX2* in bovine primary aortic endothelial cells (**BAEC**), bovine primary mammary endothelial cells (BMEC), bovine immortalized mammary epithelial cells (MAC-T), and Madin-Darby bovine kidney cells (MDBK) (n = 3). # Presented value contains sample with cycle to threshold value > 35 cycles. Values are presented as transcript abundance ($2^{-\Delta CT}$) relative to endogenous control gene *RPS9.*

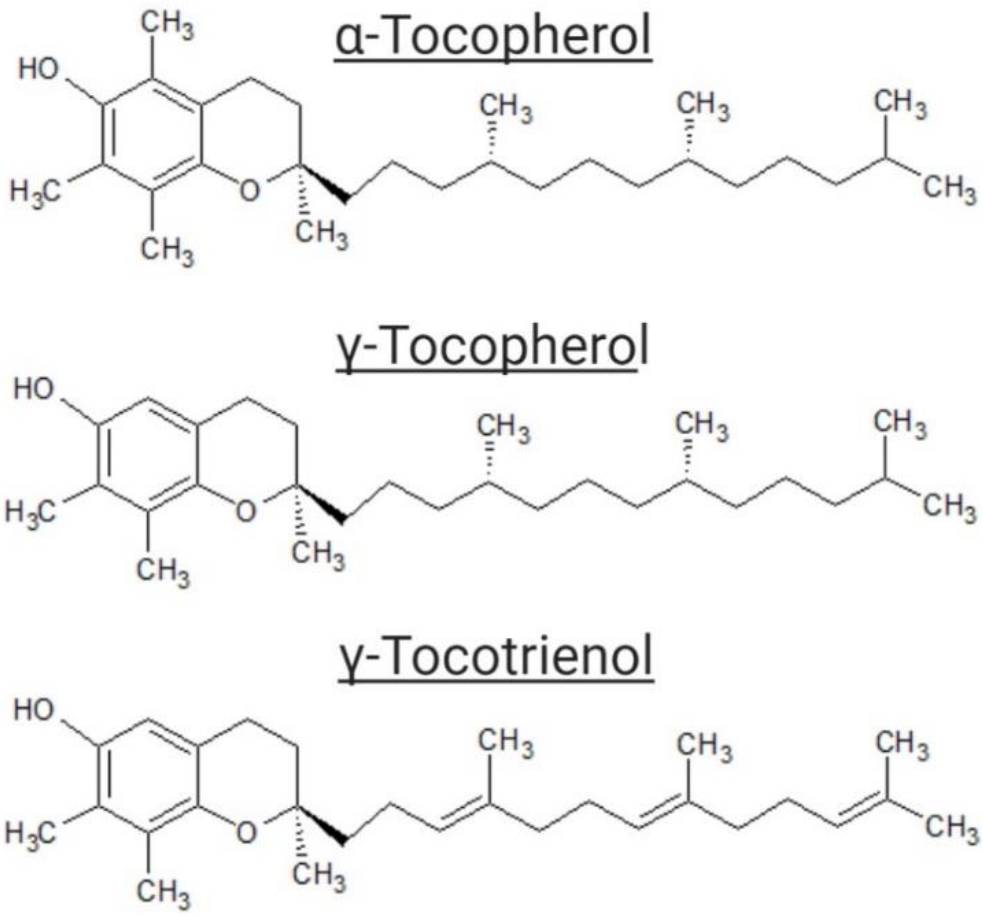

Figure 6.1. Chemical structures of the 3 analogs used for the current study, namely α-tocopherol, γ-tocopherol, and γ-tocotrienol. Figure created with ChemSketch and BioRender.com.

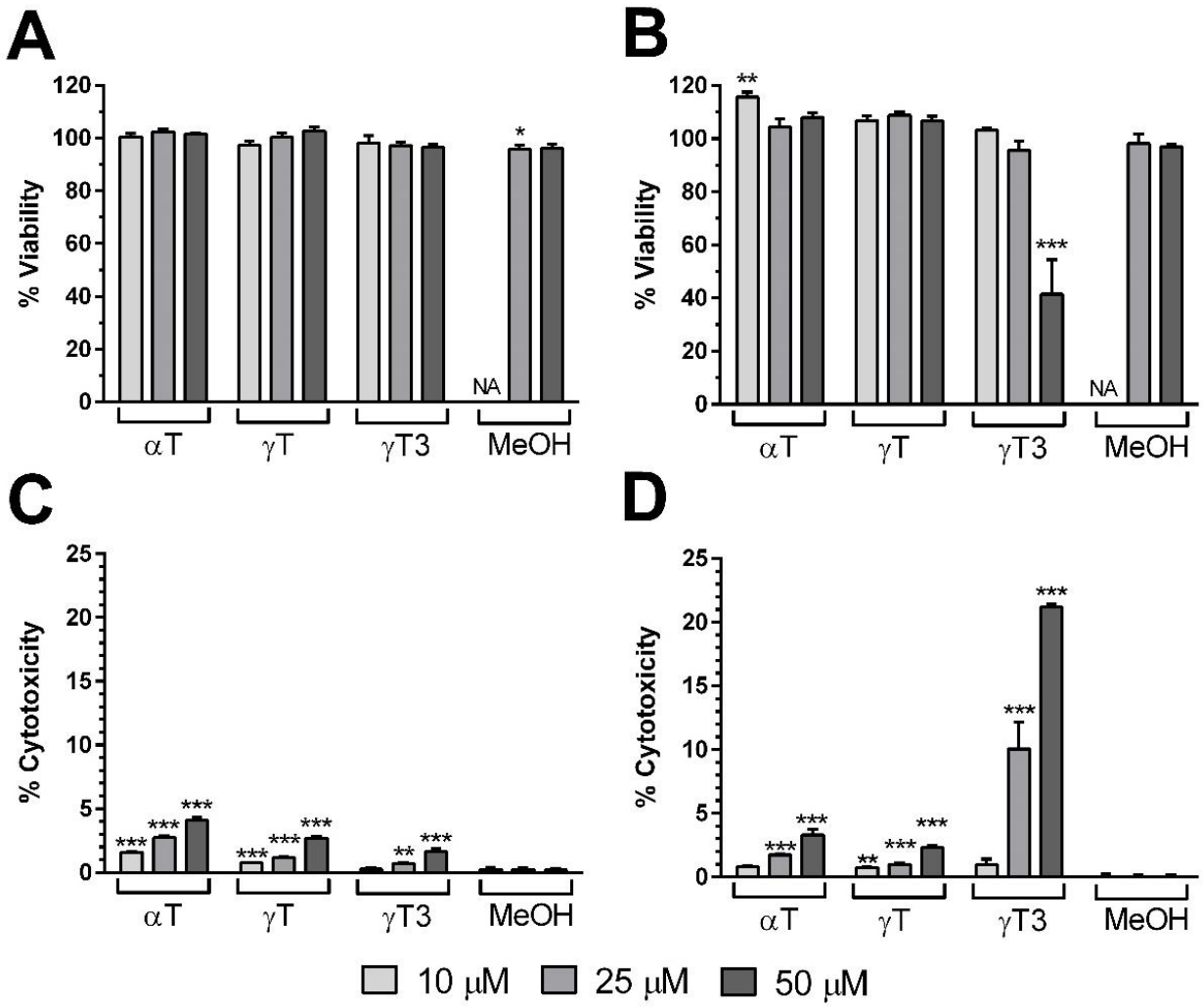

Figure 6.2: Percentage of viable cells (mean ± SEM) by ATP abundance in bovine mammary endothelial cells was not impacted after 6 h (A) or 24 h (B) treatment with vitamin E analogs α-tocopherol (αT), γ-tocopherol (γT), or γ-tocotrienol (γT3) at differing concentrations or methanol (MeOH) vehicle control aside from a 50 μM treatment of γT3 after 24 h incubation ($P < 0.05$). Methanol vehicle was not assessed for 10 μM treatments (NA). Similarly, cytotoxicity of bovine mammary endothelial cells increased slightly with increasing concentrations of vitamin E analogs after 6 h (C) or 24 h (D) of treatment although not exceeding 5% cytotoxicity aside from 25 μM and 50 μM treatments of γT3 after 24 h. Treatment concentrations: 10 μM (horizontal-left bars), 25 μM (diagonal-center bars), 50 μM (vertical-right bars) (n = 4). * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

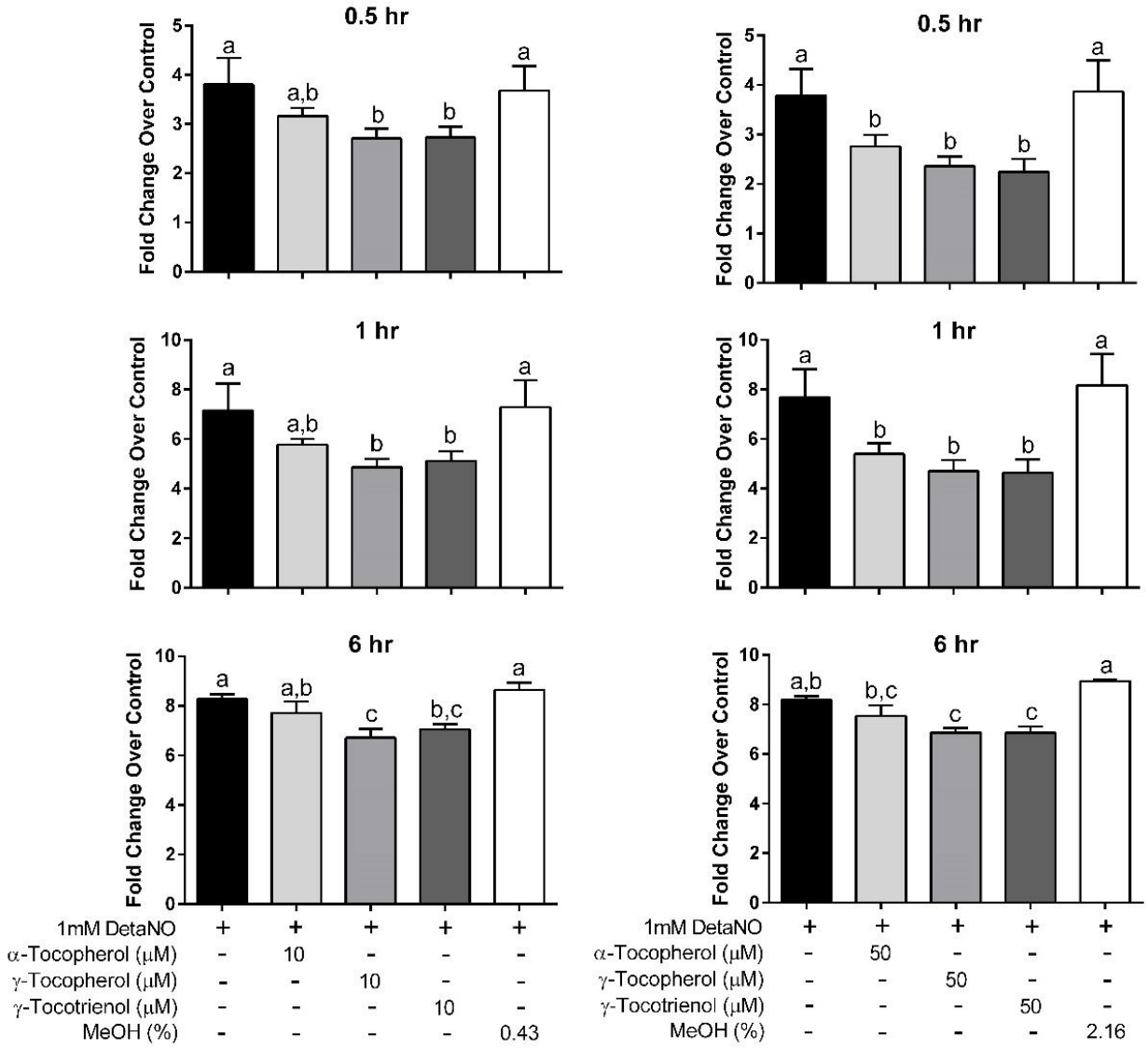

Figure 6.3: Reactive oxygen species accumulation (mean ± SEM) in bovine mammary endothelial cells was reduced by analogs γ-tocopherol and γ-tocotrienol 10 μM treatments (n = 6) after 0.5, 1, or 6 h incubations with 1 mM DetaNO as all 3 analogs reduced reactive oxygen species accumulation when supplemented at 50 μM (n = 5) ($P < 0.05$). Cells receiving vitamin E were preincubated with appropriate analog or methanol (MeOH) vehicle for 6 h and treated again with addition of DetaNO. Differing letters represent significant difference between treatments ($P < 0.05$).

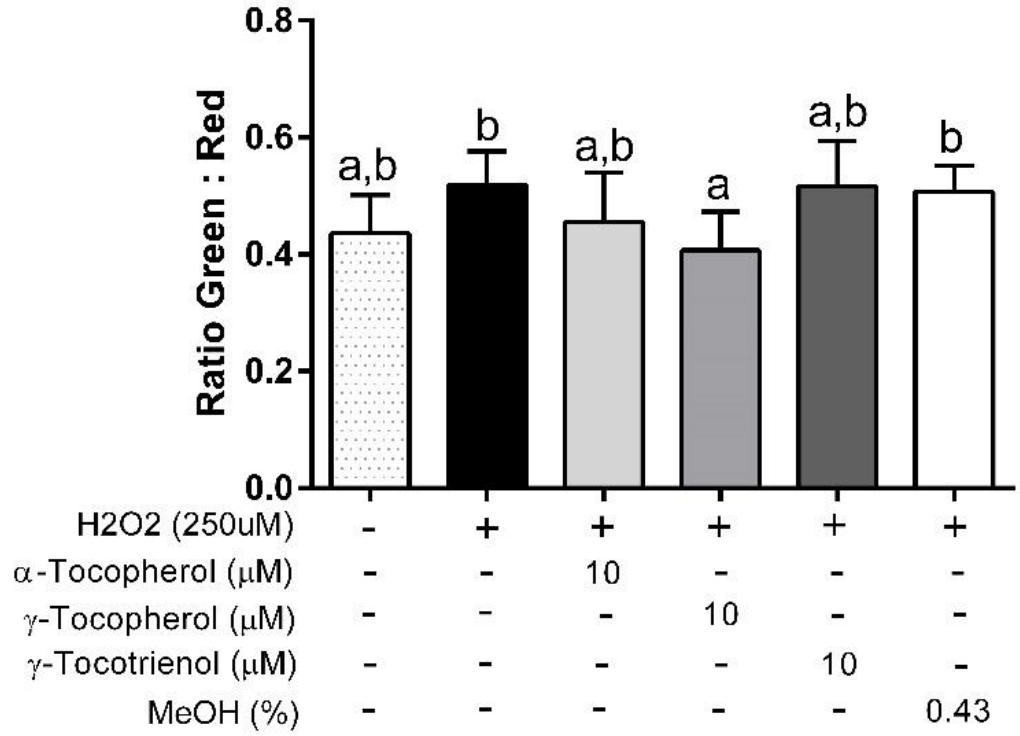

Figure 6.4: Lipid peroxidation was determined by the shift in mean cellular fluorescence from green to red by flow cytometry represented by the ratio of green : red fluorescence (mean ± SEM) where a larger ratio indicates greater lipid peroxidation. Bovine mammary endothelial cells were treated with 10 μM of appropriate analogs or methanol (MeOH) vehicle control for 6 h pre-treatment and again with 250 μM hydrogen peroxide (H_2O_2) for 30 min (n = 6). The γ-tocopherol analog was the only analog to reduce lipid peroxidation of cells ($P < 0.01$) although α-tocopherol trended towards reduction ($P = 0.06$). Differing letters represent significant difference between treatments ($P < 0.05$).

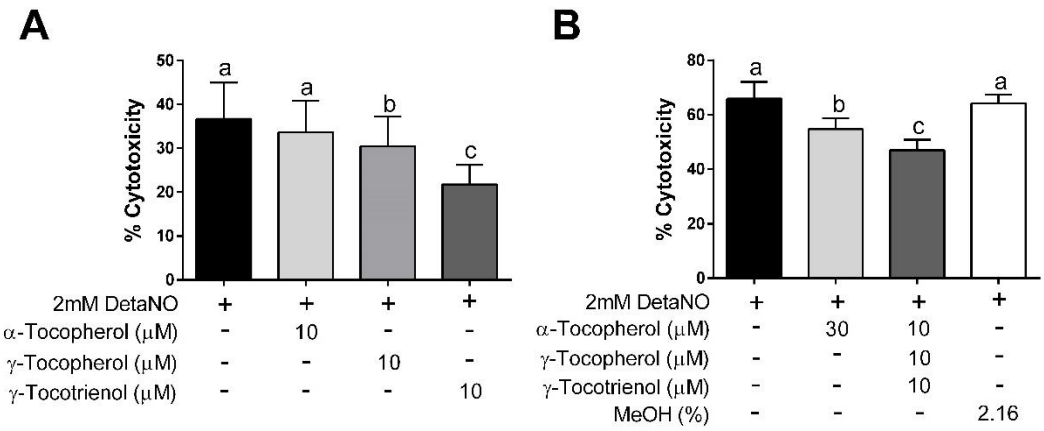

Figure 6.5: Percentage cytotoxicity (mean ± SEM) of bovine mammary endothelial cells as measured by lactate dehydrogenase release after treatment with 2mM DetaNO. Cells treated with vitamin E analogs were preincubated for 6 h with analogs prior to addition of DetaNO for an additional 24 h incubation. Analogs were either supplemented individually (A, n = 10) or as a mix (B, n = 4). Methanol (MeOH) vehicle was used in a subset of samples for panel A and was not statistically different from antagonist treatment alone in that subset. Differing letters represent significant difference between treatments ($P < 0.05$).

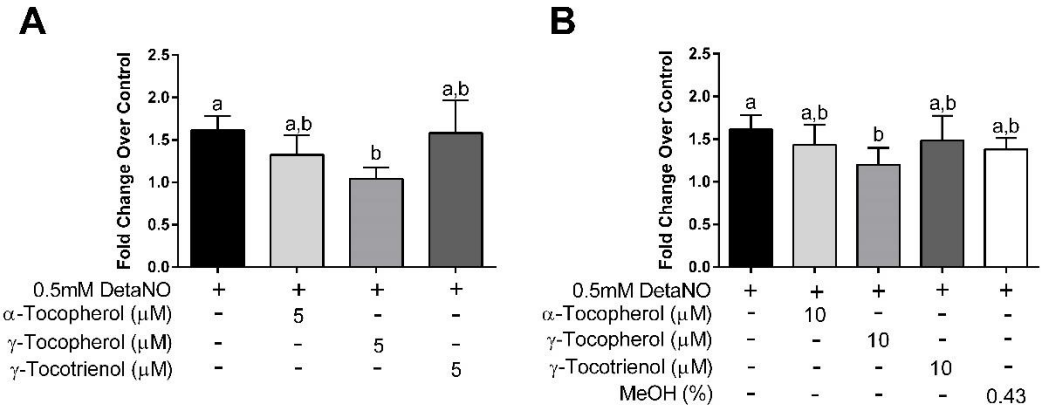

Figure 6.6: Apoptosis was quantified by flow cytometry using YoPro dye as a marker of apoptosis and exclusion of necrotic cells with propidium iodide dye. Cells treated with vitamin E analogs or methanol (MeOH) vehicle were preincubated for 6 h with analogs prior to addition of DetaNO for an additional 24 h incubation (n = 9). The γ-tocopherol treatment was the only analog to reduce apoptosis ($P < 0.05$). Differing letters represent significant difference between treatments ($P < 0.05$).

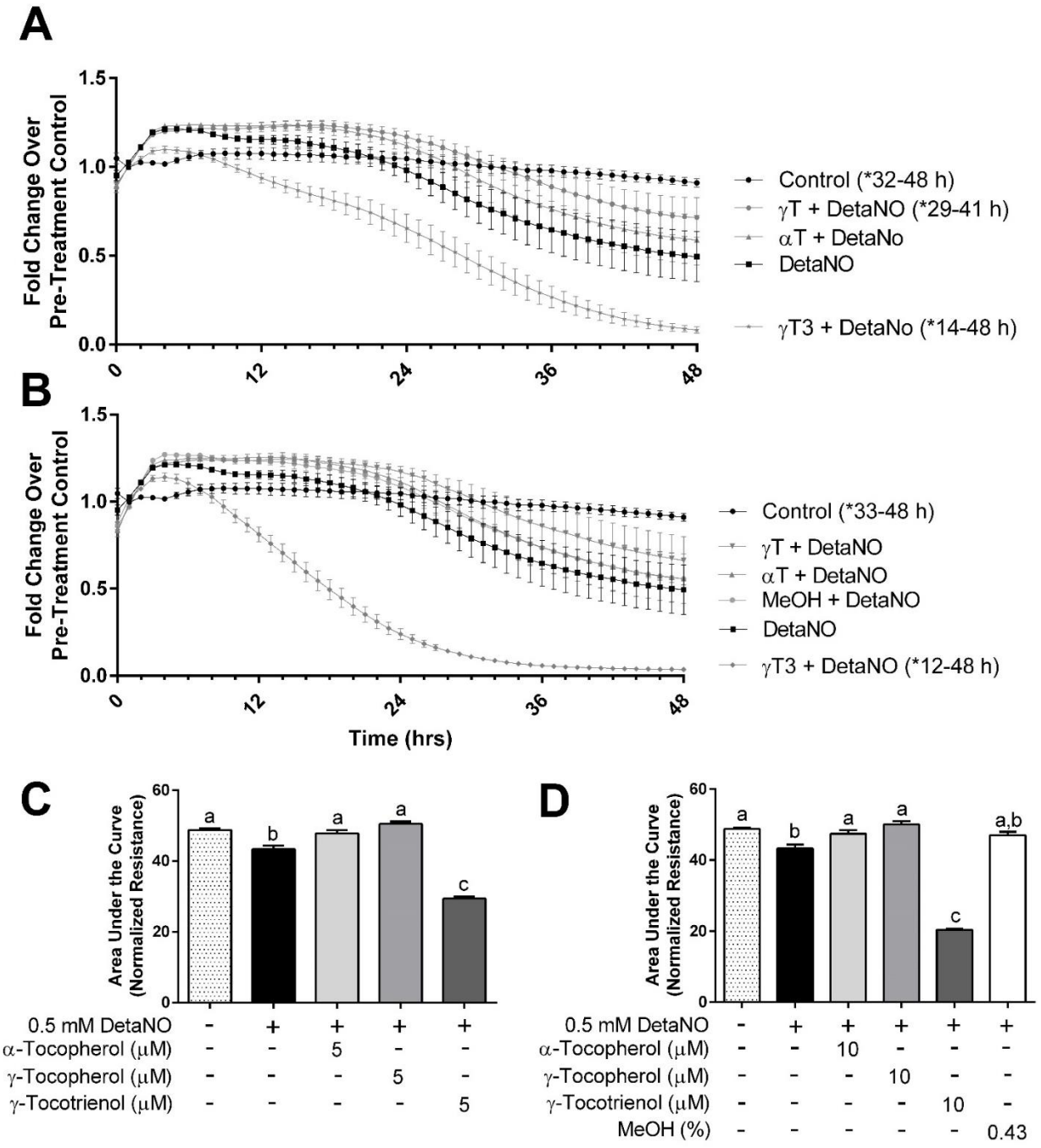

Figure 6.7: Bovine mammary endothelial cell barrier integrity was quantified by electric cell-substrate impedance sensing. Cells were grown to confluency, pretreated for 6 h with vitamin E analogs α-tocopherol (αT), γ-tocopherol (γT), or γ-tocotrienol (γT3) or methanol (MeOH) vehicle and ultimately challenged with 0.5 mM DetaNO (n = 6). Resistance was measured for analog treatments of 5 μM (A) and 10 μM (B) for 48 h (A/B). * in key followed by h represents significant differences between labeled treatment and DetaNO treatment for h listed by 2-way ANOVA ($P < 0.05$). Area under the curve quantification was derived for all treatments as measured for 48 h for analog concentrations 5 μM (C) and 10 μM (D). Differing letters represent significant difference between treatments ($P < 0.05$).

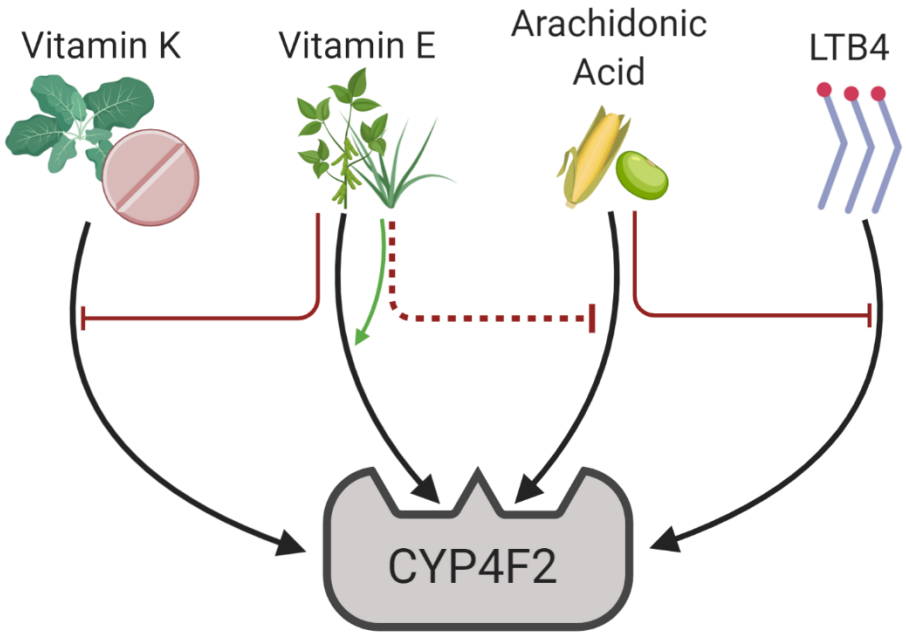

Figure 7.1: Several compounds are substrates for cytochrome P450 family 4 sub-family F member 2 (CYP4F2) listed with their common sources: vitamin K (cruciferous plants and as treatment for anti-coagulant poisoning), vitamin E (grasses and plant oils), arachidonic acid (corn, soybeans, and other plants high in linoleic acid), leukotriene B4 (LTB4) (product of arachidonic acid oxidation by lipoxygenase enzymes). Solid red lines represent known inhibitory activities of one substrate upon another. Green arrow represents heterotrophic cooperativity of α-tocopherol upon other vitamin E analogs increasing the rate of their metabolism. Dashed red line with bar represents the inhibitory activity of vitamin E analogs on arachidonic acid shown in this study.

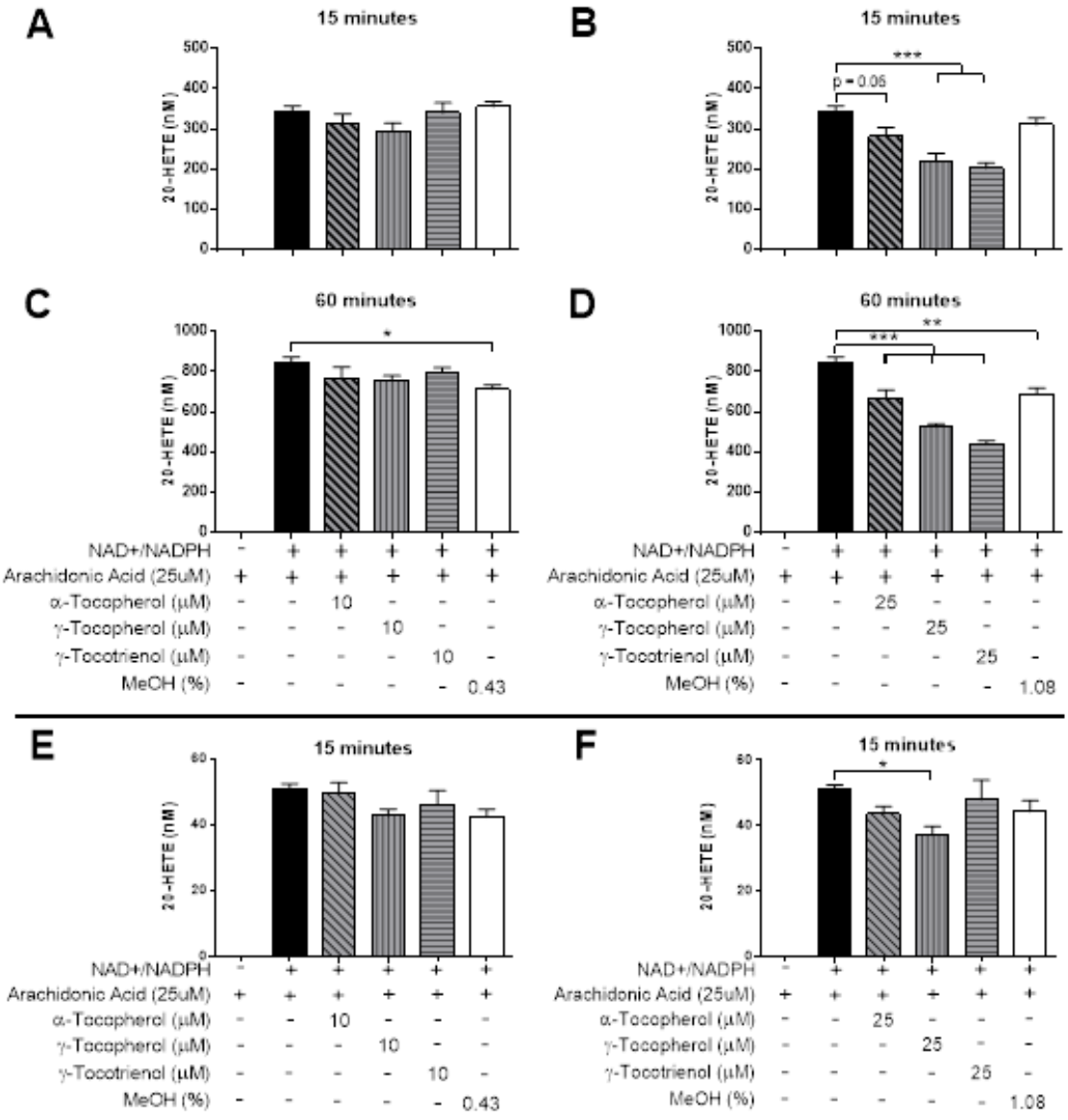

Figure 7.2: Production of 20-hydroxyeicosatetraenoic acid from human cytochrome P450 family 4 sub-family F member 2 microsomes after 15 min (A/B) or 60 min (C/D) of reaction time and bovine-kidney microsomes (E/F) after 15 min of reaction time, initiated with nicotinamide adenine dinucleotide phosphate and nicotinamide adenine dinucleotide. Vitamin E analogs were used at either 10 μM (A/C/E) or 25 μM (B/D/F) concentrations with 25 μM of arachidonic acid in each treatment (n = 5). Methanol (MeOH) used as vehicle control and represented by percentage of total reaction volume. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

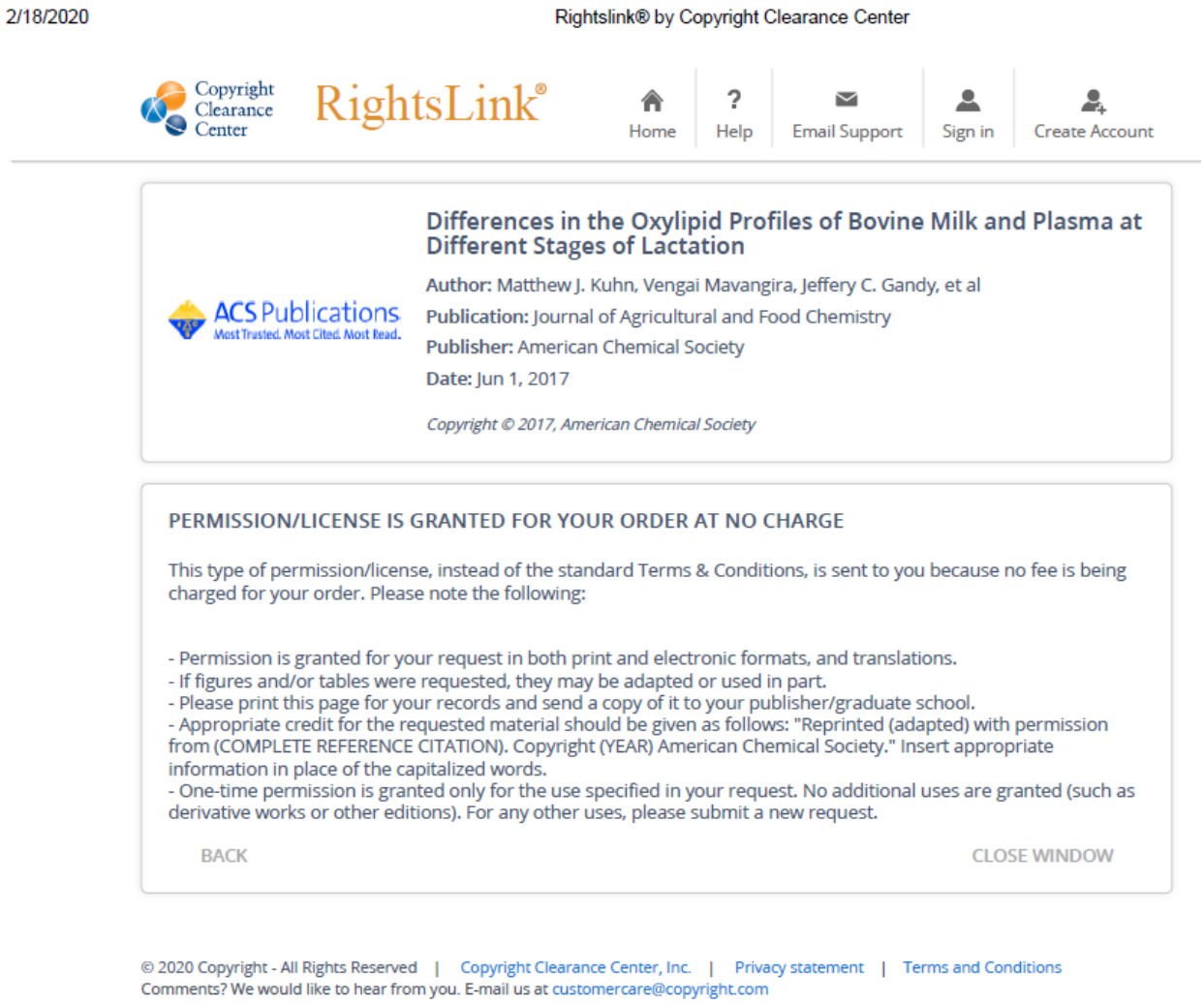

Figure 8.1: Chapter 3 authorization to publish

Figure 8.2: Chapter 4 authorization to publish

Figure 8.3: Chapter 5 authorization to publish

APPENDIX C: Supplemental Table

Table S1. MRM parameters and classifications of measured oxylipids and fatty acids

metabolite	precursor ion *(m/z)*	product ion *(m/z)*	CV (V)	CE (V)	Fatty Acid precursor	class	limit of detection (nM)
12-HHTrE	279.2	179	45	10	AA	alcohol	0.03
20-HETE	319.2	245	55	16	AA	alcohol	0.05
15-HETE	319.2	219	45	10	AA	alcohol	0.003
11-HETE	319.2	167	51	16	AA	alcohol	0.002
9-HETE	319.2	151	15	10	AA	alcohol	0.002
5-HETE	319.2	115	33	10	AA	alcohol	0.004
14,15-DHET	337.2	207	33	16	AA	diol	0.003
11,12-DHET	337.2	167	51	22	AA	diol	0.003
8,9-DHET	337.2	127	15	22	AA	diol	0.005
AEA	348.2	62	21	16	AA	endocannabinoid	0.07
2-AG	379.3	287	45	16	AA	endocannabinoid	0.002
14,15-EET	319.2	219	33	10	AA	epoxide	0.9
11,12-EET	319.2	179	27	10	AA	epoxide	0.006
8,9-EET	319.2	155	27	10	AA	epoxide	0.02
8 iso $PGF_{2\alpha}$	353.2	193	21	22	AA	isoprostane	0.006
8 iso PGA2	333.2	175	55	22	AA	isoprostane	0.03
8 iso PGA1	335.2	235	39	16	AA	isoprostane	0.003
LTB_4	335.2	195	21	16	AA	leukotriene	0.002
5(S),6(R)-	351.2	115	55	16	AA	lipoxin	0.003
15-OxoETE	317.2	113	45	16	AA	oxo	0.002
5-OxoETE	317.2	203	45	22	AA	oxo	0.002
6-keto-$PGF_{1\alpha}$	369.3	163	21	22	AA	prostanoid	0.44
PGE_2	351.2	271	55	16	AA	prostanoid	0.05
PGD_2	351.2	189	55	22	AA	prostanoid	0.1
15d-PGJ_2	315.2	271	39	10	AA	prostanoid	0.1
TXB_2	369.2	169	51	16	AA	thromboxane	0.05
17-HDoHE	343.2	281	45	10	DHA	alcohol	0.1
19,20-	361.2	273	27	16	DHA	diol	0.006
1017DiHDoHE	359.2	153	15	16	DHA	protectin	0.03
7,17-dh-DPA	361.2	143	27	16	DHA	protectin	0.07
ResolvinD2	375.2	175	21	22	DHA	resolving	0.007
17,18-DiHETE	335.2	247	39	16	EPA	diol	0.1
14,15-DiHETE	335.2	207	27	16	EPA	diol	0.02
17,18-EpETE	317.2	255	15	10	EPA	epoxide	0.006
14,15-EpETE	317.2	207	27	10	EPA	epoxide	0.007
13-HODE	295.2	195	30	16	LA	alcohol	0.007
9-HODE	295.2	171	45	16	LA	alcohol	0.007
12,13-	313.2	183	45	22	LA	diol	0.2
9,10-DiHOME	313.2	201	27	22	LA	diol	0.0001

Table S1. (Cont'd)

12,13-EpOME	295.2	195	30	16	LA	epoxide	0.004
9,10-EpOME	295.2	171	45	16	LA	epoxide	0.07
13-OxoODE	293.2	113	15	22	LA	oxo	0.002
9-OxoODE	293.2	185	27	16	LA	oxo	0.007
LTD4	495.3	177	51	22	AA	leukotriene	n/a
Resolvin D1	375.2	141	21	10	DHA	resolving	n/a
LA	279.2	59	45	16		PUFA	0.001
AA	303.2	259	21	10		PUFA	0.009
EPA	301.2	257	39	10		PUFA	0.001
DHA	327.2	283	51	10		PUFA	0.001
Stearic acid	283	283	33	22		Saturated FA	0.003
Oleic acid	281	281	45	22		Monounsaturated	0.01
Palmitic Acid	255	255	33	22		Saturated FA	0.01
PGE2-d_9	360.2	280	21	16		standard	
8,9-DHET-d_{11}	348.3	127	55	22		standard	
15-HETE-d_8	327.2	226	15	10		standard	
8,9-EET-d_{11}	330.3	268	15	10		standard	
AEA-d_8	356.3	63	33	10		standard	
2-AG-d_8	387.3	294	21	16		standard	
γ-Linolenic	277.2	233	27	10		standard	
AA_d_8	311.2	267	21	10		standard	
5-HETE-d_8	327.3	116	55	16		standard	
7Maresin1	359.2	177	51	16		standard	

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