LINOLEIC ACID AND LEUKOCYTE INFLAMMATORY MARKERS IN PERIPARTURIENT DAIRY COWS

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ABSTRACT

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Incidence and severity of dairy cow disease is greatest in the periparturient period. Mobilization of saturated and unsaturated fatty acids from adipose tissue into plasma is a well-established risk factor for periparturient disease. Lipid mobilization was associated with changes in peripheral leukocyte content of polyunsaturated fatty acids and could impact production of the mediators of inflammatory pathways known as oxylipids. Previous bovine leukocyte studies associated expression of some proinflammatory markers and enhanced inflammatory responses to endotoxin with increased disease incidence and severity during the periparturient period. However, the potential impact of oxylipids on leukocyte inflammatory marker expression during the periparturient period is unknown. The hypothesis of the Chapter 2 study was that a relationship exists between the profile of pro- and anti-inflammatory plasma oxylipids and the inflammatory phenotype of peripheral blood leukocytes during the periparturient period. Correlations were identified between concentrations of several plasma oxylipids, including some derived from linoleic acid, and expression of some proinflammatory markers by peripheral blood leukocytes. Previous reports of increased linoleic acid concentrations within mononuclear leukocytes during early lactation were confirmed. Human studies showed proinflammatory effects of linoleic acid in several cell types through oxylipid production. Early lactation monocytes were modeled *in vitro* to evaluate effects of increased linoleic acid concentrations on oxylipid production and inflammatory marker expression. The overall hypothesis of Chapter 3 studies was that increased monocyte linoleic acid concentrations enhance production of linoleic acid-derived oxylipids,

induce a pro-inflammatory phenotype, and enhance inflammatory responses to endotoxin. Results showed increased production of several linoleic acid-derived oxylipids in primary bovine and murine RAW 264.7 monocytes with linoleic acid concentrations similar to early lactation cows. Increasing linoleic acid concentrations in monocytes did not impact pro- or anti-inflammatory marker expression and did not influence endotoxin-stimulated inflammatory responses. Future studies should evaluate the impact of individual oxylipids, produced by monocytes in response to increased linoleic acid concentration, on inflammatory marker expression.

This work is dedicated to improved understanding of dairy cow disease mechanisms by animal and veterinary scientists.

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KEY TO SYMBOLS AND ABBREVIATIONS

AA; arachidonic acid

ACSL; acyl CoA synthase (ligase)

AGPAT; acylglycerol-3-phosphoacyltransferase

ALA; α-linolenic acid

AMU; atomic mass units

BHT; butylated hydroxytoluene

CDP; cytidine diphosphate

COX; cyclooxygenase

CTP; cytidine triphosphate

DGAT; diacylglycerol acyltransferase

DHA; docosahexaenoic acid

EPA; eicosapentaenoic acid

FAME; fatty acid methyl esters

GC/MS; gas chromatography/mass spectrometry

GPAT; glycerol-3-phosphoacyltransferase

HETE; hydroxyeicosatetraenoic acids

HODE; hydroxyoctadecadienoic acids

HpDHA; hydroperoxydocosahexaenoic acids

HPETE; hydroperoxyeicosatetraenoic acids

HPGD; 15-hydroxyprostaglandin dehydrogenase

HPODE; hydroperoxyoctadecadienoic acids

LA; linoleic acid

LC/MS; liquid chromatography/mass spectrometry

LOX; lipoxygenase

LPS; lipopolysaccharide

LT; leukotrienes

MaR1; 7-maresin1

NEFA; non-esterified fatty acids

NOS2; inducible nitric oxide synthase 2

OxoETE; oxoeicosatetraenoic acids

OxoODE; oxooctadecadienoic acids

PBMC; peripheral blood mononuclear cells

PD; protectins

PG; prostaglandins

PPAP; phosphatidic acid phosphatase (lipin)

PPAR-γ; peroxisome proliferator activated receptor-γ

PUFA; polyunsaturated fatty acids

RIN; RNA integrity number

TLR; toll like receptor

TNF; tumor necrosis factor-α

TX; thromboxane

CHAPTER 1

DIETARY POLYUNSATURATED FATTY ACIDS AND INFLAMMATION: THE ROLE OF PHOSPHOLIPID BIOSYNTHESIS[1]

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Abstract

The composition of fatty acids in the diets of both human and domestic animal species can regulate inflammation through the biosynthesis of potent lipid mediators. The substrates for lipid mediator biosynthesis are derived primarily from membrane phospholipids and reflect dietary fatty acid intake. Inflammation can be exacerbated with intake of certain dietary fatty acids, such as some ω-6 polyunsaturated fatty acids (PUFA), and subsequent incorporation into membrane phospholipids. Inflammation, however, can be resolved with ingestion of other fatty acids, such as ω-3 PUFA. The influence of dietary PUFA on phospholipid composition is influenced by factors that control phospholipid biosynthesis within cellular membranes, such as preferential incorporation of some fatty acids, competition between newly ingested PUFA and fatty acids released from stores such as adipose, and the impacts of carbohydrate metabolism and physiological state. The objective of this review is to explain these factors as potential obstacles to manipulating PUFA composition of tissue phospholipids by specific dietary fatty acids. A better understanding of the factors that influence how dietary fatty acids can be incorporated into phospholipids may lead to nutritional intervention strategies that optimize health.

Keywords: diet; phospholipid; fatty acid; lipid mediator; eicosanoid; inflammation; lipoxygenase; cyclooxygenase

Introduction

Dietary fatty acids can control the incidence and severity of inflammation in some diseases of humans and domestic animals. Current evidence suggests that the dietary ω-6 (n-6) to ω-3 (n-3) polyunsaturated fatty acid (PUFA) ratio is directly associated with inflammatory-based pathology in human diseases such as cancer, rheumatoid arthritis, atherosclerosis, and obesity [22, 23, 148]. Studies in production animals used for human food also suggest that dietary intake of certain PUFA can impact inflammatory responses to common infectious diseases such as mastitis and metritis [32, 153]. The associations between dietary fatty acids and diseases are partially explained by the incorporation of dietary n-6 and n-3 PUFA into membrane phospholipids [64]. The PUFA content of phospholipids influence inflammation through several mechanisms including membrane fluidity, lipid raft formation, and receptor function[147, 160], but the focus of this review is on the biosynthesis of potent phospholipid-derived lipid mediators that have pro- or anti-inflammatory functions. For example, many lipid mediators produced from n-6 PUFA have pro-inflammatory functions and the overexpression of these lipid mediators is associated with the pathogenesis of inflammation during disease. Conversely, lipid mediators derived from n-3 PUFA have inflammation-resolving properties, and partly explain the disease-protective effects that are sometimes seen with n-3 PUFA ingestion [118]. Inconsistent health benefits seen with n-3 PUFA supplementation could be due to the regulation of dietary and stored PUFA incorporation into phospholipids. Digestion, for example, can change the profile of ingested fatty acids by reducing the number of double carbon bonds [9, 19]. Additionally, fatty acid incorporation into phospholipids is sometimes selective [173]. Also, phospholipid synthetic pathways are regulated at multiple levels and are sometimes controlled by other nutrients or metabolites, such as diacylglycerol and glucose [97, 188]. The objectives of this review are to explain how dietary n-6

and n-3 PUFA are incorporated into membrane phospholipids, explain how the subsequent biosynthesis of lipid mediators can influence inflammatory responses, and describe factors confounding the influence of dietary PUFA on phospholipid composition. Future studies of these confounding factors will improve knowledge of phospholipid biosynthesis in humans and domestic animals and will reveal all health outcomes of n-3 PUFA dietary supplements.

Trends in Dietary Fatty Acid Composition

The consumption of n-3 and n-6 PUFA in Western diets changed considerably over the last several decades [15]. The n-6 PUFA content of Western human diets increased to some extent because plants or plant-derivatives, such as corn oil, are popular dietary ingredients [33]. In contrast to modern Western human diets, Northern (e.g., Greenland) and Eastern (e.g., Southeast Asian) diets contain more n-3 PUFA because proportionally more dietary fatty acid is derived from fish [8]. Marine plants and fish are rich in n-3 PUFA, relative to terrestrial plant- or animal-derived foodstuffs that are abundant in Western diets. Hence the dietary n-6 to n-3 PUFA ratio is higher in modern Western human diets compared to regions where fish remain a significant source of PUFA.

In spite of these differences in PUFA intake between regions, both n-6 and n-3 remain important dietary ingredients in all human and domestic animals. Linoleic acid (LA, C18:2 n-6) nutritional deficiency results in disease of multiple systems, including skin [50] and α-linolenic acid (ALA, C18:3 n-3) deficiency results in neurological impairment [38]. These PUFA cannot be synthesized *de novo* but, once absorbed, they can be converted to other PUFA within the same omega designation [61]. Domestic animals and humans cannot synthesize n-3 PUFA from n-6 because they lack specific desaturase enzymes, such as that transcribed from *fat-1* in lower order eukaryotes [148]. Therefore, the n-6 to n-3 PUFA ratio in tissue phospholipids is a direct reflection of dietary

composition, although the profiles of individual n-6 and n-3 PUFA are also subject to *de novo* elongation and desaturation[61].

Like humans, many species of domestic animals have experienced significant dietary change over the past several decades. This is partly due to industrialization of Western agriculture, which reduced or eliminated pasture feeding and introduced concentrated-nutrient forages into the diet of many species, including ruminants. Meat and milk derived from intensively-raised ruminants may be partly responsible for elevated n-6 to n-3 PUFA ratios in Western human diets [33] as modern ruminant diets were shown to affect the n-6 to n-3 PUFA ratio of meat [133] and milk [177] from these animals. As with humans, there is increased disease risk in ruminants fed these modern diets [42], which can be partly explained by the n-6 to n-3 PUFA ratio in phospholipids and the effect this has on severity and duration of host inflammatory responses [29].

Dietary Polyunsaturated Fatty Acids and Inflammation

Inflammation is an essential component of the innate immune response to tissue injury. Movement of serum proteins, lipids, and blood leukocytes into affected tissues eliminate or neutralize the source of tissue injury then restore normal tissue structure and function. Failure to control the magnitude and duration of the inflammatory response can cause damage to host tissues and contribute to pathology, independent of the original insult. The ratio of n-6 to n-3 PUFA in the diet has long been recognized as an important influence on the pathogenesis of inflammation because PUFA derivatives can initiate and exacerbate inflammatory responses [22]. This was demonstrated in several human and animal diseases, including atherosclerosis, sepsis, mastitis, and cancer [32, 89, 148], and involves derivatives of n-6 PUFA. In contrast, human diseases with inflammatory-based pathology were prevented with n-3 PUFA dietary supplementation. Increased fish-derived

n-3 PUFA consumption, for example, was shown to lower risk of cardiovascular diseases and improved survival from myocardial infarction [1, 57, 175]. Evidence also exists that dietary n-3 PUFA dietary supplementation can prevent several forms of cancer [131]. The inflammatory response of healthy human subjects is also affected by n-3 PUFA ingestion. For example, human mononuclear leukocytes from subjects supplemented with dietary fish-oil have decreased TNF-α expression relative to subjects consuming control diets [168]. Despite this evidence, there remains some controversy concerning the health benefits of n-3 PUFA ingestion because not all data were conclusive. Dietary supplementation with 1 g of n-3 PUFA daily, for example, did not reduce the rate of adverse cardiovascular events in patients that had or were at risk of type 2 diabetes [17]. Also a recent meta-study found no association between n-3 PUFA intake and incidence of major cardiovascular disease [129]. A better understanding of how dietary PUFA are integrated into membrane phospholipids and therefore available for biosynthesis of pro-resolving mediators of inflammation may explain these equivocal results.

Phospholipids of Inflammatory Cells

The link between dietary PUFA, inflammation, and disease susceptibility is partially due to changes in the PUFA content of phospholipids in cells involved in the inflammatory response, such as monocytes, macrophages, and vascular endothelial cells [7, 36]. Phospholipids are composed of 2 fatty acids esterified to glycerol at sn-1 and sn-2 and a phosphorylated head group esterified at sn-3 (Figure 1). The n-6 and n-3 PUFA, including arachidonic acid (AA, C20:4 n-6), LA, eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) are incorporated into the sn-2 position of phospholipids in cellular membranes [173]. Phospholipid head groups are cytidine-monophosphate, hydroxyl, choline, ethanolamine, serine, or inositols [173]. Phosphocholine and phosphoethanolamine are concentrated in macrophage plasma

membranes [6], are the major phospholipids in human plasma (76% phosphocholine and 17% phosphoethanolamine) [119], and are also the most abundant phospholipids in human erythrocytes [43]. As such, phosphocholine and phosphoethanolamine are the largest phospholipid reservoirs of dietary n-6 and n-3 PUFA in cells and fluids involved in inflammatory responses.

Other less abundant phospholipids can affect inflammatory responses, but independent of lipid mediator production. For example, diphosphoglycerol (cardiolipin) consists of 2 molecules of phosphatidic acid esterified to glycerol, is the most abundant phospholipid in the inner mitochondrial membrane [116], and can compromise cellular respiration [103] and mediate apoptosis [184] under conditions of oxidative stress. Another example is platelet activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), which directly enhances cytokine and adhesion molecules expression in vascular endothelium [34]. These are important effects of phospholipids but the objective here is to explain how major phospholipids in cells and fluids that are critical in inflammatory processes are influenced by dietary PUFA and regulate inflammatory processes when PUFAs are converted to lipid mediators of inflammation.

Inflammatory Pathways Influenced by Fatty Acid Components of Phospholipids

The fatty acid composition of membrane phospholipids in macrophages and endothelium can influence inflammatory responses in several different ways. Physical properties of membranes, such as fluidity and lipid raft formation, are influenced by specific fatty acids in membrane phospholipids and modify membrane-generated signaling cascades. For example, murine macrophage and adipocyte and human vascular endothelial cell culture studies, including some with transgenic mice, demonstrated that saturated fatty acids can directly activate proinflammatory signaling pathways through Toll-like receptor (TLR)-mediated mechanisms [80, 91, 162]. Saturated fatty acids increase proinflammatory gene expression in macrophages by inducing the

dimerization and recruitment of TLR4-related signaling proteins into lipid rafts in a way that mimics endotoxin [91, 178]. These responses to saturated fatty acids in phospholipids involve downstream pro-inflammatory signaling pathways. For example, treatment of murine macrophage cell lines with palmitic (C16:0) and lauric (C12:0) acids increased phosphorylation of JNK and ERK, resulting in enhanced expression of proinflammatory cytokines [178]. Interestingly, the saturated fatty acid-induced increase in phosphorylation of MAPK signaling subunits was attenuated by increasing the DHA content of cells [178]. This effect may be partially attributable to the direct action of n-3 PUFA and independent of conversion to lipid mediators [49, 94]. Although saturated fatty acids and PUFA have important direct effects on health, the focus of this review is on conversion of n-3 and n-6 PUFA, derived from membrane phospholipids, into the more potent lipid mediators [49].

Phospholipid-dependent Biosynthesis of Lipid Mediators

Several hundred lipid mediators have been identified and they collectively regulate the initiation, magnitude and duration of inflammatory responses [46]. Eicosanoids are the most widely studied class of lipid mediators. These are derived from AA and include hydroperoxyeicosatetraenoic acids (HPETE), prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), and lipoxins. The first step in eicosanoid biosynthesis occurs when esterified AA is released from membrane phospholipids by phospholipase enzymes. Intracellular AA may then be metabolized by the cyclooxygenase (COX), lipoxygenase (LOX), or epoxygenase (e.g. cytochrome P450) enzymatic pathways, which ultimately determine the class of eicosanoid generated (Figure 2). Two isoforms of COX enzymes, denoted as COX1 and COX2, are involved in the enzymatic oxidation pathways. Both COX isoforms first catalyze the oxidation of AA to prostaglandin G_2 (PGG_2) followed by a peroxidase reaction that reduces PGG_2 to PGH_2 [44]. From PGH_2, specific downstream PG synthases produce

PGE_2, PGD_2, PGI_2, and $PGF_{2\alpha}$. Alternatively, thromboxane synthases convert PGH_2 to TXA_2 and TXB_2. Similar to the COX family, there are several isoforms of LOX involved in the enzymatic oxidation of PUFA. For example, 5-LOX catalyzes the oxidation of AA to 5-HPETE that can be further metabolized to produce LT. Moreover, both 15-LOX1 and 15-LOX2 oxidize AA to 15-HPETE [86]. This can be further metabolized to lipoxin A_4 by 5-LOX [140]. The eicosanoid class of lipid mediators is predominantly proinflammatory, with a small number of exceptions. For example, lipoxin A_4 is pro-resolving [140] and PGE_2 has both proinflammatory and resolving effects [21].

Whereas AA is the common precursor of the eicosanoid class of lipid mediators, there are other important mediators produced from the enzymatic oxidation of LA, DHA, and EPA. For example, LA is metabolized by several enzymes including 15-LOX, COX2, and the epoxygenases into hydroperoxyoctadecadienoic acid (HPODE). This is reduced to hydroxyoctadecadienoic acid (HODE) which is dehydrogenated to form oxooctadecadienoic acid (OxoODE) [51]. The effects of LA-derived mediators can be either pro- or anti-inflammatory [5, 106, 111]. Lipid mediators derived from n-3 PUFA are the most recently discovered [140]. These mediators are synthesized from DHA by 12-LOX or 15-LOX and are referred to as resolvins, protectins, and maresin. In contrast to the mediators derived from n-6 PUFA, the function of mediators derived from n-3 PUFA are primarily anti-inflammatory or pro-resolving [140]. COX2 also synthesizes resolvins from EPA, but only in patients treated with aspirin (Figure 2). Alternatively, these EPA-derived resolvins can be synthesized by epoxygenases [140].

Enzymatic biosynthesis of lipid mediators is important because enzymes can be overexpressed in diseases such as atherosclerosis and sepsis [94, 185]. Both n-6 and n-3 PUFA, however, may also undergo non-enzymatic oxidation with reactive oxygen species, resulting in production of lipid

mediators with similar biological effects to those derived from enzymatic oxygenation [182]. For example, some 15-HPETE and 13-HPODE isomers produced by non-enzymatic oxidation are identical to those synthesized by enzymatic oxidation [47, 186]. These hydroperoxides are themselves reactive oxygen species, thus creating positive feedback loops on PUFA oxidation during an inflammatory response that can exacerbate disease pathogenesis.

Dietary PUFA Influence Inflammation Through Lipid Mediators

There is strong evidence that the dietary n-6 to n-3 PUFA ratio influences the profile of lipid mediators within tissue [78, 118, 120]. For example, a recent human study demonstrated increased plasma HODE and OxoODE with increased dietary LA [120]. Dietary supplementation with AA increased mononuclear leukocyte secretion of pro-inflammatory eicosanoids, including LTB_4 [78]. EPA and DHA dietary supplementation were shown to increase lipoxins and E and D series resolvins in mouse tissue, while also decreasing pro-inflammatory AA-derived eicosanoids [118]. Therefore *in vivo* production of lipid mediators is regulated by substrate availability. Some of these studies have confirmed that dietary n-6 to n-3 PUFA ratio does in fact influence lipid mediator biosynthesis through changes in PUFA composition of tissue phospholipids [63, 118].

Manipulation of the profile of tissue lipid mediators by dietary PUFA is significant because lipid mediators are known to influence health. For example, increased AA-derived PGE_2 biosynthesis is associated with human colon cancer [176, 193]. There is increased expression of LA-derived metabolites, 9-HODE and 13-HODE, in atherosclerotic vascular lesions [174]. Specific effects of eicosanoids on inflammation and disease pathogenesis have been demonstrated using *in vivo*, *in vitro*, and transgenic animal models of disease. For example, DHA-derived resolvin D2 was shown to ameliorate the inflammatory response in murine models of sepsis [157] and blockade of resolvin receptors in human polymorphonuclear leukocytes resulted in loss of the anti-inflammatory effects

of resolvin D1 [107]. PGE_2 receptor deletion reduced pathology in murine models of colon cancer [176]. Several studies showed that deletion of the *12/15-LOX* gene was effective in decreasing atherosclerosis pathology in mice [35, 130].

Despite cumulative evidence that changes in the profiles of eicosanoids and other lipid mediators are a major factor contributing to the health effects of PUFA intake, there are several important studies that are inconclusive. For example, a recent review of 15 human LA dietary trials found no association between intake and pro-inflammatory markers, including eicosanoids and other lipid mediators [74]. A recent meta-analysis of the protective effects of n-3 PUFA supplementation against human cardiovascular diseases failed to identify any significant health benefits [129]. There are several possible reasons why such inconsistency may exist in the literature. For example, lipid mediator biosynthesis is regulated at multiple levels in addition to supply of PUFA substrate. These include regulation of enzyme transcript and protein expression and enzyme activity [18, 30, 60, 70, 159, 185, 192]. Also, some n-6 PUFA are substrate for pro-resolving mediators, which can be displaced by n-3 PUFA supplementation [118]. Additionally, enzymes that synthesize lipid mediators are selective in their use of substrate [84, 85, 151].

Further Lipid Mediator Research

There are some important questions that remain unanswered in this field, such as "are there potentially deleterious health effects of decreasing the tissue n-6 to n-3 PUFA ratio?" The associations between PUFA content of phospholipids and lipid mediators reveal some possible problems. For example, decreased AA-derived lipoxin-A_4 biosynthesis occurs with n-3 PUFA dietary supplementation [69, 108, 118]. Effects such as this may not always be beneficial for health as, for example, decreased lipoxin A_4 is associated with severe human asthma [93]. The influence of DHA and EPA dietary supplements on asthmatics is not known. To address this, n-3 dietary

trials should quantify all lipid mediator substrates within both diet and phospholipids of immune and vascular cells, in contrast to reporting the n-6 to n-3 PUFA ratio [65, 125]. Additionally, the tissue lipid mediator profile should be assessed, in contrast to sporadic mediators in previous dietary trials [63, 118]. A comprehensive profile, rather than isolated lipid mediators, is important because the lipid mediator network can function in antagonistic, synergistic, or additive manners. Having identified interactions among lipid mediators and their substrates, future research should then investigate the possible causes and interventional targets of such relationships.

Another important question is "whether n-3 PUFA supplementation ameliorates inflammation during human and animal disease because of increased biosynthesis of resolving lipid mediators from n-3 PUFA or decreased proinflammatory lipid mediator biosynthesis from n-6 PUFA?" It was reported that decreasing the tissue n-6 to n-3 PUFA ratio increases the biosynthesis of resolving type lipid mediators from n-3 PUFA and decreases the biosynthesis of proinflammatory lipid mediators from n-6 PUFA [69, 108], but comparative health responses between low n-6 PUFA and high n-3 PUFA consumption are not clear. This question could be initially addressed in carefully designed dietary trials that measure phospholipid PUFA composition and lipid mediator biosynthesis of tissue.

The final question that this body of literature raises is "under which circumstances should inflammation be manipulated by dietary n-3 PUFA?" Inflammation is an essential component of mammalian physiology and exists to facilitate restoration of homeostasis in tissue. Anti-inflammatory effects of dietary PUFA may, therefore, have adverse consequences in disease recovery. This has in fact, been demonstrated with dietary n-3 PUFA supplementation in murine models of gastrointestinal inflammation [179]. This does not imply that treatment of uncontrolled inflammation is contraindicated, but rather, that manipulation of lipid mediator biosynthesis likely

has multiple outcomes and that some outcomes can be undesirable. Further evidence of this is the discovery that selective COX2 inhibitors affect vasoactive prostaglandin biosynthesis and increase risk of thromboembolic disease in humans [25, 54].

Delivery and Utilization of Dietary PUFA for Phospholipid Biosynthesis

A better understanding of critical regulatory steps that impact phospholipid biosynthesis from dietary PUFA will provide insight into the variable success of dietary manipulation of inflammatory conditions. For example, though phospholipid biosynthesis sometimes displays a preference for absorbed PUFA, not all dietary PUFA will be used for this purpose. Also, enzymes which synthesize phospholipids are influenced by disease and physiological states, such as reproduction. Additionally, PUFA may arise at sites of phospholipid biosynthesis from body stores, such as adipose tissue, and displace dietary PUFA as substrate.

Digestion and Absorption of Dietary PUFA

PUFA are ingested in several forms, including non-esterified fatty acid, triacylglycerol, and phospholipids. Ester bonds of ingested lipids are hydrolyzed by pancreatic lipase to produce predominantly non-esterified fatty acids and monoacylglycerol [19]. This is the primary digestive process for monogastric species, such as humans and swine and is not currently known to affect PUFA supply. However, PUFA from which lipid mediators are derived are very susceptible to non-enzymatic oxidation. For example, the anti-inflammatory effect of n-3 ingestion is increased if co-ingested with antioxidants [168], although it is not clear if the antioxidant effect occurs prior to n-3 absorption, or after, as demonstrated in other models [180]. Multi-gastric species such as ruminants experience an additional digestive process which, in contrast to lipase hydrolysis, has been demonstrated to affect dietary PUFA content [9]. This is referred to as biohydrogenation and

describes hydrogenation of PUFA by the micro-flora of the fore-stomachs. Biohydrogenation reduces the efficiency of absorption of some PUFA, particularly those present in the diet at low concentrations such as EPA and DHA. For example, *in vivo* goat models of biohydrogenation demonstrated transfer of only 3.5 to 7.6% of dietary EPA or DHA into milk [81]. Apparently this is sufficient to induce changes in tissue phospholipid composition, possibly because the quantity of absorbed n-3 PUFA is large relative to pre-supplementation tissue and milk levels [177].

The absorption process for available PUFA is similar across all domestic animal species and humans and occurs through the intestinal lymphatic system and the venous side of the vascular system [19]. In this way, absorbed PUFA are protected from hepatic metabolism in the first pass through the circulatory system, and immediately available for biosynthesis of phospholipids in organs and cells critical in determining inflammatory responses, such as bone marrow, peripheral leukocytes, and vascular tissue [19, 173].

Phospholipid Substrate from Non-dietary PUFA

There are several important sources of non-dietary PUFA that confound the influence of dietary PUFA on inflammation (Figure 3) [113] . These include PUFA synthesized *de novo* or stored in sites such as adipose tissue. The relative efficiencies of n-3 and n-6 PUFA *de novo* biosynthesis are controversial. For example, *de novo* biosynthesis of EPA and DHA from ALA has been described as modest [82] but there is evidence that humans have greater capacity for this than *de novo* biosynthesis of AA from LA [51]. Therefore *de novo* biosynthesis of some n-3 PUFA cannot be discounted as having potential health benefits if this results in increased tissue content of DHA and EPA-derived pro-resolving type lipid mediators.

Adipose-derived PUFA may also be used for phospholipid biosynthesis in inflammatory based

cells (Figure 3). This is an abundant substrate for phospholipid synthesis during dietary restriction or large, chronic expenditures of energy because the rate of lipolysis exceeds that of lipogenesis and adipose tissue is mobilized [172]. This occurs, for example, in humans and domestic animals during the periparturient period [31, 76] because of energy expenditure associated with pregnancy and lactation and a concomitant deficit in calorie intake. During adipose mobilization, the liver can utilize adipose-derived fatty acid for ketone body biosynthesis, particularly if carbohydrate ingestion or substrates for gluconeogenesis are restricted relative to the requirements for glucose [19]. Alternatively, fatty acid in the liver may be stored as triacylglycerol. In other organs, such as skeletal muscle, dietary and adipose-derived fatty acid can be energy substrates through β-oxidation, or in functional mammary glands, are excreted as milk lipid. Hence when adipose-derived fatty acids are mobilized into circulation, phospholipid biosynthesis must compete against ketogenesis, β-oxidation, and milk-lipid excretion for PUFA substrate. In spite of this competition, there is *in vitro* evidence that suggests adipose-derived fatty acids modify the composition of phospholipids in vascular endothelial tissue and induce inflammation through lipid mediators, and also evidence that this effect can be reversed with n-3 PUFA supplementation [29, 30]. This is particularly interesting as there is an exacerbated inflammatory response *in vivo* in some species coincident with adipose mobilization [145]. This suggests that decreasing the n-6 to n-3 PUFA ratio in adipose could potentially improve health during periods of adipose mobilization. Dietary PUFA are incorporated into adipose triacylglycerol by *de novo* triacylglycerol biosynthesis and remodeling through reacylation [73, 169]. The rate of *de novo* synthesis is 20% of total triacylglycerol in healthy human adipose tissue after nine-weeks [161]. The kinetics of triacylglycerol reacylation are not reported but since the half-life of all adipose triacylglycerol is 6-months to 2-years [67], it is reasonable to interpret that reacylation of adipose triacylglycerol

occurs over several months or years *in vivo*. These data suggest that a long-term change in diet is required to induce change in adipose n-3 PUFA content. Since DHA and EPA are substrate to several inflammation-resolving lipid mediators, long term ingestion of these, particularly during periods of triacylglycerol synthesis, could be therapeutic in inflammatory conditions associated with mobilization of adipose tissue.

Another potential origin of PUFA for phospholipids is recycled use of fatty acyl chains that have been hydrolyzed from existing phospholipids (Figure 3). Little is known about the role of this pool of fatty acids in biosynthesis of phospholipids. However, it is unlikely that the plasma membrane, the largest PUFA reservoir, has a major role in supplying PUFA for *de novo* phospholipids as the machinery for this are located in the endoplasmic reticulum and mitochondrion [173].

Effects of Disease, Physiological State, and Other Nutrients on Phospholipid Biosynthesis

De novo biosynthesis of phospholipids occurs in what is commonly referred to as the Kennedy pathway [79]. A brief description of this pathway is useful to understand regulation of dietary PUFA incorporation into phospholipids. The first reaction in this pathway combines long-chain fatty acid with CoA and is catalyzed by acyl CoA synthase (ligase) (ACSL, Figure 4). The fatty acyl CoA is then esterified to glycerol at sn-1 on the mitochondria by glycerol-3-phosphoacyltransferase (GPAT) [173]. The second acyltransferase is 1-acylglycerol-3-phosphoacyltransferase (AGPAT). Substrate for AGPAT is transferred from the mitochondria to the endoplasmic reticulum, and acylation occurs at sn-2 of glycerol [173]. Glycerol esterified to fatty acid at sn-1 and sn-2 is known as phosphatidic acid. Phosphatidic acid can be metabolized by phosphatidic acid phosphatase (PPAP or lipin) to synthesize diacylglycerol. This combines with cytidine diphosphate (CDP)-choline to produce phosphocholine, which may be metabolized to phosphoethanolamine or phosphoserine. The enzyme cytidine triphosphate (CTP):phosphocholine

cytidylyltransferase (CCT) catalyzes CDP-choline biosynthesis, and CDP-choline:1,2-diacylglycerol cholinephosphotransferase catalyzes the diacylglycerol reaction with CDP-choline [173]. Triacylglycerol is the alternative diacylglycerol product, catalyzed by diacylglycerol acyltransferase (DGAT) [95]. If phosphatidic acid is not utilized for biosynthesis of diacylglycerol, then it can be utilized by CDP-diacylglycerol synthase for synthesis of phosphoinositol, phosphoglycerol, or cardiolipin.

Expression of some phospholipid synthesizing enzymes is influenced by pathogen associated molecular patterns and so is likely influenced by bacterial infection. For example, expression of *ACSL1*, *3*, and *4* is increased in RAW 264.7 murine macrophages in response to the TLR-4 agonist, Kdo_2 [2], an endotoxin-like molecule involved in sepsis and mastitis [145]. Monocyte expression of *ACSL1* is also increased in mouse models of type 1 diabetes, and is associated with an inflammatory phenotype and atherosclerosis [75]. Other rodent models indicate that insulin resistance is associated with *AGPAT2* and PPAP1 deficiency [163]. The clinical relevance of these data is not currently understood but transcriptional effects suggest *de novo* biosynthesis of some phospholipids may change during these diseases and therefore affect how dietary PUFA are incorporated into membrane phospholipids in some patients.

Expression of some phospholipid synthesizing enzymes is also affected by physiological state. For example, *ACSL1* hepatic and mammary tissue expressions are increased in dairy cattle during the postpartum period, relative to prepartum [13, 98]. *GPAT, AGPAT,* and *PPAP* expressions are also increased after bovine parturition [13, 98, 172]. Activities of some enzymes also change with physiological state. Activity of CCT, the rate limiting enzyme in phosphocholine biosynthesis, is regulated by diacylglycerol-induced intracellular translocation to membranes [173]. Translocation results in 1.8-fold increased hepatic activity in the period immediately following parturition in

cows [14]. This may also occur when plasma and hepatic non-esterified fatty acid and di- and triacylglycerol are increased, such as in obese humans [181]. In fact, human adipose expression of a *CCT* gene, *Pcyt1a*, is positively correlated with adipose mass [142]. These findings indicate that the capacity for *de novo* biosynthesis of phospholipids may change by reproductive state and adipose mass, and warrant further investigation at all levels of enzyme regulation. Identifying certain physiological states where dietary PUFA is more successful in influencing the PUFA composition of tissue phospholipids could be used to mitigate disease risk.

Glucose has an important influence on biosynthesis of phospholipids. Glucose availability positively affects cyclin-dependent kinase cdc28, which up-regulates triacylglycerol lipase-4 activity [97]. This results in stored fatty acids being available to phospholipids when glucose is abundant and there is a need for new membrane formation and cell proliferation. Loewen (2012) reports that glucose metabolism also influences biosynthesis of phospholipids through phosphatidic acid and the repressive transcription factor Opi1 [97]. Specifically, under conditions of glucose starvation, intracellular pH declines and results in the release of bound Opi1 from phosphatidic acid. The released Opil translocates to the nucleus resulting in repression of genes involved in biosynthesis of phospholipids, and thus a conservation of stored fatty acid for more critical needs such as β-oxidation [188]. These data indicate that phospholipid biosynthesis is affected by glucose homeostasis and suggest that dietary PUFA incorporation into phospholipids be investigated with consideration of dietary carbohydrate ingredients.

After *de novo* biosynthesis in the Kennedy pathway, phospholipids are continuously susceptible to post-synthetic deacylation and reacylation at the sn-1 and sn-2 fatty acyl positions (Figure 3) in what is referred to as the Lands' cycle [88]. This allows absorbed PUFA to quickly change the composition of tissue phospholipids, apparently within several weeks [102]. Manipulation of

PUFA in tissue phospholipids by the Lands' cycle may be particularly successful during bacterial disease as expression of some acyltransferase enzymes is induced by TLR ligands [144]. Further investigation of this effect may identify therapeutic applications for dietary PUFA during these diseases.

Regulation of the Acyl Composition of Phospholipid

Phospholipids have a diverse fatty acid composition. For example Quehenberger *et al* (2010) measured 31 fatty-acyl variants of phosphocholine and 38 fatty-acyl variants of phosphoethanolamine in human plasma [119]. This diversity is attributable to different combinations of long chain fatty acids at sn-1 and sn-2 of glycerol [119] and is determined by enzyme preference for specific fatty acid substrate. This is important because enzymes in the Kennedy pathway will define the milieu of lipid mediators produced in tissue when they show preference for substrate of lipid mediators. For example, ACSL-3 and -4 show preference for AA and EPA [28]. AGPAT-1, prefers myristic acid (C14:0), palmitic acid, and LA-CoAs, and AGPAT-2, prefers AA-CoA [144]. These enzymes are differentially expressed in models of sepsis and diabetes, and because of their selection of substrate, may partly explain lipid mediator biosynthesis and inflammation during these diseases [2, 75]. Substrate selection by enzymes involved in tissue phospholipid biosynthesis has not been evaluated in PUFA dietary studies, but should be considered when interpreting future studies that examine the influence of dietary PUFA on lipid mediator biosynthesis.

Some Lands' cycle enzymes are selective between fatty acids. For example, the conversion of phosphoethanolamine to phosphocholine by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase favors biosynthesis of sn-1 palmitic acid/ sn-2 DHA. This phospholipid then undergoes rapid reacylation with stearic acid (C18:0) at sn-1, and AA, LA, or

DHA at sn-2. In fact, the sn-1 stearic acid/sn-2 AA forms of phosphocholine and phosphoethanolamine are usually synthesized by replacement of other acyl chains in existing phospholipids, rather than directly [173]. Therefore the Lands' cycle can result in rapid and preferential incorporation of DHA and other PUFA into pre-existing phospholipids. This indicates that dietary supplementation with some n-3 PUFA could have especially rapid effects on health, in light of what is known about the effect of dietary n-3 PUFA supplements on inflammation [118].

As mentioned previously, adipose-derived fatty acid can be a major substrate for biosynthesis of phospholipids in some physiological and disease states. In this context, selectivity of fatty acyl incorporation into triacylglycerol could affect the composition of the fatty pool available for phospholipid synthesis during mobilization of adipose. The final step in triacylglycerol synthesis involves DGAT, which adds fatty acyls to the sn-3 position of diacylglycerol. Unfortunately little research has been conducted into substrate selectivity of mammalian DGAT, but in plants it appears that DGAT-2 demonstrates preference for the less abundant, *trans* isomers of PUFA, and there is similar DGAT-1 and -2 specificity for palmitic acid, oleic acid (C18:1), LA, and ALA fatty acyls [95]. In yeast, both DGAT-1 and -2 have broad substrate preferences [170]. In reviews of these enzymes, Yen *et al* (2008) suggest DGAT-1 prefers oleic over saturated fatty acyls [183], while others indicate DGAT-2 is required for essential fatty acyl (i.e. LA and ALA) incorporation into triacylglycerol [191]. In summary, it appears that much work must be done before the selectivity of mammalian DGAT, and their influence on PUFA incorporation into adipose tissue, will be fully understood.

Conclusions

Human and animal research indicate that for dietary n-6 and n-3 PUFA to modify the pathology

of disease, the PUFA content of phospholipids in cells involved in the inflammatory response must reflect the dietary PUFA composition [63, 118] and specific phospholipid-derived PUFA must be utilized for biosynthesis of pro-inflammatory or pro-resolving lipid mediators [118]. The roles of lipid mediators in maintenance of health and regulation of inflammation during disease have been introduced in the literature. Whether lipid mediators are pro-inflammatory or pro-resolving is partly determined by the specific PUFA from which the lipid mediator is derived. There are trends in the literature supporting a role for n-3 PUFA in control and prevention of inflammation during disease but it is also important to recognize that some n-6 PUFA are essential nutrients and produce pro- and anti-inflammatory lipid mediators. A number of clinical trials with patients supplemented with fish-oil showed no clear health benefit, and could be explained by factors that affect dietary PUFA incorporation into phospholipids. In conclusion, future studies of the impact of dietary PUFA on tissue phospholipid biosynthesis should examine digestive processes, competition for absorbed PUFA by other physiological processes, supply of PUFA from sources other than diet, obesity and reproductive state, glucose homeostasis, and selectivity of phospholipid synthesizing enzymes for fatty acids. Such research will improve knowledge of phospholipid biosynthesis in humans and domestic animals. Complementary studies that measure inflammatory outcomes and lipid mediator biosynthesis should reveal the true health benefit or cost of n-3 PUFA dietary supplements.

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Conflicts of Interest

The authors declare no conflict of interest.

CHAPTER 2

ASSOCIATION BETWEEN POLYUNSATURATED FATTY ACID-DERIVED OXYLIPID BIOSYNTHESIS AND LEUKOCYTE INFLAMMATORY MARKER EXPRESSION IN PERIPARTURIENT DAIRY COWS[2]

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Interpretive Summary

Unregulated inflammatory responses in cows can enhance disease pathogenesis during the periparturient period. Previous research showed that oxylipids regulate inflammation, but their role in periparturient inflammatory responses is not known. This study describes changes in both plasma oxylipids concentrations and their substrates across the periparturient period and correlates these profiles with inflammatory gene expression in peripheral leukocytes. Future studies will investigate sources of polyunsaturated fatty acid substrates of plasma oxylipids and roles of specific plasma oxylipids in inflammatory pathways.

Abstract

Peripheral blood mononuclear leukocytes from periparturient cows can have exacerbated inflammatory responses that contribute to disease incidence and severity. Oxylipids derived from the oxygenation of polyunsaturated fatty acids (PUFA) can regulate the magnitude and duration of inflammation. Although PUFA substrate for oxylipid biosynthesis in leukocytes is known to change across the periparturient period, the plasma oxylipid profile and how this profile relates to leukocyte inflammatory phenotype is not clear. The objective of this study was to determine if a relationship exists between the profile of pro- and anti-inflammatory plasma oxylipids and the inflammatory phenotype of peripheral blood leukocytes during the periparturient period. Seven multiparous Holsteins were sampled from the prepartum period through peak lactation. Plasma oxylipids were measured by liquid chromatography-mass spectrometry, peripheral leukocyte mRNA expression was measured by qPCR, and PUFA content of peripheral blood mononuclear cells was measured by gas chromatography-mass spectrometry. Concentrations of several hydroxyl products of linoleic and arachidonic acid changed over time. Linoleic acid and arachidonic acid concentrations in leukocytes increased during early lactation, suggesting that substrate availability for hydroxyoctadecadienoic and hydroxyeicosatetraenoic acid biosynthesis may influence the oxylipid profile. Leukocyte mRNA expression of *IL-12B, IL-1B*, inducible nitric oxide synthase 2, and cyclooxygenase 2 were correlated with several plasma oxylipids. These are the first observations linking leukocyte inflammatory gene responses with shifts in oxylipid biosynthesis in periparturient dairy cows.

Keywords: inflammation, periparturient, eicosanoid, oxylipid, lipid mediator

Introduction

A major factor contributing to decreased productivity and longevity of dairy cows is the incidence of disease in the first month after parturition [117]. Dysfunctional inflammatory responses during the periparturient period can contribute to disease incidence and severity [154]. For example, bovine peripheral blood mononuclear cells (PBMC) have an exacerbated inflammatory response to lipopolysaccharide in the periparturient period, compared with cells from mid-lactation cows [152]. Uncontrolled inflammatory responses also were associated with increased severity of clinical mastitis [145]. Control and prevention of periparturient disease is currently difficult, partly because it is unclear why dysfunctional inflammatory responses occur around parturition.

Oxylipids are defined as the class of PUFA-derived metabolites that regulate all aspects of the inflammatory response. The biosynthesis of oxylipids involves a number of complex pathways that start with the peroxidation of cell membrane-derived PUFA including linoleic (C18:2 n6), arachidonic (C20:4 n6), eicosapentaenoic (C20:5 n3), and docosahexaenoic acids (C22:6 n3). Initial peroxidation of these PUFA can occur enzymatically through the cyclooxygenases (COX), lipoxygenases (LOX), and epoxygenases, but are also mediated by free oxygen radicals. Oxylipids produced by PUFA peroxidation include 9- and 13- hydroperoxyoctadecadienoic acid (HPODE), prostaglandin (PG) H_2, 5- and 15-hydroperoxyeicosatetraenoic acid (HPETE) and 14- and 17-hydroperoxydocosahexaenoic acids (HpDHA) [140, 150]. These are unstable lipid mediators that are quickly reduced to more stable hydroxyls, such as hydroxyoctadecadienoic acid (HODE) and hydroxyeicosatetraenoic acid (HETE). The hydroxyl oxylipids can serve as substrates to a vast number of downstream metabolites, such as oxooctadecadienoic acids (OxoODE) [120], leukotrienes (LT) [105], thromboxanes (TX), PGD_2, PGE_2, PGF_2, PGI_2 [150], 7-maresin1 (MaR1), and protectins (PD) [140]. Thus, the rate of PUFA oxidation and the extent of metabolism into

downstream products determine the relative abundance of oxylipids with different functional capabilities.

A major determinant of whether oxylipids initiate or resolve inflammation is the PUFA from which they are derived. Both C20:5 n3 and C22:6 n3-derived oxylipids predominantly abrogate inflammation. For example, MaR1 is produced from C22:6 n3 and reduces neutrophil recruitment during peritoneal inflammation [141]. In contrast to the n-3 PUFA-derived oxylipids, the downstream products of n-6 PUFA peroxides have multiple effects in inflammatory pathways. Different PG, for example, can stimulate (e.g. PGF_2) or abrogate (e.g. PGD_2, PGE_2) inflammatory events [138]. The effects of some n-6 derived oxylipids are dependent on species and cell types. For example, HODE activates adhesion molecule expression on endothelial cells [56], thus facilitating leukocyte extravasation during inflammation. However, HODE also binds to nuclear receptors, and abrogates tumor necrosis factor-α (TNF-α) induced inflammatory responses in human epithelial tissue [5]. Several studies showed that dietary changes in PUFA substrate supply could effectively change oxylipid profiles [123]. For example, n-3 PUFA dietary supplementation in humans and rodents was shown to increase tissue phospholipid content of these PUFA and increase n-3 PUFA-derived oxylipids in tissue [118, 120]. Recent studies in periparturient dairy cows identified C18:2 n6 as more abundant in several tissues after parturition, including PBMC [4, 31], but the influence of C18:2 n6 on oxylipid production and leukocyte inflammatory pathways in cows is not known.

Concentrations and relative potency of individual oxylipids [104, 107] determine the net effect of the tissue oxylipid profile on inflammatory processes. The abundance of pro-inflammatory oxylipids relative to pro-resolving oxylipids impacts the chronicity and resolution of disease by initiating or resolving segments of the inflammatory pathway [140]. Describing the

plasma concentrations of oxylipids in periparturient cows, in particular those oxylipids which may affect circulating immune cells and vascular endothelium, may assist in explaining why periparturient PBMC may have enhanced inflammatory responses following exposure to bacterial agonists [152]. The hypothesis of this study was that there is a relationship between the profile of pro- and anti-inflammatory plasma oxylipids and the inflammatory phenotype of peripheral blood leukocytes during the periparturient period.

Materials and methods

Animals

This study was conducted in the autumn months of 2012 in a 1500-cow, intensively housed commercial dairy herd located in Michigan. The herd was fed total mixed rations that contained corn silage and alfalfa haylage as predominant forages. Seven Holstein cows were randomly selected from all healthy cows within 8 to 12 d of expected parturition at study commencement. Median parity for the observed parturition was 3, and ranged from 2 to 4. Blood (50 mL) was aseptically collected in EDTA vacutainers (Becton, Dickinson and Co., Franklin Lakes, NJ) by coccygeal venipuncture, and immediately stored on ice. The distribution of sampling time relative to parturition was 14 d prepartum (range = 6 to 17), immediately after parturition (range = 1 to 3 d postpartum), 10 d postpartum (range = 7 to 10), 28 d postpartum (range = 21 to 32), and 84 d postpartum (range = 77 to 88). Plasma non-esterified fatty acids (NEFA) were measured in 3 randomly selected cows by an enzymatic colorimetric method (Wako NEFA HR(2), Wako Chemicals USA, Richmond, VA) conducted at the Diagnostic Center for Population and Animal Health, Lansing, MI. Michigan State University Animal Care and Use Committee preapproved all animal use and care.

Plasma Oxylipid Quantification

Oxylipid Extraction

Plasma (500 µL) was separated from whole blood prior to PBMC isolation (931 *g* for 30 min, 4°C). Formic acid (1.7 uL) and antioxidants/reducing agents were added (4 µL of combined 2.4 mg EDTA in 3 mL water, 2.4 mg butylated hydroxytoluene (BHT) 1.5 mL ethanol, 24 mg triphenylphosphine in 6 mL methanol (Sigma-Aldrich, St. Louis, MO), and 24 mg indomethacin in 1.5 mL ethanol (Cayman Chemical, Ann Arbor, MI)). Deuterated internal standards ($LTB_{4\text{-}d4}$, $TXB_{2\text{-}d4}$, $PGF_{2\alpha\text{-}d4}$, $PGE_{2\text{-}d4}$, $PGD_{2\text{-}d4}$, 13(S)-$HODE_{\text{-}d4}$, 6-keto $PGF_{1\alpha\text{-}d4}$, 12(S)-$HETE_{\text{-}d8}$, and 15(S)-$HETE_{\text{-}d8}$, Cayman Chemical) were combined in ethanol:water (1:1), to achieve 0.1 ng / uL for each standard, and 200 uL of this mixture (20 ng of each standard) was added to samples. Methanol was then added to samples (1 mL, -20°C), and protein precipitated by brief vortexing and freezing (-20°C for 3 h). Samples were then centrifuged at 18,000 *g* for 15 min (4°C). The supernatant was diluted in 9.2 mL water containing 0.1% formic acid (v/v). Oxylipids were extracted by solid phase methods as previously reported [53], with the following modifications. Extraction columns (Strata-X 60 mg, 3 mL, Phenomenex, Torrance, CA) were conditioned (3 mL methanol then 3 mL water), washed (3 mL 50% methanol (v/v) in high performance liquid chromatography grade water), dried, and samples eluted in 3 mL methanol:acetonitrile (1:1, v/v). Samples were then vacuum dried, suspended in 200 uL acetonitrile:water:formic acid (1850:3150:1, v/v/v), and centrifuged at 18,000 *g* for 30 min (4°C), immediately prior to chromatography.

Liquid Chromatography/Mass Spectrometry (LC/MS)

Oxylipids were measured using LC/MS as we reported previously [53] but with quantification of 8-iso $PGF_{2\alpha}$, PGE_2, PGD_2, $PGF_{2\alpha}$, TxB_2, 6-keto $PGF_{1\alpha}$, resolvin D_1 (RD1), and RD2 utilizing an isocratic mobile phase of acetonitrile:water:0.1% formic acid (35:55:10; v/v/v). 9-HODE, 13-

HODE, 15-oxoeicosatetraenoic acid (OxoETE), 5-OxoETE, 5-HETE, 8-HETE, 11-HETE 12-HETE, 15-HETE, 20-HETE, 9-oxoODE, 13-oxoODE, MaR1, PD1, and LTD_4 were measured as previously described [53]. Oxylipids were identified in samples by matching their deprotonated (i.e., [M-H]-) m/z values and LC retention times with those of pure standards. Oxylipids were quantified relative to internal standard abundance, and calibration against standard curves.

PBMC Fatty Acid Methyl Ester Quantification

Lipid Extraction

PBMC in 45 mL whole blood were isolated using a Ficoll-Paque Plus gradient (GE Healthcare, Waukesha, WI), as previously described [31]. Pelletized PBMC were frozen in liquid nitrogen and stored at -80°C until completion of sample collections. Total lipid was extracted using methanol and chloroform, as previously reported [16, 55]. Briefly, PBMC pellets were suspended in 1.0 mL 1x PBS, then proteins precipitated by adding 3.0 mL of -20°C methanol (500 mg / L BHT). After 30 min, 1.0 mL 1x PBS, 2.0 mL methanol (500 mg / L BHT), 7.0 uL formic acid, and 7.5 µg C13:0 fatty acid (1 mg / mL in hexane) were added. Samples were then sonicated (30 min, 25°C), and 2.5 mL chloroform added. Samples were then shaken for 16 h (25°C), and a further 2.5 mL of chloroform, and 2.5 mL 2 % sodium chloride (w/v, in high performance liquid chromatography grade water) added. Following centrifugation, (2095 *g* for 15 min, 8°C), chloroform was aspirated and stored, and extraction repeated with 2.5 mL chloroform and centrifugation (2095 *g* for 15 min, 8°C). Samples were then dried under vacuum. Contaminant lipids were measured in cell-free samples, concurrent to PBMC samples.

Derivation

Sample lipids were saponified in 0.5 mL methanol, utilizing 1.0 mL freshly made 0.5 M potassium hydroxide in methanol, and heat (100° C for 10 min). After cooling to 4° C, derivation of PUFA

to methyl esters (FAME) was performed under nitrogen gas, utilizing 3.0 mL fresh 20% v/v methanolic hydrochloric acid [115], and heat (90° C for 16 h). After cooling to 4° C, acid was neutralized using 10.0 mL of 6 % potassium carbonate (w/v, in high performance liquid chromatography grade water). FAMES were then extracted with 3.0, 1.5, and 1.5 mL n-hexanes by cetrifugation (524 *g* for 5 min at 8° C). Samples were then vacuum dried, resuspended in 35 uL n-hexanes, and transferred to a GC microinsert vial. Finally, 7.5 µg C15:0 FAME (5 mg / mL in hexane) was added, and samples immediately analyzed by gas chromatography/mass spectrometry.

Gas Chromatography/Mass Spectrometry (GC/MS)

The gas chromatograph was Agilent Technologies model 6890N (Agilent Technologies, Santa Clara, CA), fitted with a DB-23 column (30 m x 0.25 mm internal diameter x 0.25 um film thickness, Agilent Technologies). Inlet temperature was 240^{o} C. The method utilized helium gas at 60.2 kPa psi, a 20:1 split ratio, and a combined flow rate of 25.9 mL / min. Column temperature was optimized and set at 50^{o} C for 1 min, then increased at 25^{o} C / min to 175^{o} C, then increased at 4^{o} C / min to 240^{o} C and held constant for 5 min. Total run time was 27.25 min. Injection volume was 1.0 uL. A pre- and post-injection delay of 1 s was applied.

The mass spectrometer was Agilent Technologies model 5975B inert XLMSD (Agilent Technologies). Transfer line, source, and quadrupole temperatures were 240^{o}, 230^{o}, and 150^{o}C, respectively. Detection criteria were 40 to 400 amu, and a 3 min solvent delay was applied. Total ion chromatograms and mass spectra were generated using Agilent ChemStation software (Agilent Technologies). A 37-component FAME standard mix was utilized to determine retention times (Sigma-Aldrich). Analytes were identified with at least 90% similarity between analyte and reference mass spectra (National Institute of Standards and Technology). Total ion chromatograms

were created for FAMES of C13:0, C15:0, C18:2 n6, and C20:4 n6. Extracted ion chromatograms were created for FAMES of C15:0 (74 atomic mass units [amu]), C20:5 (79 amu) and C22:6 (79 amu) at the appropriate retention times.

GC/MS was validated in several ways. The within-assay and between-assay coefficient of variation for standard grade C18:2 n6 FAME was calculated to be 5 and 15 %, respectively. Second, a single cow sample was divided into 3 aliquots and analyzed separately at the beginning, midway, and completion of chromatography. The coefficient of variation for C18:2 n6 to C15:0 FAMES ratio across these 3 aliquots was 5.4%. Finally, because chromatograms were performed in 2 batches, variance in chromatogram intensity of the C15:0 FAME internal standard between batches was assessed by calculating the ratio of intensity in samples to that of calibration standards for each batch (coefficient of variation = 0.9 %). Batch variance in methylation efficiency was also evaluated and the coefficient of variation was 10.3 %.

FAME Quantification

Sample internal standards were added prior to extraction (C13:0 fatty acid) and following derivation (C15:0 FAME). Calibration curves for C18:2 n6, C20:4 n6, C20:5 n3, and C22:6 n3, and C13:0 FAMEs were created using freshly prepared standard-grade FAMES, analyzed before and after the samples. Standard dilutions were 0.7 fold in n-hexanes, and concentrations extended 2 dilutions above the maximum sample concentration and below the minimum sample concentration. Calibration equations were calculated by linear regression of the known standard concentration against the ratio of analyte to C15:0 FAME ($R^2 > 0.975$). This ratio was calculated from analyte and C15:0 FAME chromatogram intensities.

Analyte concentration in samples was calculated by comparing the intensity ratio of analyte

to C15:0 FAME in samples to that of the linear regression equation from calibration. Analyte concentrations in samples were corrected for methylation efficiency, by comparing the C13:0 FAME abundance in samples to the expected abundance from C13:0 fatty acid internal standard. Analyte concentrations also were corrected for FAME abundance in cell free samples.

Leukocyte mRNA Quantification by qPCR

Total RNA was extracted from 3 mL whole blood for quantification of mRNA using the QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA) with DNase. cDNA was synthesized using the High Capacity cDNA reverse transcriptase kit with RNase inhibitor (Applied Biosystems, Foster City, CA). A single sample was excluded for poor RNA integrity (RIN < 6, Agilent Bioanalyzer). Primers were derived from the *Bos taurus* genome (Table 1). The cDNA was amplified using TaqMan PreAmp Kit (Applied Biosystems). The qPCR was conducted as previously reported [29]. Briefly, PCR was performed in triplicate, using 20 µL reaction mixture per replicate, comprising 11.7 µL TaqMan Gene Expression PCR Master Mix (2x, Applied Biosystems), 1.2 µL (20x) TaqMan Gene Expression Assay Mix (Applied Biosystems), 5 µL amplified cDNA, and 2.1 µL nuclease-free water. Pre-designed (20x) TaqMan Gene Expression Assays from Applied Biosystems for bovine *TBP*, *PGK1*, and *ACTB* were selected as reference genes. Each PCR plate included a non-template control. Two-step PCR thermal cycling conditions were utilized: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; stage 3, 95°C for 15 s; stage 4, 60°C for 1 min, with 40 replicates of stages 3 and 4. Quantification was carried out with the relative quantification method [96]. Briefly, the abundance of target genes is normalized to the average of the reference genes, and calculated relative to calibrator samples by $2^{-\Delta\Delta Ct}$, where C_t is the cycle number at which the fluorescence signal of the product crosses an arbitrary threshold set with exponential phase of the PCR, and $\Delta\Delta Ct = (Ct_{\text{target gene unknown sample}} - Ct_{\text{average of endogenous control genes unknown sample}}) - (Ct$

target gene calibrator sample − Ct average of endogenous control genes calibrator sample). Samples collected at 84 d were the calibrator samples.

Statistical Evaluation

Temporal oxylipid, mRNA (mean ΔCt), and FAME concentrations were evaluated in a mixed analysis of variance model, with cow as a random source of variance, the dependent variables repeated across time and within cow, and the model solved for the fixed effect of time. Covariance structures (compound symmetry, unstructured, or first order autoregressive) were selected according to Aikake's information criterion. Differences between least square means were calculated with Sidak's adjustment. Pearson correlations were calculated to measure associations between plasma oxylipid concentrations and leukocyte mRNA expression (23 oxylipids x 8 genes = 184 correlations), and between PBMC PUFA and plasma oxylipid concentrations (3 PUFA x 23 oxylipids = 69 correlations), and between PBMC PUFA concentrations and leukocyte mRNA expression (3 PUFA x 8 genes = 24 correlations). Mean ΔCt was multiplied by -1 prior to calculation of Pearson coefficients. Significance of correlation was adjusted for false discovery associated with multiple tests.

Results

Animal Production and Health

All cows remained free from clinical disease during the period of study. Mean (± standard deviation) 305 d mature equivalent milk production was 12,324 (± 2,518) kg. Mean (± standard error) plasma NEFA concentration in 3 randomly selected cows was significantly greater at 0 days (0.70 (± 0.15) mEq / L, $P < 0.0136$) than -14 days (0.08 (± 0.01) mEq / L) and 10 days (0.43 (± 0.09) mEq / L).

Plasma Oxylipid Profile

Plasma oxylipid concentration differed across the periparturient period for 3 of the 23 oxylipids measured (Figures 5 and 6). Early lactation (10 d) was associated with increased 13-HODE relative to prepartum. Samples at 84 d had greatest 9-HODE and 11-HETE concentrations. Plasma concentrations of 9-HODE were approximately 2-fold higher than 13-HODE. Both 9- and 13-HODE concentrations were approximately 10-fold higher than 11-HETE. No significant changes over time were observed in plasma concentrations of 20 oxylipids (Table 2). The limit of detection for oxylipids was 0.01 ng.

Leukocyte mRNA Expression

The inflammatory phenotype of peripheral leukocytes showed minor changes across the period of study. The expression of *15-LOX1* and 15-hydroxyprostaglandin dehydrogenase (*HPGD*) mRNA in peripheral leukocytes were decreased significantly at 10 d postpartum, relative to prepartum samples, and also relative to 84 d samples for *15-LOX1* (Figure 7). The expression of *IL-12B* mRNA was significantly increased at parturition and 10 d, relative to 28 d samples (Figure 8). No significant changes were observed in the gene expression of *IL-10*, inducible nitric oxide synthase 2 (*NOS2*), *IL-1β, TNF-α,* or *COX2* during the entire experimental period.

PBMC FAME Content

C18:2 n6 and C20:4 n6 FAMEs were consistently detected in PBMC, across all cows and sample periods. The concentrations of C18:2 n6 and C20:4 n6 were significantly increased at 10 d relative to prepartum samples (Figure 9). PBMC C20:5 n3 FAME was not detected in 20 of 35 PBMC samples. Mean (± standard error) sample mass of C20:5 n3 FAME was 899 (± 132) ng. Minimum mass of C20:5 n3 FAME standard utilized and detected was 4 ng. C22:6 n3 FAME was detected

in one sample therefore no statistical analysis for this FAME was performed. The limit of detection of C22:6 n3 FAME standard was 0.57 ng. Samples contained a chromatogram peak at the identical retention time as C22:6 n3 did in standards, but this peak was abundant in 239 amu, was not the mass spectra of C22:6 n3 nor typical of any methyl ester, and was not identified. C18:2 n6 was the only FAME of interest detected in cell free control samples (mean ± SE of 5 extraction batches = 3912 ± 860 ng).

Statistical Relationships

There were no significant correlations between C18:2 n6, C20:4 n6, or C20:5 n3 in PBMC and plasma oxylipids. Among the leukocyte genes assessed, *IL-12B*, *IL-1B*, *COX2*, and *NOS2* expression were associated with plasma oxylipids (Table 3). Expression of *HPGD* was associated with C20:5 n3 concentrations ($r = -0.75$, $p = 0.0052$).

Discussion

This study showed for the first time how concentrations of select pro- and anti-inflammatory plasma oxylipids change in dairy cows that successfully transitioned from late gestation to early lactation without clinical illness. Until now, only select bovine oxylipids were studied in physiological processes, such as luteolysis and maintenance of pregnancy [90, 149], and also in case-control studies of bovine pathology [126, 136], but little was known about the relationships between plasma oxylipids and peripheral leukocyte inflammatory responses. Leukocytes are a primary target for the actions of oxylipids so changes in PBMC inflammatory gene expression in the absence of clinical disease may be due to the actions of these potent lipid mediators. Results from this study revealed several notable features of the periparturient oxylipid profile. For example, hydroxyl forms of C18:2 n6 (13-HODE) was the most abundant plasma oxylipid

especially during early lactation. There are several proinflammatory effects of HODE, such as induction of adhesion molecule expression on human vascular tissue [56]. HODEs also were the most abundant oxylipids found in human atherosclerotic lesions [60] and therefore have been investigated as potential contributors to chronic, uncontrolled inflammatory conditions. In contrast, HODEs can be important activators of peroxisome proliferator activated receptors [104], which can lead to decreased expression of inflammation-based genes such as inducible nitric oxide synthase [128]. Thus, the current literature suggests that HODE could be pro- or anti-inflammatory oxylipids in different situations. The significance of enhanced HODE biosynthesis during the periparturient period on bovine inflammatory responses is not known. The possibility that HODEs may contribute to dysfunctional inflammatory reactions in early lactation cows with metabolic or infectious diseases would be a logical question to ask in future studies.

The enhanced expression of HODE in periparturient cows could be due to several factors including substrate availability. The substrate for HODE, C18:2 n6, increased in PBMC during early lactation (Figure 9). These findings are consistent with earlier studies that reported an increase in plasma and liver C18:2 n6 concentrations of recently calved dairy cows [4, 31]. Both i*n vivo* and *in vitro* studies support the contention that increased HODE biosynthesis is at least partly regulated by substrate availability. For example, increased human dietary C18:2 n6 intake is associated with increased plasma HODE [120]. Increased HODE biosynthesis was observed in cultured bovine endothelial cells exposed to a NEFA mixture with high C18:2 n6 content that is typically found in bovine plasma during the periparturient period [30]. Therefore, increased plasma HODE may be a reflection of increased tissue, leukocyte, or plasma contents of C18:2 n6. The tissue and cellular source of plasma HODES measured in this study were not assessed. Oxidation of C18:2 n6 may occur in leukocytes or other cell types by COX, LOX, epoxygenase, and non-

enzymatic oxidation. Previous studies showed increased *COX2* and *15-LOX1* mRNA and COX2 protein expression in bovine vascular endothelial cells in response to NEFA typical of those present in bovine plasma during the periparturient period [30]. Chemical inhibition of COX and 15-LOX enzymes, however, did not abrogate endothelial HODE biosynthesis from NEFA and suggests that non-enzymatic oxidation of plasma C18:2 n6 could contribute to endothelial cell production of plasma HODE [29]. Reduced transcript expression of leukocyte *15-LOX1* in this study also may suggest that there is a decreased capacity for the enzymatic oxidation of C18:2 n6 in early lactation. Identifying the primary cellular sources of HODE and the mechanism of oxidation could have future implications of how to not only regulate its biosynthesis, but also influence the relative contribution to inflammatory responses.

The ratios of HODE to OxoODE reveal changes to C18:2 n-6 metabolism in early lactation. Mean 13-HODE to 13-OxODE ratios (Figure 6d) indicate lesser abundance of the ketone derivative than hydroxyl derivative of C18:2 n-6 during the first weeks of lactation (ratio > 1.0) relative to prepartum, parturition, and 84 days (ratio < 1.0). Moreover, the range of both 13-HODE to 13-OxODE and 9-HODE to 9-OxoODE ratios (Figure 6c) were greatest in early lactation. HODE is the reduced product of HPODE, which is more stable and can be converted into OxoODE by dehydrogenation [120]. The extent of HPODE metabolism is directly associated with anti-inflammatory potential [56, 104], therefore increasing HODE to OxoODE ratios may indicate a change in the net effect of C18:2 n6 derived oxylipids on inflammatory pathways. If future studies prove the HODE to OxoODE ratio to be influential in bovine inflammatory responses, then the precise pathways by which HODES are degraded in cows should be investigated as a potential target of inflammatory disease intervention [62, 187].

Temporal changes in the plasma oxylipid profile can provide some insight as to how bovine

leukocytes may respond within that microenvironment. Whereas the HODEs increased during early lactation, it is important to also note those oxylipids that did not change concentration in this study. For example, the steady state biosynthesis of certain anti-inflammatory oxylipids could influence leukocyte phenotype when considered as a key component of the overall oxylipid profile. In this study, several potent anti-inflammatory oxylipids (PD1, RD1, RD2, and 7-MaR1) did not significantly change throughout the experimental period in the absence of clinical disease. Several recent studies documented the significance of these oxylipids in resolving inflammation and restoring tissue to normal function following infections [138, 140]. The precise role of these anti-inflammatory oxylipids on bovine leukocytes during the periparturient period is not known. The present study does, however, provide baseline information of the overall oxylipid profile in cows that successfully transition into lactation without succumbing to disease. Plasma oxylipids will likely differ by health state as oxylipid enzyme expression is influenced by disease [135].

Correlations between plasma oxylipids and peripheral leukocyte mRNA expression were conducted to assess the potential impact that certain pro-inflammatory and anti-inflammatory bovine oxylipids may have in the absence of clinical disease. In the present study, no dramatic changes were observed in several markers used previously to assess leukocyte pro-inflammatory responses during the periparturient period. This is in contrast to previous studies that documented significant cytokine gene expression in PBMC obtained from periparturient cows subjected to metabolic, environmental or microbial challenge [101, 152, 165]. The present study, however, used healthy animals and the results are consistent with previous reports noting no change in PBMC pro-inflammatory cytokine mRNA expression in periparturient cows that remain free of clinical disease [112]. Pearson coefficients did identify several correlations between oxylipids and PBMC gene expression although no clear conclusion can be drawn from the inconsistent findings

(Table 3). Although some oxylipids with known pro-inflammatory functions (PGE_2 and LTD_4) were positively correlated with pro-inflammatory gene expression as would be expected, there were several instances where pro-inflammatory oxylipids (TXB_2, 9-HODE, and 13-HODE) were negatively correlated with the pro-inflammatory markers (Table 3). One explanation is that cytokine expression and pro-inflammatory oxylipid biosynthesis may form negative feed-back loops with each other. Expression of epoxygenase enzymes, for example, can be suppressed by TNF-α [10, 11]. Another possibility is that certain oxylipids may be more effective in controlling leukocyte inflammatory responses than other lipid mediators within the profile. Without conducting more controlled cause-effect studies, it is not possible to determine which oxylipids may be the most potent in regulating PBMC gene responses. Based on the finding presented in this study, however, it is clear that the oxylipid network is complex and the relative impact on leukocyte responses during the periparturient period will likely be determined by the relative composition of the oxylipid profile.

In conclusion, this study showed for the first time how concentrations of a select group of oxylipids change during the periparturient period in healthy animals. The most abundant oxylipids during early lactation were those derived from C18:2 n6 and C20:4 n6 with significant increases in their respective hydroxyl products (HODE and 11-HETE) as lactation progressed. Although plasma oxylipids are likely derived from many cellular sources, an interesting observation in this study was that the significant increases in HODEs and 11-HETE corresponded to significant increases in PBMC content of their respective PUFA substrates (C18:2 n6 and C20:4 n6). These findings suggest that it may be feasible to manipulate oxylipid concentrations during the periparturient period by altering the dietary content of available PUFA that can be metabolized through various peroxidation pathways. Future studies that focus on how individual oxylipids may

directly impact the pro-inflammatory responses of bovine leukocytes would provide the foundation for targeted therapies used to optimize innate immune defenses during the periparturient period.

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CHAPTER 3

IMPACT OF INCREASED LINOLEIC ACID CONCENTRATION ON INFLAMMATORY MARKER EXPRESSION IN BOVINE MONOCYTES

Abstract

Incidence and severity of dairy cow disease is greatest in the periparturient period. Disease risk is increased with mobilization of adipose-derived fatty acids, such as linoleic acid, into plasma. Tissue and cellular linoleic acid concentrations are increased in early lactation, including within peripheral monocytes. Expression of proinflammatory markers by monocytes in the periparturient period was associated with incidence of some diseases. Enhanced monocyte inflammatory responses to endotoxin in the periparturient period were associated with severity of some diseases. Metabolism of linoleic acid into inflammatory mediators known as oxylipids impacts the incidence and severity of human disease but the impacts of increased linoleic acid on bovine monocyte phenotype and inflammatory responses are unknown. The hypothesis of this research was that increased bovine monocyte linoleic acid concentration enhances production of linoleic acid-derived oxylipids, induces a proinflammatory phenotype, and enhances inflammatory responses to bacterial endotoxin. Primary bovine monocytes and murine RAW 264.7 monocytes were cultured *in vitro* in 25 and 50 uM linoleic acid to emulate *in vivo* monocyte linoleic acid content in early lactation. Inflammatory phenotype and endotoxin responses, defined by cytokine transcript expression and measured by qPCR, were not influenced by monocyte content of linoleic acid. Oxylipid production was measured in cell culture media by liquid chromatography / mass spectrometry. Monocyte enrichment with linoleic showed increased production of hydroxyoctadecadienoic and oxooctadecadienoic acids, and no change in arachidonic or n-3 fatty acid-derived oxylipids. In conclusion, monocyte inflammatory markers were not influenced by cell linoleic acid concentration. Future studies are required to assess the specific impact of individual linoleic acid-derived oxylipids on monocyte inflammatory pathways.

Keywords: monocyte; linoleic acid; hydroxyoctadecadienoic acid; oxooctadecadienoic acid; inflammation; bovine; oxylipid

Introduction

Incidence and severity of several forms of clinical disease in dairy cows peak in the periparturient period [117]. The most common diseases are mastitis and metritis and incidence of these peaks within the first 2 weeks of lactation [37, 59, 114]. Despite previous research into disease prevention and pathogenesis, periparturient disease continues to have negative impacts on farm economics [45] and animal welfare [146]. However, previous research has identified important risk factors that are related to host inflammatory responses.

Epidemiological studies showed that mobilization of adipose-derived lipid into plasma as non-esterified fatty acids (NEFA) during the periparturient period increased risk of several diseases, including metritis [24, 77, 137]. Mobilization of adipose-derived lipid is an adaptive response to increased energy requirements for lactation [32]. Studies have identified potential mechanisms by which NEFA may influence disease risk. For example, early lactation plasma had increased concentrations of linoleic acid (LA, C18:2 n6), and this change in plasma fatty acid was associated with increased LA concentrations in peripheral mononuclear leukocytes (PBMC) [31].

LA is associated with increased incidence and severity of some human diseases with inflammatory based pathology. For example, the risk of chronic inflammatory vascular conditions, such as coronary artery disease, was associated with increased dietary LA [121]. Rodent models of inflammatory bowel disease showed increased disease severity with increasing dietary LA [171]. *In vitro* models of human breast cancer demonstrated pro-tumorigenic effects of LA that involved inflammatory pathways [100]. These associations between LA and inflammatory based pathology in humans and rodents suggest that increased LA concentration in PBMC from early lactation cows could influence monocyte inflammatory phenotype and responses.

Monocytes are critical cells in innate immune responses during diseases such as mastitis and metritis. Previous studies identified changes in some monocyte inflammatory responses during the periparturient period. For example, similar doses of lipopolysaccharide (LPS) elicited greater tumor necrosis factor (TNF)-α expression in monocytes from early lactation cows compared to monocytes from mid-lactation cows [152]. TNF-α influences multiple inflammatory processes, including fever [40]. Subsequent research in cows demonstrated a greater febrile response with intramammary *Escherichia coli* challenge in early lactation relative to mid-lactation [145]. Therefore some clinical signs of gram negative mastitis are more severe in early lactation than mid-lactation and this difference in disease severity is partly due to an exacerbated monocyte inflammatory response.

Subsequent research showed that the inflammatory phenotype of monocytes from periparturient cows was predictive of future disease incidence. For example, monocyte *TNF-α* expression was greater in the first two weeks of lactation in cows that went on to develop clinical metritis, than in cows that remained healthy[58]. Therefore *in vivo* expression of some proinflammatory cytokines are predictive of future disease in cows. It is not clear why periparturient monocyte phenotype differs between groups of cows that are more or less likely to develop clinical disease, or why periparturient monocyte inflammatory responses to LPS differ by stage of lactation. An objective of these studies was to evaluate the impact of increased cell LA concentration on monocyte phenotype and inflammatory responses. Previous research of human diseases suggests that metabolism of LA into oxylipids could be involved with changes in phenotype and inflammatory responses [71].

Oxylipids are mediators of inflammatory processes derived from some polyunsaturated

fatty acids (PUFA). Major substrates are LA, arachidonic (AA, C20:4 n6), eicosapentaenoic (EPA, C20:5 n3), and docosahexaenoic (DHA, C20:6 n3) acids that are stored within cell membranes and released during inflammatory responses [108]. While many EPA and DHA-derived oxylipids have anti-inflammatory effects [156], AA and LA-derived oxylipids are balanced between proinflammatory and anti-inflammatory effects [123]. For example, the LA-peroxide, 13-hydroperoxyoctadecadienoic acid (HPODE), stimulated proinflammatory marker expression in human vascular smooth muscle cells [48]. Hydroxyoctadecadienoic acid (HODE) stimulated proinflammatory cytokine production in human mononuclear leukocytes [83] but attenuated clinical signs of inflammation in a skin disease model [20]. The HODE product, oxooctadecadienoic acid (OxoODE) reduced TNF-α stimulated inflammatory responses in epithelial cells [5]. The net effect of the milieu of oxylipids on monocyte inflammatory phenotype and responses depends on the relative concentrations, receptor affinity, and efficacy of each oxylipid [104, 107]. Production of many oxylipids is regulated by the supply of PUFA substrate within cells [108, 118]. However the impact of increased LA in bovine monocytes on oxylipid production is not known. An objective of these studies was to describe the profile of oxylipids produced by bovine monocytes in response to increased LA. The overall hypothesis of this research was that increased monocyte LA concentration enhances production of LA-derived oxylipids, induces a pro-inflammatory phenotype, and enhances inflammatory responses to bacterial endotoxin.

Materials and Methods

Cell Culture

An *in vitro* model was developed to emulate PBMC concentration of LA in early lactation. The

model utilized PBMC from mid-lactation cows that had lower PBMC concentrations than early-lactation cows (Figure 10). PBMC were cultured in media containing various concentrations of supplementary LA (6.25, 25, and 50 uM). LA was mixed in ethanol, diluted in media (0.01%), and filtered (0.22 um). PBMC were utilized immediately after extraction from bovine whole blood by Ficoll-Paque Plus gradient (GE Healthcare, Waukesha, WI), as previously described [31]. All animal use and care was preapproved by Michigan State University Animal Care and Use Committee.

LA stimulated oxylipid biosynthesis and cytokine transcript expression were assessed in primary bovine monocytes and murine-derived monocyte-like macrophages (RAW 264.7, ATCC, Manassas VA). Primary monocytes were isolated from PBMC by plate adhesion, as previously described [110]. PBMC were seeded immediately after isolation from blood, and monocytes used immediately after isolation from PBMC. Ethanol (0.01%) associated oxylipid biosynthesis and cytokine transcript expression was assessed to control for non-specific effects of the fatty acid vehicle. Arachidonic acid (50 uM) was utilized to control for non-specific PUFA effects.

RPMI media was utilized, with 5% (RAW 264.7) or 10% (primary cells) fetal bovine serum, L-glutamine (Life Technologies, Grand Island NY), and 1X antibiotic/antimycotic (Life Technologies). Cells were incubated at 37°C with 5% carbon dioxide and the start of experiments defined as 0 hour or when cells were first exposed to LA. Culture within LA mixtures occurred from hour 0 to 12 (PBMC), hour 0 to 16 (primary monocytes), and 0 to 13 (RAW 264.7). LPS (25 ng / mL, Sigma-Aldrich, St. Louis MO) was added to primary monocyte media from hour 12 to 16, Kdo2 Lipid A (100 ng / mL, Avanti Polar Lipids, Alabaster AL) to RAW 264.7 cell media from hour 12 to 13. Viability was measured in PBMC and monocytes by trypan blue exclusion under light microscopy.

Flow cytometry

Enrichment of monocytes from PBMC was assessed by fluorescent antibody staining and flow cytometry methods as previously described [110]. Murine-derived primary monoclonal antibodies against bovine CD172a (monocyte/granulocyte, 1:100, v/v), CD14 (monocyte, 1:50, v/v), CD3 (T-cell receptor, 1:100, v/v), and CD18 (integrin beta-2, negative control, 1:100, v/v, VMRD, Pullman WA) and secondary goat anti-mouse antibody (1:2000, Life Technologies) were utilized. Results were corrected for non-specific secondary antibody binding.

PBMC Fatty Acid Methyl Ester Quantification

Lipid Extraction

Adherent and non-adherent PBMC were collected by centrifugation of media and gentle plate scraping, rinsed in 1X PBS, pelletized, then frozen in liquid nitrogen and stored at -80°C until completion of sample collections. Total lipid was extracted using methanol and chloroform, based on published methods [16, 55] and used previously by our group [122]. Contaminant LA was measured in cell-free samples, concurrent to PBMC samples.

Derivation

Sample lipids were saponified using 1.0 mL freshly made 0.5 M potassium hydroxide in methanol, and heat (100° C for 10 min), as previously reported [122]. After cooling to 4° C, derivation of PUFA to methyl esters (FAME) was performed under nitrogen gas, utilizing 3.0 mL fresh 20% v/v methanolic hydrochloric acid [115], and heat (90° C for 16 h). FAMES were then extracted with 6.0 mL n-hexanes by cetrifugation (524 g for 5 min at 8° C). Samples were then vacuum dried, resuspended in 35 uL n-hexanes, and transferred to a GC microinsert vial.

Gas Chromatography/Mass Spectrometry (GC/MS)

The gas chromatograph was Agilent Technologies model 6890N (Agilent Technologies, Santa Clara, CA), fitted with a DB-23 column (30 m x 0.25 mm internal diameter x 0.25 um film thickness, Agilent Technologies). Inlet temperature was 240° C. The method utilized helium gas at 8.73 psi, a 20:1 split ratio, and a combined flow rate of 25.9 mL / min. Column temperature was optimized and set at 50° C for 1 min, then increased at 25° C / min to 175° C, then increased at 4° C / min to 240° C and held constant for 5 min. Total run time was 27.25 min. Injection volume was 1.0 uL. A pre- and post-injection delay of 1 s was applied.

The mass spectrometer was Agilent Technologies model 5975B inert XLMSD (Agilent Technologies). Transfer line, source, and quadrupole temperatures were 240°, 230°, and 150°C, respectively. Detection criteria were 40 to 400 amu, and a 3 min solvent delay was applied. Total ion chromatograms and mass spectra were generated using Agilent ChemStation software (Agilent Technologies). A 37-component FAME standard mix was utilized to determine retention times (Sigma-Aldrich). Analytes were identified with at least 90% similarity between analyte and reference mass spectra (National Institute of Standards and Technology). Total ion chromatograms were created for FAMES of C13:0, C15:0, and C18:2 n6. The within-assay and between-assay coefficient of variation for standard grade C18:2 n6 FAME was calculated to be 5 and 15 %, respectively.

FAME Quantification

Sample internal standards were added prior to extraction (C13:0 fatty acid) and following derivation (C15:0 FAME). Calibration curves for C18:2 n6 and C13:0 FAMEs were created using freshly prepared standard-grade FAMES, analyzed before and after the samples. Standard dilutions were 0.7 fold in n-hexanes, and concentrations extended 2 dilutions above the maximum sample concentration and below the minimum sample concentration. Calibration equations were

calculated by linear regression of the known standard concentration against the ratio of analyte to C15:0 FAME (R^2 > 0.975). This ratio was calculated from analyte and C15:0 FAME chromatogram intensities.

Analyte concentration in samples was calculated by comparing the intensity ratio of analyte to C15:0 FAME in samples to that of the linear regression equation from calibration. Analyte concentrations in samples were corrected for methylation efficiency and FAME abundance in cell free sample. Methylation efficiency was calculated by comparing the C13:0 FAME abundance in samples to the expected abundance from C13:0 fatty acid internal standard. Treatment groups were normalized by cell count.

Plasma Oxylipid Quantification

Oxylipid Extraction

Solid phase extraction methods were identical to those previously used by our group [122]. Deuterated internal standards were utilized (leukotriene B_4-d4, thromboxane B2-d4, prostaglandin (PG) $F_{2\alpha}$-d4, PGE_2-d4, PGD_2-d4, 13(S)-HODE-d4, 6-keto $PGF_{1\alpha}$-d4, 12(S)-hydroxyeicosatetraenoic acid-d8 (12(S)-HETE-d8), and 15(S)-HETE-d8, Cayman Chemical, Ann Arbor MI).

Liquid Chromatography/Mass Spectrometry (LC/MS)

Oxylipids were measured using LC/MS as previously reported [122]. Chiral HODE isomers were separated using a Lux 3u Cellulose-1, 150mm x 2 mm column (Phenomenex, Torrance, CA) held at 35º C. Isocratic elution at 0.225 mL / min lasted 30 m and utilized acetonitrile:methanol:water (39/3/58, v/v/v), all with 0.1% acetic acid.

Abundance of PGD_2, PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, 8-iso-$PGF_{1\alpha}$, thromboxane B2,

leukotriene D_4, resolvin D1 and resolvin D2, protectin D1, 7-maresin1, 5-, 8-, 11-, 12-, and 15-HETE, 9- and 13-HODE, 9- and 13-OxoODE were quantified in culture media. Oxylipids were identified in samples by matching their deprotonated (i.e., [M-H]-) m/z values and LC retention times with those of pure standards. Oxylipids were quantified relative to internal standard abundance, and calibration against standard curves. Treatment groups were normalized by sample volume, DNA mass (Quant-iT dsDNA broad range, Life Technologies), and protein mass (Bradford assay).

Monocyte mRNA Quantification by qPCR

Total RNA was extracted from monocytes for quantification of mRNA using the RNeasy Mini Kit (Qiagen, Valencia, CA) with DNase. cDNA was synthesized using the High Capacity cDNA reverse transcriptase kit with RNase inhibitor (Applied Biosystems, Foster City, CA). RNA concentration was measured by Nanodrop (Thermo Fisher Scientific, Waltham, MA) and integrity by Agilent Bioanalyzer (Santa Clara, CA). Primers were derived from the *Bos taurus* and *Mus musculus* genome (Table 4). Primary monocyte cDNA was amplified using TaqMan PreAmp Kit (Applied Biosystems). The qPCR was conducted as previously reported [29]. Briefly, PCR was performed in triplicate, using 20 µL reaction mixture per replicate of primary monocyte cDNA, comprising 11.7 µL TaqMan Gene Expression PCR Master Mix (2x, Applied Biosystems), 1.2 µL (20x) TaqMan Gene Expression Assay Mix (Applied Biosystems), 5 µL amplified cDNA, and 2.1 µL nuclease-free water. RAW 264.7 cDNA was not amplified and PCR performed in triplicate using 10 uL reaction mixture per replicate, comprising 5 µL TaqMan Gene Expression PCR Master Mix (2x, Applied Biosystems), 0.5 µL (20x) TaqMan Gene Expression Assay Mix (Applied Biosystems), 0.75 µL cDNA, and 3.75 µL nuclease-free water. Pre-designed (20x) TaqMan Gene Expression Assays from Applied Biosystems for bovine *TBP*, *PGK1*, and *ACTB* and murine

GUSB, *PGK1*, and *TBP* were selected as reference genes. Each PCR plate included a non-template control. Two-step PCR thermal cycling conditions were utilized: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; stage 3, 95°C for 15 s; stage 4, 60°C for 1 min, with 40 replicates of stages 3 and 4. Quantification was carried out with the relative quantification method [96]. Briefly, the abundance of target genes is normalized to the average of the reference genes, and calculated relative to calibrator samples by $2^{-\Delta\Delta Ct}$, where Ct is the cycle number at which the fluorescence signal of the product crosses an arbitrary threshold set with exponential phase of the PCR, and $\Delta\Delta Ct = (Ct_{\text{target gene unknown sample}} - Ct_{\text{average of endogenous control genes unknown sample}}) - (Ct_{\text{target gene calibrator sample}} - Ct_{\text{average of endogenous control genes calibrator sample}})$.

Statistical Evaluation

Oxylipid, mRNA (mean ΔCt), and FAME concentrations were evaluated in a mixed analysis of variance model, with RAW 264.7 cell clone or primary monocyte donor as random sources of variance, and the model solved for the fixed effects of cell treatments. Differences between least square means were calculated with Sidak's adjustment.

Results

Cell culture

Mean viability of PBMC after Ficoll separation and 12 h culture ranged from 89% (media control group) to 95% (6.25 and 50 uM LA, n = 3). Mean viability of primary monocytes after enrichment procedures and 16 h culture ranged from 81% (50 uM AA, 25 ng / mL LPS) to 94 % (media control group, n = 3). Proportion of total monocytes positive for CD172a, CD14, CD3, and CD18 were 91%, 79%, 3%, and 4% respectively. Culture of PBMC in media with 25 uM or 50 uM supplementary LA increased cell LA concentrations to levels similar to peak *in vivo* concentrations

[122]. Results of statistical tests were similar for oxylipid samples normalized by DNA, protein, and volume. Oxylipids concentrations are reported as ng / mL media.

Transcriptional responses to increased cell LA concentration

Transcript expression of *TNF-α*, *IL-10*, *IL-12B*, 15-lipoxygenase (*LOX*) 1, and cyclooxygenase (*COX*) 2 in primary monocytes and *TNF-α*, *IL10*, *IL-1β*, *IL-6*, *15-LOX1*, and *COX2* in RAW 264.7 cells were unchanged with increasing cell LA ($p > 0.05$, $n = 3$). In contrast, 50 uM AA increased *TNF-α* expression in primary monocytes and *IL-1β* in RAW 264.7 cells ($p < 0.05$, Figure 11). Ethanol had no effect on transcript expression at the concentration used (0.01 %). Expression of murine *IL-12B* mRNA was not detected by the utilized methods.

LA-derived oxylipid production with increased cell LA concentration

Production of 9- and 13-HODE and 9- and 13-OxoODE in primary monocyte culture media increased with 25 uM and 50 uM LA supplementation (Figure 12). Concentrations of 9-OxoODE were approximately twice that of 13-OxODE under similar culture conditions, although 9- and 13-HODE concentrations did not differ. 9-OxODE was consistently more abundant than 9-HODE with LA supplementation of 25 uM and 50 uM. Ratios of 9-, 13-, and total HODE / OxoODE did not change significantly with LA supplementation ($p > 0.05$). The HODE chiral ratio (R / S) increased with 6.25 uM and 25 uM LA supplementation (Figure 13). Production of 9- and 13-HODE and 9-OxoODE also increased in RAW 264.7 cell culture media containing 50 uM supplementary LA (Figure 14). Ratios of 9-, 13-, and total HODE / OxoODE did not change significantly in RAW 264.7 cell culture media with LA supplementation ($p > 0.05$).

Monocyte production of PGF_{2a}, thromboxane B_2, 5-, 11-, and 15-HETE increased with AA supplementation ($p < 0.05$, $n= 3$), but was not affected by LA supplementation or ethanol. Protectin

D1, 7-maresin1, and leukotriene D_4 production was independent of fatty acid treatment.

Proinflammatory challenge with increased cell LA concentration

Primary monocyte transcript expression of *TNF-α, IL-10, IL-12B,* and *COX2* were induced by LPS (25 ng / mL, $p < 0.05$, $n = 3$), but LPS induced expression was not modified by 6.25, 25, or 50 uM LA, 50 uM AA, or ethanol ($p > 0.05$, $n = 3$). Expression of *15-LOX1* did not differ significantly between LPS, LA, AA, or ethanol cell treatment groups.

LA-derived oxylipid production was not influenced by LPS stimulation of monocytes enriched with 0 or 25 uM LA, but 9-HODE production increased with LPS stimulation in monocytes enriched with 50 uM LA and 13-HODE production decreased with LPS stimulation in monocytes enriched with 6.25 uM LA ($p < 0.05$, $n = 3$). HODE and OxoODE production response to LA was similar between LPS stimulated cell culture media and unstimulated cell culture media. However, the ratio of 9-HODE / 9-OxoODE in culture media from LPS stimulated monocytes was significantly less with 25 uM or 50 uM LA supplementation than 0 uM (Figure 15, $p < 0.05$, $n = 3$). Influence of fatty acids on production of other oxylipids was similar to unstimulated monocytes, except production of PGF_{2a} and 5-HETE was not increased by AA in LPS stimulated monocytes. ($p > 0.05$, $n = 3$).

Discussion

Despite previous reviews [27, 113], *in vivo* [121, 134], and *in vitro* studies [39, 66, 166, 167, 189] that suggest increasing concentrations of LA enhanced the risk of some diseases by exacerbating inflammatory responses, results showed that monocyte LA concentration was not associated with inflammatory phenotype in either primary bovine monocytes or RAW 264.7 monocyte-like macrophages and did not impact LPS-stimulated inflammatory responses in primary bovine

monocytes. These experiments are the first, to the best of our knowledge, which measure the impact of cell LA concentration on monocyte inflammatory markers of any type. Previous ruminant studies utilized primary ovine lymphocytes and reported no impact of LA (0.8 – 100 uM) on antibody secretion [87]. Some previous monocyte studies focused on conjugated linoleic acid effects but also utilized single concentrations of LA as a fatty acid treatment control. For instance, interferon-γ stimulated RAW 264.7 cells expressed less TNF-α transcript and protein when cells were cultured in 200 uM LA [190]. According to these results (Figure 10), doses of this magnitude could possibly exceed bovine physiological concentrations. Other studies utilized primary human monocytes with 30 uM LA and failed to change activity of the critical pro-inflammatory transcription factor, nuclear factor-κβ [158].

This study defined inflammatory phenotype at the transcript level because previous studies showed that the selected cytokines, including TNF-α and IL-1β, are transcriptionally regulated within the time frames of the cell culture experiments reported here [41, 52]. The rationale for gene selection was to describe a profile of pro- and anti-inflammatory cytokines involved in early inflammatory responses during several types of periparturient bovine disease [58, 145] or murine models of fatty acid-induced inflammation [171]. Some cytokines, such as IL-12B, TNF-α, and IL-1β, were also selected because expression was associated with *in vivo* oxylipid profiles in normal and diseased cows and mice [122, 164]. Murine-derived RAW 264.7 monocytes are used to model fatty acid metabolism in some human diseases, such as sepsis [108], but the impact of LA concentration on such models was not previously investigated. Hence the results from these studies enhance understanding of the impact of LA on monocyte inflammatory pathways in humans, in addition to ruminant species.

The second objective was to describe the profile of oxylipids produced by bovine monocytes in response to increased cell LA concentrations. Production of 9- and 13-HODE and 9- and 13-OxoODE increased with LA concentration in primary bovine and RAW 264.7 monocytes. This suggests that monocyte production of 13-HODE may increase in periparturient cows as cell LA concentrations increase and could, therefore, contribute to increased plasma 13-HODE concentrations that were recently described in early lactation [122]. The magnitude of production responses to LA differed between bovine and murine cells (Figures 12 and 14), but similar LA-dose effects were seen across species. The ratio of HODE to OxoODE did not differ by cell LA concentration in bovine primary or RAW 264.7 monocytes, and also did not change *in vivo* during the periparturient period (Figure 6). Production of other oxylipids, such as HETES and anti-inflammatory ω-3 derived oxylipids, were not influenced by monocyte LA content, and also did not change *in vivo* [122]. Therefore, the oxylipid profiles in primary bovine monocyte and murine monocyte culture media have several similar features to the periparturient plasma oxylipid profile. However, *in vitro* production of some oxylipids, such as leukotrienes, may not completely mimic *in vivo* production as biosynthesis of some oxylipids is dependent on monocyte-endothelial cell interaction [105].

The oxylipid profile associated with increasing LA concentration in monocytes was further defined by measurement of the ratio of (R) to (S)-HODE chiral isomers. Previous research showed non-enzymatic oxidation of LA produced equal proportions of (R) and (S) isomers [186]. Hence increased (R) / (S)-HODE ratios associated with increasing primary monocyte LA concentration (Figure 13) could indicate changes in enzymatic oxidation. Transcript expression of COX2 and 15-LOX1 was not influenced by cell LA concentration ($p > 0.05$, $n = 3$), but protein expression and post translational regulation were not evaluated. These experiments were designed to describe

the profile of oxylipids produced in response to increased monocyte availability of LA but were not designed to investigate the specific pathway by which the metabolites were derived. Therefore, specific enzyme pathways by which metabolites are produced in these experiments cannot be determined.

Only production of LA-derived oxylipids changed with increasing cell LA concentration. HODES and OxoODES both increased, but how either individual oxylipids impact the phenotype or inflammatory responses of monocytes cannot be determined from these studies. Future studies should investigate the individual effects of HODES and OxoODES in bovine monocytes, as previous research suggests they have opposing effects on inflammatory phenotype that could potentially be counteractive when present in similar concentrations [5, 83]. Future studies could, for example, utilize purified oxylipids, such as 13-HODE, in primary bovine monocyte culture, and assess time and dose related effects on inflammatory phenotype.

Oxylipid production in LPS-stimulated monocytes was largely similar to production in unstimulated monocytes. However, 9-HODE production was greater in LPS-stimulated monocytes (50 uM LA, $p < 0.05$, $n = 3$). Expression of some oxylipid producing enzymes, such as *COX2*, was increased by LPS in this and previous studies [72]. COX2 is one of the enzymes that metabolizes LA to HPODES [68], therefore could be involved with increased 9-HODE production in the LPS-stimulated monocytes, although the results of these experiments are not definitive.

In conclusion, the results showed that increasing LA concentration within bovine and murine monocytes did not impact inflammatory phenotype and did not influence LPS-stimulated inflammatory responses. Monocyte production of 9- and 13-HODE and 9- and 13-OxoODE increased with increasing cell LA concentration, as did the ratio of (R)-HODE / (S)-HODE chiral

isomers. Future studies are required to assess the specific impact of individual LA-derived oxylipids on monocyte inflammatory pathways.

Acknowledgements

The author acknowledges the resources and staff of the Michigan State University Research Technology Support Facility Mass Spectrometry Core, especially Drs. Dan Jones and Scott Smith. The author also acknowledges Jeff Gandy and Dr. Edward Kabara for their assistance. This work was funded, in part, through a grant (2012-67011-20019) from the Agriculture and Food Research Initiative Competitive Grants Programs of the USDA National Institute for Food and Agriculture and by an endowment from the Matilda R. Wilson Fund (Detroit, MI, USA).

CHAPTER 4

CONCLUSIONS

Plasma oxylipid profile changes across the periparturient period of dairy cattle

The objective of Chapter 4 is to describe significance of the results and new gaps in knowledge. Chapter 2 shows that the oxylipid profile of dairy cows is dynamic across the periparturient period. Previous studies showed that plasma content of non-esterified fatty acids (NEFA) are also dynamic across the periparturient period [24, 31]. Changes in circulating NEFA could influence the fatty acid pool available for oxylipid production within plasma, cells, and organs [123]. In fact, peripheral blood mononuclear cell (PBMC) concentrations of linoleic acid (LA) are increased in early lactation relative to prepartum concentrations [31]. Changes in polyunsaturated fatty acid (PUFA) composition of murine monocytes impacts oxylipid production [108]. The results from Chapter 3 indicate that a consequence of bovine monocyte enrichment with LA is increased production of 9- and 13-hydroxyoctadecadienoic (HODE) acids. Circulating monocytes may secrete oxylipids such as HODES in response to increased supply of LA, which could ultimately affect plasma HODE concentrations. Previous *in vitro* studies also showed enhanced HODE production by bovine vascular endothelial cells in response to a mixture of NEFA that mimicked periparturient plasma [30]. Therefore, monocytes, endothelial cells and possibly other cell populations should be considered as potential contributors to plasma HODES. Previous human studies showed that plasma oxylipid substrate was influenced by dietary fatty acid composition [120]. Although periparturient dairy cows usually experience a diet change at the onset of lactation, the potential impact of this on plasma fatty acids and oxylipid production has not been researched.

Periparturient changes in plasma oxylipid concentration could be influenced by changes in oxylipid enzyme expression and activity in various cell types. Previous research showed increased 15-lipoxygenase transcript expression in bovine mammary tissue in early lactation [3]. Cyclooxygenase-2 transcript and protein expression were increased in bovine vascular endothelial

cell culture in response to NEFA that mimicked periparturient plasma [30]. Chapter 2 showed decreased 15-lipoxygenase and 15-hydroxyprostaglandin dehydrogenase transcript expression in peripheral leukocytes which could impact oxylipid production and secretion into plasma. Although enzyme activity in mammary tissue, peripheral leukocytes, vascular endothelium, and other sites, could result in biosynthesis of oxylipids that are secreted into plasma, the relative rates of oxylipid biosynthesis in each cell type is unclear.

Non-enzymatic peroxidation of plasma NEFA could contribute to some oxylipid changes in the periparturient period. Previous research showed that early lactation plasma has increased oxidative capacity [12] and increased concentration of LA [31]. Research in other species showed that PUFA readily react with free-radicals to produce hydroperoxy-type oxylipids [187]. Therefore, non-enzymatic peroxidation of plasma LA should be investigated as a potential cause of increased plasma HODE in early lactation. Should non-enzymatic lipid peroxidation occur in bovine plasma or cells, the influence of antioxidant pharmaceuticals or nutraceuticals on oxylipid production should be evaluated in periparturient cows.

Previous studies indicate that incidence and severity of disease are greatest in the periparturient period [117, 145]. Relationships between periparturient disease and the oxylipid profile are not known. Recent mouse studies showed that the oxylipid profile was associated with the severity of disease after viral challenge but similar studies have not been conducted in cows [164]. Bovine case-control or challenge studies could be useful in describing the oxylipid profile associated with disease. Once the oxylipids that are associated with disease in cows are identified, oxylipids could be used as markers that predict future disease incidents or trigger early intervention strategies.

Plasma oxylipids are associated with leukocyte transcript

Specific components of the bovine oxylipid profile that potentially influence periparturient disease incidence and severity are not known. Previous *in vitro* research showed that some oxylipids do influence monocyte and endothelial cell inflammatory transcript and protein expression in humans and cows [56, 83, 155]. However, the effects of specific oxylipids that changed concentration during the periparturient period, or were associated with leukocyte transcript expression in Chapter 2, have not been evaluated in cows. Future studies of bovine monocytes that use controlled *in vitro* exposure to oxylipids will enhance understanding of the correlations presented in Chapter 2. Having identified the specific oxylipids that are relevant to periparturient health, subsequent research should identify how such oxylipids are produced or exert such effects so that disease intervention strategies can be implemented. Oxylipid effects in some human and animal diseases are abrogated by inhibitors of oxylipid receptors [92] or steroidal and non-steroidal anti-inflammatory drugs that affect expression and activity of oxylipid producing enzymes [109, 139, 143]. Antioxidants are used to reduce free-radical mediated production of oxylipids [187]. Production of some oxylipids is controlled by increasing or decreasing polyunsaturated NEFA supply [108, 118, 120]. Omega-3 NEFA supplementation, for example, increased production of some ω-3 and ω-6 derived anti-inflammatory oxylipids in bovine endothelial cells [29]. The same study showed that ω-3 fatty acids abrogated inflammatory responses induced by a NEFA mixture that mimicked periparturient plasma [29]. Which intervention strategies are utilized in periparturient cows will be determined after the biological effects and biosynthetic pathways of the periparturient oxylipid profile have been identified.

Cellular linoleic acid concentration does not impact monocyte inflammatory phenotype

There are a preponderance of reviews [27, 113], *in vivo* [121, 134], and *in vitro* studies [39, 66, 166, 167, 189] that suggest increasing concentrations of LA enhanced the risk of some diseases by exacerbating inflammatory responses. Peripheral blood mononuclear cells from early lactation cows were recently reported to have increased LA content relative to cells from prepartum cows [31]. Peripheral blood mononuclear cells from early lactation cows were shown to have exacerbated inflammatory responses to lipopolysaccharide (LPS) [152]. Until now, the relationship between increased LA and LPS-induced inflammatory responses in PBMC was unknown. Chapter 3 shows that the bovine monocyte inflammatory cytokine response to LPS is not associated with cellular concentration of LA. Although previous research showed pro-inflammatory effects of HODES in human monocytes [83], enhanced HODE production in this study was not associated with changes in cytokine transcript. However, Chapter 3 transcript expression must be interpreted in the context of all LA-derived metabolites. For example, oxooctadecadienoic acid (OxoODE) production also increased in response to LA enrichment. Human and mouse cell culture studies indicate that OxoODEs are ligands of nuclear receptors including peroxisome proliferator activated receptor (PPAR)-γ [104]. Activation of PPAR-γ can lead to inhibition of nuclear factor–κβ mediated transcript responses, such as those induced by LPS [26]. Cytokine transcript of bovine monocytes could be regulated by balanced production of proinflammatory HODEs and anti-inflammatory OxoODEs, but further research is required to confirm this.

Summary of strengths and limitations

Several features of the experiments are novel and advance knowledge of bovine physiology. For example, this dissertation is the first report of quantitative assessment of PUFA in ruminant cells

by gas chromatography/mass spectrometry. Quantification by these methods could be superior to qualitative assessment when samples differ in total lipid content. Total lipid in monocytes and macrophages increases in some pathophysiological states [99], so quantitative assessment of PUFA is important for monocyte studies, such as those in Chapter 2 and 3. A disadvantage of quantitative assessment, compared to qualitative assessment, is that not all fatty acids are measured [108]. Qualitative chromatography, in contrast, *requires* that all chromatogram peaks are identified [127]. Disadvantages such as this can be overcome in quantitative assessment by creating calibration curves for the complete fatty acid profile. However, these calibrations were not required for Chapter 2 and 3 objectives.

Chapter 2 provides the first report of changes in monocyte AA concentration during the periparturient period. Arachidonic acid produces oxylipids that were proinflammatory in bovine endothelial cell studies [155], but oxylipid effects have not been evaluated in bovine monocytes. Additionally, the impact of bovine monocyte AA concentration on inflammatory phenotype has not been evaluated. AA was used in Chapter 3 to control for the non-specific effects of PUFA and results showed that AA directly induced inflammation in monocytes. However, the significance of these results is unclear because the *in vitro* AA dose required to mimic *in vivo* AA concentrations was not calculated. Subsequent experiments should address potential pro-inflammatory effects of AA at specific concentrations present in periparturient monocytes.

Chapter 2 also showed, for the first time, simultaneous changes in LA and 13-HODE, and relationships between 13-HODE and inflammatory phenotype of monocytes from dairy cows that successfully transitioned into lactation. Subsequent studies were conducted *in vitro* to identify the potential impact of monocyte LA concentration on 13-HODE production and inflammatory phenotype. Results showed that increasing monocyte LA concentrations resulted in increased 13-

HODE production and, therefore, it is likely that monocytes are the source of some plasma 13-HODE *in vivo*.

Linoleic acid did not induce inflammation in bovine monocytes or modify the monocyte inflammatory response to LPS. These results are conclusive within the context of the experimental conditions, and how these conditions relate to *in vivo* conditions impacts the significance of these results to dairy cow health. Additional *in vitro* modeling of the complexity of periparturient physiology would enhance the significance of results. Reactive oxygen metabolites, for example, are more abundant in plasma after parturition compared to prepartum [12]. Such conditions could be mimicked *in vitro* by inducing oxidative stress, and could enhance understanding of LA peroxidation by non-enzymatic means during the periparturient period. Also, the complexity of paracrine mediated oxylipid production could be lost in the current *in vitro* model. Endothelial-monocyte co-culture experiments could enhance understanding of oxylipid biosynthesis in periparturient dairy cows that is mediated by interactions between these cell types [105].

APPENDICES

Appendix 1. Authorization to publish Chapter 2 in Dissertation.

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March 10, 2014

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Sincerely,

Susan Pollock
Managing Editor
Journal of Dairy Science

Appendix 2. Tables**.**

Table 1. Gene targets for quantitative PCR in Chapter 2.

Gene[1]	Reference Sequence[2]	TaqMan Assay ID[3]
IL-10	NM_174088.1	Bt03212724_m1
IL-12B	NM_174356.1	Bt03213923_m1
NOS2	NM_001076799.1	Bt03249598_m1
IL-1β	NM_174093.1	Bt03212741_m1
TNF-α	NM_173966.3	Bt03259156_m1
15-LOX1	NM_174501.2	Bt03214775_m1
COX2	NM_174445.2	Bt03214492_m1
HPGD	NM_001034419.2	Bt03226254_m1
TBP[4]	NM_001075742.1	Bt03241946_m1
PGK1[4]	NM_001034299.1	Bt03225857_m1
ACTB[4]	NM_173979.3	Bt03279174_g1

[1]*NOS2* = inducible nitric oxide synthase 2; *TNF-α* = tumor necrosis factor-α; *15-LOX1* = 15-lipoxygenase1; *COX2* = cyclooxygenase2; *HPGD* = 15-hydroxyprostaglandin dehydrogenase; *TBP* = TATA box binding protein; *PGK1* = phosphoglycerate kinase ; *ACTB* = beta actin
[2]National Center for Biotechnology Information reference sequence
[3]Life Technologies, Grand Island, NY
[4]Endogenous controls

Table 2. Plasma oxylipids with stable concentration across the periparturient period (mean ± SE, pg / uL, n = 7).

Oxylipid[1]	Days in Milk				
	-14	0	10	28	84
5-HETE	0.02 ± 0.02	0.09 ± 0.04	0.06 ± 0.04	0.02 ± 0.02	0.06 ± 0.06
8-HETE	0.11 ± 0.06	0.09 ± 0.06	0.05 ± 0.03	0.02 ± 0.02	0.06 ± 0.06
12-HETE	0.08 ± 0.06	0.13 ± 0.07	0.06 ± 0.06	0.02 ± 0.02	0.04 ± 0.04
15-HETE	0.15 ± 0.06	0.23 ± 0.05	0.15 ± 0.05	0.23 ± 0.05	0.21 ± 0.05
20-HETE	0.19 ± 0.06	4.15 ± 0.76	2.80 ± 0.46	0.82 ± 0.24	0.33 ± 0.14
5-OxoETE	0.70 ± 0.32	0.17 ± 0.06	0.51 ± 0.20	0.57 ± 0.23	0.78 ± 0.13
15-OxoETE	0.12 ± 0.06	0.08 ± 0.04	0.06 ± 0.06	0.14 ± 0.03	0.06 ± 0.03
PGD_2	0.19 ± 0.04	0.38 ± 0.11	0.29 ± 0.05	0.17 ± 0.06	0.32 ± 0.10
PGE_2	0.41 ± 0.19	0.48 ± 0.15	0.36 ± 0.08	0.32 ± 0.09	0.36 ± 0.08
PGF_2	32.14 ± 18.86	23.12 ± 11.36	7.85 ± 3.64	12.04 ± 5.58	25.22 ± 15.64
6-keto-$PGF_{1\alpha}$	0.38 ± 0.05	0.34 ± 0.03	0.38 ± 0.05	0.34 ± 0.05	0.40 ± 0.04
8-iso-$PGF_{2\alpha}$	3.28 ± 1.67	3.01 ± 1.41	1.60 ± 0.98	0.72 ± 0.37	1.07 ± 0.50
TXB_2	0.13 ± 0.05	0.13 ± 0.07	0.36 ± 0.10	0.46 ± 0.20	0.36 ± 0.15
LTD_4	37.73 ± 13.66	54.84 ± 18.86	60.10 ± 20.74	62.55 ± 16.49	119.26 ± 70.29
9-OxoODE	3.58 ± 0.41	6.04 ± 1.53	5.98 ± 1.34	4.95 ± 0.97	7.77 ± 2.03
13-OxoODE	2.44 ± 0.34	4.61 ± 1.07	3.54 ± 0.77	3.39 ± 0.85	5.16 ± 0.97
PD1	2.10 ± 0.44	2.99 ± 0.59	3.79 ± 0.40	3.87 ± 0.60	11.47 ± 4.91
RD_1	0.11 ± 0.06	0.06 ± 0.06	0.15 ± 0.06	ND[2]	0.29 ± 0.20
RD_2	0.04 ± 0.02	0.15 ± 0.07	0.17 ± 0.05	0.17 ± 0.05	0.17 ± 0.05
7-MaR1	1.43 ± 0.24	1.05 ± 0.20	0.70 ± 0.12	0.98 ± 0.12	1.92 ± 0.73

[1]HETE = hydroxyeicosatetraenoic acid; OxoETE = oxoeicosatetraenoic acid; PG = prostaglandin; TX = thromboxane; LT = leukotriene; OxoODE = oxooctadecadienoic acid; PD = protectin D; RD = resolvin D; MaR = maresin. [2]ND = not detected

Table 3. Correlations between plasma oxylipid concentrations and peripheral leukocyte mRNA expression, adjusted for multiple tests.

Oxylipid[1]	Gene	Pearson Coefficient	*P*
PGE_2	*IL-12B*	0.575	< 0.0001
LTD_4	*IL-12B*	0.553	0.0131
PGD_2	*IL-12B*	0.481	0.0460
9-HODE	*IL-12B*	-0.524	0.0131
13-HODE	*IL-12B*	-0.554	0.0131
5-OxoETE	*IL-12B*	-0.637	<0.0001
PGF_2	*IL-12B*	-0.66	< 0.0001
8-iso-$PGF_{2\alpha}$	*IL-12B*	-0.69	< 0.0001
9-HODE	*NOS2*	0.631	< 0.0001
13-HODE	*NOS2*	0.629	< 0.0001
PGF_2	*NOS2*	0.626	< 0.0001
8-iso-$PGF_{2\alpha}$	*NOS2*	0.618	< 0.0001
5-OxoETE	*NOS2*	0.594	< 0.0001
15-HETE	*NOS2*	0.532	0.0131
TXB_2	*COX2*	-0.565	< 0.0001
TXB_2	*IL-1B*	-0.489	0.0368

[1]PG = prostaglandin; LT = leukotriene; HODE = hydroxyoctadecadienoic acid; OxoETE = oxoeicosatetraenoic acid; HETE = hydroxyeicosatetraenoic acid; TX = thromboxane

Table 4. Gene targets for quantitative PCR in Chapter 3.

Species	Gene[1]	Reference Sequence[2]	TaqMan Assay ID[3]
Bovine	*IL-10*	NM_174088.1	Bt03212724_m1
Bovine	*IL-12B*	NM_174356.1	Bt03213923_m1
Bovine	*TNF-α*	NM_173966.3	Bt03259156_m1
Bovine	*15-LOX1*	NM_174501.2	Bt03214775_m1
Bovine	*COX2*	NM_174445.2	Bt03214492_m1
Bovine	*TBP*[4]	NM_001075742.1	Bt03241946_m1
Bovine	*PGK1*[4]	NM_001034299.1	Bt03225857_m1
Bovine	*ACTB*[4]	NM_173979.3	Bt03279174_g1
Murine	*IL-1β*	NM_008361.3	Mm01336189_m1
Murine	*IL-6*	NM_031168.1	Mm00446190_m1
Murine	*IL-10*	NM_010548.2	Mm00439614_m1
Murine	*IL-12B*	NM_008352.2	Mm00434174_m1
Murine	*TNF-α*	NM_013693.3	Mm00443258_m1
Murine	*15-LOX1*	NM_009660.3	Mm00507789_m1
Murine	*COX2*	NM_011198.3	Mm01307329_m1
Murine	*GUSB*[4]	NM_010368.1	Mm00446953_m1
Murine	*TBP*[4]	NM_013684.3	Mm00446971_m1
Murine	*PGK1*[4]	NM_008828.2	Mm00435617_m1

[1]*TNF-α* = tumor necrosis factor-α; *15-LOX1* = 15-lipoxygenase1; *COX2* = cyclooxygenase2; *TBP* = TATA box binding protein; *PGK1* = phosphoglycerate kinase ; *ACTB* = beta actin; *GUSB* = beta glucuronidase

[2]National Center for Biotechnology Information reference sequence

[3]Life Technologies, Grand Island, NY

[4]Endogenous controls

Appendix 3. Figures.

Figure 1. Phospholipids consist of glycerol, usually esterified to a saturated long chain fatty acid at sn-1 and to an unsaturated long chain fatty acid at sn-2, and to a phosphorylated head group at sn-3.

This example is 1-hexadecanoyl-2-(9*Z*,12*Z*-octadecadienoyl)-*sn*-glycero-3-phosphocholine.

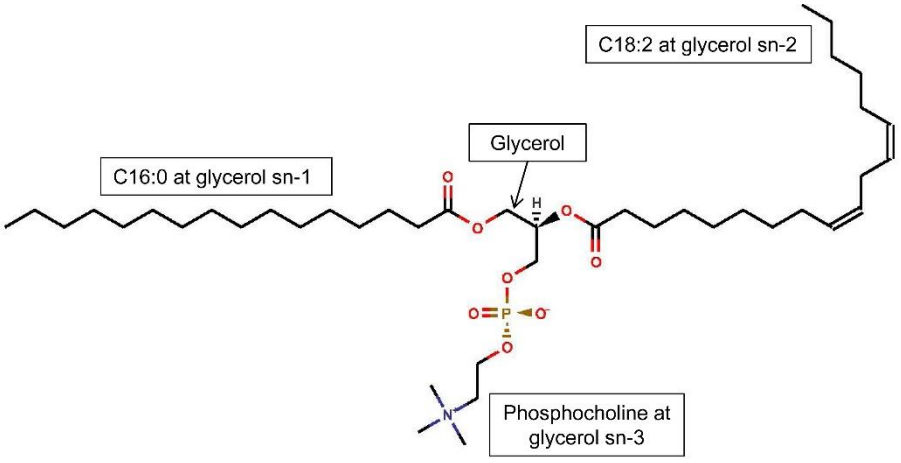


Figure 2. Fatty acyl chains of phospholipids located within the membranes of cells and organelles may be enzymatically oxidized into lipid mediators.

Examples of proinflammatory (orange), resolving (blue), and variable function (green) lipid mediators are illustrated by fatty acid substrate (yellow) and biosynthetic enzyme. *: aspirin acetylated; LA: linoleic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; COX: cyclooxygenase; Cyt-P450: cytochrome-P450 complex; HEPE: hydroxyeicosapentaenoic acid; HETE: hydroxyeicosatetraenoic acid; HODE: hydroxyoctadecadienoic acid; LOX: lipoxygenase; LT: leukotriene; LX: lipoxin; MaR: maresin; PG: prostaglandin; Rv: resolvin; PD: protectin; TX: thromboxane.

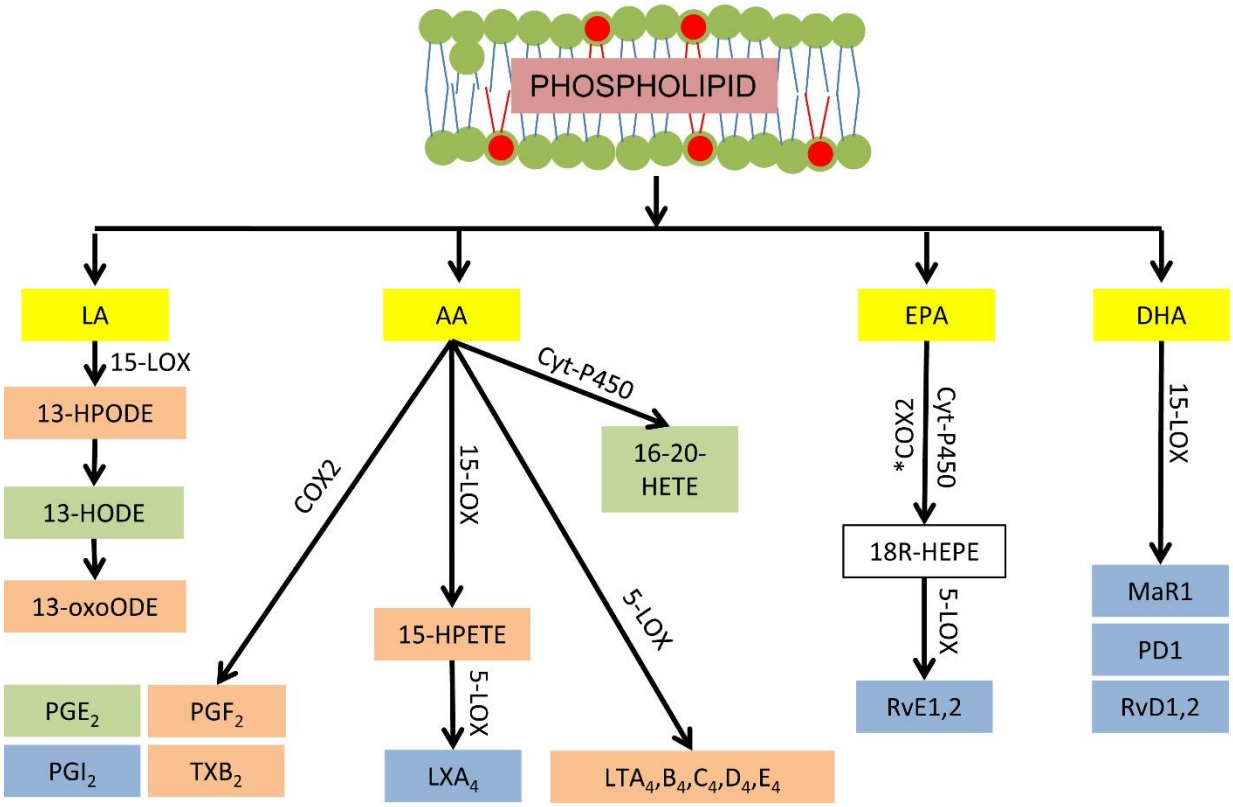


Figure 3. The utilization of long chain fatty acids for *de novo* phospholipid biosynthesis (Kennedy pathway, red arrows and boxes), post-synthetic modification of phospholipids by reacylation (Lands' cycle, green arrow), and the metabolism of phospholipids to oxidized lipid mediators of inflammation (orange arrow and box) in critical cells during inflammatory responses, such as vascular endothelial cells and mononuclear leukocytes.

Origins of fatty acid substrate addressed in this review are (solid blue arrows and boxes) adipose triacylglycerol-derived fatty acid[1], fatty acid synthesized *de novo*[2], recently absorbed dietary fatty acid[3], and phospholipid-derived fatty acid, cleaved by phospholipases then recycled for phospholipid biosynthesis[4] (broken blue arrow).

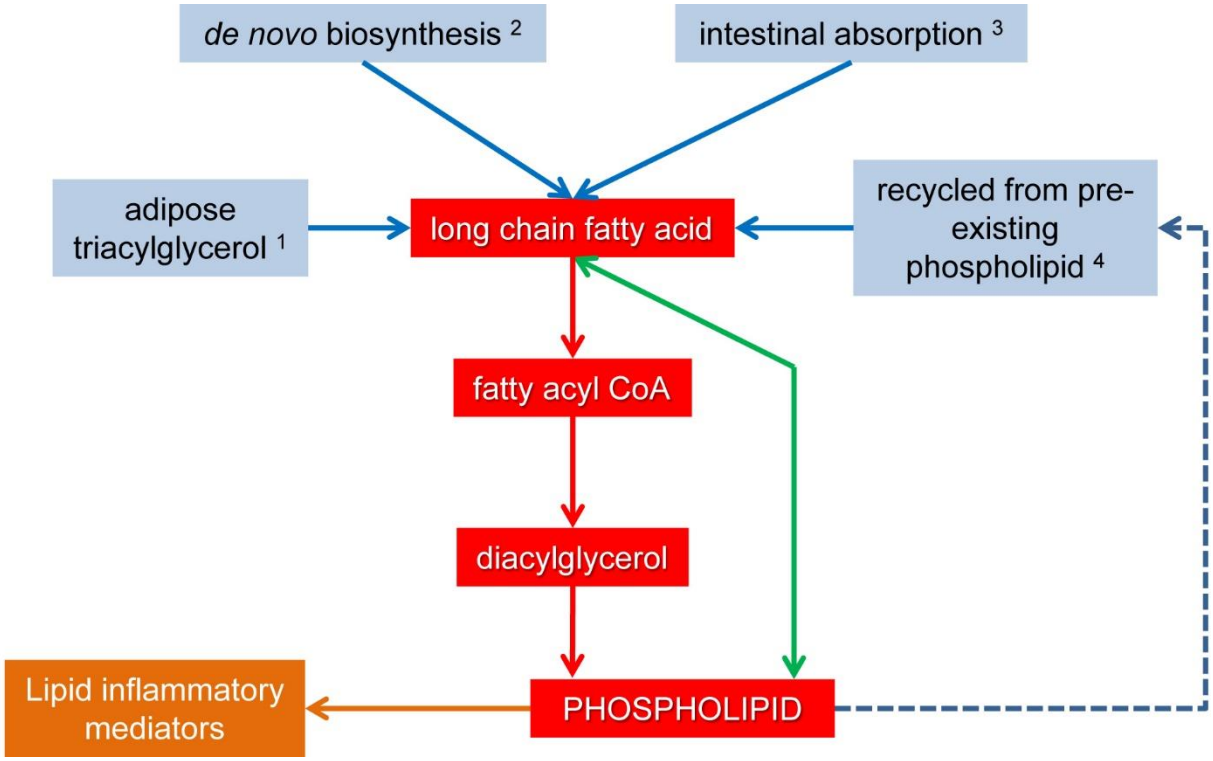


Figure 4. Biosynthetic pathway of major phospholipids in vascular endothelial tissue and mononuclear leukocytes.

Metabolites are boxed, enzymes are unboxed. Note that diacylglycerol is a substrate for both triacylglycerol and phospholipids. Key: ACSL: acyl CoA synthase (ligase); AGPAT: 1-acylglycerol-3-phosphoacyltransferase; CDP-choline: cytidine-diphosphate choline; CDS: cytidine-diphosphate diacylglycerol synthase; CL: cardiolipin; CCT: cytidine-triphosphate:phosphocholine cytidylyltransferase; DG: diacylglycerol; DGAT: diacylglycerol acyltransferase; GPAT: glycerol-3-phosphoacyltransferase; PA: phosphatidic acid; PC: phosphocholine; PE: phosphoethanolamine; PG: phosphoglycerol; PI: phosphoinositol; PPAP: phosphatidic acid phosphatase; PS: phosphoserine; TG: triacylglycerol.

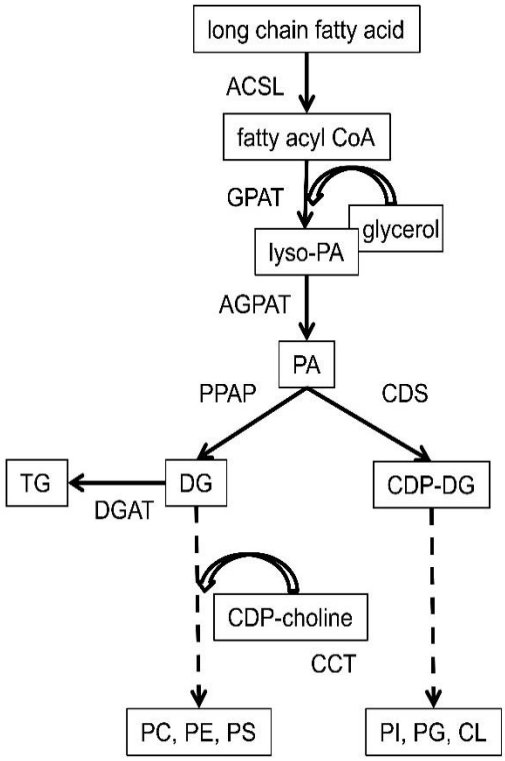


Figure 5. Plasma concentrations of arachidonic acid-derived oxylipid, 11-hydroxyeicosatetraenoic acid (11-HETE) changed across the periparturient period. Reported as least square mean ± SE ($p \leq 0.05$, differences labelled with different letters, n = 7).

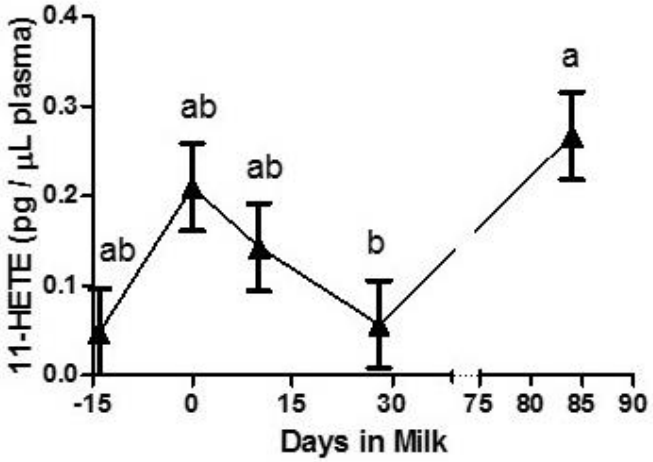


Figure 6. Plasma concentration of linoleic acid-derived oxylipid, 9-hydroxyoctadecadienoic acid (HODE) (A) and 13-HODE (B), changed in the periparturient period.

The ratios of 9-HODE to 9-oxooctadecadienoic acid (OxoODE) (C) and 13-HODE to 13-OxoODE (D) show increased variance in the early lactation period and the ratio is higher for 9-isomers than 13- at similar stages of lactation. The ratio of 13-HODE to 13-OxoODE inverted in early lactation (D), indicating that mean 13-HODE concentrations exceeded mean 13-OxoODE concentration in early lactation. Reported as least square mean ± SE, $p \leq 0.05$, n = 7.

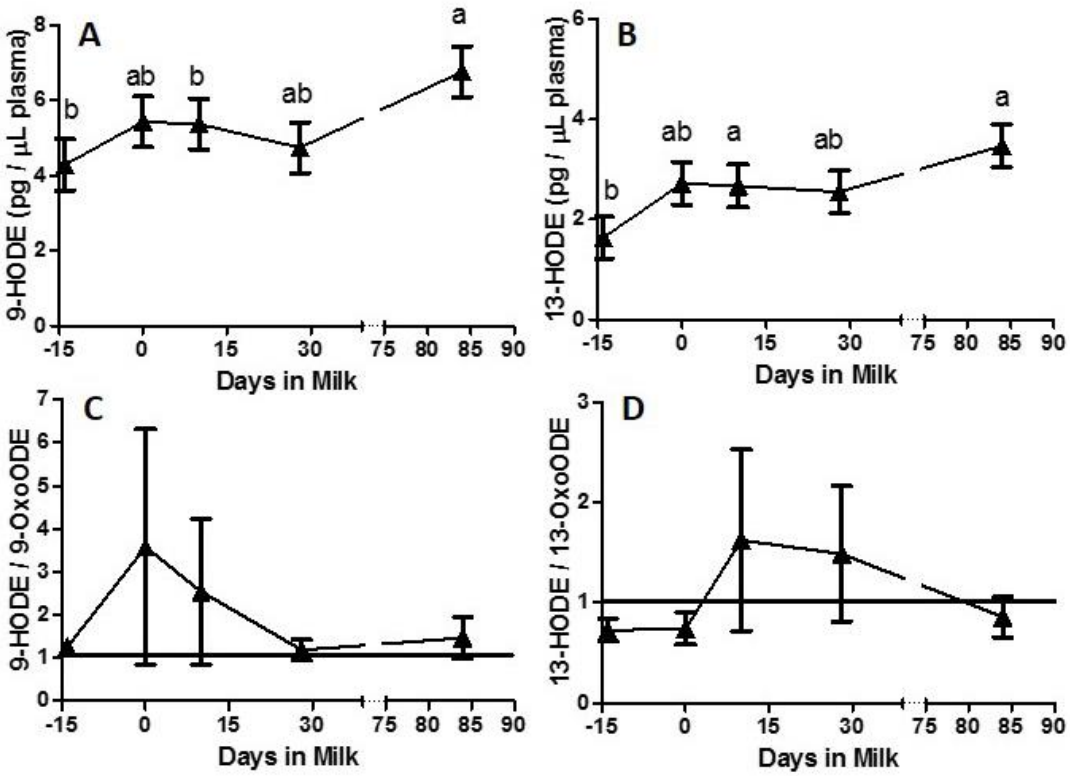


Figure 7. Relative (±SE) 15-lipoxygenase (*LOX*) 1 (A) and 15-hydroxyprostaglandin dehydrogenase (*HPGD*) (B) mRNA expression from peripheral leukocytes, calibrated to expression at 84 d ($p < 0.05$, $n = 7$)**.**

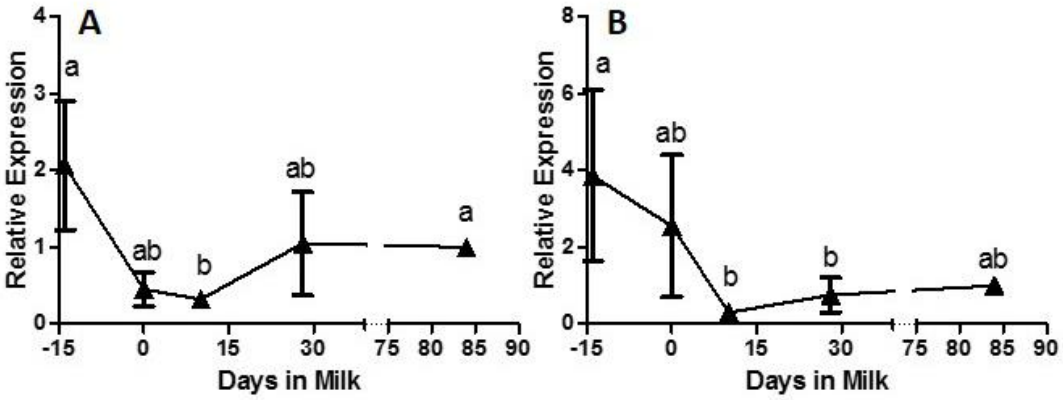


Figure 8. Relative (±SE) *IL-12B* mRNA expression from peripheral leukocytes, calibrated to expression at 84 d ($p \leq 0.05$, n = 7).

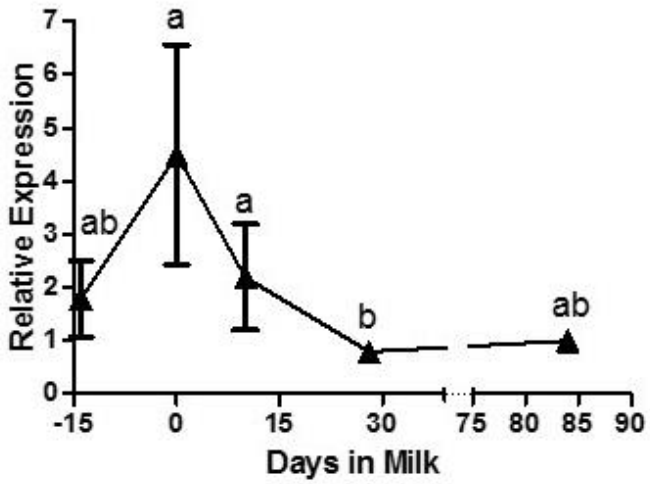


Figure 9. Linoleic (C18:2 n6, ▲) and arachidonic acid (C20:4 n6, ○) concentrations in peripheral blood mononuclear cells increased in early lactation relative to prepartum samples, while eicosapentaenoic (C20:5 n3, ◊) acid concentrations were unchanged.

Reported as least square mean ± SE, $p \leq 0.05$, n = 7.

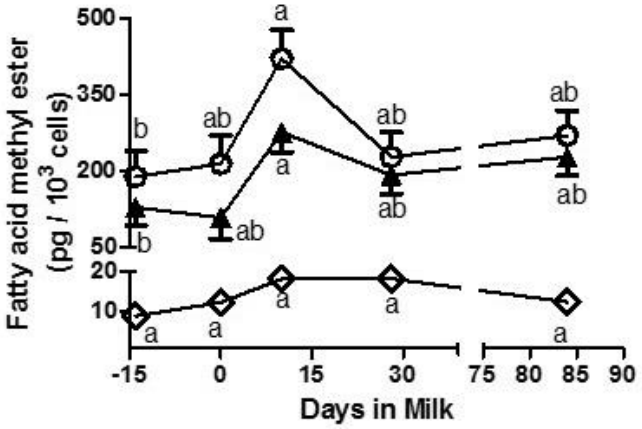


Figure 10. Linoleic acid methyl ester concentration in peripheral blood mononuclear cells at 10 d postpartum (*in vivo*, n = 7) versus cells from mid-lactation cows cultured *in vitro* (n = 6) for 12 h in media supplemented with various concentrations of linoleic acid.

Letters indicate significant ($p < 0.05$) differences between cells by culture conditions and * indicates significant ($p < 0.05$) difference between *in vitro* and *in vivo* cell concentrations.

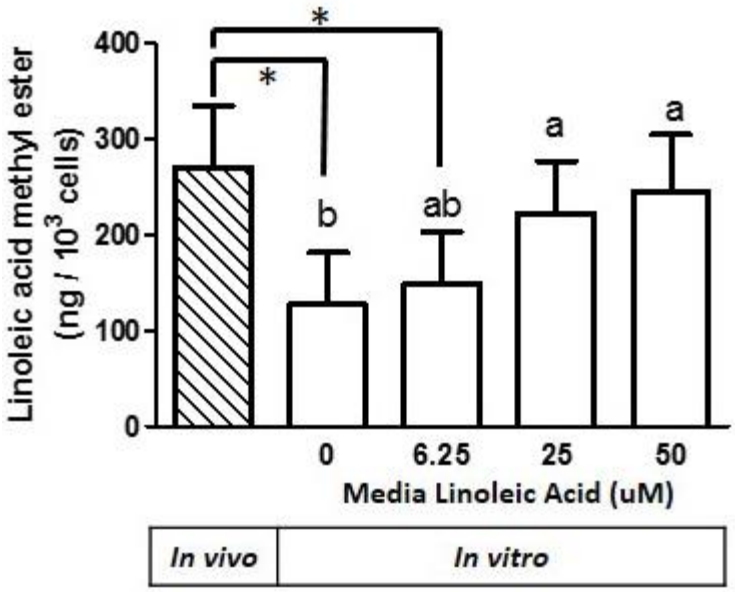


Figure 11. Transcript expression of tumor necrosis factor (*TNF*)-α in primary bovine monocytes (A) and *IL-1β* in murine RAW 264.7 monocytes (B) after 16 h incubation in media supplemented with various concentrations of linoleic acid (LA).

The effect of the fatty acid vehicle (ethanol, EtOH) and an alternative omega-6 fatty acid (arachidonic acid, AA) were also evaluated ($p < 0.05$, $n = 3$).

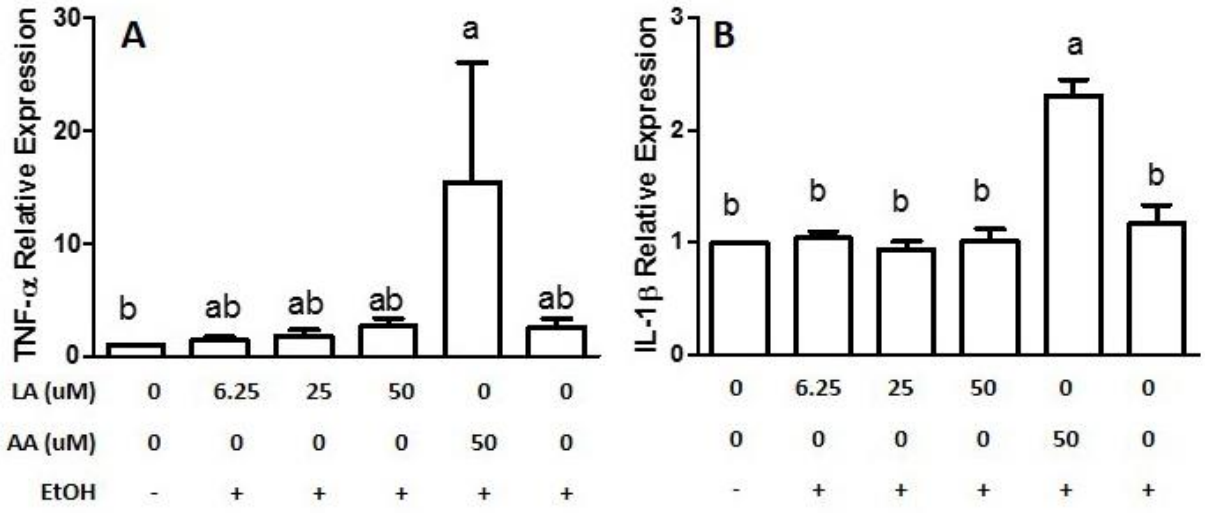


Figure 12. Fold changes in primary bovine monocyte culture media concentrations of 9-hydroxyoctadecadienoic (HODE, A), 13-HODE (B), 9-oxooctadecadienoic acid (OxoODE, C), and 13-OxoODE (D).

Reference groups are cells cultured in 0 uM linoleic acid (LA) and no ethanol (EtOH). Increased cellular concentrations of LA were associated with increased production of 9- and 13-HODE and 9- and 13-OxoODE ($n = 3$, $p < 0.05$). The effect of the fatty acid vehicle (EtOH) and an alternative omega-6 fatty acid (arachidonic acid, AA) were also evaluated.

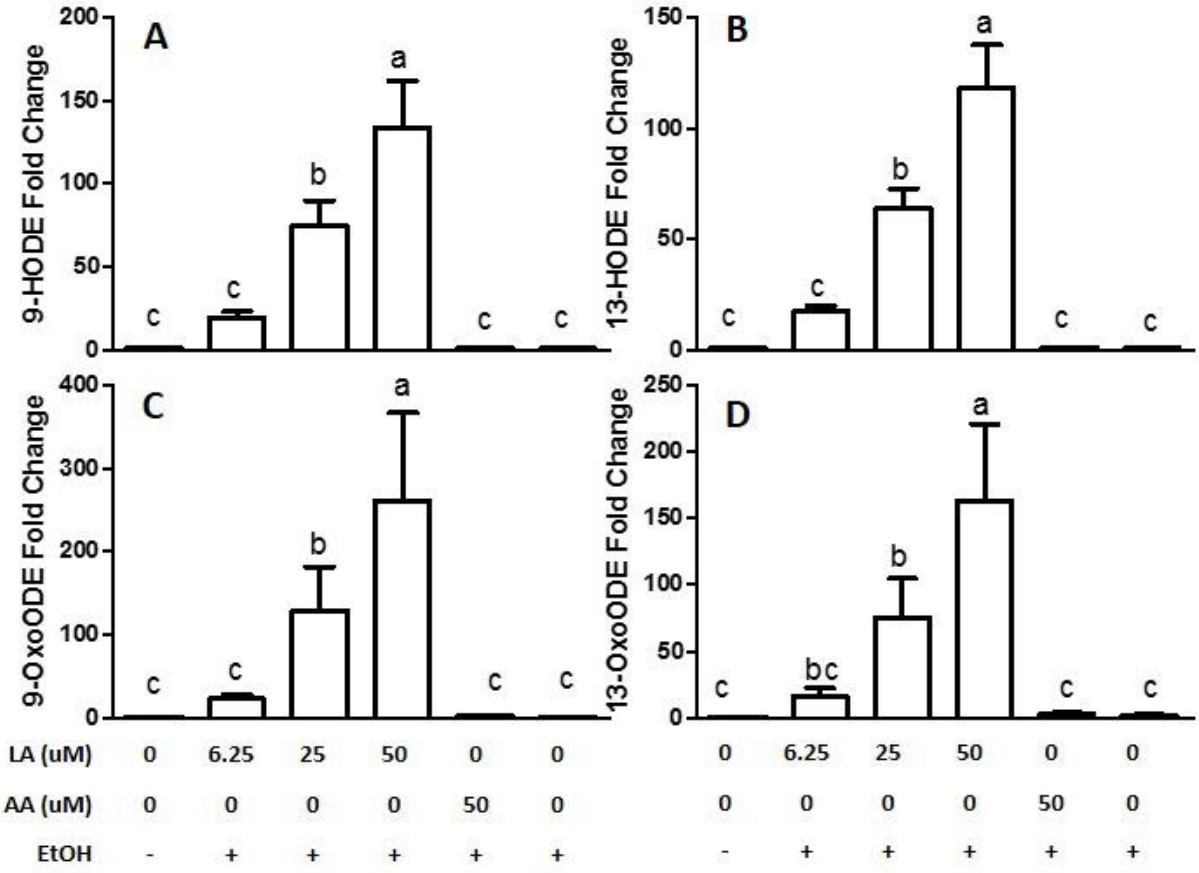


Figure 13. Primary bovine monocyte culture media concentrations of (R)-hydroxyoctadecadienoic acid ((R)-HODE, Fig. 13A) and (S)-HODE (Fig. 13B), and the ratio of (R)-HODE / (S)-HODE (Fig. 13C).

Production of (R)- and (S)-HODE isomers, and the (R)-HODE / (S)-HODE ratio all increased with cellular LA concentration ($n = 3$, $p < 0.05$).

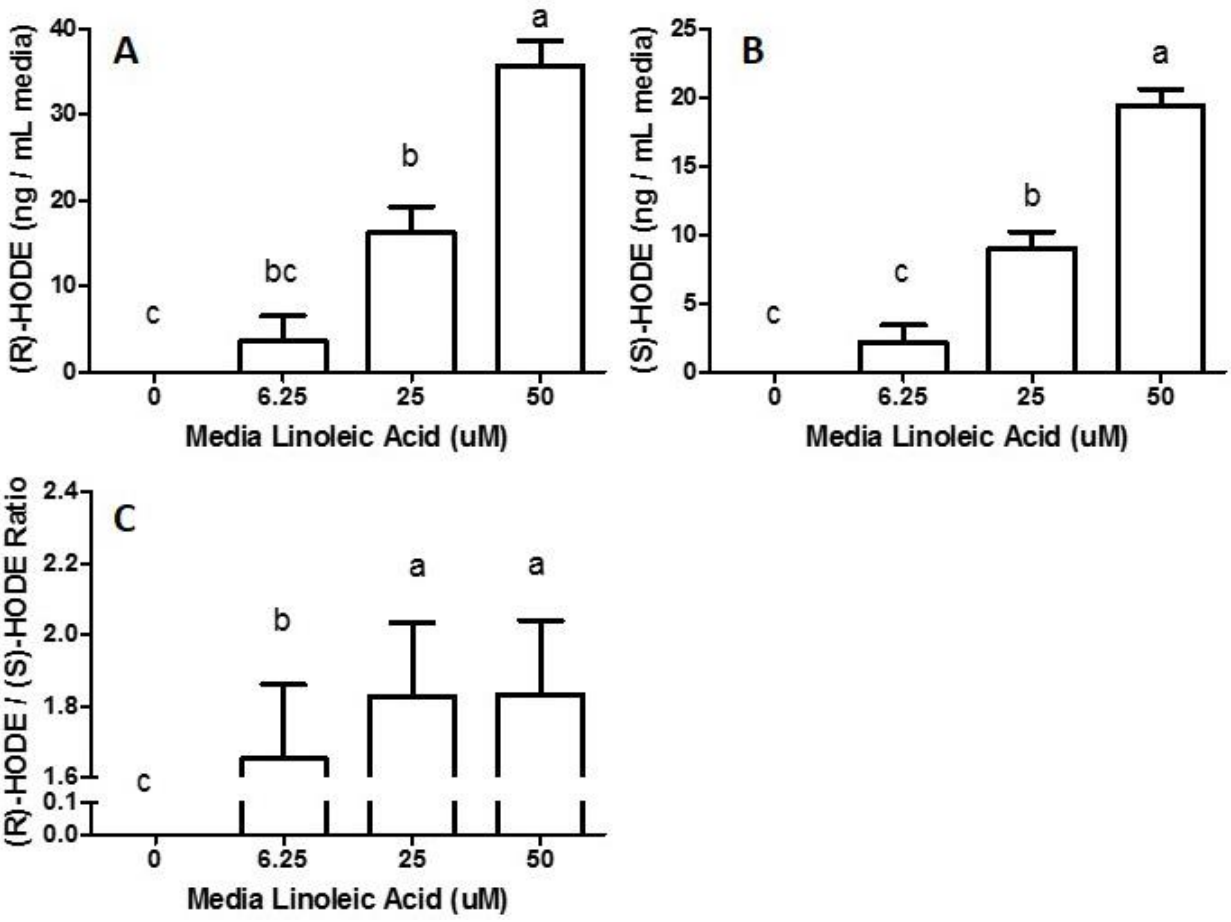


Figure 14. Fold changes in murine RAW 264.7 monocyte culture media concentrations of 9-hydroxyoctadecadienoic (HODE, A), 13-HODE (B), 9-oxooctadecadienoic acid (OxoODE, C), and 13-OxoODE (D).

Reference groups are cells cultured in 0 uM linoleic acid (LA) and no ethanol (EtOH). Increased cellular concentrations of LA were associated with increased production of 9- and 13-HODE and 9-OxoODE ($n = 3$, $p < 0.05$). The effect of the fatty acid vehicle (EtOH) was also evaluated.

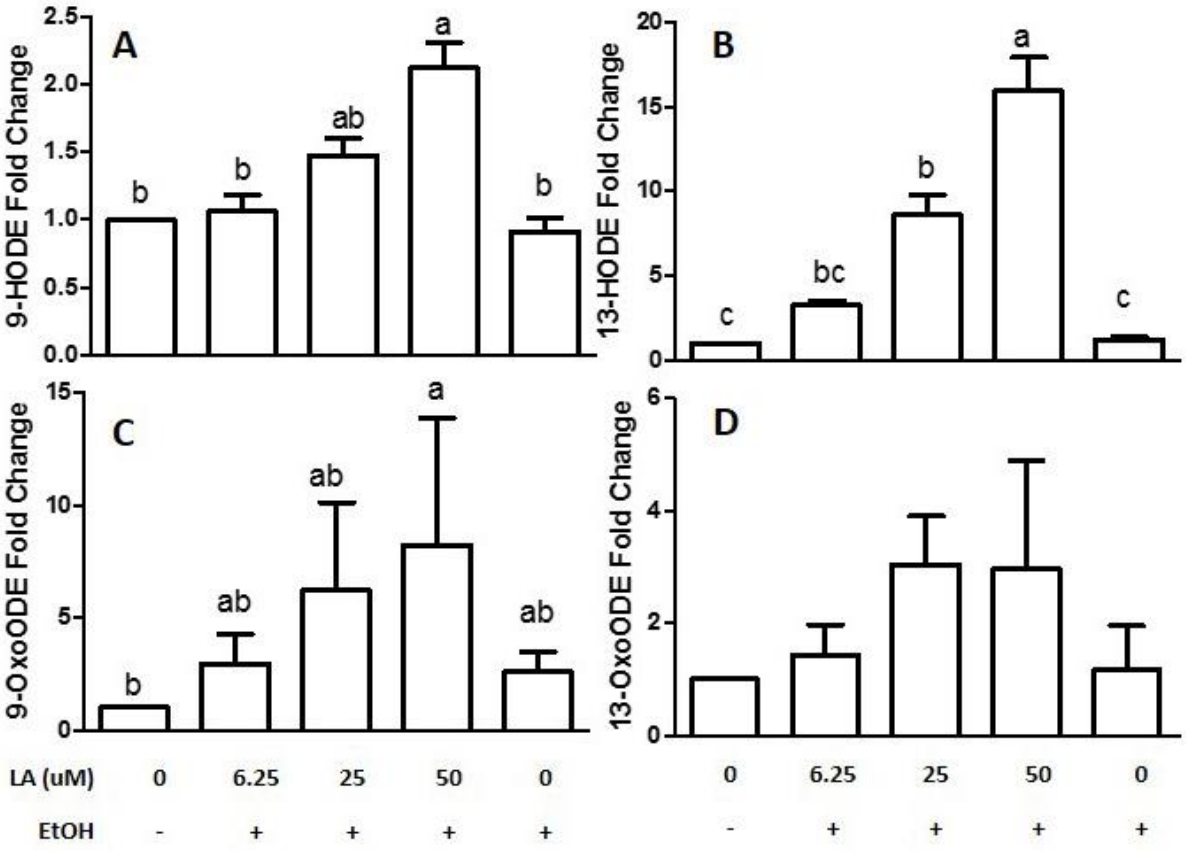
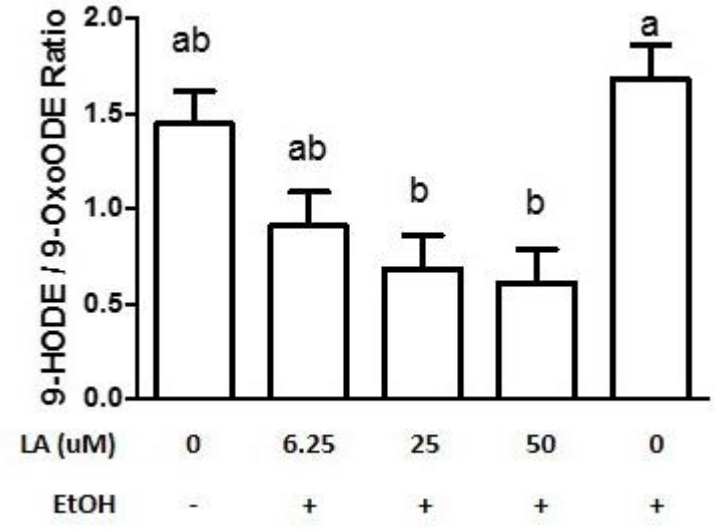


Figure 15. Ratio of 9-hydroxyoctadecadienoic (HODE) / 9-oxooctadecadienoic (OxoODE) acids in lipopolysaccharide stimulated (25 ng / mL) primary monocyte cultures supplemented with various concentrations of linoleic acid (LA). LA was delivered in ethanol (EtOH) ($n = 3$, $p < 0.05$).



Appendix 4. Supplementary Figures.

Figure 16. Unidentified compound in lipid extracts of RAW 264.7 monocytes elutes at identical retention time as pure docosahexaenoic acid methyl ester (C22:6 n3 FAME) standard.

(A). Mass spectra of pure C22:6 n3 FAME standard (Sigma-Aldrich, St. Louis, MO). Gas chromatograph/mass spectrometry methods are described in Chapter 2.

(B). Gas chromatogram of RAW 264.7 cell lipid extract at retention time of pure standard in (A). Extractions methods are described in Chapter 1.

(C). Mass spectra of chromatogram peak in (B).

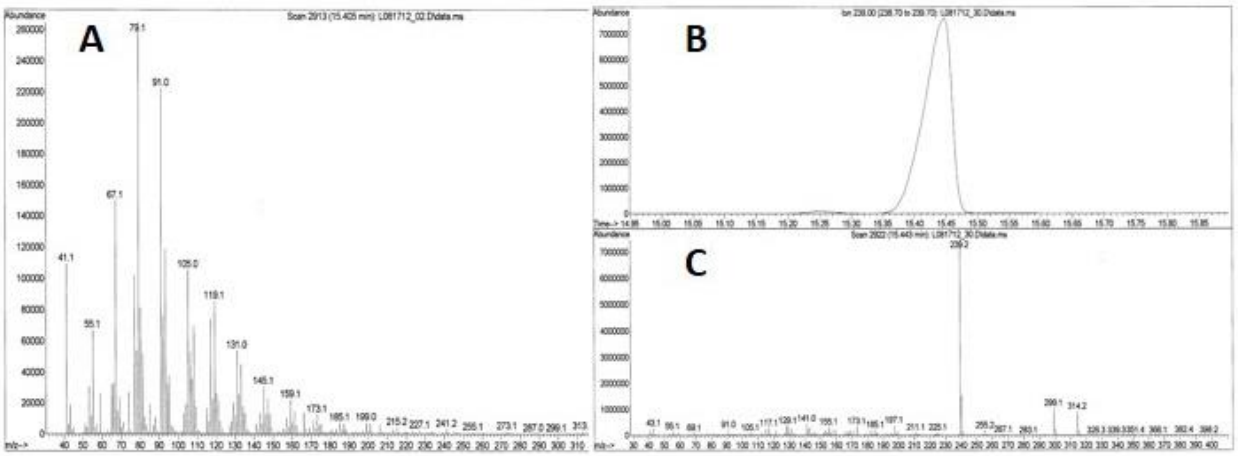


Figure 17. Linoleic acid (LA, C18:2 n6) methyl ester abundance in solvent controls (n = 3, open bars) and RAW 264.7 cell lipid extracts (n = 3, cross hashed bars) spiked with 50 ug LA fatty acid (Sigma-Aldrich, St. Louis, MO) either at start of experiment or between lipid extraction and fractionation of lipid types. Other samples were spiked with 50 ug LA methyl ester (Sigma-Aldrich) at end of experiment.

Lipid extraction and methylation methods are similar to Chapter 2, except these samples were not saponified prior to methylation. Fractionation utilized 500 mg aminopropyl cartridges (Strata NH2, Phenomenex, Torrance, CA) and methods by Ruiz *et al*., (2004) [132]. Abundance is reported as gas chromatogram peak area.

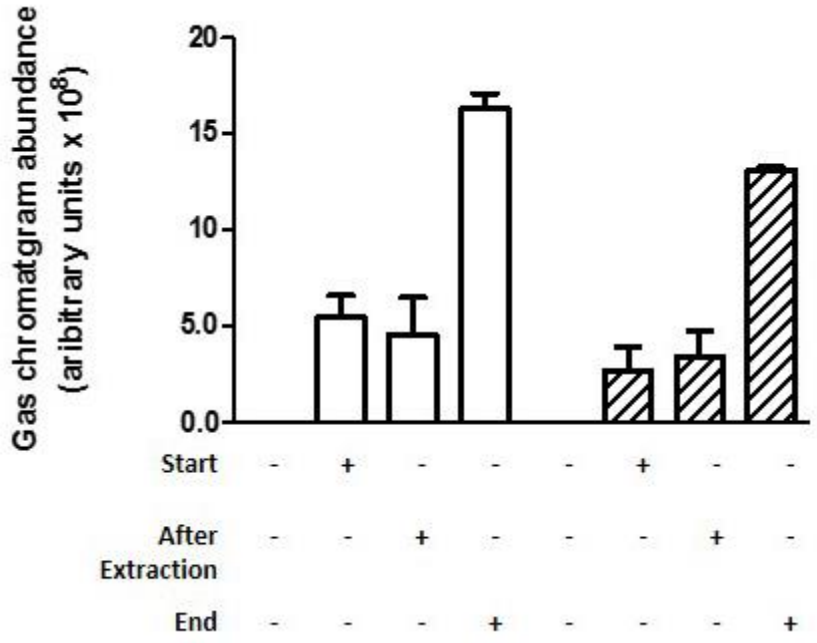


Figure 18. Decreased 15-lipoxygenase1 (15-LOX1) and tumor necrosis factor (TNF)-α mRNA expression in lipopolysaccharide stimulated primary bovine monocytes associated with increased concentrations of fatty acid free-bovine serum albumin (BSA).

(A) 15-LOX1 mRNA expression in lipopolysaccharide (LPS)-stimulated cells versus supplemental BSA concentration. Results reported relative to unstimulated cells (closed bar). Cells were cultured for 17 hours in 1XRPMI, with 10% fetal bovine serum and 0.1 uM sodium selenite. LPS utilized at 25 or 33 ng / mL media during last 4 hours of culture. qPCR methods are described in Chapter 2 (n = 2, 0 and 1.67 uM BSA; n = 1, 0.11 to 1.25 uM BSA).

(B) Similar data for TNF-α expression.

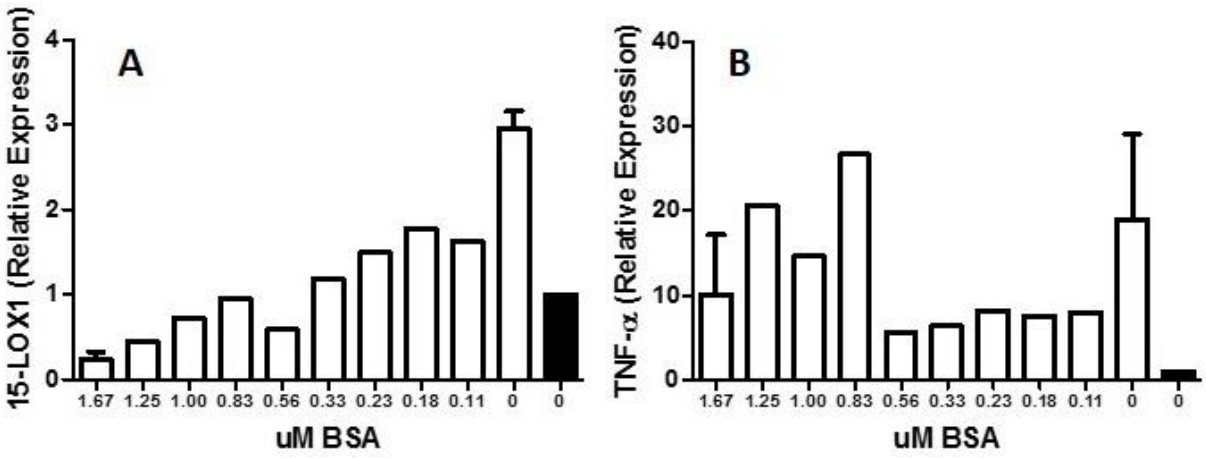


Figure 19. Decreased antioxidant potential induced by 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH, Sigma-Aldrich, St. Louis, MO) in RAW 264.7 monocytes.

Cells were cultured in 1XRPMI with 5% fetal bovine serum and 50 uM linoleic acid (LA, C18:2 n6, Sigma-Aldrich, St. Louis, MO, 0.01% ethanol) for 12 hours. AAPH was then added to the original media or replacement 0 uM LA media then cells were cultured for an additional 18 h. Kdo2-lipidA (100 ng / mL, Avanti Polar Lipids, Alabaster, AL) was added to media for the last 1 hour of culture ($n = 3$, $p < 0.05$). Antioxidant potential was measured as previously reported [124].

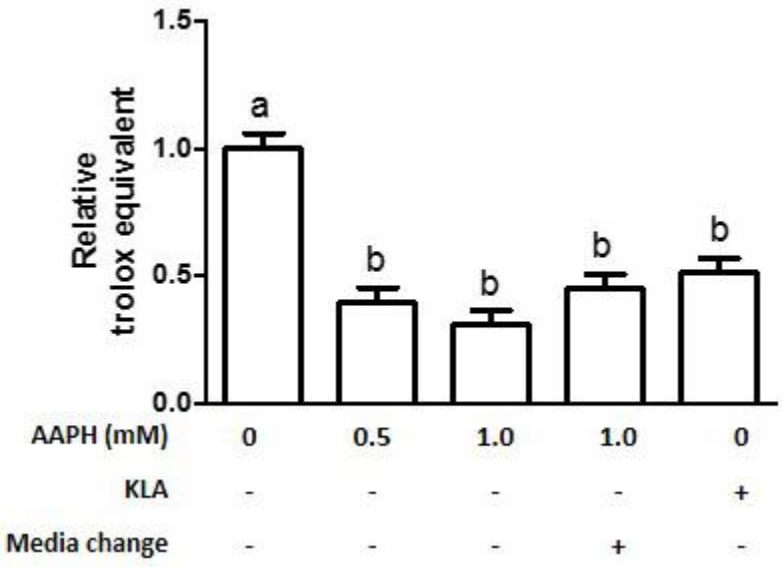


Figure 20. Induction of 9-hydroxyoctadecadienoic acid (9-HODE) production by 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH, Sigma-Aldrich, St. Louis, MO) in RAW 264.7 monocytes.

Cells were cultured in 1XRPMI with 5% fetal bovine serum and 50 uM linoleic acid (LA, C18:2 n6, Sigma-Aldrich, St. Louis, MO, 0.01% ethanol) for 12 hours. AAPH was then added to the original media or replacement 0 uM LA media then cells were cultured for an additional 18 h. Kdo2-lipidA (100 ng / mL, Avanti Polar Lipids, Alabaster, AL) was added to media for the last 1 hour of culture ($n = 3$, $p < 0.05$). 9-HODE was measured by liquid chromatography/mass spectrometry, as described in Chapter 2.

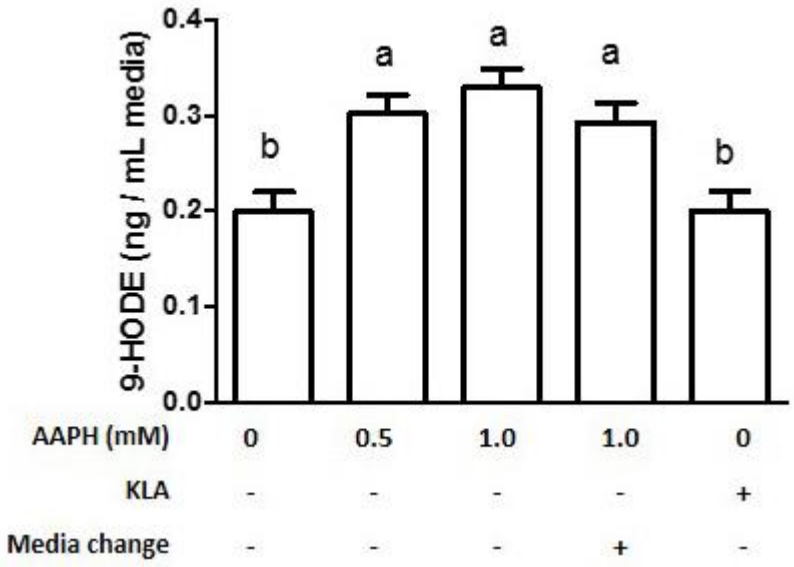


Figure 21. Viability is decreased by 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH, Sigma-Aldrich, St. Louis, MO) in RAW 264.7 monocytes.

(A) Cells were cultured in 1XRPMI with 5% fetal bovine serum and 50 uM linoleic acid (LA, C18:2 n6, Sigma-Aldrich, St. Louis, MO, 0.01% ethanol) for 12 hours. AAPH was then added to the original media or replacement 0 uM LA media then cells were cultured for an additional 18 h. Kdo2-lipidA (100 ng / mL, Avanti Polar Lipids, Alabaster, AL) was added to media for the last 1 hour of culture ($n = 3$, $p < 0.05$). Cell viability was measured by the CellTiter-Glo assay for adenosine triphosphate (Promega, Madison, WI).

(B) Similar data for APPH culture times of 10 to 16 h.

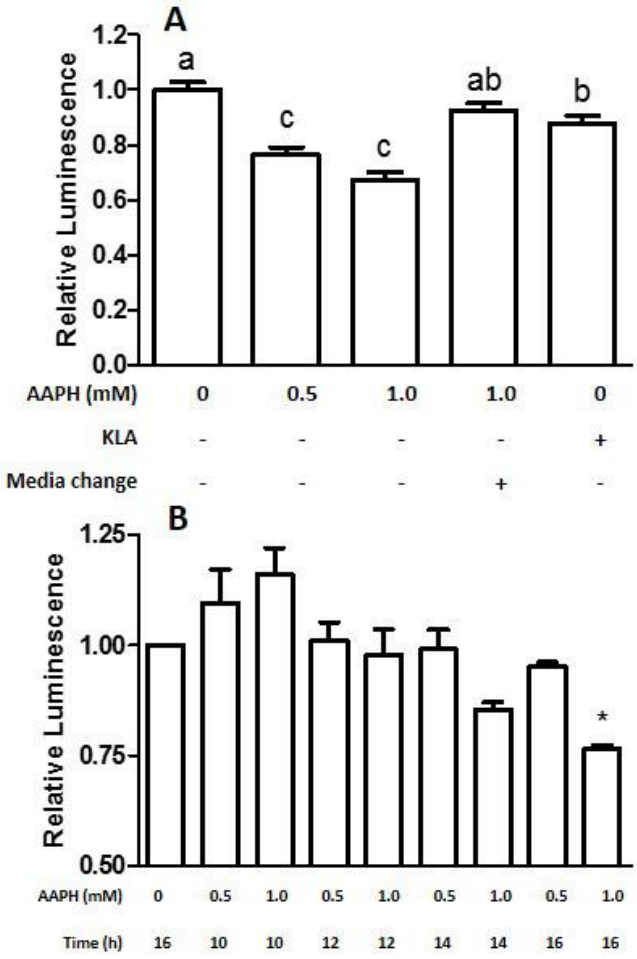
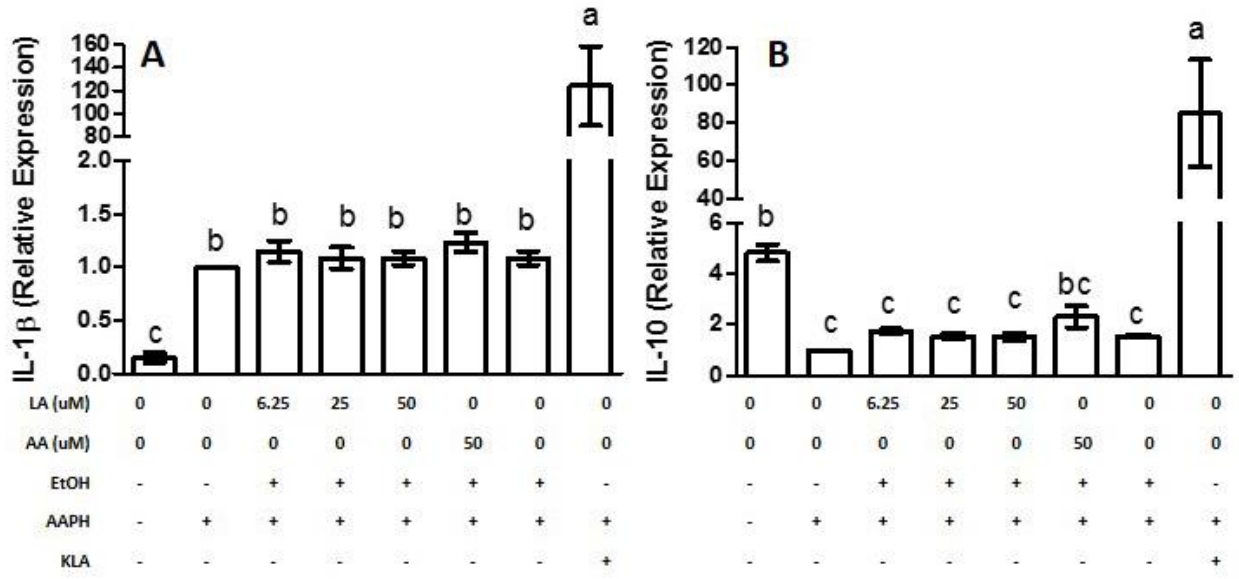


Figure 22. Linoleic acid does not affect IL-1β (A) or IL-10 (B) transcript induction by 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH, Sigma-Aldrich, St. Louis, MO) in RAW 264.7 monocytes.

Cells were cultured in 1XRPMI with 5% fetal bovine serum and supplemented with various concentrations of linoleic acid (LA, C18:2 n6, Sigma-Aldrich, St. Louis, MO, 0.01% ethanol), 50 uM arachidonic acid (AA, C20:4 n6, Sigma-Aldrich, 0.01% ethanol) or ethanol for 12 hours. AAPH was then added to the original media and cells were cultured for an additional 12 h. Kdo2-lipidA (100 ng / mL, Avanti Polar Lipids, Alabaster, AL) was added to media for the last 1 hour of culture ($n = 3$, $p < 0.05$). qPCR methods are described in Chapter 2.



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