LIPID MOBILIZATION AND LIPID MEDIATORS BIOSYNTHESIS IN PERIPARTURIENT DAIRY COWS

By

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ABSTRACT

LIPID MOBILIZATION AND LIPID MEDIATORS BIOSYNTHESIS IN PERIPARTURIENT DAIRY COWS

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The transition period of dairy cattle is characterized by changes in metabolism and host defense mechanisms that are associated with increased disease susceptibility. Intense lipid mobilization is a metabolic adaptation common to periparturient cows that results in significant release of non-esterified fatty acids (NEFA) into circulation. Whereas these fatty acids are important sources of energy during times of increased metabolic demands, elevated plasma NEFA may disrupt several immune and inflammatory functions. ***The main hypothesis of this dissertation is that shifts in plasma fatty acid composition induced during lipid mobilization can influence inflammatory responses and alter cyclooxygenase and lipoxygenase lipid mediator biosynthesis.*** The hypothesis was tested through the following objectives. The first objective determined the influence of lipid mobilization on plasma lipid fractions and leukocyte phospholipid fatty acid profiles. Around parturition and during early lactation, the proportion of palmitic acid significantly increased in plasma NEFA and phospholipid fractions with a concomitant increase in leukocyte phospholipids. In contrast, long chain polyunsaturated fatty acids content was reduced in leukocytes phospholipids, especially during the first two weeks following parturition. The second objective evaluated the effects of lipid mobilization on vascular cells inflammatory responses. Bovine aortic endothelial cells (BAEC) were cultured with different concentrations of a NEFA mixture that reflected plasma NEFA composition of periparturient cows during the first week of lactation. Gene expression and protein quantification of inflammatory markers were used to evaluate inflammatory response. Changes in

cyclooxygenase (COX) and 15 lipoxygenase (15LOX) gene expression and metabolite biosynthesis in response to NEFA exposure were also assessed. Addition of NEFA altered BAEC phospholipid fatty acid content by increasing the concentration of stearic acid and decreasing arachidonic acid and other long chain polyunsaturated fatty acids. These changes induced a significant increase of mRNA expression of COX2, interleukin 6, interleukin 8, inter-cellular adhesion molecule 1 (ICAM1), and vascular adhesion molecule 1. Changes in gene expression were reflected in protein expression by a significant increment in the protein expression of COX2 and ICAM1. Significant increases in the biosynthesis of COX metabolite prostaglandin E_2, and the 15LOX derivatives 9- and 13-hydroxyoctadecadienoic acids, resulted after treatment with NEFA complexes. The third objective of this study was to assess in vitro changes of inflammatory responses in BAEC induced by supplementation with a NEFA mixture that included eicosapentaenoic acid (EPA) and docosahexapentaenoic acid (DHA). Changes in inflammatory responses were assessed similar to objective 2. In vitro supplementation of EPA and DHA to BAEC successfully increased the concentration of both fatty acids in cellular phospholipids. Furthermore, EPA and DHA reduced endothelial inflammatory responses by diminishing the expression of pro-inflammatory cytokines and adhesion molecules. There were also shifts in the biosynthesis of COX and 15LOX derived eicosanoids. This research demonstrated for the first time that in dairy cows lipid mobilization induces endothelial inflammatory responses and alters eicosanoid biosynthesis and that EPA and DHA supplementation may reduce such responses. Further studies should examine in detail how altered eicosanoid biosynthesis may affect vascular inflammatory responses during lipid mobilization, possibly identifying pharmaceutical or nutraceutical targets that would improve health not only in dairy cows, but also in humans under the concept of one health one medicine.

DEDICATION

To my parents Genaro and Veronica, my wife Monica, and my children Samuel and Santiago.

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CHAPTER 1

Lipid mobilization and inflammatory responses during the transition period of dairy cows [1]

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Abstract

The transition period of dairy cattle is characterized by dramatic changes in metabolism and host defense mechanisms that are associated with increased disease. Intense lipid mobilization from tissue stores is an important metabolic adaptation during the transition period that results in significant release of non-esterified fatty acids (NEFA) into the blood stream. Whereas these fatty acids are important sources of energy during times of increased metabolic demands, elevated concentrations of NEFA are known to disrupt several immune and inflammatory functions. This review will discuss the implications of lipid mobilization on inflammatory responses with special emphasis on leukocytes and endothelial cell functions during the transition period of dairy cows.

Keywords: Lipid mobilization, Dairy cows, Transition period

Introduction

The transition period in the dairy cow is defined as the change from a gestational non-lactating to a non-gestational lactating state. This period, which spans from 3 weeks before through 3 weeks after parturition, is associated with an elevated incidence of diseases such as milk fever, retained placenta, metritis, ketosis, left displaced abomasum, lameness, and clinical mastitis (Kelton et al., 1998). Transition dairy cows experience sudden changes in both metabolic and immune functions. Some of these alterations are related to increases in energy requirements driven by both fetal needs and lactogenesis. At the same time, dairy cows show a marked decrease in dry matter intake (DMI) that is related to physical, behavioral, metabolic and hormonal changes around parturition (Grummer et al., 2004; Allen et al., 2005). As a result, dairy cows enter a negative energy balance (NEB) that is further aggravated by the nutrient prioritization towards the mammary gland (Leroy and Vanholder, 2008). Dairy cows adapt to NEB by mobilizing adipose energy depots and by turning skeletal muscle into the major site for use of fat-derived fuels, such as non-esterified fatty acids (NEFA) and ketone bodies, allowing glucose to be redirected for fetal metabolism and lactose synthesis (Herdt, 2000). Metabolic adaptations such as lipid mobilization are accompanied by alterations in inflammatory responses that modify immune function. Although previous reports linked lipid mobilization and impaired immunity in the transition dairy cow, the underlying mechanisms are inconclusive and our current knowledge in this area relies on alterations of lipid homeostasis common to human diseases such as obesity, metabolic syndrome, type-2 diabetes, and atherosclerosis.

Lipid Mobilization

Lipid mobilization is a physiological adaptation mammals acquired to survive times of reduced nutrient and energy availability. It is defined as an imbalance between lipogenesis and

lipolysis within the adipose tissue (Figure 1). Lipogenesis initially involves the biosynthesis of diacylglycerol from fatty acid acyl-CoAs and then the process is completed by adding a final fatty acid forming a triglyceride (TG) molecule. There are 3 major pathways that provide diacylglycerol for TG production including: glycerol phosphate pathway, monoacylglycerol pathway, and glyceroneogenesis. After a diacylglycerol molecule is formed, 2 major enzymes complete TG production within the adipocyte: acyl-CoA diacylglycerol acyltransferase 1 and 2. The details of TG biosynthesis are beyond the scope of this review, but the interested reader is referred to comprehensive reviews on lipogenesis for additional information (Nye et al., 2008; Yen et al., 2008).

During lipolysis, adipose tissues lipases including hormone sensitive lipase, adipose triglyceride lipase, and monoacylglycerol lipase initiate the catabolism of TG into glycerol and NEFAs (Langin et al., 2005). Upon NEFA release from TG, these molecules are targeted for export by the adipocyte fatty acid binding protein (FABP)-4 (Shen et al., 1999). Once in the extracellular space, NEFA are transported in blood typically by albumin, although a small portion is transported as unbound monomers in aqueous solution (Richieri and Kleinfeld, 1995). Throughout the transition period, lipolysis is favored by a series of hormonal changes that include reduced levels of plasma insulin and glucose, impaired insulin sensitivity in adipose and other peripheral tissues, an uncoupling of the somatotropic axis, and increased plasma concentrations of catecholamines, growth hormone and glucocorticoids (Herdt, 2000). Increased rates of lipolysis have profound impact on body condition score, an important guideline for transition cow management (Roche et al., 2009). Excessive body condition score reduction is linked directly with a greater incidence of transition cow diseases and reduced lactation performance (Bewley and Schutz, 2008). Remarkably, recent research suggests the inclusion of

body condition score and NEB markers evaluation as an additional tool for dairy cow welfare assessment (Roche et al., 2009).

The net outcome of increased lipolysis during the transition period is the augmentation of plasma NEFA concentrations. In fact, quantification of plasma NEFA levels is used as a diagnostic tool to assess the degree of NEB in transition cows. During late lactation and the dry period, plasma NEFAs average less than 0.2 mM/L. Levels start to increase 2 weeks before parturition, then peak between 0 to 10 days after parturition with concentrations greater than 0.75 mM/L, depending on the degree of lipid mobilization. If the cow develops ketosis, NEFA values will surpass 1.0 mM/L (Adewuyi et al., 2005). Elevated plasma NEFA concentrations, as seen in transition dairy cows, also reflect what is observed in humans with type-2 diabetes, metabolic syndrome or those considered obese (Blaak, 2003; Desideri-Vaillant et al., 2004; Pilz and Marz, 2008). In contrast to the increase in plasma NEFA, the concentrations of other plasma lipid fractions such as phospholipids (PL) and TG, decrease during the first week following parturition (van den Top et al., 1996). Plasma TG drop from concentrations over 200 µM during the dry period to values below 50 µM in the first 10 days after parturition, at the same times, PL decrease from 200 µM a month before parturition to concentrations under 120 µM following calving (Blum et al., 1983; van den Top et al., 1996).

Enhanced lipolysis during the transition period not only increases plasma NEFA, but it also alters the fatty acid profiles of plasma lipid fractions. Moreover, the overall composition of total plasma lipids is not reflected in each of the plasma lipid fractions: NEFA, PL, cholesterol esters (CE), and TG. In the dairy cow, for example, the main fatty acid in plasma total lipids is linoleic acid (C18:2n6c), in contrast plasma NEFAs are mainly composed of saturated fatty acids including palmitate (C16:0) and stearate (C18:0), and the monounsaturated, oleic acid

(C18:1n9c). Furthermore, the concentrations of these fatty acids vary depending on the diet, days in lactation, and plane of nutrition (Leroy et al., 2005; Tyburczy et al., 2008). For example, Loor et al (Loor et al., 2006) demonstrated that transition cows fed ad libitum moderate energy diets incorporated palmitate (C16:0) into TG at a higher rate than cows under restricted feeding. In the days following parturition, the concentration of palmitate (C16:0) and stearate (C18:0) within the plasma NEFA fraction are increased significantly (Contreras et al., 2010c). Another important change is the decrease in the concentration of the n-3 series (omega 3) long chain polyunsaturated fatty acids in both plasma NEFA and PL fractions (Lock et al., 2009). These alterations in plasma fatty acid composition rapidly are reflected in the fatty acid profiles of cellular membrane PL of blood cells including erythrocytes and peripheral blood mononuclear cells (Lock et al., 2009; Contreras et al., 2010c). Other plasma lipid fractions including PL, CE and TG also are altered in their composition. The content of saturated fatty acids such as palmitate (C16:0) and stearate (C18:0) is increased in the PL and CE fractions around parturition. Similarly, the main omega-6 polyunsaturated fatty acid in dairy cows, linoleic acid (C18:2n6c) increases in the CE fraction. At the same time, omega-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA, C20:5n3c)) and docosahexanoic acid (DHA, C22:6n3c) in PL, CE and TG are diminished during the first week of lactation (Tyburczy et al., 2008; Contreras et al., 2010c). These changes in the concentrations of saturated, omega-3 and omega-6 polyunsaturated fatty acids could potentially affect inflammatory responses in transition dairy cows.

Cellular Metabolism of NEFA

Lipid mobilization makes NEFA an alternate energy substrate readily available to cells in different tissues. NEFAs are internalized by leukocytes and endothelial cells by free diffusion or

by protein receptor mediation (Hajri and Abumrad, 2002). Once in the cytoplasm, NEFAs can take different metabolic pathways. Some can be used as energy substrate for β oxidation in the mitochondria or anabolic pathways in cellular organelles such as the endoplasmic reticulum. When used as an energy substrate, cell specific fatty acid acyl-CoAs are formed by acyl-CoA synthetases (Li et al., 2010). Different isoforms of acyl-CoA synthetases were identified in leukocytes. Human monocytes express a medium chain acyl-CoA synthetase and accessory acyl-CoA transporters including acyl-CoA binding protein (Klapper et al., 2006). Lymphocytes also are capable of expressing specific acyl-CoA synthetases as demonstrated in mouse lines (Tebbey and Buttke, 1993). Similarly, endothelial cells express both acyl-CoA synthetases and acyl-CoA binding protein, facilitating utilization of NEFAs (Weis et al., 2005). Currently, the effects of transition period metabolic adaptations on endothelial and leukocyte acyl-CoA synthetases and binding proteins are unknown. A recent study, however, described reduced gene expression of liver acyl-CoA binding proteins in periparturient dairy cows (Loor et al., 2007). Further studies are required to elucidate changes in fatty acid acyl-CoA metabolism in vascular cells and leukocytes.

When NEFA are not transformed into their specific acyl-CoA, they are transported to specific organelles within the cytosol by FABP (Storch and Corsico, 2008). Leukocytes and endothelial cells have specific isotypes of FABP. For example, monocytes and macrophages express FABP3, FABP4, which is analogue to the adipocyte's binding protein, and FABP5 (Rolph et al., 2006; Elmasri et al., 2009). Similarly, dendritic cells express FABP3 and FABP4, and lymphocytes express FABP5 which also is known as epidermal FABP (Furuhashi and Hotamisligil, 2008; Li et al., 2009). Whereas changes in endothelial and leukocyte FABP expression and function are not described for transition dairy cows, Loor et al (2007) described

up-regulation of liver FABP3 around parturition. Once bound to the specific FABP, some fatty acids are targeted to the cellular secretory pathway that includes the endoplasmic reticulum and the Golgi apparatus. Within these organelles, fatty acids either may be incorporated into phospholipids (Fagone and Jackowski, 2009) or in the case of liver and adipose tissue, included in TG molecules as detailed in the lipid mobilization section.

Besides being part of cellular membrane structure as the main component of the phospholipids, certain fatty acids in the cytosol can be attached to proteins and thereby modifying their structure and functions. The most common example of this process is palmitoylation (Smotrys and Linder, 2004). In this reaction, a molecule of palmitic acid (C16:0) is included in the molecular structure of certain proteins. Through this process, proteins change their ability to bind to lipid bilayers, and alter their intracellular trafficking and targeting patterns (Resh, 2006). Palmitoylation is an important process that is necessary to activate leukocyte receptors during immune responses (Bijlmakers, 2009). Fatty acids can also bind to intracellular receptors to modulate gene expression such as the peroxisome proliferator-activated receptors (PPARs). These nuclear receptors regulate gene expression by binding DNA sequence elements localized in the promoter region of target genes (Kliewer et al., 1997). PPARs and their ligands, which include saturated fatty acids, polyunsaturated fatty acids and select lipid mediators derived from phospholipids like 15-deoxy-12,14-prostaglandin J2, modulate inflammatory reactions in many cellular types including endothelial cells, monocytes and adipocytes (Hira et al., 2010).

Certain saturated fatty acids such palmitate (C16:0) and stearate (C18:0) can interact both directly and indirectly with intracellular signaling pathways that modify the inflammatory response in different type of cells including myotubes, adipocytes, leukocytes and endothelial cells. One example is the nuclear factor κ B (NF-κB) signaling pathway. Both palmitate (C16:0)

and stearate (C18:0) can activate the inhibitor of κB kinase (IKKβ) in endothelial cells therefore resulting in increased activity of the NF-κB transcription factor (Kim et al., 2005; Staiger et al., 2006b). Activation of IKKβ is mediated by the toll like receptor 4 (TLR-4) pathway, which can be induced directly by palmitate (C16:0) and stearate (C18:0) (Maloney et al., 2009). IKKβ then promotes a wide variety of pro-inflammatory effects in endothelial and leukocytes including: decreasing the production of nitric oxide, activating of NF-κB and impairing insulin signaling by phosphorylation of the insulin receptor substrate 1.

Effects of NEFA on Leukocyte and Endothelial Function

Lipid mobilization during the transition period of dairy cows alters both plasma NEFA concentration and content. Previously, different authors described changes in leukocyte and endothelial function activity during periods of lipid mobilization. For example, Lacetera et al. (2004), reported that DNA synthesis, secretion of interferon γ, and IgM synthesis by bovine peripheral blood mononuclear cells (PBMC) is decreased as plasma NEFA concentrations increase. The same group also described how bovine polymorphonuclear cell function and viability were affected by plasma NEFA levels, where neutrophils exhibited increased reactive oxygen species (ROS) production and decreased viability in a NEFA concentration-dependent manner (Scalia et al., 2006). Plasma NEFA content and composition is not the only factor involved in altering leukocyte function; the duration of exposure to high NEFA also plays a key role. In humans, prolonged exposure (over 24 h) to elevated NEFA concentrations enhanced monocyte inflammatory behavior. Some of the observations in this study included elevated gene transcription and cellular surface expression of adhesion molecules such as β integrins; these changes enhanced monocyte adhesion to endothelial cells, which is one of the triggering events to the development of atherosclerotic lesions (Zhang et al., 2006).

Despite recent advances in the understanding of lipid mobilization in dairy cows, veterinary literature is scarce in describing possible mechanisms that link alterations in lipid homeostasis with leukocyte and endothelial dysfunction. Human and rodent studies demonstrated that lipid mobilization could induce alterations in endothelial and leukocyte function, affecting their inflammatory responses in several ways. Direct actions include lipotoxicity. Indirectly, fatty acids could modify intracellular signaling, induce oxidative stress, and alter lipid mediator biosynthesis.

Lipotoxicity

Accumulation of lipids in the cytoplasm of non-adipose cells is defined as ectopic fat deposition or steatosis. The most common lipid metabolites associated with steatosis include NEFA, their corresponding acyl CoAs, diacylglycerol, TG and ceramides. This process is described in human liver, skeletal muscle, pancreas, heart, and vascular tissue (van Herpen and Schrauwen-Hinderling, 2008). In dairy cows, fatty liver is the most common form of steatosis and is almost always related to intense lipid mobilization (Bobe et al., 2004). Excessive accumulation of TG and other lipid metabolites in the hepatocytes and other cells could cause physical damage including compression and reduction of size and number of organelles (Bobe et al., 2004). In human atherosclerosis, physical damage triggered by lipid droplet accumulation in endothelial, smooth muscle cells and macrophages induces inflammatory responses that perpetuate the pathophysiology of the disease (Hansson, 2005).

A second consequence of excessive NEFA and other lipid metabolites accumulation is the induction of programmed cell death or apoptosis. Although not described in leukocytes, lipoapoptosis was characterized in cardiomyocytes and pancreatic ß cells (Listenberger and Schaffer, 2002). Fatty acyl CoAs and fatty acid derived ceramides, inactivate and reduce the

expression of the antiapoptotic factor Bcl_2, thereby, enhancing apoptosis events (Shimabukuro et al., 1998). NEFA and acyl CoAs also can induce apoptosis through a process called endoplasmic reticulum stress. This adaptive response is characterized by an attenuation of protein translation, an up-regulation of endoplasmic reticulum's folding capacity and a degradation of misfolded proteins. When endoplasmic reticulum stress becomes a chronic event, apoptosis is triggered (Cnop et al., 2008). Interestingly, the major fatty acids in transition dairy cows plasma NEFA, palmitate (C16:0) and oleate (C18:1n9c), were reported to induce apoptosis by endothelial reticulum stress in rodent pancreatic cells (Kharroubi et al., 2004). The same fatty acids can induce apoptosis by altering the mitochondrial membrane potential and the composition of cardiolipin. This molecule is the anchoring structure of cytochrome-c, a known pro-apoptotic mitochondrial organelle. If cytochrome-c is released to the cytoplasm, the apoptotic cascade is irreversible (Newsholme et al., 2007).

Intracellular Signaling

As introduced before, NEFA or their corresponding acyl-coAs are capable of altering signaling pathways directly by occupying specific receptors or indirectly by altering protein structure and function by processes such as palmitoylation. In dairy cows, palmitic acid (C16:0) concentration in the cellular membrane PL layer of leukocytes, hepatocytes and adipocytes increases significantly during the transition period (Douglas et al., 2007; Contreras et al., 2010c). Elevation of the concentration of palmitic acid (C16:0) in the plasma membrane could enhance leukocyte activation during the transition period and early lactation through palmitoylation. When omega-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA, C20:5n3c) and docosapentaenoic acid (DHA, C22:6n3c) replace palmitic acid (C16:0), activation of selected leukocytes, such as T-cells, is delayed (Webb et al., 2000). Interestingly, both EPA (C20:5n3c)

and DHA (C22:6n3c) decrease in the PL fraction of cellular membranes during the transition period (Douglas et al., 2007).

Saturated NEFA, including palmitic (C16:0) and stearic (C18:0) acids or their corresponding acyl-coA, can activate NF-κB signaling pathways directly. NF-κB induces transcription of several adhesion molecules including: endothelial leukocyte adhesion molecule (ELAM-1), vascular cell adhesion molecule-1 (VCAM1), intercellular adhesion molecule (ICAM1), and E-selectin (Collins et al., 1995; Ahn and Aggarwal, 2005). NF-κB also can induce the expression of numerous cytokines, chemokines, and their receptors, enhancing the cellular inflammatory response. Whereas saturated fatty acids activate NF-κB, omega-3 polyunsaturated fatty acids can inhibit this inflammatory pathway.

As noted previously, saturated fatty acids are able to activate TLR-4 and through this action, initiate the NF-κB signaling pathway. TLR-4 activation impairs some of the insulin mediated responses in endothelial cells including increased production of nitric oxide that enhances blood flow to skeletal muscle (Muniyappa et al., 2008). In dairy cows, plasma insulin concentrations are at their lowest point right after parturition and this characteristic could enhance the pro-inflammatory activity of TLR-4. Similarly to TLR-4, TLR-2, which recognizes gram positive bacteria cell wall components, can also become activated by saturated fatty acids (Lee et al., 2004; Nguyen et al., 2007). Omega-3 polyunsaturated fatty acids on the other hand, are able to inhibit the activation of TLR-4 (Lee et al., 2003). Changes in the expression of TLR due to lipid mobilization may be linked to excessive inflammatory responses in transition cow diseases such as coliform mastitis and metritis.

Another intracellular signaling pathway directly activated by saturated NEFAs is the mitogen activated protein kinase (MAPK) pathway. A recent study demonstrated that palmitate

(C16:0) can initiate the activation of the p38 MAPK in human aortic endothelial cells inducing apoptosis in a dose dependent manner (Chai and Liu, 2007). This MAPK is responsible for the phosphorylation and down regulation of the anti-apoptotic protein, Bcl-$_{xL}$, a member of the Bcl-2 mitochondrial protein family, which play an important role in the intrinsic mitochondrial apoptotic pathway (Grethe et al., 2004). Although recent studies analyzing gene expression in transition cows reported down regulation of certain MAPK during an intramammary challenge with *Streptococcus uberis* (Moyes et al., 2010), further research is needed to characterize the effects of lipid mobilization on the activity of MAPK.

In monocytes, which increase significantly as a percentage of the circulating leukocyte population during the transition period (Shafer-Weaver et al., 1999), PPARγ is activated by certain omega-3 polyunsaturated fatty acids such as α-linolenic acid and DHA. As a result, the transcription of certain pro-inflammatory signaling pathways such as NFκB is inhibited (Li et al., 2005). Reducing the transcription of NFκB provides anti-inflammatory effects such as limiting the production of inflammatory cytokines like IL-1β, IL-6 and TNFα (Zhao et al., 2005). Paradoxically, the concentrations of omega-3 polyunsaturated fatty acids capable of binding PPARγ decline during the transition period. This could further aggravated by reduced gene expression of PPARγ during infectious events in highly susceptible organs like the mammary gland (Moyes et al., 2010).

Oxidative Stress

Human in vivo studies reveal that plasma NEFA or their corresponding acyl-CoA augment ROS and reactive nitrogen species production by leukocytes and endothelial cells at the mitochondrial or at the cytosolic level (Tripathy et al., 2003; Valko et al., 2007). ROS are derived from molecular oxygen and superoxide anion by the mitochondrial electron transport

chain or from NADPH oxidase. Similarly, reactive nitrogen species are derived from nitric oxide by nitric oxide synthases (Valko et al., 2007). In the mitochondria, NEFA and acyl CoAs can increase the production of ROS by slowing the electron flux within the mitochondrial electron transport chain, and when used as energy substrate, fatty acids enhance the production of ROS during β oxidation (Schönfeld and Wojtczak, 2008). Both effects are increased markedly during the transition period because of the augmented availability of fatty acids due to lipid mobilization, and can be further aggravated in cows with high body condition score that mobilize fatty acids at higher rates when compared with those having a lower body condition score (Bernabucci et al., 2005). Furthermore, obese cows are known to have depleted availability of antioxidant defenses (O'Boyle et al., 2006). Certain types of fatty acids can enhance ROS production during β oxidation; palmitate (C16:0), for example, generates increased amounts of superoxide compared to oxidation of other substrates, such as pyruvate and malate (St-Pierre et al., 2002). Increased concentrations of ROS and RNS impair leukocyte function and their capacity to initiate an effective immune response.

Lipid Mediators

Fatty acids are the substrate for the biosynthesis of lipid mediators (LMs). Variations in the concentration and the fatty acid profiles of plasma lipid fractions and leukocyte's cellular membranes could affect the expression of pro-inflammatory mediators through LM biosynthetic pathways (Calder, 2006). LMs include eicosanoids, lysophospholipids, phosphoinositides, sphingolipids, diacylglycerol, phosphatidic acid, and ceramide (Serhan et al., 2008). LMs are involved in the development and resolution of inflammatory responses. For example, the lysophospholipid, lyso platelet activating factor (*lyso*-PAF), is an early promoter of the expression of inflammatory cytokines IL-1β, IL-8, and ICAM1 (Corl et al., 2008b). Production

of the same cytokines is inhibited by lipoxin A_4, another LM that promotes resolution of inflammatory events (Wu et al., 2008).

Among LMs, eicosanoids are known to mediate chronic and acute inflammatory responses. These compounds are synthesized from omega-3 and omega-6 polyunsaturated fatty acids of 18- and 20- carbons and include prostaglandins, prostacyclins, leukotrienes, lipoxins and thromboxanes. Eicosanoids can activate endothelial cells and leukocyte populations. During the transition period and early lactation, the activity of the eicosanoid-producing enzymes cyclooxygenase (COX) and 15 lipoxygenase (15LOX) is increased, as demonstrated by their enhanced gene expression in peripheral blood mononuclear cells, uterus, and mammary tissue (Silva et al., 2008; Aitken et al., 2009). In dairy cows, the COX pathway is composed of 2 enzyme isoforms, COX1 and COX2. COX pathway products are related to inflammatory events and some prostanoids are powerful vasoactive mediators that induce endothelial responses through cAMP, calcium and other intracellular pathways (Bogatcheva et al., 2005). Besides acting as substrate, fatty acids can also modify the gene and protein expression of COX enzymes. For example, palmitate (C16:0) and stearate (C18:0) are capable of inducing the expression of COX2 in leukocytes through the activation of TLR-4 (Lee et al., 2001). In contrast, omega-3 polyunsaturated fatty acids, such as DHA, can down regulate the protein expression of COX2 in endothelial cells by attenuating the activation of the NFκB pathway (Massaro et al., 2006). Although these observations were replicated in bovine cells, it is likely that increased concentrations of saturated fatty acids and decreased content of omega-3 polyunsaturated fatty acids during the transition period of dairy cows could enhance the expression and activity of COX2.

Like COX enzymes, LOX enzymes metabolize polyunsaturated fatty acids of 18 and 20 carbons (Kühn, 1996). LOX products include hydroxyperoxieicosanoid acids (HPETEs), hydroxyeicosanoid acids (HETEs), hydroperoxyoctadecadienoic acids (HPODE), hydroxyoctadecadienoic acids (HODEs), lipoxins and leukotrienes (Kühn and O'Donnell, 2006). Aitken et al. (2009) demonstrated for the first time that 15LOX1 gene expression increases markedly in mammary tissue during early lactation, but its impact on bovine health is unknown. A possible explanation for such increase in 15LOX1 transcription activity is the augmentation of plasma palmitate (C16:0) in transition dairy cows. Although not demonstrated in endothelial cells or leukocytes of either bovine or human origin, palmitate (C16:0) is capable of enhancing the expression of 15LOX1 in adipocytes in vitro (Chakrabarti et al., 2009). Previous studies in humans suggested that 15LOX1 metabolites induce specific responses that could explain, at least in part, some physiological characteristics observed in dairy cows during the transition period. For example, human neutrophils exposed to 15 HETE exhibit delayed migration. This phenomenon, which is not clearly understood, is observed in transition dairy cows and is thought to increase their susceptibility to coliform mastitis (Burvenich et al., 2003). Other responses generated by 15LOX1 metabolites in humans and rodents include: an enhanced expression of adhesion molecules and an increased oxidation of membrane lipids. *In vitro* studies in bovine endothelial cells demonstrated that 15HPETE induces expression of intercellular adhesion molecule 1 (ICAM1), inhibits prostacyclin (PGI_2) function, and induces apoptosis (Weaver et al., 2001; Sordillo et al., 2005; Sordillo et al., 2008).

NEFAs and Albumin

There are other factors associated with lipid mobilization during the transition period that could affect and modify inflammatory responses in dairy cows. A known consequence of

increased concentrations of NEFAs in plasma is the alteration of the physiological functions of their main transporter, albumin. In transition dairy cows, plasma levels of albumin are known to decrease especially during the first weeks after parturition (Carlson et al., 2007; Seifi et al., 2007; Trevisi et al., 2009). Albumin is considered a negative acute phase protein, implying that its blood concentrations decrease during systemic inflammation (Murata et al., 2004). Reduced concentrations of albumin during the transition period, accompanied by intense lipid mobilization, rapidly increase the NEFA to albumin ratio in plasma. In humans, elevated NEFA:albumin ratios predispose and are part of pathological changes of diseases that include endothelial dysfunction in their pathology. Some examples include pre-eclampsia (Endresen et al., 1992), coronary artery disease (Narang et al., 1997) and metabolic diseases like type 2 diabetes (Cnop et al., 2001). An increased NEFA:albumin ratio also affects the antioxidant properties of albumin that include copper binding. An overload of fatty acids bound to albumin induces a conformation change in the protein; thus altering the antioxidant activity of copper bound to the molecule into prooxidant activity (Gryzunov et al., 2003).

Conclusions

Transition cow metabolic adaptations to NEB have profound consequences not only to energy availability, but also to inflammatory responses and immune function. Lipid mobilization may alter the balance between inflammation development and resolution, thereby favoring excessive inflammatory responses that are major pathophysiological events in some transition cow diseases, including mastitis, metritis, and laminitis (Mateus et al., 2002; Bergsten, 2003; Burvenich et al., 2003). Furthermore, concentration and composition changes in plasma lipids associated with lipid mobilization directly affect leukocyte activity enhancing susceptibility to disease (Burvenich et al., 2007; Contreras et al., 2010c). Ideally on farm management of dairy

cows transitioning from gestation to lactation should restrict lipid mobilization, minimize dry matter intake reductions and assure an adequate and effective supplementation of polyunsaturated fatty acids.

Because of the peculiar characteristics of the transition period in dairy cows, this time will continue to have a heightened incidence of disease. However, understanding adaptation mechanisms initiated by the cow to cope with gestational and lactation requirements can help producers improve transition cow performance. A key regulator of energy demand and supply is lipid metabolism; therefore, future research should focus on interventions that will minimize the need for lipid mobilization and diminish the pro-inflammatory consequences of exacerbated adipose lipolysis. The biology of transition dairy cows offer a unique opportunity to study intense lipid mobilization and other alterations in lipid homeostasis, both being common features of human diseases such as atherosclerosis, metabolic syndrome, obesity, and type-2 diabetes. Progress in lipid science could develop novel nutritional approaches and the discovery of pathway targets that will help dairy cows to enhance lactation productivity with better health and improved welfare.

Conflict of Interest Statement

No conflicts of interests exist with this manuscript.

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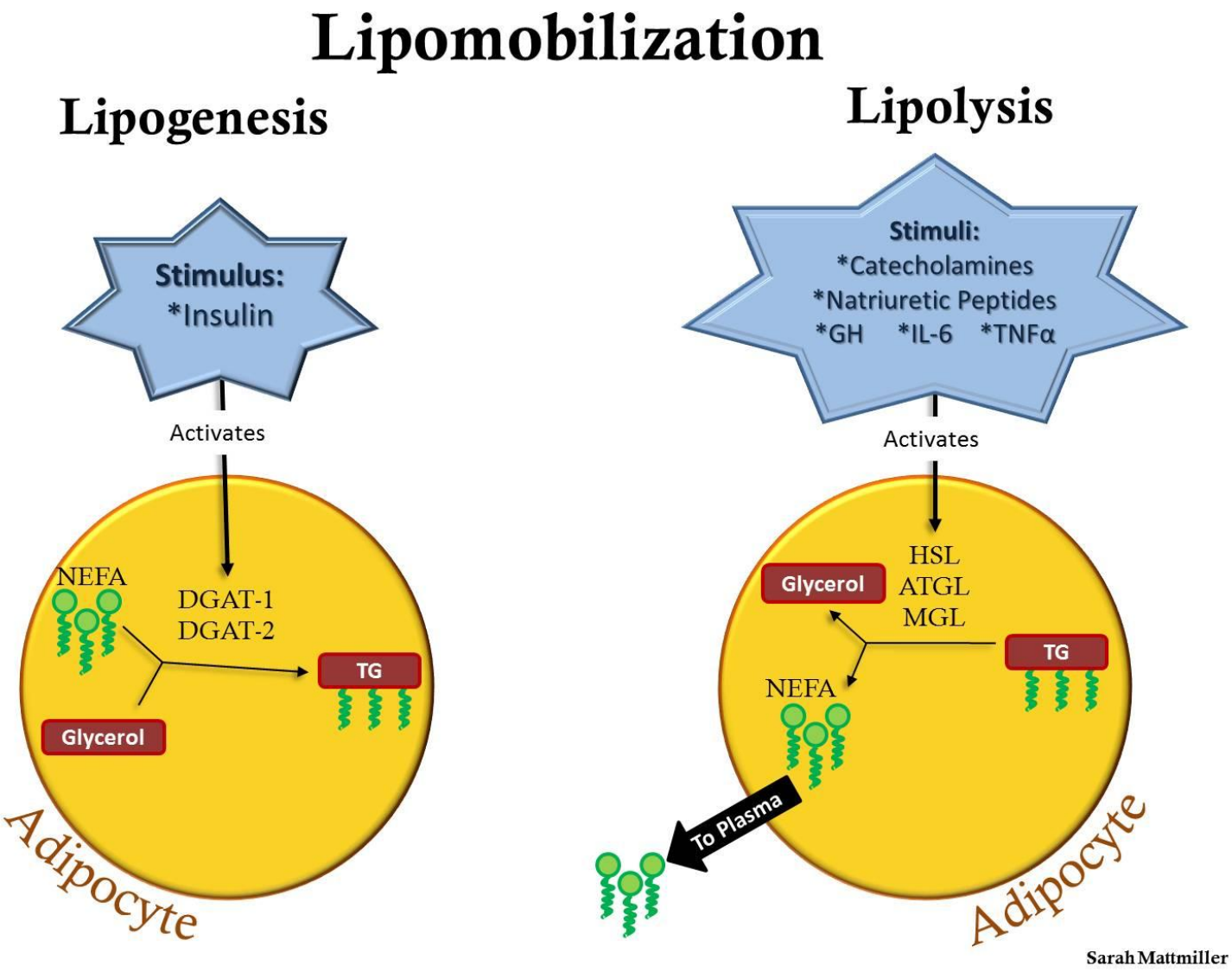

Figure 1. Lipid mobilization in the adipocyte.

The process is divided in lipogenesis and lipolysis. Lipogenesis synthesizes triacylglycerol molecules from non-esterified fatty acids and glycerol. The opposite, lipolysis releases non-esterified fatty acids from triacylglycerol molecules. Lipolysis is enhanced during the transition period by the action of specific hormones including: catecholamines, growth hormone and cytokines (TNFα and IL-6).
For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Table 1. Fatty acids alter immune function through different mechanisms including intracellular signaling and lipid mediator biosynthetic pathways.

DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, αLNA=α-linolenic acid, γLNA=γ-linolenic acid

Fatty Acid	Intracellular Signaling	Lipid Mediator Biosynthesis
Saturated Palmitic, Stearic	○ Palmitoylation ○ Activation of TLR-4 and TLR2 ○ Activation of NFκB ○ Activation of MAPK	○ Activation of COX2 through NFκB
Polyunsaturated Omega-3 DHA, EPA, αLNA	○ Inhibition of NFκB ○ Activation of PPARα, β, γ	○ Inhibitors of COX2 expression ○ Substrate for anti-inflammatory LM e.g. lipoxins, resolvins, protectins
Polyunsaturated Omega-6 Linoleic, γLNA, Arachidonic	○ Activation of PPARα, β, γ	○ Substrate for both anti and pro-inflammatory LM. e.g. prostaglandins, thromboxanes, lipoxins

CHAPTER 2
Lipomobilization in Periparturient Dairy Cows Influences the Composition of Plasma Nonesterified Fatty Acids and Leukocyte Phospholipid Fatty Acids[1]

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Abstract

The periparturient period is characterized by sudden changes in metabolic and immune cell functions that predispose dairy cows to increased incidence of disease. Metabolic changes include alterations in the energy balance that lead to increased lipomobilization with consequent elevation of plasma nonesterified fatty acids (NEFA) concentrations. The objective of this study was to establish the influence of lipomobilization on fatty acid profiles within plasma lipid fractions and leukocyte phospholipid composition. Blood samples from 10 dairy cows were collected at 14 d and 7 d before due date, at calving, and at 7, 14, and 30 d after calving. Total lipids and lipid fractions were extracted from plasma and peripheral blood mononuclear cells. The degree of lipomobilization was characterized by plasma NEFA concentration measurement. The fatty acid profile of plasma NEFA, plasma phospholipids, and leukocyte phospholipids differed from the composition of total lipids in plasma where linoleic acid (C18:2n6c) was the most common fatty acid. Around parturition and during early lactation, the proportion of palmitic acid (C16:0) significantly increased in the plasma NEFA and phospholipid fractions with a concomitant increase in the phospholipid fatty acid profile of leukocytes. In contrast, the phospholipid fraction of long chain polyunsaturated fatty acids in leukocytes was diminished during the periparturient period, especially during the first 2 wk following parturition. This study showed that the composition of total plasma lipids does not necessarily reflect the NEFA and phospholipid fractions in periparturient dairy cows. These findings are significant since it is the plasma phospholipid fraction that contributes to fatty acid composition of membrane phospholipids. Increased availability of certain saturated fatty acids in the NEFA phospholipid fractions may contribute to altered leukocyte functions during the periparturient period.

Key words: NEFA, peripheral blood mononuclear cells, periparturient, lipomobilization

Introduction

The onset of lactation imposes vast energy demands on the dairy cow, involving considerable changes in both dietary requirements and metabolic functions. In dairy cows, the energy requirements of early lactation generally are not met by the diet and mobilization of tissue energy reserves is required. Mobilization of lipids from tissue stores involves the release of fatty acids into the blood stream. Fatty acids are transported in circulation by various lipid fractions that include neutral lipids (**NL**), phospholipids (**PL**), and nonesterified fatty acids (**NEFA**). The NL fraction is composed of triglycerides, diglycerides, monoglycerides, and cholesteryl esters. Both NL and PL are carried by lipoproteins that include very low density lipoprotein, low density lipoprotein, and high density lipoprotein, that function to maintain the lipids in aqueous solution. Fatty acids from these molecules are made available to cell metabolism by the action of lipoprotein lipases (Chung et al., 1995). In contrast, NEFA are held in solution in combination with albumin, although a small portion are transported as unbound monomers in aqueous solution (Richieri and Kleinfeld, 1995). Circulating NEFA are readily available for complete oxidation by a variety of tissues. However, a large portion of them are either partially oxidized to ketone bodies or re-esterified to form triglycerides in the liver (Grummer, 1993; Hocquette and Bauchart, 1999; Jean-François and Dominique, 1999).

In humans, the fatty acid profiles of both plasma lipid fractions and erythrocyte cellular membranes vary greatly during the last trimester of gestation through the first weeks of lactation (Al et al., 1995; Otto et al., 2001). These shifts are a consequence of elevated requirements for fatty acids by the growing fetus and mammary gland (Al et al., 1995; Otto et al., 2001; Hanebutt et al., 2008). Similarly, dairy cows experience a sharp increase in the concentrations of lipids in plasma during the periparturient period in response to higher metabolic demands, driven by fetal

needs and the onset of lactation (Drackley, 1999). This characteristic was described quantitatively for the NEFA fraction. Plasma NEFA in late lactation and the dry period average less than 0.2 mM/L. Concentrations start to increase 2 wks before parturition reaching its highest point during the first 10 d of lactation with concentrations of approximately 0.750 mM/L depending on the degree of lipomobilization. Concentrations may surpass 1.0 mM/L, especially in cows that are destine to develop ketosis (Adewuyi et al., 2005). These alterations in serum lipids have consequences not only for energy redistribution but also for cell metabolism and function.

Disturbances in adipose tissue function and lipid homeostasis (lipodystrophies and dyslipidemias) in human and animal models can lead to insulin resistance and metabolic disorders including altered cellular immune status (Montecucco and Mach, 2009). Fatty acids can affect cellular immune function by modifying intracellular signaling, associating with lipid-raft proteins, binding to specific toll-like receptors (TLR), controlling gene expression, activating transcription factors, inducing apoptosis, and modifying lipid mediator production (Calder and Yaqoob, 2007). Therefore the fatty acid composition of immune cells directly affects their activity. The effect of specific fatty acids on immune cell function was described in humans (Haversen et al., 2009). However, changes in the fatty acid profile of the plasma NEFA and PL fractions during the periparturient period of dairy cows are not defined in the literature. Furthermore, the implications of shifts in the fatty acid composition of bovine immune cell lipid fractions are largely unknown. The objective of this study was to describe the variations in the fatty acid profile of NEFA, plasma PL fractions, and PL content of peripheral blood mononuclear cells (PBMC) obtained from periparturient dairy cows.

Materials and Methods

Animals and Diets

All animal procedures were approved by the Michigan State University Animal Care and Use Committee. Ten healthy, mature, multiparous, Holstein cows were selected at the moment of dry-off from a large commercial Michigan dairy herd. Animals were chosen based on the following criteria: more than 210 d of gestation, a last DHI test with SCC less than 250,000 cells/mL, and a body condition score of 3.5 to 3.75. During the trial, cows were monitored for health status and exhibited no lameness or other disease. Animals were housed in free stalls and fed two different rations based on lactation status, a transition diet and a lactation diet. The ration composition and fatty acid profile of each diet is shown in Tables 2 and 3. Samples were collected when dry cows entered the close-up pen at 14 d before expected calving, a wk later at 7 d before due date, at calving, and at 7, 14, and 30 d after calving.

Peripheral Blood Mononuclear Cells Isolation

Samples were collected in the morning between 0800 and 0900 h after feed was delivered. Blood (150 mL) was obtained by jugular venipuncture and immediately mixed with a 15 mL 40 mM EDTA solution containing ascorbic acid (5 g/L). The PBMC were isolated by differential gradient using Ficoll-Paque Plus (GE Healthcare, Upsala, Sweden), collected at the interface after centrifugation (456 g for 30 min), and washed 3 times with Hank's balanced salt solution. PBMC and plasma samples were stored at -80°C until lipid extraction procedure. A sub-sample of PBMC was collected to determine cellular populations using flow cytometry as described by (Shafer-Weaver et al., 1999). Cells were incubated with each of the following lineage-specific bovine monoclonal antibodies (VMRD, Pullman, WA): CD2 (T lymphocytes BAQ95A at 1:100 vol/vol), CD3 (T-cell receptor MM1A at 1:100 vol/vol), CD4 (T-helper

CACT83B at 1:160 vol/vol), B-B2 (B-lymphocytes BAQ44A at 1:150 vol/vol), MG (monocyte/granulocyte CD172a DH59B at 1:100 vol/vol), CD14 (monocyte M-M8 at 1:50 vol/vol).

Lipid Extractions

Total lipids from plasma and PBMC were extracted using a hexane:ethanol (1:1) solvent mix. Plasma or a saline solution containing at least $1x10^7$ PBMC per ml were combined with the solvent mix and vortexed for 10 min, and then centrifuged at 2,095 g for 10 min at room temperature. Finally the top hexane layer was collected for total fatty acid composition and lipid fractioning. Nonadecanoic acid was used as internal standard.

Lipids were further separated into NL, NEFA and PL fractions by solid-phase extraction using Strata cartridges containing aminopropyl (NH_2) sorbent (Phenomenex, Torrance, CA) as described by others (Kaluzny et al., 1985). Briefly, after conditioning of the cartridges with hexane, the NL fraction was eluted with chloroform:2-propanol (2:1), the NEFA with 2% acetic acid in diethyl ether, and the phospholipids fraction with methanol. 1,2-Diheneicosanoyl-*sn*-Glycero-3-phosphocholine (21:0 PC) was used as internal standard for lipid fractioning. Fatty acids from total extractions and each lipid fraction were transesterified and methylated using 20% methanolic hydrochloric acid for 2 h at 90°C (Browse et al., 1986). Methylated extractions were analyzed using a Perkin Elmer - Clarus 500 gas chromatography apparatus with a Supelco SP-2560 column (100m x 25 mm with a 0.2um film thickness). Fatty acid peaks were identified by using a mix of pure methyl ester standards (Nu Check, Elysian, MN).

Serum total NEFA concentrations were quantified with the NEFA-HR-2 kit (Wako, Richmond, VA), following the manufacturers protocol. Nine standards were prepared from the supplied stock (0.1 to 1.3 mEq/L). Samples, standards, and blanks were run in triplicate in a 96-

well microplate at 6 µL per well. The microplate was incubated twice for 5 min after adding each of the 2 reagents. The samples were read at 550 nm using a Victor^{TM} microplate reader (Perkin Elmer, Waltham, MA).

Statistical Analysis

Variables were analyzed as repeated measures using a mixed model procedure (PROC MIXED; SAS Inst. Inc., Cary NC). The following model was used to estimate the sampling day effect on each of the measured variables:

$$Y_{ij} = \mu + S_i + e_{ij}.$$

Where Y_{ij} was the dependent variable for cow_i in $sample_j$ relative to calving; μ is the overall mean of the population; S_i is the fixed effect of time as the repeated factor; and e_{ij} is the random error, assumed to be correlated. Least square means were calculated and adjusted using the Tukey-Kramer method. The CORR procedure of SAS was used to determine correlations between specific fatty acid concentrations in different lipid fractions in plasma and PBMC, total plasma NEFA (quantified in mEq/L), and the percentage values for specific PBMC phenotypes. Significance was set at α=0.05.

Results

PBMC Populations

Changes in leukocyte populations were observed when comparing phenotype distributions at different sampling stages (Figure 2). As described in previous reports (Shafer-Weaver et al., 1999), monocyte/granulocytes populations (CD172a) increased significantly as a proportion of PBMC at calving and 7 d in milk when compared to early lactation (14 d and 30 d). Similarly, the expression of CD14 (a co-receptor expressed on monocytes) increased at

parturition when compared to values at other sampling time points. In contrast, the T cell subpopulation (CD3) presented a significant decrease at calving and at 7 d in milk when comparing values to 14 d prior to calving and 30 d in lactation.

Lipid Fractions in Plasma and PBMC

NEFA concentrations are shown in Figure 3. Lipomobilization was demonstrated by a significant increase of plasma NEFAs at calving (0.54 mEq/L) and 7 d (0.67 mEq/L), when compared to prepartum (-14 d and -7 d) and early lactation levels (14 and 30 d).

The fatty acid composition of plasma lipids is shown in Table 4. Linoleic acid (C18:2n6c) was the main fatty acid and its weight percentage increased significantly during early lactation (14 d and 30 d) when compared to values before parturition and during the first wk after calving. In contrast, stearic acid (C18:0) values decreased from 14 d before parturition through early lactation (30 d) and values at 7 d before due date (16.36g / 100g) were significantly higher than those observed at 30 d (12.86g/100g). Oleic acid (C18:1n9c) values increased from 7 d prior to calving until 14 d in milk, coinciding with plasma NEFA increment. Oleic acid (C18:1n9c) concentration decreased, however, by 30 DIM. Values for myristic (C14:0), palmitic (C16:0), arachidonic (C20:4n6c) and docosahexanoic acids (DHA) (C22:6n3c) were constant through the periparturient period. Eicosapentanoic acid (C20:5n3c) (EPA) concentrations were undetected in the total plasma lipid samples.

The plasma PL fraction composition is presented in Table 5. The predominant fatty acids in this lipid fraction were palmitic (C16:0) and stearic (C18:0) acids. The concentration of palmitic acid (C16:0) was lower at 14 d before due date (26.01g/100g) increasing gradually until reaching its highest point at 14 d after calving (37.76g/100g). Linoleic acid (C18:2n6c) concentration was significantly higher during the first wk after calving (10.67g/100g), compared

to other time points. Whereas EPA (C20:5n3c) concentrations were significantly higher during early lactation when compared to pre-calving samples, arachidonic acid (C20:4n6c) and DHA (C22:6n3c) concentrations were found to be steady through the periparturient period.

The plasma NEFA composition is presented in Table 6. Similar to the PL fraction, palmitic (C16:0) and stearic (C18:0) acids were the predominant fatty acid. Palmitic acid (C16:0) values increased significantly from 14 d (25.86g/100g) and 7 d (25.29g/100g) before parturition when compared to 7 d in lactation (32.81g/100g). Oleic (C18:1n9c), linoleic (C18:2n6c), and DHA (C22:6n3c) concentrations remained unchanged through the 6 wk follow-up period. Arachidonic (C20:4n6c) and EPA (C20:5n3c) were detected in very low concentrations in this fraction.

The fatty acid composition of the PBMC PL fraction is showed in Table 7. Reflecting the profiles of plasma NEFAs and PL fractions, the predominant fatty acids were palmitic (C16:0) and stearic (C18:0). Palmitic acid (C16:0) significantly increased from the wk prior to calving (-7d, 30.96g/100g) when compared to 7d (34.75g/100g), 14d (35.79g/100g), and 30d (35.04g/100g). Myristic (C14:0) and stearic (C18:0) acids remained constant through the sample period. Linoleic acid (C18:2n6c) concentrations significantly increased after parturition. Measures of long chain polyunsaturated fatty acids (**PUFA**), including arachidonic (C20:4n6c), EPA (C20:5n3c) and DHA (C22:6n3c), were variable throughout the sampling period.

Correlation coefficients were calculated to establish interactions between plasma NEFAs concentration, the most common fatty acids in plasma NEFA and PL (palmitic (C16:0) and stearic (C18::0) acids), and the percentage of monocytes within the PBMC population (Table 8). NEFA concentration was positively correlated with palmitic acid (C16:0) concentrations in PBMC ($r = 0.394$), palmitic acid (C16:0) in plasma PL ($r = 0.326$), and the percentage of

monocytes in PBMC (r = 0.424). The NEFA value was negatively correlated with values for stearic acid (C18:0) in plasma NEFA (r = -0.35). The values of stearic acid (C18:0) in the PL fraction of PBMC were correlated with palmitic acid (C16:0) in PL of PBMC and plasma PL (r = 0.305). Within the plasma NEFA fraction, values for stearic (C18:0) and palmitic (C16:0) acids were highly correlated (r = 0.825).

Discussion

Common physiological events associated with mammalian parturition and the onset of lactation drive changes in lipolysis and lipogenesis. Lipomobilization is a physiological adaptation by mammals in response to reduced nutrient and energy availability. Previous research showed that lipomobilization not only affects relative concentrations of total plasma lipids and corresponding fractions, but also causes major shifts in fatty acid composition (Douglas et al., 2007). Results from the present study confirmed this observation as total lipid fatty acid profiles varied from prepartum to early lactation sampling periods. Linoleic acid (C18:2n6c) concentrations increased after parturition in contrast to stearic acid (C18:0) values which decreased as lactation progressed. These modifications in the plasma total lipid fatty acid profile reflected changes in the diet and the increased rate of lipomobilization. However, fatty acid composition of total plasma lipids does not reflect the fatty acid profile of certain plasma lipid fractions. This is due, in part, to differences in fatty acid distributions among lipid classes. For example, linoleic acid (C18:2n6c) is preferentially found in the NL fraction within cholesterol esters, whereas PUFA (arachidonic (C20:4n6c) and EPA (C20:5n3c)) that are found in the PL fraction. These particularities also were demonstrated by others in late (Tyburczy et al., 2008) and early lactation cows (Leroy et al., 2005).

The fatty acids of the various serum lipid fractions have different metabolic fates and functions. Neutral lipids (triglycerides, diglycerides, monoglycerides, and cholesterol esters) are intermediate molecules of lipid metabolism with dense energy content, but require the activity of lipoprotein lipases to provide such energy to different tissues. Phospholipids on the other hand, are the main components of biological membranes, including the unilamellar membrane of serum lipoproteins. Phospholipids provide lipid substrates for the biosynthesis of potent proinflammatory mediators, including the eicosanoids and platelet activating factor (Henneberry et al., 2002). Finally, NEFAs are involved in intracellular signaling processes, modification of cellular functions, or as energy substrates.

In general, reductions in the body content of adipose tissue (lipomobilization) as reflected by an increase in NEFA, leads to impaired metabolic and immune cell functions (Mora and Pessing, 2002). In humans, increased NEFA concentrations were linked to inflammatory based diseases such as asthma, atherosclerosis, metabolic syndrome, and type 2 diabetes (Boden, 2008; Wood et al., 2009). Previous studies in dairy cows also demonstrated that elevated NEFA during the periparturient period largely affect immune cell functions (Lacetera et al., 2004; Scalia et al., 2006). In the present study, plasma NEFA concentrations were correlated positively with changes in the PBMC populations, particularly with the relative percentage of monocytes in circulation. The activity of this subset of mononuclear cells is especially modified during the periparturient period exhibiting enhanced production of pro-inflammatory cytokines (Sordillo et al., 1995). A recent report in humans demonstrated that increased cytokine production by macrophages is influenced greatly by palmitic (C16:0) and stearic (C18:0) fatty acids (Håversen et al., 2009). Therefore, the immune cell activity during the periparturient period may not only be affected by the quantity of NEFA in blood, but also by the profile of its fatty acids.

A known consequence of increased concentrations of NEFAs in plasma is the alteration of the physiological functions of their main transporter, albumin. In periparturient dairy cows, plasma concentrations of this protein are known to be diminished especially during the first weeks after parturition (Trevisi et al., 2009). Albumin is considered a negative acute phase protein, implying that its blood concentrations decrease during systemic inflammation (Murata et al., 2004). Reduced concentrations of albumin during periparturient period accompanied by intense lipomobilization rapidly increase the NEFA to albumin ratio. In humans, the consequences of elevated NEFA to albumin ratio predispose subjects to immune-endothelial dysfunction diseases such as pre-eclampsia (Endresen et al., 1992) and to metabolic diseases like type 2 diabetes (Cnop et al., 2001). The physiological consequences of altered NEFA to albumin ratio in the dairy cow warrants further investigation.

In contrast to total lipid fatty acid profile in plasma where linoleic acid (C18:2n6c) was the main fatty acid, the principal components of plasma NEFA and PL were palmitic (C16:0), stearic (C18:0), and oleic (C18:1n9c) acids. During lipomobilization events, hormone-sensitive lipases and triglyceride lipase release fatty acids from triglyceride depots in the adipose tissue (Kershaw et al., 2006). These molecules are released into circulation and transported bounded to albumin. A logical conclusion is that during periods of negative energy balance, NEFA profiles may directly reflect the fatty acid composition of adipose tissue. In fact, a recent study described the fatty acid profiles of subcutaneous adipose tissue in periparturient cows finding that the main fatty acids were palmitic (C16:0), stearic (C18:0), and oleic (C18:1n9c) acids (Douglas et al., 2007). Although our study did not evaluate fatty acid profiles in adipose tissue, differences between plasma NEFA and adipose tissue composition should be expected. In human adipose tissue for example, long chain unsaturated fatty acids are preferentially mobilized from, and re-

extracted by adipose tissue during lipolysis and lipogenesis (Raclot, 2003), respectively. In dairy cattle, the patterns of fatty acid trafficking from adipose tissue during lipomobilization are unknown. Another potential explanation for differences between adipose tissue and plasma NEFA profiles is that the fatty acid composition of adipose tissue varies depending on anatomical localization (e.g. visceral vs. subcutaneous depots). Therefore, the composition of lipid deposits that poses higher mass and blood irrigation has higher influence on the final NEFA profile during lipomobilization events. Indeed, human visceral fat is higher in saturated fatty acids including palmitic (C16:0) and stearic (C18:0) acids when compared to subcutaneous fat (Phinney et al., 1994). Further studies are required to improve our knowledge with respect to lipomobilization, including the effect of adipose tissue site, fatty acid composition of adipose deposits, and the preferential mobilization of specific fatty acids in periparturient dairy cows. Despite the importance of plasma NEFA composition on the inflammatory process, there is a lack of knowledge about variations in plasma NEFA profiles during different production stages in dairy cows. Indeed, the majority of studies in periparturient cows that include dietary or pharmacological interventions to improve periparturient dairy cows' health only describe the total fatty acid concentration.

The NEFA profile composition was reflective of the fatty acid contents of plasma and PBMC PL fractions. Various research groups demonstrated in humans that the lipid composition of the PL fraction of immune cells directly affects their physiological function and response to inflammatory stimuli (Kew et al., 2003; Trebble et al., 2004). The saturated palmitic (C16:0) and stearic (C18:0) fatty acid components of this fraction increased during weeks of intense lipomobilization. Both of these saturated fatty acids are known for their pro-inflammatory characteristics, including the ability to activate different intracellular inflammatory signaling

pathways including NF-κB and MAPK (Calder and Yaqoob, 2007; Serhan et al., 2008). Palmitic (C16:0) and stearic (C18:0) acids also can promote inflammation by enhancing the production of inflammatory cytokines and lipid mediators. For example, palmitic acid (C16:0) through its activated molecule palmitoyl-coA is able to enhance the production of cytokines TNF-α, IL-1β and IL-8 in monocytes. Additionally, stearate (C18:0) and palmitate (C16:0) can induce the expression of cyclooxygenase 2, an inflammatory eicosanoid enzyme (Lee et al., 2001) via activating TLR-4. Several recent studies reported an increased expression of several pro-inflammatory genes in periparturient cows (Loor et al., 2005; Sordillo et al., 2005; Aitken et al., 2009). Although conjecture, the increased concentrations of saturated fatty acids in the different plasma and cellular lipid fractions may contribute to increase inflammatory responses of periparturient dairy cows.

In contrast to saturated fatty acids, the concentrations PUFA in plasma NEFA and PL, as well as in the PL fraction of PBMC were low when compared to values reported in other species (Kew et al., 2003; Fritsche, 2007). Ruminants have limited availability of PUFA due to biohydrogenation of these compounds in the rumen and the limited synthesis of such acids by ruminal bacteria (Jenkins, 1993). Dairy cows have partially offset this limited availability by their very efficient intestinal fatty acid absorption of PUFA (Bauchart, 1993). Reduced levels of PUFA are detrimental to immune cell function (Harbige, 2003). These fatty acids are the substrate for the synthesis of several lipid mediators. Depending on the fatty acid used as substrate and the biosynthetic enzyme, these molecules act as initiators, modulators and/or resolvers of inflammation (Serhan et al., 2008). Previous studies in humans demonstrated that PUFA supplementation, specifically omega-3 fatty acids, improves the outcome of inflammatory diseases such as rheumatoid arthritis, asthma, and atherosclerosis (Kris-Etherton

et al., 2003; Wong, 2005; Galarraga et al., 2008). In dairy cows, various research groups have evaluated PUFA supplementation effects on immune cell function during the periparturient period (Lessard et al., 2003; Lessard et al., 2004; Rodriguez-Sallaberry et al., 2007). None of these studies, however, evaluated the effectiveness of PUFA supplementation to change the fatty acid composition of immune cells, though it has been proven to change the lipid profile of liver and adipose tissue (Douglas et al., 2007). This study elucidated the implications of lipomobilization during the periparturient period on fatty acid composition of plasma lipid fractions and PL of immune cells. Based on our findings and observations in human medicine, future evaluations of fatty acid supplementation in periparturient dairy cows should include a critical evaluation of the shifts in these fractions.

Conclusion

Lipomobilization is a common physiological process during the periparturient period of mammalian species and functions to provide energy to the dam during periods of increased energy requirements due to fetal growth and lactogenesis. However, this process is exacerbated in dairy cows because of their homeorhetic drive to meet genetic potential for milk production. Although lipomobilization increases energy availability, it also affects lipid homeostasis and promotes changes in the fatty acid profiles of different plasma lipid fractions. In the present study, palmitic (C16:0) and stearic (C18:0) acids were the most prominent components of plasma NEFA and PL. The composition of both plasma lipid fractions was reflected in the fatty acid profile of PL fraction of PBMC. Increased concentrations of saturated fatty acids and diminished amounts of PUFA in the PL fraction of PBMC could negatively affect the immune cell response during the periparturient period. Further studies are needed to better characterize

lipomobilization during the periparturient period and its consequences on lipid homeostasis, metabolism and immune cell function.

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Table 2. Ingredient composition of pre-calving and lactation diets

Ingredient	Diet	
	Transition	Lactation
Alfalfa haylage[1]	0	6.52
Corn Silage[2]	14.27	23.53
Wheat Straw	4.24	0.46
Wet Corn Gluten	1.23	1.14
Dry Corn Gluten	0	1.28
Bakery byproduct	0	1.23
Canola Meal	0	1.00
Corn Grain Medium Grind	0	1.78
Soybean meal solvent	0	2.01
Dried Citrus Pulp	0	1.28
Wet Beet Pulp	0	3.97
High moisture corn	0	2.22

Continues next page

Table 2. (cont'd)

Ingredient	Diet	
	Transition	Lactation
Supplements and mineral mix	0.55	1.60
Vitamin ADE mix[3]	0.04	0.02
Trace Mineral Mix[4]	0	0.02
Selenium blend[5]	0.05	0
Vitamin E[6]	0.005	0
Sodium Selenate	0.003	0
Sodium Sesquicarbonate	0	0.52
Calcium Carbonate	0.134	0.47
Sodium chloride	0.078	0.26
Ground Soya Bean Hulls	0.096	0
Wheat Midds	0	1.2
Magnesium Sulfate	0.187	0
Calcium Sulfate	0.311	0
Magnesium Oxide	0.062	0.067
Blood Meal	0.187	0.552
Fishmeal	0.062	0.247
Biotin 1%	0.005	0.004
Mepron[7]	0	0.01
Tallow	0.024	0.1
Rumensin 80	0.004	0.003
Chemical analysis, % of DM		
NDF	50.9	29.2
ADF	29.9	16.89
Ether Extract	3.08	3.9
NEl MJ/Kg of DM[8]	5.49	7.27

Values expressed in kg of dry matter

[1]Corn silage 31% DM (as fed), [2]Alfalfa haylage 42% DM (as fed), [3]Vitamin ADE mixture contained (g/kg) 10.8 retinyl acetate, 0.18 cholecalciferol, and 0.047 DL-α-tocopherol. [4]Trace mineral mix contained (g/100g): 13.0 calcium, 0.3 magnesium, 2.0 copper, 8.8 magnesium, 12.0 sulfur, 10.5 zinc, 0.3 manganese, 0.25 cobalt and 0.19 iodine, [5]Selenium blend contains 0.006% sodium selenite. [6]Vitamin E contained 68.0 g/kg of DL-α-tocopherol. [7]Rumen-protected methionine; Evonik Industries AG, Essen, Germany. [8]Net energy for lactation.

Table 3. Fatty acid composition of pre-calving and lactation diets

Fatty acid, g per day	Diet	
	Transition	Lactation
C12:0	0.99	7.45
C14:0	1.24	7.72
C16:0	41.67	113.81
C16:1	1.08	4.11
C18:0	7.34	28.92
C18:1	40.18	141.96
C18:2	105.85	264.36
C18:3	34.00	49.78
Other	7.43	20.55

Table 4. Fatty acid composition of total fatty acids in plasma of dairy cows (n=10)

Date	C14:0	C16:0	C18:0	C18:1	C18:2	C20:4	C20:5	C22:6
-14	0.71 ± 0.36	14.22 ± 0.91	18.88 ± 2.13 ^{ab}	11.64 ± 1.14 ^{a}	26.03 ± 1.84 ^{b}	2.97 ± 0.22	. ± .	1.10 ± 0.37
-7	1.02 ± 0.25	15.56 ± 0.56	16.36 ± 0.58 ^{a}	12.10 ± 0.64 ^{ab}	27.42 ± 1.55 ^{b}	2.98 ± 0.11	. ± .	1.22 ± 0.25
0	1.02 ± 0.22	15.91 ± 0.35	15.46 ± 0.88 ^{ab}	14.96 ± 1.16 ^{bd}	28.02 ± 1.40 ^{b}	3.15 ± 0.12	. ± .	0.67 ± 0.07
7	0.79 ± 0.22	15.47 ± 0.42	14.69 ± 0.64 ^{ab}	14.99 ± 0.55 ^{c}	32.01 ± 1.33 ^{b}	2.78 ± 0.16	. ± .	0.77 ± 0.12
14	0.52 ± 0.15	15.18 ± 0.53	13.33 ± 0.67 ^{ab}	12.81 ± 0.80 ^{cd}	35.79 ± 1.45 ^{a}	2.67 ± 0.20	. ± .	1.00 ± 0.24
30	1.11 ± 0.06	12.46 ± 0.70	12.86 ± 0.27 ^{b}	9.36 ± 0.88 ^{cd}	38.86 ± 1.32 ^{a}	2.11 ± 0.32	0.21 ± 0.13	0.96 ± 0.14

Values are expressed as grams per 100 grams of total fatty acids

abc Means in a column without a common letter differ, P< 0.05

C14:0 myristic acid, C16:0 palmitic acid, C18:0 stearic acid, C18:1 oleic acid, C18:2 linoleic acid

C20:4 arachidonic acid, C20:5 eicosapentanoic acid, C22:6 docosahexanoic acid

Table 5. Fatty acid composition of plasma phospholipid fraction of dairy cows (n=10)

Date	C14:0	C16:0	C18:0	C18:1	C18:2	C20:4	C20:5	C22:6
-14	1.47 ± 0.07	26.01 ± 0.90 [a]	36.74 ± 2.89	8.37 ± 1.55	6.14 ± 1.39 [a]	1.28 ± 0.46	1.13 ± 0.06 [ac]	0.73 ± 0.34
-7	1.52 ± 0.06	30.24 ± 1.37 [ab]	34.81 ± 2.07	6.92 ± 1.45	5.46 ± 1.33 [a]	1.27 ± 0.33	1.22 ± 0.10 [ac]	0.83 ± 0.33
0	1.67 ± 0.11	35.14 ± 1.86 [ab]	35.44 ± 1.60	5.29 ± 0.81	4.02 ± 0.91 [a]	1.06 ± 0.20	1.63 ± 0.13 [bc]	0.69 ± 0.10
7	1.22 ± 0.08	33.46 ± 2.38 [ab]	32.71 ± 2.35	7.72 ± 1.36	10.67 ± 1.24 [b]	1.24 ± 0.19	1.45 ± 0.17 [abc]	0.59 ± 0.05
14	1.39 ± 0.12	37.76 ± 1.80 [b]	34.77 ± 1.52	4.05 ± 0.58	4.47 ± 1.19 [a]	0.58 ± 0.13	1.73 ± 0.08 [b]	1.10 ± 0.17
30	1.80 ± 0.10	31.91 ± 1.53 [ab]	36.18 ± 2.03	4.19 ± 0.82	6.63 ± 1.40 [ab]	0.98 ± 0.33	1.76 ± 0.09 [b]	0.96 ± 0.16

Values are expressed as grams per 100 grams of total fatty acids

abc Means in a column without a common letter differ, $P < 0.05$

C14:0 myristic acid, C16:0 palmitic acid, C18:0 stearic acid, C18:1 oleic acid, C18:2 linoleic acid
C20:4 arachidonic acid, C20:5 eicosapentanoic acid, C22:6 docosahexanoic acid

Table 6. Fatty acid composition of plasma NEFA fraction of dairy cows (n=10)

Date	C14:0	C16:0	C18:0	C18:1	C18:2	C20:4	C20:5	C22:6
-14	2.50 ± 0.22	25.86 ± 0.76 [a]	40.46 ± 0.57	10.20 ± 3.02	0.89 ± 0.27	. ± .	. ± .	0.72 ± 0.11
-7	2.49 ± 0.11	25.29 ± 1.09 [a]	36.96 ± 1.91	8.95 ± 2.54	1.30 ± 0.23	. ± .	0.56 ± 0.38	1.33 ± 0.62
0	2.17 ± 0.12	27.20 ± 1.21 [ab]	35.75 ± 2.35	13.51 ± 3.54	1.07 ± 0.19	. ± .	0.74 ± 0.05	1.96 ± 0.42
7	2.04 ± 0.35	32.81 ± 1.59 [b]	43.52 ± 2.74	3.19 ± 2.24	0.68 ± 0.28	. ± .	0.26 ± 0.26	2.48 ± 0.56
14	2.15 ± 0.30	27.77 ± 1.56 [ab]	39.48 ± 2.48	6.57 ± 2.10	1.28 ± 0.55	. ± .	0.78 ± 0.04	2.08 ± 0.44
30	1.60 ± 0.68	30.91 ± 2.45 [ab]	49.39 ± 4.60	3.75 ± 1.79	0.84 ± 0.51	. ± .	. ± .	2.00 ± 1.38

Values are expressed as grams per 100 grams of total fatty acids

ab Means in a column without a common letter differ, $P < 0.05$

C14:0 myristic acid, C16:0 palmitic acid, C18:0 stearic acid, C18:1 oleic acid, C18:2 linoleic acid
C20:4 arachidonic acid, C20:5 eicosapentanoic acid, C22:6 docosahexanoic acid

Table 7. Fatty acid composition of PBMC phospholipid fraction of dairy cows (n=10)

Date	C14:0	C16:0	C18:0	C18:1	C18:2	C20:4	C20:5	C22:6
-14	1.57 ± 0.43	30.86 ± 1.85 ^{ab}	38.61 ± 1.53	1.92 ± 0.67	0.32 ± 0.03 ^{a}	0.38 ± 0.11	1.68 ± 0.38	1.16 ± 0.35
-7	1.45 ± 0.23	30.96 ± 0.82 ^{a}	34.82 ± 1.82	4.21 ± 0.98	1.02 ± 0.50 ^{ab}	0.61 ± 0.26	1.12 ± 0.24	1.04 ± 0.19
0	1.33 ± 0.15	31.87 ± 1.76 ^{ab}	32.63 ± 1.87	3.67 ± 0.66	0.88 ± 0.28 ^{ab}	1.15 ± 0.40	1.21 ± 0.26	0.96 ± 0.14
7	1.68 ± 0.26	34.75 ± 0.81 ^{b}	35.39 ± 1.11	3.71 ± 0.62	0.77 ± 0.18 ^{b}	1.11 ± 0.43	0.77 ± 0.10	1.04 ± 0.21
14	1.64 ± 0.22	35.79 ± 1.12 ^{b}	34.49 ± 1.60	4.16 ± 0.49	0.89 ± 0.18 ^{b}	1.23 ± 0.44	0.60 ± 0.08	1.10 ± 0.15
30	1.38 ± 0.24	35.04 ± 0.92 ^{b}	36.67 ± 1.31	4.19 ± 0.39	0.97 ± 0.28 ^{b}	1.63 ± 0.35	0.84 ± 0.09	0.99 ± 0.19

Values are expressed as grams per 100 grams of total fatty acids

ab Means in a column without a common letter differ, $P < 0.05$

C14:0 myristic acid, C16:0 palmitic acid, C18:0 stearic acid, C18:1 oleic acid, C18:2 linoleic acid

C20:4 arachidonic acid, C20:5 eicosapentanoic acid, C22:6 docosahexanoic acid

Table 8. Pearson correlation coefficients for selected fatty acids in different lipid fractions and monocyte population values of periparturient dairy cows (n=10)

	NEFA	Palmitic PBMC	Palmitic PL	Palmitic NEFA	Stearic PBMC	Stearic PL	Stearic NEFA	MG[1]
NEFA	1	0.394 0.01	0.326 0.05	-0.094 NS[2]	-0.097 NS	-0.116 NS	-0.353 0.02	0.424 0.002
PalmiticPBMC	0.394 0.01	1	0.135 NS	0.132 NS	0.305 0.04	-0.245 NS	0.160 NS	-0.044 NS
PalmiticPL	0.326 0.05	0.135 NS	1	0.113 NS	-0.448 0.004	0.286 NS	0.043 NS	0.102 NS
PalmiticNEFA	-0.094 NS	0.132 NS	0.113 NS	1	0.015 NS	-0.169 NS	0.825 <.0001	-0.063 NS
StearicPBMC	-0.097 NS	0.305 0.04	-0.448 0.004	0.015 NS	1	0.007 NS	0.137 NS	-0.048 NS
StearicPL	-0.116 NS	-0.245 NS	0.286 NS	-0.169 NS	0.007 NS	1	0.074 NS	-0.143 NS
StearicNEFA	-0.353 0.02	0.160 NS	0.043 NS	0.825 <.0001	0.137 NS	0.074 NS	1	-0.240 NS
MG	0.424 0.002	-0.044 NS	0.102 NS	-0.063 NS	-0.048 NS	-0.143 NS	-0.240 NS	1

[1] Percentage of mononuclear cell population reactive to monocyte/granulocyte antibody (CD172)
[2] P>0.05

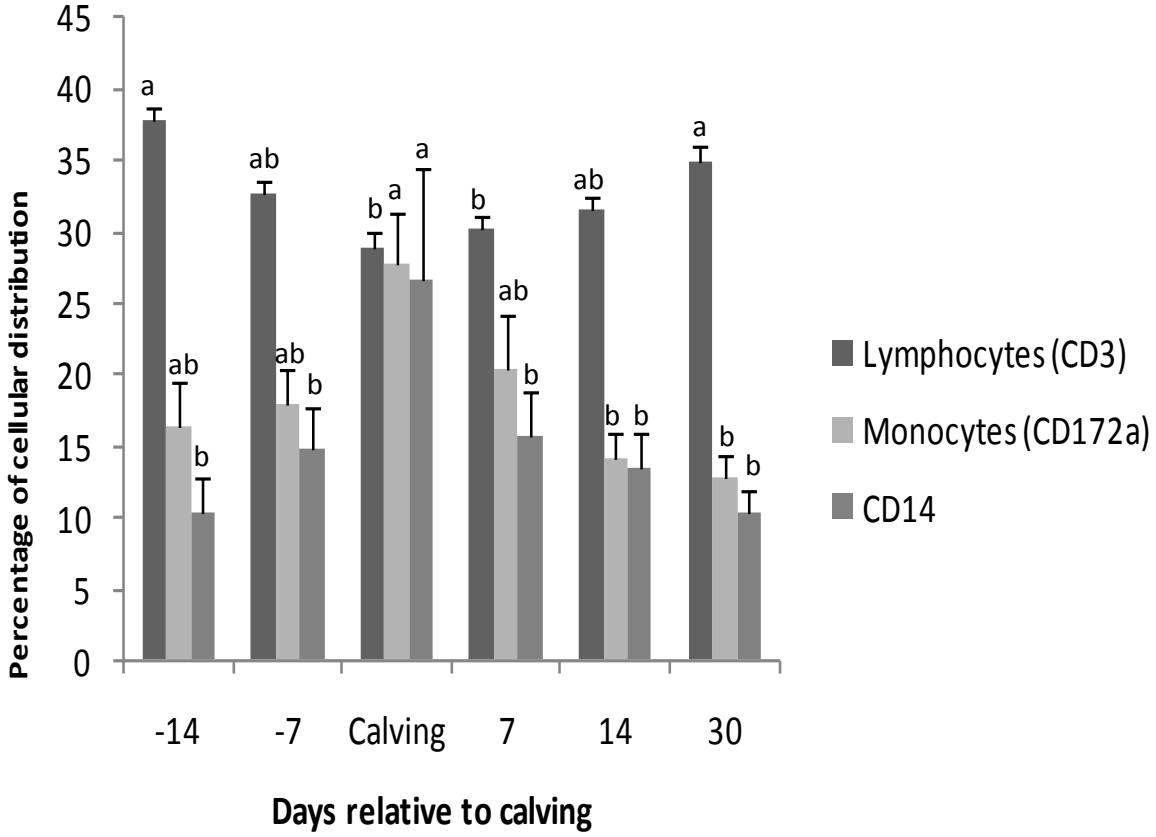

Figure 2. Peripheral blood mononuclear cells (PBMC) phenotype distribution changes relative to calving date in periparturient dairy cows (n=10).

Values are percentage of the population ± SEM as measured by flow cytometry using specific bovine monoclonal antibodies. Phenotypes were characterized by the expression of CD3 for lymphocytes and CD172a for monocytes. CD14 is a co-receptor that also identifies monocytes in the total PBMC population. Means without a common letter differ across the time points, $P<0.05$.

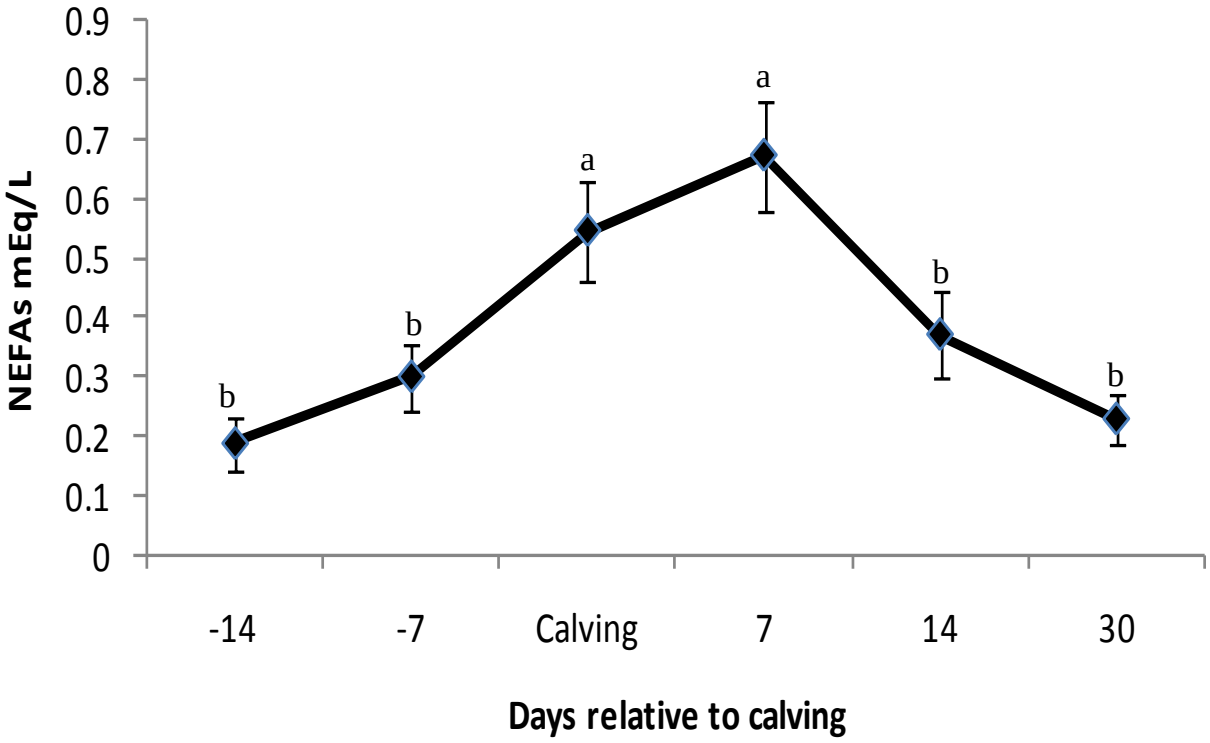

Figure 3. Plasma NEFA concentrations (mEq/L) ± SEM in dairy cows during the periparturient period (n=10). Means without common letters differ, $P<0.05$

CHAPTER 3
Non-esterified Fatty Acids Modify Inflammatory Response and Eicosanoid Biosynthesis in Bovine Endothelial Cells[1]

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Abstract

Intense lipid mobilization during the transition period in dairy cows alters plasma nonesterified fatty acids (NEFA) concentrations and composition. However, less is known about how lipid mobilization alters vascular inflammatory responses and how these changes contribute to dairy cows' increased susceptibility to disease. The objective of this study was to evaluate if shifts in NEFA could modify vascular inflammatory responses in vitro by changing the expression of cytokines and adhesion molecules and alter the biosynthesis of specific COX and LOX eicosanoids. Bovine aortic endothelial cells (BAEC) were cultured with different concentrations of a NEFA mixture that reflected the plasma NEFA composition during the first week of lactation. Gene expression or/and protein quantification of IL-6, IL-8, ICAM1, and VCAM1 were used to assess changes in BAEC inflammatory response. Eicosanoid biosynthesis was evaluated by gene and protein expression of COX1, COX2, and 15LOX, and by quantifying their metabolites using UPLC-MS. Addition of NEFA complexes altered the fatty acid profile of BAEC by increasing the concentration of stearic acid (C18:0) and decreasing the content of arachidonic acid (C20:4n6c) and other long chain polyunsaturated fatty acids in the phospholipid fraction. There was also a significant increase of mRNA expression of IL-6, IL-8, ICAM1, and VCAM1. Changes in gene expression were reflected in protein expression by a significant increment in the protein expression of ICAM1. Gene and protein expression of COX2 were increased in a NEFA concentration dependent manner. The biosynthesis of COX metabolite PGE_2 as well as 15LOX metabolites 9- and 13-HODE was increased significantly after treatment with NEFAs. This research described for the first time specific changes in vascular inflammatory response during in vitro exposure to NEFA mixtures mimicking the transition period. Future studies should elucidate specific mechanisms by which identified NEFA and eicosanoids alter

inflammatory responses in endothelial cells. Understanding the dynamics of lipid mobilization during the transition period may lead to novel nutraceutical or pharmacological interventions that modulate inflammatory responses, potentially improving the health of dairy cows during the transition period.

Introduction

A hallmark of the transition period in dairy cows is intense lipid mobilization. This mechanism of adaptation is necessary to fulfill the energy deficits experienced by cows in late gestation and early lactation (Drackley, 1999). Lipid mobilization is a dynamic process involving lipolysis and lipogenesis. During the transition period, the rate of lipolysis surpasses that of lipogenesis inducing the release of non-esterified fatty acids (NEFA) into the blood stream (Herdt, 2000). As a consequence, increased NEFA concentrations disrupt systemic lipid homeostasis not only quantitatively, but also relative to composition. Previous research demonstrated that enhanced lipolysis during the transition period altered the fatty acid composition of different organs and cell populations including blood, liver, adipose tissue, and peripheral blood mononuclear cells (Douglas et al., 2007; Contreras et al., 2010c). A common change was the increment in the concentrations of saturated fatty acids (palmitic (C16:0) and stearic (C18:0)). In humans, alterations in plasma NEFA are part of the pathophysiology of several diseases with a chronic inflammatory component such atherosclerosis, arthritis and asthma (Fraser et al., 1999; Egan et al., 2008; Wood et al., 2009). Similarly, previous studies in dairy cows link plasma NEFA changes with impaired inflammatory and immune responses (Lacetera et al., 2004; Scalia et al., 2006).

Interactions between leukocytes and the vascular endothelium determine efficiency of host inflammatory responses during disease. Indeed, endothelial cells regulate the trafficking of leukocyte migration to infected tissues (Ley et al., 2007). In normal conditions, endothelium resists leukocyte adhesion and therefore migration, however, during early stages of inflammation, the vasculature expresses several chemotactic pro-inflammatory cytokines including interleukins (IL) 6 and 8, and different adhesion molecules including intercellular

adhesion molecule (ICAM1) and vascular adhesion molecule (VCAM1) (Sprague and Khalil, 2009). As the inflammatory process progresses resolution cytokines are produced and adhesion molecule expression is inhibited to reduce leukocyte migration and promote tissue remodeling and healing (Frangogiannis, 2008). In humans, the development of certain chronic diseases is linked directly to imbalances between inflammation development and resolution. For example, over-expression of both adhesion molecules ICAM1 and VCAM1, and IL-6 and IL-8 is linked directly with the pathophysiology of atherosclerosis and peripheral arterial disease (Weber et al., 1995{Signorelli, 2003 #1133; Signorelli et al., 2003)).

Another component of inflammation development and resolution is the biosynthesis of eicosanoids. These molecules are produced by endothelial cells and leukocytes from phospholipid derived polyunsaturated fatty acids. The main pathways for eicosanoid biosynthesis are cyclooxygenases (COX) and lipoxygenases (LOX). Certain COX and LOX metabolites, such as prostaglandins, thromboxanes and leukotrienes, are potent vasoactive mediators that modulate directly endothelial cells inflammatory responses (Bogatcheva et al., 2005). At the same time, other COX and LOX metabolites like lipoxins, resolvins, and certain prostaglandins (i.e. PGJ_2) have anti-inflammatory and pro-resolving functions that promote the resolution of inflammatory processes (Serhan et al., 2008). Imbalances in the biosynthesis of pro and anti-inflammatory eicosanoids during inflammatory processes are part of the pathogenesis of vascular diseases (Wittwer and Hersberger, 2007). In human atherosclerosis for example, enhanced biosynthesis of 15LOX metabolites such as 9- and 13- hydroxyoctadecadienoic acid (9-, 13-HODE) promotes the development of foam cells, one of the main cellular populations within the atheroma lesion (Barlic and Murphy, 2007). In contrast, 15-hydroxyeicosatetraenoic acid (15-HETE) reduces superoxide production and activation of circulating immune effectors including

polymorphonuclear cells (Wittwer and Hersberger, 2007). The same metabolite is substrate for the biosynthesis of lipoxins, a group of eicosanoids that reduces mononuclear cell infiltration to atheroma lesions (Merched et al., 2008).

Alterations in plasma NEFA concentrations also are known to increase human atherosclerosis susceptibility. Elevated plasma NEFA concentrations promote adhesion molecule expression and inhibit certain anti-inflammatory processes such as insulin mediated vasodilation (Pilz and Marz, 2008). Similarly, in dairy cows elevations in plasma NEFA increase expression of pro-inflammatory cytokines linked to transition cow diseases such as mastitis and metritis (Aitken et al., 2009; Sordillo et al., 2009). However, less is known about the consequences of shifts in plasma NEFA composition and how these changes contribute to alterations in vascular inflammatory responses that may lead to increased disease susceptibility during the transition period. The objective of this study was to evaluate if sifts in NEFA concentration and composition associated with the transition period in dairy cows could modify the biosynthesis of specific COX and LOX eicosanoids and alter endothelial cell inflammatory responses in vitro by changing the expression of cytokines and adhesion molecules.

Materials and Methods

Materials

Antibiotics and antimycotics, trypsin-EDTA, HEPES buffer, and Ham's F-12K (Kaighn's Nutrient Mixture F-12) were purchased from Cellgro (Herndon, VA, USA). Hyclone Laboratory (Logan, UT, USA) supplied fetal bovine serum (FBS). The RNeasy Mini Kit for isolation of RNA was purchased from Qiagen (Valencia, CA, USA). Reagents for quantitative real-time PCR (qPCR) were purchased from Applied Biosystems (Foster City, CA, USA) and Qiagen (Valencia, CA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

Cell Culture

Bovine aortic endothelial cells (BAEC) were isolated from the bovine aorta using collagenase as a digestion method as described previously (Sordillo et al., 1998). BAEC were cloned to a single-cell level by the limiting dilution method and maintained until confluent as previously described (Sordillo et al., 2008). BAEC were exposed 4 or 24 h to 3 different NEFA complex concentrations emulating different degrees of lipid mobilization during lactation: 0.25 mM late lactation, 0.5 mM peak lactation, and 0.75 mM for the transition period. BAEC were exposed to culture media without addition of NEFA complex 0 mM as a negative control, culture media plus albumin without any NEFA bound as the NEFA complex control, and culture media plus lipopolysaccharide (LPS) at a dose of 50 ng/mL as a positive inflammation control. The BAEC were used at passage 9 or less for all experiments.

Cell Viability Assay

Trypan blue exclusion was used to evaluate cell death. Following treatment of BAEC with the different controls and NEFA complex concentrations for 4 and 24 h, cells were collected using trypsin, diluted 1:4 in trypan blue, and counted on a hemocytometer. Viable and non-viable cells were counted using a hemocytometer to obtain the percentage of viable cells. Metabolic activity, and thereby cell viability, was measured using Promega CellTiter-Glo Viability Assay (Promega, Madison, WI, USA) which quantifies the amount of ATP, an indicator of metabolically active cells. Endothelial growth factor (30 μg/mL) was used as a positive control for this assay since it sustains cell growth. The mixture of substrate and buffer results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of viable cells. Luminescence was measured using Wallac Victor3^{TM} 1420 Multilabel Plate Reader

(PerkinElmer, Waltham, MA, USA). Data were expressed as percent relative luminescence units.

Non-esterified Fatty Acids-Albumin Complex Solution

NEFA complex composition was calculated based upon a previous study that characterized the fatty acid profiles of plasma NEFA in periparturient cows (Contreras et al., 2010b). The complex resembled the fatty acid profile in the NEFA fraction during the first week of lactation and included: myristic (C14:0) (3%), palmitic (C16:0) (30%), stearic acid (C18:0) (45%), oleic (C18:1n9c) (16%), linoleic (C18:2n6c) (5%) and docosahexaenoic (DHA, C22:6n3c) (1%) fatty acids. NEFA were bound to albumin, which is their natural transporter in plasma to mimic in vivo physiological conditions. NEFA-albumin preparations were produced by saponification (Kim et al., 2005). Briefly, palmitic (C16:0) and stearic (C18:0) acids were dissolved in ethanol at 90°C and further sonicated with a Branson Sonifier 250 for 10 sec at an output of 10%. Once readily dissolved, the solution was dried under nitrogen, and then resuspended in 0.1 mol/L NaOH at 90°C. Oleic (C18:1n9c), linoleic (C18:2n6c), and DHA (C22:6n3c) acids were dissolved in 0.1 mol/L NaOH at 70°C and then mixed with the palmitic (C16:0) and stearic (C18:0) acid NaOH solution. NEFA mixture solution was complexed with 10% bovine serum albumin (fatty acid-free) at 55°C for 10 min. The NEFA-albumin complexes were then filter sterilized (0.44 μm) and stored under argon atmosphere at -20°C for future use. The final concentration of the complex was 100 μM of NEFA, with an albumin molar ratio of 5:1.

Lipid Extractions

Total lipids from media and BAEC were extracted using a hexane:ethanol (1:1) solvent mix as previously described (Contreras et al., 2010b). BAEC were harvested by gentle scraping,

washed in Hank's buffered salt solution (HBSS) and centrifuged at 233 g. Prior to lipid extraction BAEC were resuspended in HBSS to a minimum concentration of $5.5x10^6$ cells/mL. To obtain total lipids, media or BAEC were combined with the solvent mix and vortexed for 10 min, and then centrifuged at 2,095 g for 10 min at room temperature. The hexane layer was collected for total fatty acid composition and lipid fractioning. Nonadecanoic acid (C19:0) was used as internal standard.

Lipids were further separated into neutral lipids, NEFA and phospholipids fractions by solid-phase extraction using Strata cartridges containing aminopropyl (NH_2) sorbent (Phenomenex, Torrance, CA, USA) as described by (Kaluzny et al., 1985). Briefly, after conditioning of the cartridges with hexane, the neutral lipid fraction was eluted with chloroform:2-propanol (2:1), the NEFA with 2% acetic acid in diethyl ether, and the phospholipids fraction with methanol. 1,2-diheneicosanoyl-*sn*-glycero-3-phosphocholine (21:0 phosphatidyl choline) was used as internal standard for lipid fractioning. Fatty acids from total extractions and each lipid fraction were transesterified and methylated using 20% methanolic hydrochloric acid for 2 h at 90°C (Browse et al., 1986). Methylated extractions were analyzed using a Perkin Elmer - Clarus 500 gas chromatography apparatus with a Supelco SP-2560 column (100m x 25 mm with a 0.2um film thickness). Fatty acid peaks were identified by using a mix of pure methyl ester standards (Nu Check, Elysian, MN, USA).

Gene Transcript Quantification by qPCR

Total RNA was extracted from BAEC for quantification of IL-6, IL-8, ICAM1, VCAM1, COX1, COX2, 15LOX1, and β-actin mRNA transcript expression by qPCR as formerly described (Corl et al., 2008a). Briefly, total RNA was extracted utilizing the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA). DNase digest was done during the RNA extraction using

the RNase-Free DNase Set (Qiagen, Valencia, CA, USA). Purified RNA was converted to cDNA using the Applied Biosystems High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). All qPCR assays were conducted utilizing TaqMan gene expression assays from Applied Biosystems. TaqMan primer and probe sets (Table 9) were designed from bovine sequences with the Applied Biosystems Pipeline software and synthesized by Applied Biosystems. Samples were assayed in triplicate with 50 ng of cDNA per reaction along with 10 μL of TaqMan Fast Universal PCR Master Mix (2X), and 1 μL of the appropriate TaqMan Gene Expression Assay Mix (20X) on the Applied Biosystems 7500 Fast Real-Time PCR System. A nonreverse transcriptase control was run to ensure genomic DNA was not being amplified. The relative quantification of each gene was calculated utilizing the 7500 Fast SDS Software (version 1.3.1) (Livak, 2001). Data were calculated based on the comparative Ct method ($2^{-\Delta\Delta Ct}$) of relative quantification using β-actin as the control gene (Steibel et al., 2009). The calibrator was set as the 0 mM NEFA complex supplementation.

Western Blot Analyses

Protein quantification was performed by protein blot analysis. BAEC cells were harvested by gentle scraping. Whole cell lysates were harvested in M-Per reagent (Pierce, Rockford, IL, USA) and centrifuged at 10,000 g for 10 min at 4°C to remove membrane fractions. Supernatant were collected and total protein was quantified using the Coomassie brilliant blue method. Equal amounts of protein (30 μg) were electrophoresed on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The Millipore Snap i.d. Protein Detection System (Millipore, Billerica, MA, USA) was utilized to carry out the remaining steps. The membrane was blocked in 0.5% dry milk in Tris-buffered saline (TBS) with 0.01% Tween-20 and washed 3 times with TBS. Membranes were incubated for 10 min

with anti-bovine COX1 (1:400 dilution, Abcam, Cambridge, MA, USA), anti-human COX2 (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-rabbit 15LOX1 (1:2000, Cayman, Ann Arbor, MI, USA), and anti-human ICAM1 (1:100 dilution, Invitrogen, Carlsbad, CA, USA) in 1% bovine serum albumin in TBS with 0.01% Tween-20. Following 3 washes with TBS, membranes were incubated with the correspondent anti-host IgG secondary antibody labeled with horseradish peroxidase (HRP; 1:3,000 dilution in 0.5% dry milk, Pierce, Rockford, IL, USA) for 10 min at room temperature, washed 3 times with TBS, exposed to HRP substrate (Pierce), and visualized by chemiluminescence using the ChemiDoc™ XRS (Bio-Rad, Hercules, CA, USA) and Quantity One® software (Bio-Rad, Hercules, CA, USA). Anti-human actin (1:3,000 dilution, Millipore, Billerica, MA, USA) served as the loading control. Density of the bands was quantified by the Quantity One® software. The ratio of specific antibodies:actin was calculated and the values were expressed as a fold change over the 0 mM NEFA supplemented BAEC.

Eicosanoid Detection and Quantification

BAEC eicosanoid production was detected and quantified using ultra-high pressure liquid chromatography and mass spectrometry (UPLC-MS). In brief, BAEC and media were harvested by gentle scraping and additioning of EtOH to a 10% solution by volume. Internal standards were added prior to harvesting cells and supernatants. Specimens were then sonicated while in water bath at 4°C for 3 min at output 10% using a Misonix S-400 sonicator (Newton, CT, USA). Samples were then centrifuged at 4°C for 5 min at 3000g and the supernatant was used for extraction. Lipid mediators were isolated from specimens by solid phase extraction using Strata X SPE columns (Phenomenex, Torrance, CA, USA). Cartridges were first conditioned with 2mL of MeOH then washed with 2mL of H_2O. Samples were loaded into the cartridge, washed with 2

mL of MeOH (20% v/v), and finally eluted with MeOH:acetonitrile (50:50 v/v). Eluates were dried using a Savant Speedvac (Thermo, West Palm Beach, FL, USA) and then redissolved in acetonitrile:water:formic acid (36:64:0.2 v/v) to perform UPLC-MS using a Waters H-class Acquity UPLC® chromatographer (Waters, Milford, MA, USA). Lipid mediators were separated using an Acquity UPLC® BEH C18 1.7µm 2.1 ^{x} 100 mm column (Waters, Milford, MA, USA). 15LOX derived eicosanoids were separated at a flow rate of 600 µL/min at 37ºC with the column equilibrated in methanol-acetonitrile- water-acetic acid (15.8:47.4:36.8:0.02; v/v/v/v) and 20 µl of sample were injected. Eicosanoids were isolated during a 15 min run using the same solvent mixture. COX derived eicosanoids were separated at a flow rate of 600 µL/min at 37ºC. The column was equilibrated in water-acetonitrile-acetic acid (65:35:0.02; v/v/v) and 20 µl of sample were injected. Eicosanoids were isolated during an 18 min run using acetonitrile-acetic acid (65:35:0.02; v/v/v). Eicosanoids were analyzed using a mass spectrometer Acquity SQD (Waters, Milford, MA, USA). UPLC-MS results were quantified using Empower-Pro 2 software (Waters, Milford, MA, USA). Measured eicosanoids included: prostaglandins (PG) D_2, E_2, F_1, $F_2\alpha$, and thromboxane B_2 (TXB_2) for the COX pathway. For the 15LOX1 pathway, linoleic acid (C18:2n6c) derived metabolites included: 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE) and arachidonic acid (C20:4n6c) derived metabolites included: 5-, 12- and 15-hydroxyeicosatetraenoic acids (5, 12, and 15-HETE). Results are expressed as ng of lipid mediator per mL of media.

Statistical Analyses

Statistical evaluation was conducted using proc GLM procedure (SAS v9.1, SAS Institute Inc, Cary, NC, USA). Comparisons of the means were made by analysis of variance followed by Tukey–

Kramer's HSD *post hoc* test as necessary. Data are shown as means ±SEM. Differences with $p < 0.05$ were considered statistically significant.

Results

Cell Viability

At the beginning of the study, NEFA complex supplementation effect on endothelial cell viability was established. As assessed by trypan blue exclusion, no significant changes in viability were observed at 4 h and 24 h of incubation when BAEC were exposed to 0mM, albumin, 0.25 mM, 0.5 mM, and 0.75 mM of the NEFA complex with cell death counts below 10%. After 4 h and 24 h of culture with either the NEFA complexes (up to 0.75 mM) or albumin control, BAEC were determined to be viable by measuring ATP production. No differences in cell viability were detected among treatments (data not shown).

NEFA Supplementation Increased Saturated Fatty Acid and Decreased Polyunsaturated Fatty Acid Concentrations in BAEC Phospholipids

Addition of NEFA-albumin complex to culture media did not alter significantly the fatty acid composition of the phospholipids membrane of BAEC at 4 h (results not shown). After 24 h in culture with the NEFA complex, however, there were significant compositional changes as shown in Table 2. At this time, there was a significant increase in the concentrations of stearic acid (C18:0) when cells were supplemented with all concentrations of NEFA complex when compared to 0 mM or albumin control treatments. Similarly, pentadecanoic acid (C15:0) content increased significantly with NEFA supplementation at 0.5 mM, but not at 0.75 mM when compared to the other treatments. At the same time, there was a significant decrease in the concentration of some polyunsaturated fatty acids. Vaccenic acid (C18:1n7c) concentration decreased significantly at 0.25 mM, 0.5 mM and 0.75 mM of NEFA complex when compared to

albumin and 0mM. Arachidonic (C20:4n6c) and docosatrienoic (C22:3n3c) acids content decreased at 0.5mM and 0.75mM of NEFA complex when compared to 0mM, 0.25mM, and albumin supplementation. Finally the concentrations of docosaenoic acid (C22:1n9c) diminished significantly when BAEC were supplemented with 0.75 mM of NEFA complex.

NEFA Supplementation Altered Pro-Inflammatory Cytokine and Adhesion Molecules Gene and Protein Expression

After 24 h of culture, exposure to the 0.75 mM NEFA complex increased gene expression of IL-6 when compared to values for the 0 mM and 0.25 mM NEFA complex groups (Figure 4a). Similarly, IL-8 gene expression in BAEC was significantly altered when supplemented with 0.5 mM, and 0.75 mM NEFA complex when compared to 0 mM and 0.25 mM NEFA complex (Figure 4b). NEFA complex supplementation also altered the gene expression of adhesion molecules (Figure 4c and d). When exposing BAEC to the different treatments, there was a significant increase in the gene expression of ICAM1 and VCAM1 at 0.5 mM and 0.75 mM of NEFA complex. Similarly, ICAM1 protein expression was enhanced in BAEC exposed to 0.5mM and 0.75mM when compared to 0mM, 0.25mM, and albumin control (Figure 5).

NEFA Supplementation Altered Lipid Mediator Biosynthesis in BAEC

Eicosanoid Enzymes.

Changes in NEFA concentrations and content in cultured media did not induce significant changes in the gene expression of COX1 (Figure 6a) at any NEFA complex concentration or when supplemented with albumin or stimulated with LPS (positive control). Similarly, there were no changes in the protein expression of COX1 of BAEC cells when cultured for 24 h with different NEFA complex supplementations or both controls albumin and LPS (Figure 6b). In contrast to COX1, COX2 mRNA expression significantly increased in a concentration dependent

manner (Figure 6c), where 0.5 mM, and 0.75 mM treatment groups had significantly higher gene expression than the 0 mM or 0.25 mM treatment groups. Changes in gene expression were directly reflected in protein expression by a significant NEFA complex concentration dependent increment in COX2. The protein expression was significantly augmented when BAEC where exposed to 0.5 mM, 0.75 mM NEFA complex (Figure 6d). 15LOX1 gene expression was enhanced significantly by stimulation with LPS when compared to 0 mM. Comparing BAEC supplemented with any NEFA complex concentration and LPS stimulated, however, showed no significant differences in 15LOX1 gene expression.

Lipid Mediator Biosynthesis.

The concentrations of COX (Figure 7) and 15LOX (Figure 8) metabolites were determined by UPLC-MS. At 24 h of supplementation with different concentrations of NEFA complex, there were no significant changes in the production of PGD_2, PGF_1 and TXB_2 when compared to 0 mM NEFA complex treatment. At the same time, only the production of PGF_1 was significantly augmented when BAEC were stimulated with LPS when compared to control, albumin control, or any NEFA complex supplementation. PGE_2 biosynthesis was significantly augmented when cells were supplemented with 0.75mM of NEFA complex when compared to other NEFA concentrations, including 0 mM.

Changes were observed in the BAEC production of linoleic acid (C18:2n6c) derived 15LOX1 metabolites after exposure to the treatments. Both 9-HODE and 13-HODE biosynthesis was enhanced after NEFA complex supplementation in dose a dependent manner (Figure 8), however there were no changes in the production of these metabolites when BAEC were exposed to the positive control (LPS) compared to both the negative control (0mM) and albumin treatment groups. As for 15LOX arachidonic acid (C20:4n6c) metabolites, including 5, 12, and

15 HETE, there were no significant changes in their biosynthesis as a result of exposure to different concentrations of NEFA complex (Figure 8).

Discussion

Intense lipid mobilization during the transition period of dairy cows releases high amounts of NEFA from adipose tissue inducing alterations in plasma NEFA content and concentrations (Contreras et al., 2010b). In humans, increased plasma NEFA are part of the pathophysiology of several chronic inflammatory diseases including atherosclerosis and asthma (Egan et al., 2008; Wood et al., 2009). In dairy cows, previous work reported changes in leukocyte function when those cells were exposed high NEFA concentration (Lacetera et al., 2004; Scalia et al., 2006). However, to our knowledge this is the first study to report changes in endothelial inflammatory responses when supplemented with NEFA complexes mimicking transition cow plasma NEFA concentration and content.

Significant changes in BAEC phospholipid fatty acid content were observed only after exposing BAEC for 24 h to different NEFA complex concentrations. This is in agreement with other studies where the phospholipid content of endothelial cells changed significantly only after at least 24 h of culture (Ferguson et al., 1975; Couloubaly et al., 2007). Héliès-Toussaint et al. (Héliès-Toussaint et al., 2006) reported that supplementation with saturated fatty acids increased their concentrations in endothelial phospholipids and at the same time this addition was balanced by a decrease in the concentrations of polyunsaturated fatty acids of both omega 3 and 6 series. Similarly, our study showed that there was significant increase in the concentration of stearic acid (C18:0) and a significant decrease in polyunsaturated fatty acids such as vaccenic (C18:1n7c), arachidonic (C20:4n6c), docosatrienoic (C22:3n3c), and docosaenoic (C22:1n9c) acids. Endothelial cells have a reduced ability to store fatty acids as triglycerides and it was

expected that the majority of fatty acids would either be directed to phospholipid biosynthesis or become beta oxidized in the mitochondria (Héliès-Toussaint et al., 2006). Interestingly, of the supplied saturated fatty acids (palmitic (C16:0) and stearic (C18:0)), only stearic (C18:0) increased significantly in the phospholipid content. These changes could be related to the higher supplementation of stearate ((C18:0) (45%)) in comparison to palmitate ((C16:0) (30%)). Moreover, palmitic acid (C16:0) is more efficient in generating acyl-coA molecules for mitochondrial beta oxidation than stearic acid (C18:0) due to its shorter chain length, therefore it may be used mainly as energy substrate over stearic acid (C18:0) (Crunkhorn et al., 2007). It is important to note that our results for in vitro phospholipid fatty acid incorporation contrast with our previous in vivo study in transition dairy cows, where despite higher plasma NEFA stearate (C18:0) concentrations in comparison to palmitate (C16:0), leukocyte phospholipids had only significant increases in palmitate (C16:0) (Contreras et al., 2010b). Further research needs to address if there are in vivo differences in phospholipid fatty acid incorporation between leukocytes and endothelial cells, or if there are other factors influencing palmitate (C16:0) and stearate (C18:0) uptake in BAEC during in vitro supplementation with NEFA complexes.

Alterations in the fatty acid composition in cellular membranes have detrimental effects on endothelial function. For example, both high concentrations of saturated fatty acids and reduced content of polyunsaturated fatty acids decrease cellular membrane fluidity (Artwohl et al., 2003; Hahn and Schwartz, 2008). In transition dairy cows, trafficking processes are impaired during the transition period by changes in leukocyte adhesion molecule expression and specific leukocyte cellular distribution (Weber et al., 1995; Shafer-Weaver et al., 1999). Currently, it is unknown if changes in the physical properties of vascular phospholipid bilayers may enhance alterations in leukocyte migration patterns.

After exposing BAEC to concentrations over 0.5 mM for 24 h, both ICAM1 and VCAM1 increased their gene and protein expression. These pro-inflammatory responses may be explained by different factors. First, there was a significant increase in the production of COX derived PGE_2 when cells were exposed to 0.75 mM NEFA concentrations. This prostaglandin induces the expression of both ICAM1 and VCAM1 in endothelial cells during inflammatory responses and in physiological processes including cervical relaxation during parturition (Winkler et al., 1997; Winkler et al., 1998). Second, there was an increased production of 15LOX linoleic acid (C18:2n6c) derived eicosanoids 9- and 13-HODE. Both metabolites can induce the expression of ICAM1 and VCAM1 in a concentration dependent manner (Friedrichs et al., 1999; Viita et al., 1999). The mechanism involved is the activation of the pro-inflammatory signaling pathway NF-κB (Viita et al., 1999). Finally, the same pathway could have been activated directly by fatty acids. The NEFA complex supplemented to BAEC had high concentrations of stearic (C18:0) and palmitic acids (C16:0), both saturated fatty acids can activate the inhibitor of κB kinase (IKKβ) in endothelial cells therefore increasing the activity of NF-κB (Kim et al., 2005; Staiger et al., 2006b). The present study did not evaluate directly NFκB activity during NEFA supplementation; therefore, future studies are required to elucidate direct implications of specific 15LOX and COX metabolites in the activation of this pro-inflammatory pathway in vascular cells after exposure to high NEFA concentrations.

IL 6 and 8 transcription also was up-regulated after 24 h of NEFA exposure. IL-6 activation can be related to NF-κB activation by palmitic (C16:0), stearic (C18:0) and linoleic (C18:2n6c) acid as demonstrated previously in pulmonary artery (Toborek et al., 1996) and coronary artery endothelial cells (Staiger et al., 2006a). Furthermore, palmitic acid (C16:0) plasma levels in humans were directly correlated to IL-6 content in blood (Staiger et al., 2006a).

Similarly, IL-8 transcription enhancement in human aortic cells is also mediated by palmitic (C16:0) and oleic (C18:1n9c) acid (Packard and Libby, 2008). Although an induction mechanism for increased IL-8 expression was not established in vascular cells, palmitate (C16:0) and stearate (C18:0) metabolism induce IL-8 expression through de novo ceramide synthesis and the activation of p38 and JNK kinases in human macrophages (Håversen et al., 2009).

Reductions in the concentration of both omega 3 and 6 polyunsaturated fatty acids, as observed after exposure to high NEFA concentrations, have deleterious consequences in the control and resolution of inflammatory processes. For example, these fatty acids and some of their specific eicosanoid derivatives are ligands for peroxisome proliferator-activated receptors (PPAR) α, γ, and β/δ (Daynes and Jones, 2002). When activated, PPARα will inhibit the production of IL-6 and VCAM1 as well as repress the transcriptional expression of NF-κB in endothelial cells (Barbier et al., 2002). In our experiments, the concentration of certain polyunsaturated fatty acids such as arachidonic acid (C20:4n6c) and docosatrienoic (C22:1n9c) that can activate PPARα was reduced as NEFA complex concentrations were augmented, at the same time biosynthesis of alternative ligands such as 5, 12, and 15-HETE was not enhanced. Although conjecture, alterations in lipid homeostasis caused by intense lipid mobilization in transition dairy cows may impair some of the BAEC anti-inflammatory mechanisms including PPARs. At this time the impact of altered NEFA content and composition in the activity of different PPARs is unknown. Furthermore, research should evaluate the effects of specific polyunsaturated fatty acid supplementation, including arachidonic (C20:4n6c), on the activity and metabolite production of 15LOX and COX.

In the present study we described how changes in NEFA content and concentration altered BAEC phospholipid fatty acid profiles. A possible consequence to these changes is the

alteration of substrate availability for eicosanoid biosynthetic pathways including COX and 15LOX. Indeed, after NEFA supplementation we observed a reduction in the phospholipid content of arachidonic acid (C20:4n6c) and other polyunsaturated fatty acids of 20 or more carbon structure. Decreased availability of this group of polyunsaturated fatty acids may have induced an increase in the use of alternate substrates such as 18 carbon chain polyunsaturated fatty acids. In fact, we obtained a significantly higher production of linoleic acid (C18:2n6c) metabolites of the 15LOX pathway, 9 and 13-HODE and no changes in 5, 12, and 15-HETE which are arachidonic (C20:4n6c) acid derivatives. Besides lower arachidonic acid (C20:4n6c) availability, these shifts in 15LOX pathway final products also could be explained by the higher affinity of the enzyme for linoleic acid (C18:2n6c) over arachidonic acid (C20:4n6c) and other fatty acids of 20 carbons (Brash et al., 1997). Although not demonstrated in endothelial cells, 9 and 13-HODE are known to interfere with the anti-inflammatory actions of PPARs, for example, 13-HODE down-regulates PPARγ activity (Hsi et al., 2002). Furthermore, high levels of the same 15LOX metabolites are highly correlated with endothelial dysfunction and atherosclerosis in humans (Verma et al., 2003). Thus elevations in the production of 15LOX linoleic acid (C18:2n6c) derivatives due to increased NEFA concentrations may extend endothelial inflammation processes. Complementary studies should look at the effect of increased plasma NEFA in other factors that determine polyunsaturated fatty availability to eicosanoid enzymes including phospholipases activity and specific availability of selected fatty acids depending on their biochemical properties.

The biosynthesis of downstream COX1 and 2 metabolites is dependent on secondary PG synthases. After 24 h of NEFA supplementation, the concentrations of PGD_2, PGF_1, and TXB_2 remained unchanged. A possible explanation is that reduction of arachidonic acid (C20:4n6c)

availability could have impaired biosynthesis of these metabolites, since COX1 and COX2 have a high affinity for arachidonic acid (C20:4n6c) over other fatty acids (Fritsche et al., 2001). Nevertheless there was a significant increment in the concentration of PGE_2 which is directly related to enhanced expression of COX2 at both the transcription and protein expression level (Samuelsson et al., 2007). Further studies are necessary to characterize the time of expression and activity of different PG synthases during lipid mobilization and their adaptations to dyslipidemic events.

Stages of energy imbalance in mammals are linked directly with inflammatory-based diseases. In humans, several studies established that alterations in lipid homeostasis induced by overnutrition are part of the pathophysiology of atherosclerosis, type 2 diabetes and metabolic syndrome (Scaglione et al., ; Boden, 2008). Similarly, dairy cows face severe energy imbalances during the transition period, the lactation stage with higher incidence of inflammatory based diseases such as mastitis, metritis and laminitis (Sordillo et al., 2009). In the present study, we demonstrated that alterations in NEFA content and concentration can enhance the pro-inflammatory phenotype of endothelial cells. NEFA supplementation altered the BAEC phospholipid fatty acid profile, enhanced expression of adhesion molecules and cytokines, and promoted the biosynthesis of specific 15LOX and COX derived metabolites with known pro-inflammatory functions including 9- and 13-HODE and PGE_2. All these factors could modify adjacent tissue inflammatory responses and alter leukocyte migration patterns. Future studies should analyze specific mechanisms by which NEFA induce inflammatory responses. It is necessary to evaluate the activation of pro-inflammatory intracellular signaling pathways upon NEFA supplementation and their interaction with eicosanoid biosynthetic pathways. At the substrate level, by identifying changes induced by lipid mobilization in polyunsaturated fatty

acid availability and phospholipase activity. At the enzyme level, by characterizing transcriptional and post-transcriptional changes. Finally, at the metabolite level, by evaluating the activity of specific eicosanoid products on the expression of endothelial inflammatory markers. This work has identified specific metabolites such as linoleic acid derived 9 and 13-HODE as possible key players in the development and maintenance of inflammatory phenotype. A final step would be to include in the NEFA complex supplementation of selected polyunsaturated fatty acids such as EPA (C20:5n3c) and DHA (C22:6n3c) that are known to ameliorate the production of inflammatory markers in other species including rodents and humans.

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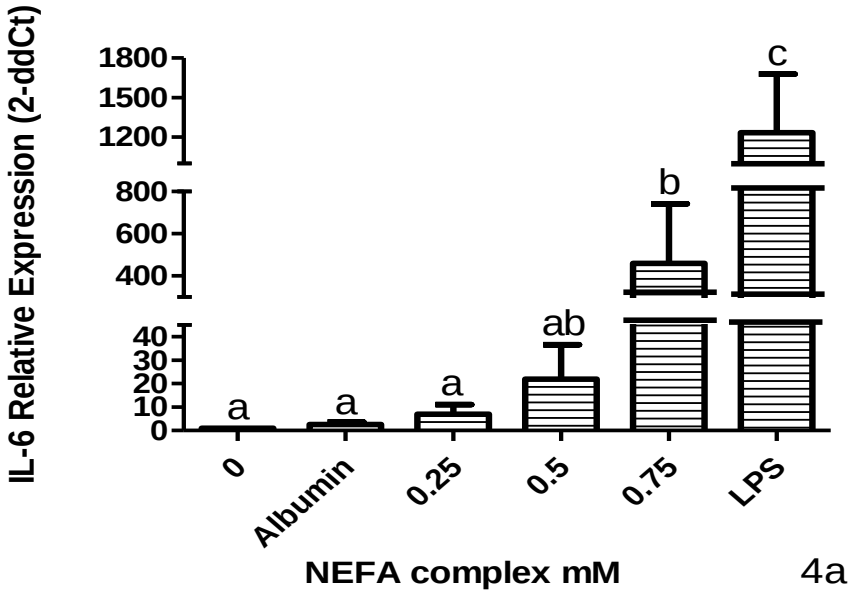
Table 9. Gene targets and primer sequences for qPCR

Target	Accession#		Sequence (5' to 3')
ICAM1	NM-174348	Forward	GCAGGTGGTCCACAAACAC
		Reverse	GCAATCCCGCTGGTCTAGTC
		Probe	ATGTCCTGTACGGCCCC
VCAM1	NM-174484	Forward	ACAAAGGCAGAGTACACAAACACTT
Il-6	X57317	Forward	AGGACGGATGCTTCCAATCTG
		Reverse	GAAGACCAGCAGTGGTTCTGAT
		Probe	CAATCAGGCGATTTGC
Il-8	NM-173925	Forward	GCTCTCTTGGCAGCTTTCCT
		Reverse	GGCATCGAAGTTCTGTACTCATTCT
		Probe	CAGAACTGCAGCTTCAC
COX1	NM-001105323.1	Forward	ATGGAGTTCAACCAGCTTTACCA
		Reverse	CAGAAACTGCTCGTAGCTGTAGTC
		Probe	CATGCCCGACTCCTTC
COX2	NM-174445.2	Forward	GGCGATGAGCAGTTGTTCCA
		Reverse	TGCTGTACGTAGTCTTCAATCACAAT
		Probe	CAAGCAGGCTAATCCT
15LOX1	NM-174501	Forward	GTGCCTTCCGTCTATACATCCTATG
		Reverse	CCCGGATGTTAATTTCCATGGTGTA
		Probe	CCCGGATGTTAATTTCCATGGTGTA
β-actin	NM-173979	Forward	CCGCCCCGCTAGCA
		Reverse	AACTGGTTGCGGTGTCGA
		Probe	CCTTCGCCGCTCCGC

Table 10. Fatty acid composition of bovine aortic endothelial cells (BAEC) phospholipid fraction after 24 h of culture with different concentrations of NEFA complex or albumin.

N.D. non detected

Fatty acid, g/100 g	NEFA complex supplementation					
	0mM	Albumin	0.25mM	0.5mM	0.75mM	P value
C14:0	2.27 ± 0.26	2.59 ± 0.22	1.99 ± 0.20	1.66 ± 0.28	2.15 ± 0.19	0.135
C14:1n5c	2.50 ± 0.50	2.55 ± 0.52	4.01 ± 0.91	3.95 ± 0.63	2.88 ± 0.52	0.159
C14:1n5t	1.40 ± 0.28	1.34 ± 0.24	1.57 ± 0.37	1.75 ± 0.21	1.30 ± 0.24	0.254
C15:0	2.08 ± 0.54 a	1.20 ± 0.25 a	3.13 ± 0.80 ab	3.52 ± 0.64 b	2.31 ± 0.39 ab	0.029
C16:0	23.47 ± 1.89	25.34 ± 1.56	20.15 ± 2.26	18.41 ± 2.37	22.56 ± 1.67	0.158
C16:1n7c	0.80 ± 0.09	0.83 ± 0.03	0.84 ± 0.15	0.83 ± 0.12	0.80 ± 0.23	0.562
C17:0	1.69 ± 0.19	1.42 ± 0.17	1.64 ± 0.32	1.82 ± 0.15	1.34 ± 0.20	0.354
C18:0	24.41 ± 1.31 a	26.69 ± 1.89 ab	28.57 ± 2.44 bc	28.87 ± 1.63 bc	30.85 ± 1.18 c	0.021
C18:1n7c	1.07 ± 0.36 a	1.16 ± 0.32 a	0.55 ± 0.18 b	0.40 ± 0.17 b	0.36 ± 0.11 b	<0.01
C18:1n9c	5.00 ± 1.35	5.72 ± 1.54	4.55 ± 1.32	3.84 ± 1.11	3.94 ± 1.14	0.080
C18:1n9t	0.93 ± 0.33	1.55 ± 0.35	1.05 ± 0.45	0.73 ± 0.33	1.20 ± 0.25	0.180
C18:2n6c	0.65 ± 0.23	0.59 ± 0.23	1.46 ± 0.52	1.48 ± 0.62	1.33 ± 0.44	0.620
C18:3n3c	0.24 ± 0.13	0.16 ± 0.10	0.12 ± 0.06	0.08 ± 0.06	0.14 ± 0.08	0.360
C18:3n6c	0.48 ± 0.05	0.48 ± 0.04	0.54 ± 0.04	0.69 ± 0.10	0.47 ± 0.04	0.060
C20:0	0.10 ± 0.07	0.11 ± 0.07	0.13 ± 0.10	0.25 ± 0.10	0.15 ± 0.06	0.780
C20:3n3c	0.18 ± 0.08	0.11 ± 0.07	0.23 ± 0.13	0.25 ± 0.11	0.27 ± 0.18	0.370
C20:4n6c	3.46 ± 0.91 a	2.26 ± 0.72 ab	1.70 ± 0.44 ab	1.24 ± 0.43 b	1.41 ± 0.28 b	0.016
C20:5n3c	N.D.	N.D.	N.D.	N.D.	N.D.	N.D
C22:1n9c	0.50 ± 0.10 a	0.49 ± 0.11 ab	0.26 ± 0.11 ab	0.31 ± 0.12 ab	0.31 ± 0.06 b	0.04
C22:3n3c	1.31 ± 0.43 a	0.97 ± 0.11 ab	0.98 ± 0.59 ab	0.99 ± 0.50 b	0.85 ± 0.36 b	<0.01
C22:5n3c	0.73 ± 0.28	0.62 ± 0.28	0.49 ± 0.13	0.44 ± 0.13	0.45 ± 0.24	0.090
C22:6n3c	4.37 ± 1.28	5.04 ± 2.32	2.01 ± 1.08	3.29 ± 1.15	3.44 ± 1.11	0.137
Unidentified	12.01 0.90	10.44 0.68	11.74 1.16	12.22 1.23	10.52 1.17	0.636



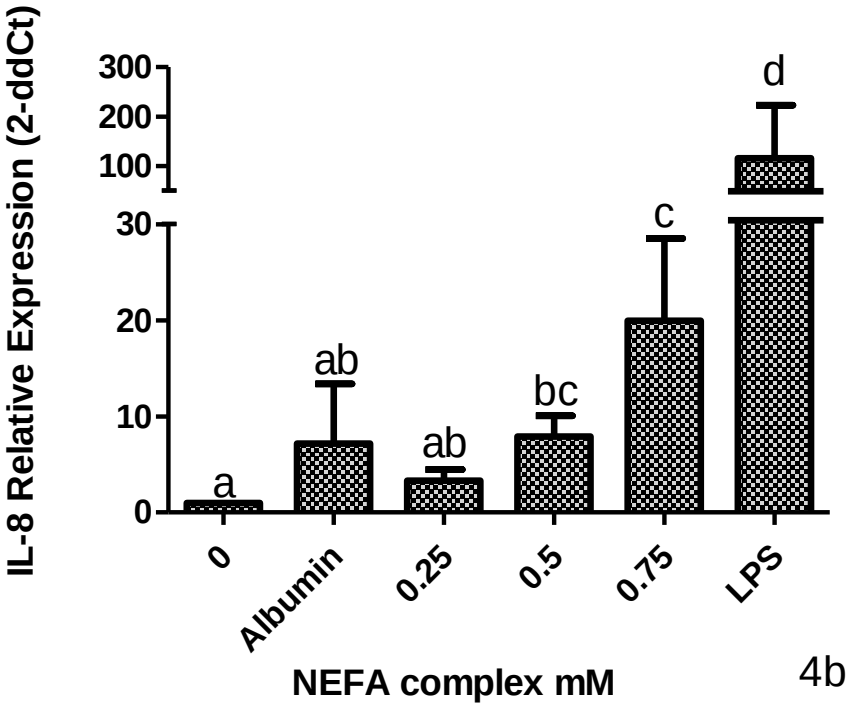
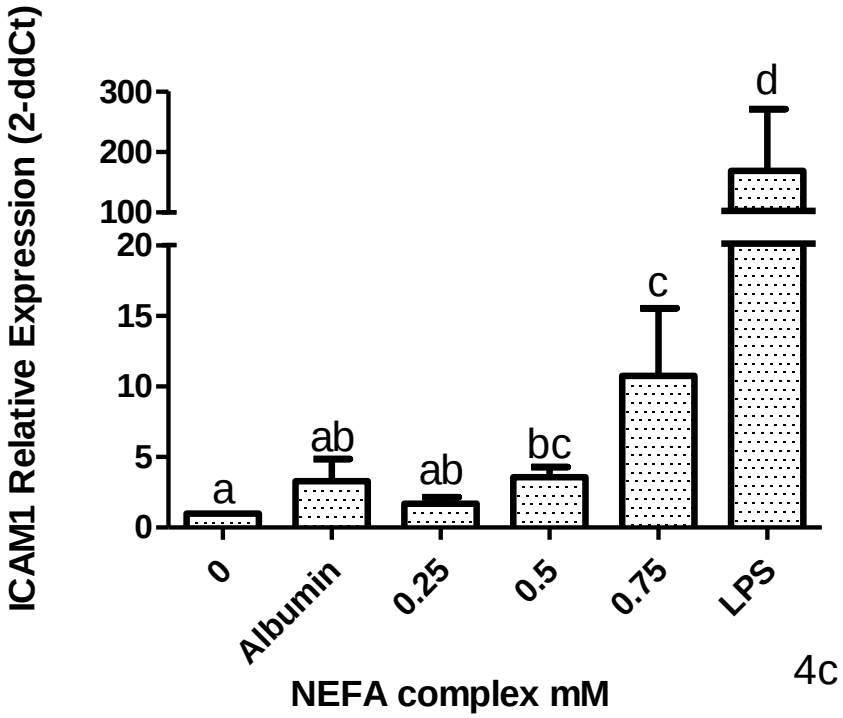


Figure 4. NEFA complex supplementation for 24 h at different concentrations induced significant changes in the gene expression of (a) IL-6, (b) IL-8, (c) ICAM1, and (d) VCAM1. Values are shown as $2^{-\Delta\Delta CT}$ with 0 mM set as calibrator. Interleukin (IL), intercellular adhesion molecule 1(ICAM1), vascular adhesion molecule 1 (VCAM1).

Figure 4 (cont'd)



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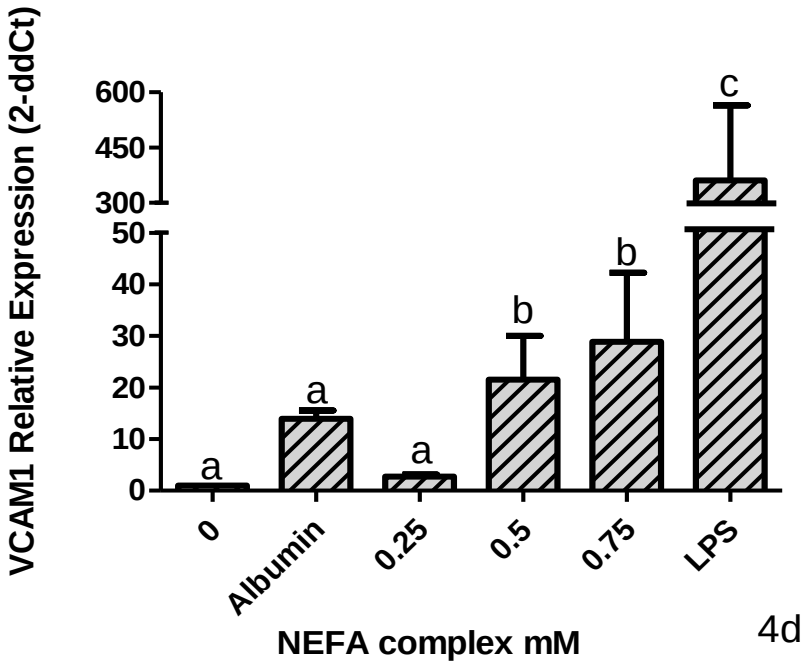

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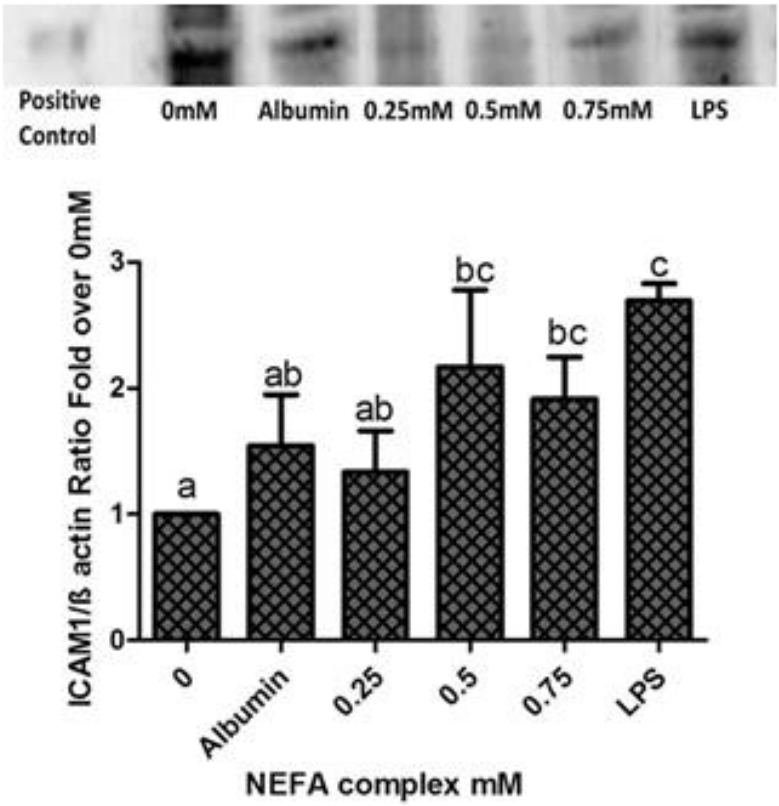


Figure 5. NEFA complex supplementation for 24 h at different concentrations induced significant changes in the protein expression of ICAM1.

Values are expressed as the ratio of ICAM1 to actin as a fold change over the 0 mM NEFA supplemented BAEC. Bovine aortic endothelial cells (BAEC), intercellular adhesion molecule (ICAM1).

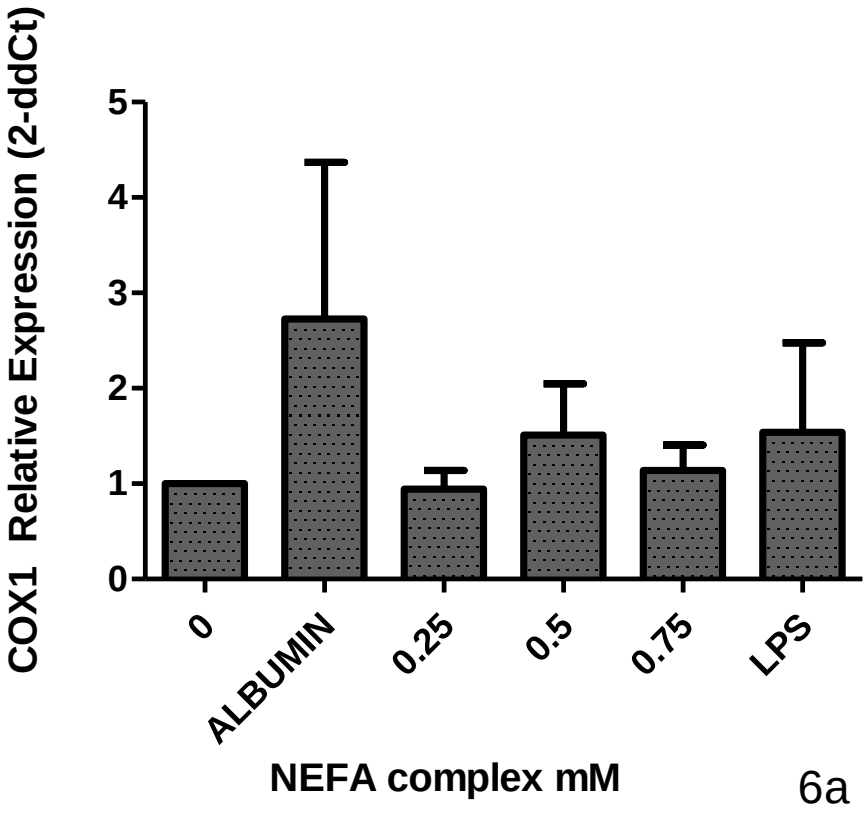
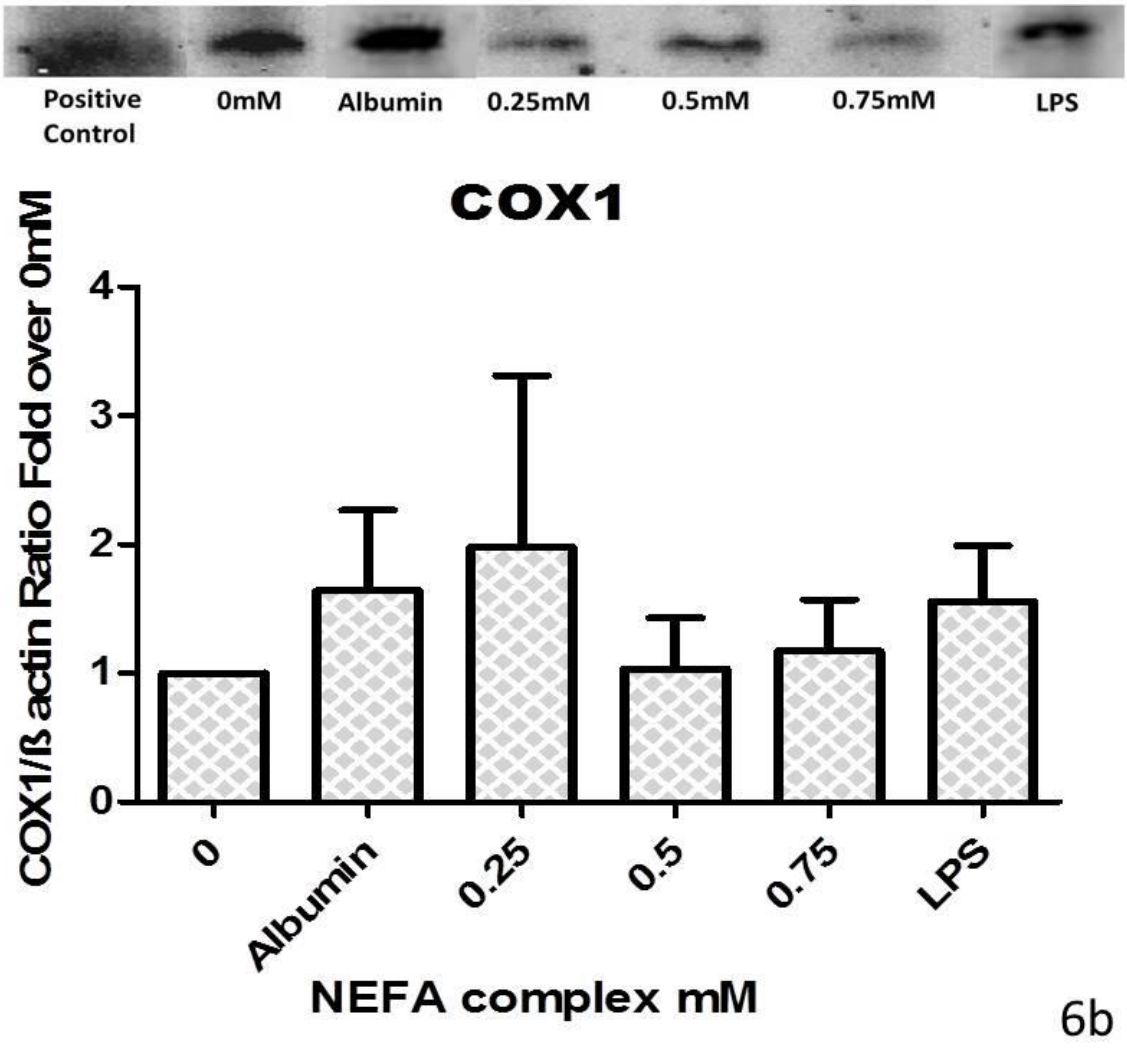


Figure 6. NEFA complex supplementation for 24 h at different concentrations induced significant changes in the gene and protein expression of cyclooxygenases.

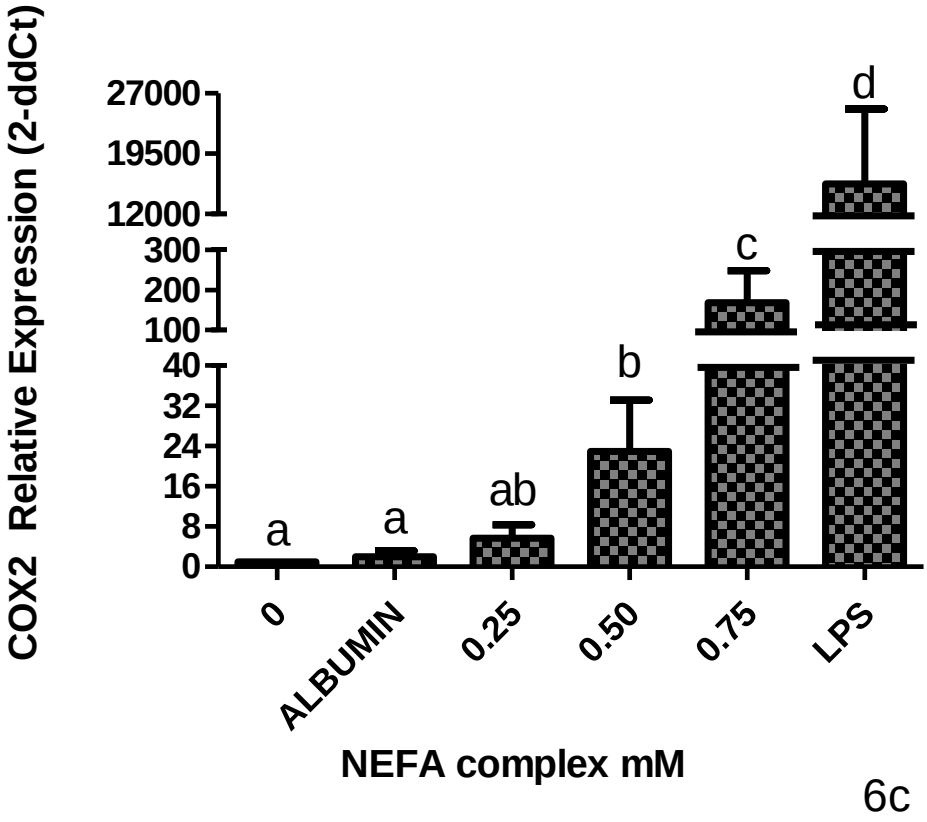
Relative gene expression of (a) COX1 and (b) COX2 in BAEC for different treatments, values are shown as $2^{-\Delta\Delta CT}$ with 0 mM set as calibrator. Protein expression of (b) COX1 and (d) COX2 in BAEC for different treatments. Values are expressed as the ratio of COX1 or COX2 to actin as a fold change over the 0 mM NEFA supplemented BAEC.

Figure 6. (cont'd)



6b

Figure 6. (cont'd)



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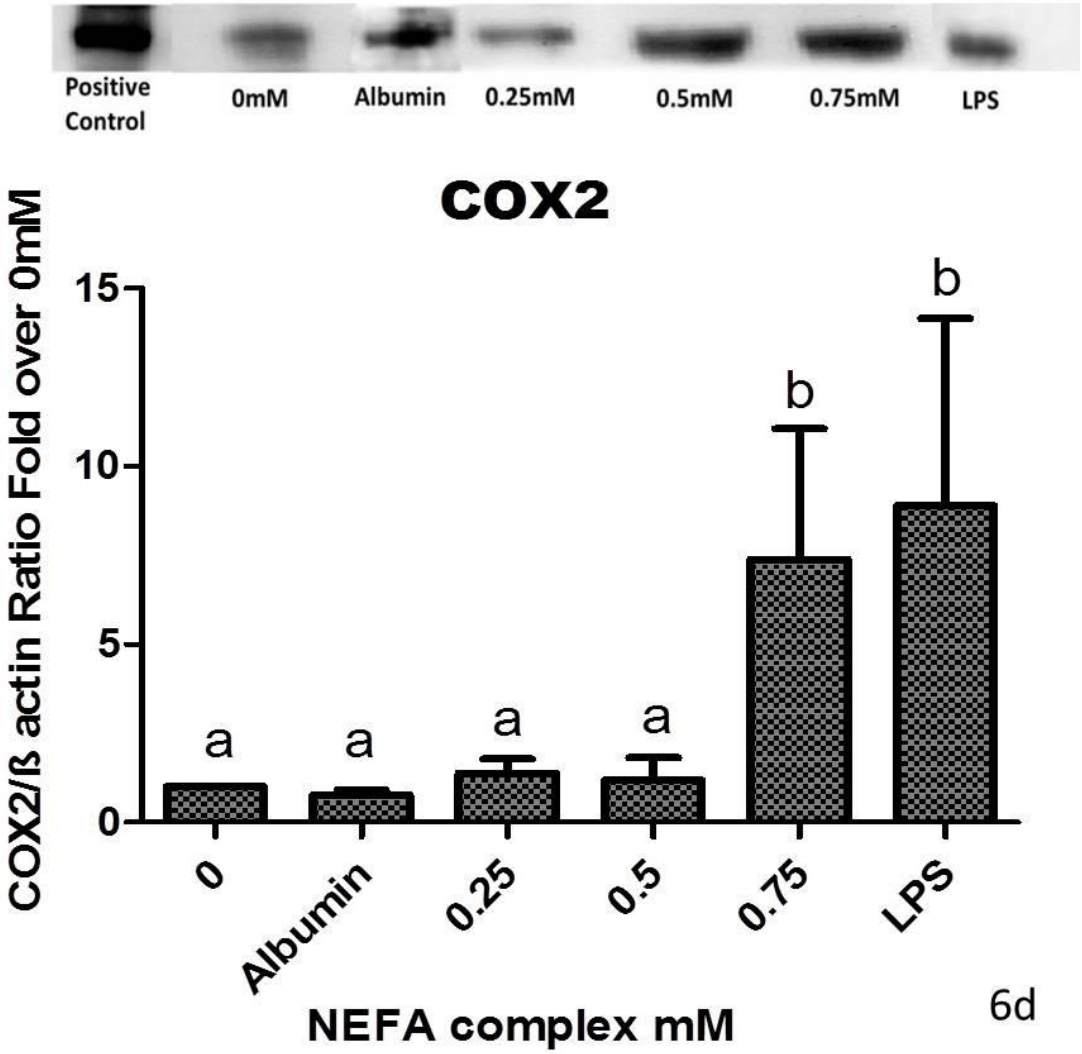
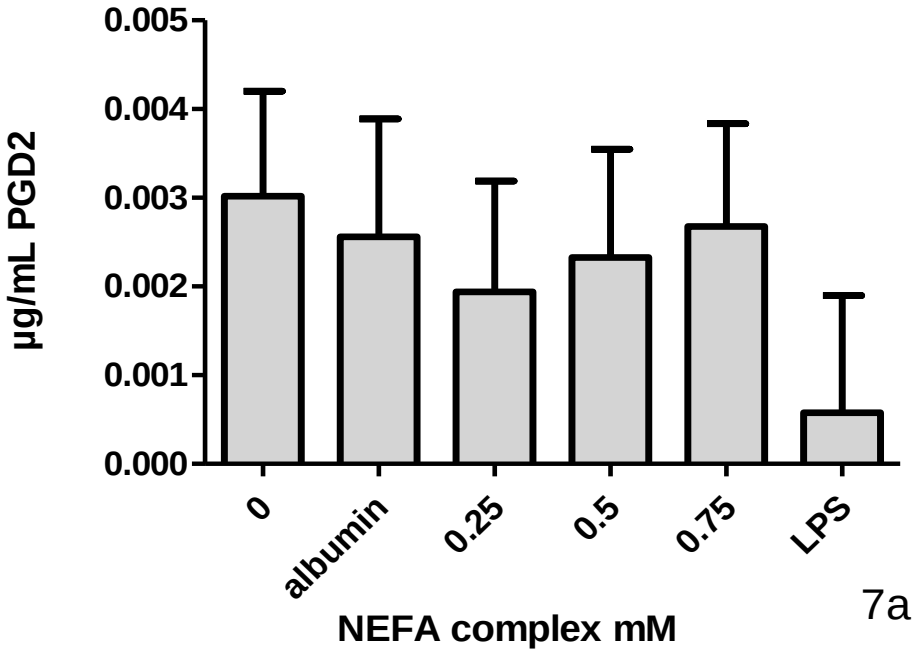




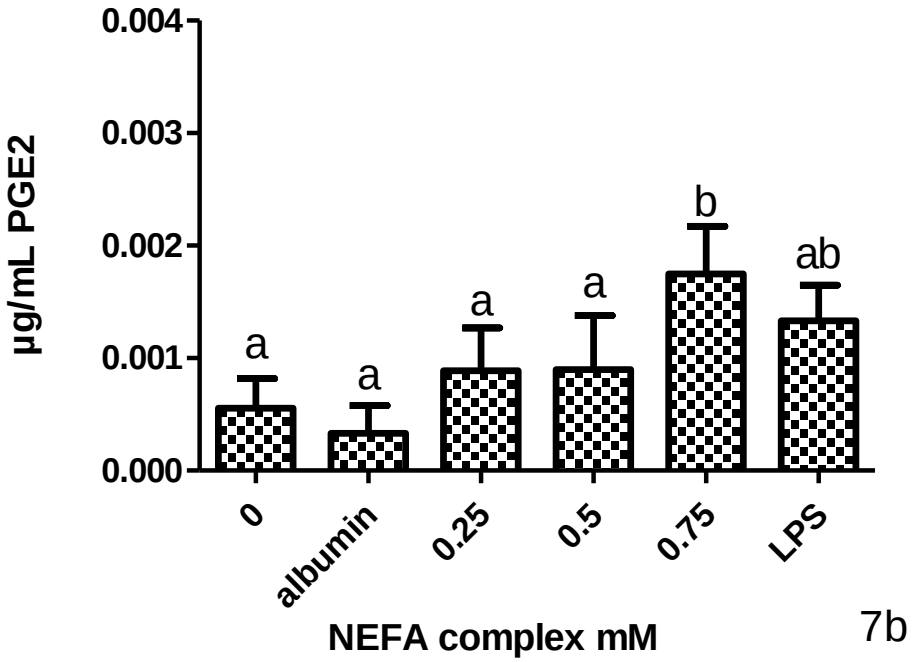
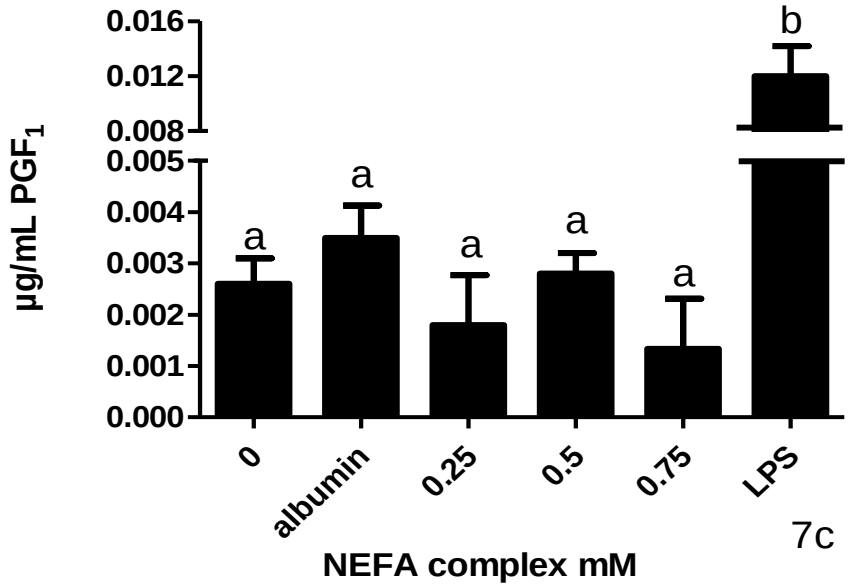
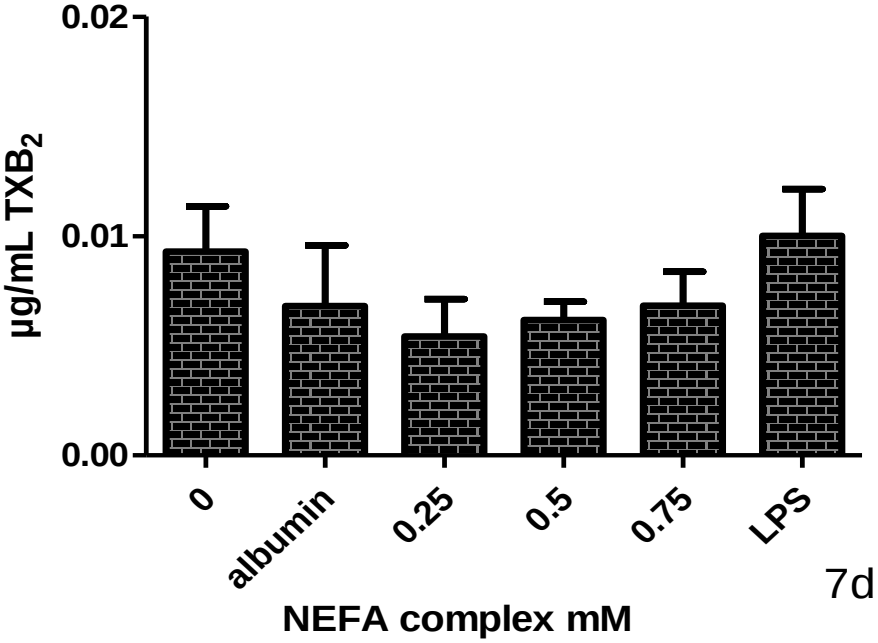


Figure 7. Effect of NEFA complex supplementation for 24 h at different concentrations in eicosanoid biosynthesis by COX enzymes.

Values are expressed in µg/mL. Prostaglandins (a) D2, (b) E2, (c) F1, and (d) thromboxane B_2 (TXB2) (d) were isolated, identified and quantified by UPLC-MS.

Figure 7. (cont'd)





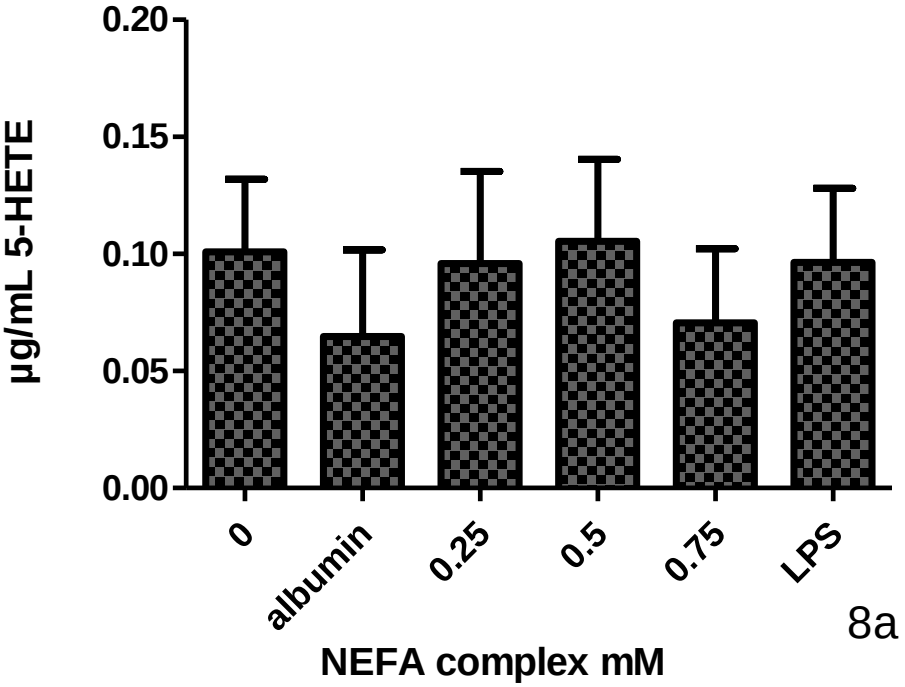
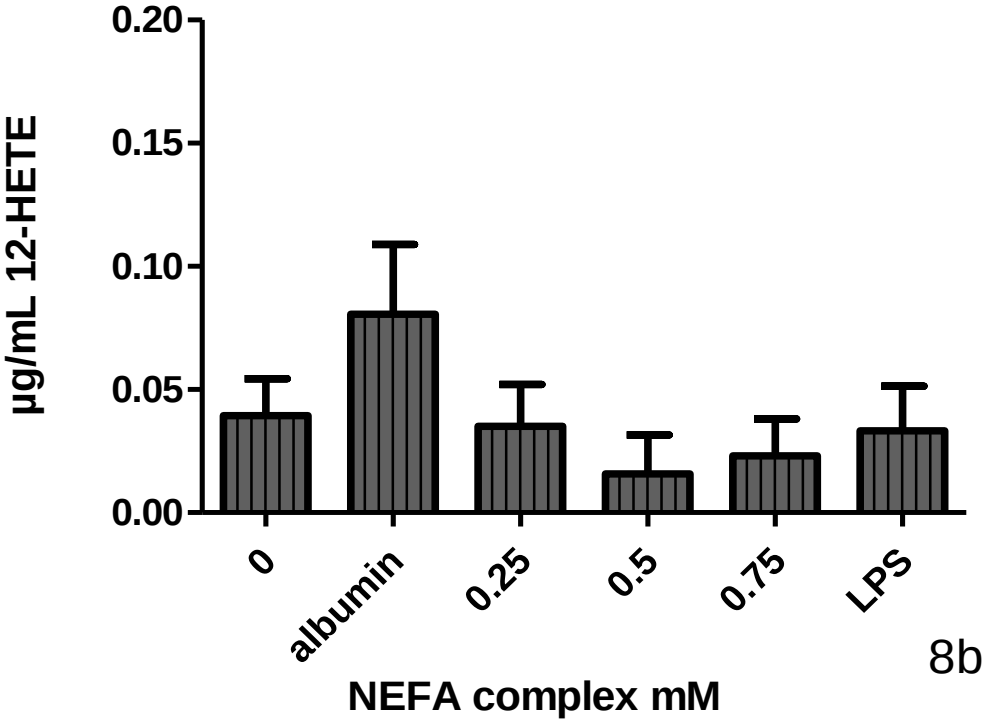


Figure 8. Effect of NEFA complex supplementation for 24 h at different concentrations in eicosanoid biosynthesis by 15LOX.

Values are expressed in µg/mL. Arachidonic acid metabolites included: (a) 5-HETE, (b) 12-HETE, (c) 15-HETE. Linoleic acid (C18:2n6c) metabolites included (d) 9-HODE and (e) 13-HODE. Hydroxieicosatetraenoic acid (HETE), hydroxioctadecadienoic acid (HODE).

Figure 8. (cont'd)



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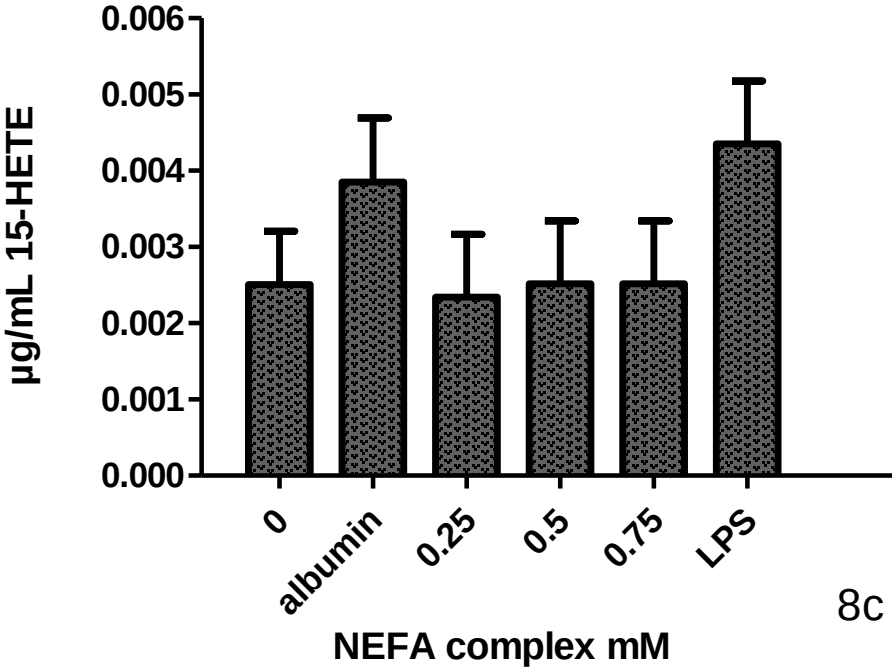

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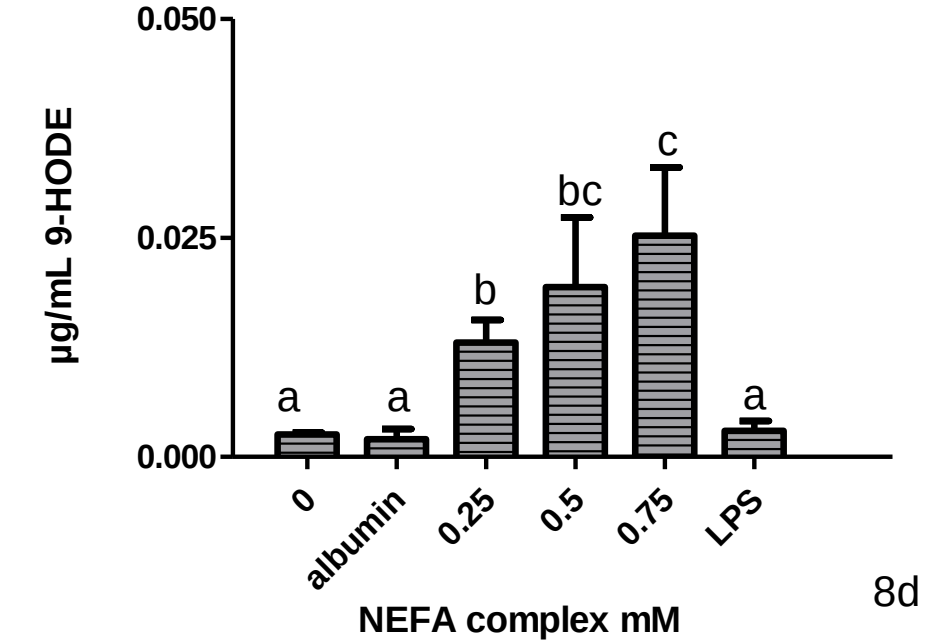
Figure 8. (cont'd)



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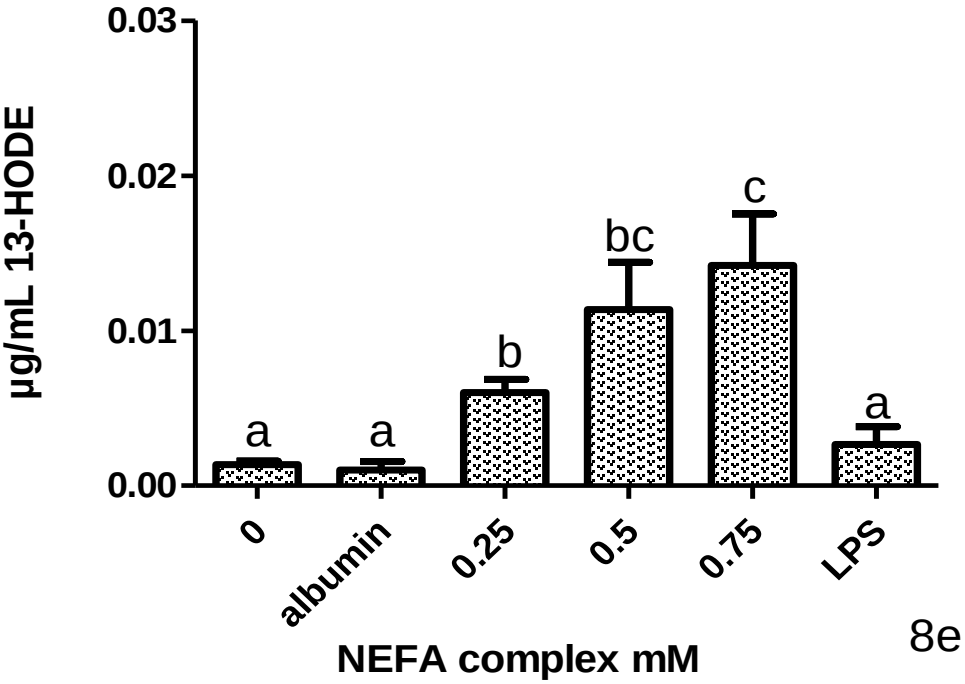

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CHAPTER 4
Eicosapentaenoic and Docosahexaenoic Acids Supplementation Improve Inflammatory Response Markers in Bovine Endothelial Cells Exposed to High Non-esterified Fatty Acids Concentrations[3]

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Abstract

A hallmark of the transition period in dairy cows is intense lipid mobilization. This mechanism of adaptation is necessary to fulfill the energy deficits experienced by cows in late gestation and early lactation. As a consequence, increased non-esterified fatty acids (NEFA) concentrations disrupt systemic lipid homeostasis not only quantitatively, but also relative to composition. Previous research demonstrated that vasculature endothelial cells exposed to increased concentrations of NEFA mimicking that off the transition period have altered expression of some inflammatory markers and changes in eicosanoid biosynthetic pathways. Consumption of omega-3 polyunsaturated fatty acids eicosapentaenoic (EPA, C20:5n3c) and docosahexaenoic (DHA, C22:6n3c) improves endothelial function and cardiovascular health in humans by attenuation of inflammatory responses and enhancement of its resolution. The objective of this study was to assess the in vitro anti-inflammatory effects of omega-3 fatty acids on bovine endothelial cells when exposed to increased NEFA concentrations that emulate lipid mobilization during the transition period of dairy cows. Bovine aortic endothelial cells (BAEC) were cultured with 2 different 0.75 mM concentrations of NEFA mixture complexed to bovine serum albumin. The 0.75 mM NEFAcomplex mixture reflected the plasma NEFA fraction during the first wk of lactation, whereas 0.75Ω3 included higher proportions of EPA (C20:5n3c) and DHA (C22:6n3c). To evaluate shifts in the fatty acid profile of BAEC, phospholipids were analyzed. Pro-inflammatory cytokine, adhesion molecule, and eicosanoid enzymes expression were assessed by qPCR and protein blot analysis. BAEC eicosanoid production was quantified using liquid chromatography. In vitro supplementation of EPA (C20:5n3c) and DHA (C22:6n3c) to BAEC while exposed to NEFA concentrations that mimicked intense lipid mobilization successfully increased the concentration of both fatty acids in BAEC phospholipids.

Supplying EPA (C20:5n3c) and DHA (C22:6n3c) also reduced the expression of pro-inflammatory cytokines. Furthermore, increased proportions of DHA (C22:6n3c) and EPA (C20:5n3c) decreased the activity of COX2 and induced expression of resolution mediators like lipoxin D_1 with known anti-inflammatory effects. The present study suggest that EPA (C20:5n3c) and DHA (C22:6n3c) could offer a nutraceutical option to diminish the pro-inflammatory state and improve endothelial function in transition dairy cows during intense lipid mobilization stages. Further studies are needed to determine in vivo effects of omega 3 polyunsaturated fatty acids supplementation on inflammatory responses.

Key words: lipid mobilization, endothelial dysfunction, eicosapentaenoic acid, docosahexaenoic acid

Introduction

Around the time of parturition, dairy cows experience metabolic challenges that are linked with the development of low grade inflammation and increased susceptibility to disease (Sordillo et al., 2009). The main metabolic change during this time is the mobilization of lipids from adipose tissue in order to offset the negative energy balance induced by the onset of lactation. Lipid mobilization alters both concentration and composition of plasma nonesterified fatty acids (NEFA), with increments in the plasma concentration of saturated fatty acids and reduction in the content of polyunsaturated fatty acids (Contreras et al., 2010b). Omega-3 polyunsaturated fatty acids were shown to exert anti-inflammatory effects in rodent and human studies leading to the inclusion of fat supplements with high content of these acids including fish oil and flax derivatives in the diet of humans and more recently in transition cow diets (Lessard et al., 2004; Wall et al., 2010).

Research in humans and rodents supports the concept that consumption of marine fish oils improves endothelial function and cardiovascular health by modulating inflammatory responses (Calder, 2004). Most benefits of including fish oil in human diet are linked to the presence of high concentrations of omega-3 polyunsaturated fatty acids including eicosapentaenoic acid (EPA, C20:5n3c) and docosapentaenoic acid (DHA, C22:6n3c) (Siscovick et al., 1995; Gorjão et al., 2009). These fatty acids modulate several endothelial and immune cell functions including intracellular signaling pathways, nuclear receptor activity, and eicosanoid biosynthesis, Previous studies that supplemented dairy cattle diets rich in EPA (C20:5n3c) and DHA (C22:6n3c) found diverse benefits. For example, after feeding fish oil to transition and mid lactation cows, Mattos et al (2004) induced a concentration dependent decrease in the uterine production of prostaglandin (PG) $F_{2\alpha}$. These findings lead the authors to suggest that

lower PG concentrations may have improved conception rate. Other studies enhanced milk content of omega-3 polyunsaturated fatty acids in mid lactation cows by supplementing fish oil either as a rumen protected source or by infusing it directly into the abomasum (Castañeda-Gutiérrez et al., 2007). Despite well-known anti-inflammatory properties of EPA (C20:5n3c) and DHA (C22:6n3c), there is no previous research in transition cows looking at the effects of supplementing these fatty acids during periods of intense fat mobilization such as the transition period and their impact in vascular inflammatory responses. The objective of this study was to assess in vitro changes of inflammatory responses in bovine endothelial cells following exposure to EPA (C20:5n3c) and DHA (C22:6n3c) together with NEFA concentrations that emulate conditions of lipid mobilization during the transition period of dairy cows.

Materials and Methods

Cell Culture

Bovine aortic endothelial cells (BAEC) were isolated from the bovine aorta using collagenase as a digestion method as described previously (Contreras et al., 2010a). BAEC were cloned to a single-cell level by the limiting dilution method and maintained until confluent as previously described (Sordillo et al., 2008). BAEC were cultured for 24 h exposed to 2 different treatments. The 0.75 mM NEFA supplementation emulated intense lipid mobilization during lactation and a 0.75 NEFA supplementation with EPA (C20:5n3c) and DHA (C22:6n3c) added (O.75Ω3). Cells were exposed to culture media without addition of NEFA complex 0 mM as a negative control, culture media plus albumin without any NEFA bound as the NEFA complex control, and culture media plus lipopolysaccharide (LPS) at a dose of 50 ng/mL as a positive inflammation control. The BAEC were used at passage 9 or less for all experiments.

Cell Viability Assay

Cell death was evaluated using Trypan blue exclusion. Cell viability, was measured using Promega CellTiter-Glo Viability Assay (Promega, Madison, WI, USA) which quantifies the amount of ATP, an indicator of metabolically active cells. Both assays were performed as previously described (Contreras et al., 2010a).

Non-esterified Fatty Acids-Albumin Complex Solutions

NEFA complex composition was calculated based upon a previous study that characterized the fatty acid profiles of plasma NEFA in periparturient cows (Contreras et al., 2010a). The complex mimicked the fatty acid profile in the NEFA fraction during the first wk of lactation and included: myristic (C14:0) (3%), palmitic (C16:0) (30%), stearic acid (C18:0) (45%), oleic (C18:1n9c) (16%), linoleic (C18:2n6c) (5%) and DHA (C22:6n3c), C22:6n3c (1%) fatty acids. For the 0.75Ω3 treatment, the complex included DHA (C22:6n3c) and EPA (C20:5n3c) as follows: myristic (C14:0) (3%), palmitic (C16:0) (30%), stearic acid (C18:0) (45%), oleic (C18:1n9c) (9%), linoleic (C18:2n6c) (5%), EPA (C20:5n3c) (4%), and DHA (C22:6n3c) (4%). NEFA-albumin preparations were produced by saponification, NEFA were included into the media culture using albumin as a transporter emulating what is observed in vivo, where this protein binds fatty acids in circulation (Kim et al., 2005). Briefly, palmitic (C16:0) and stearic (C18:0) acids were dissolved in ethanol at 90°C solution and further sonicated with a Branson Sonifier 250 for 10 s at an output of 10%. Once readily dissolved, the solution was dried under nitrogen, and then resuspended in NaOH 0.1 mol/L at 90°C. Oleic (C18:1n9c), linoleic (18:2n6c), DHA (C22:6n3c), and EPA (C20:5n3c) acids were dissolved in NaOH 0.1 mol/L at 70°C and then mixed with palmitic (C16:0) and stearic (C18:0) acids NaOH solution. The NEFA mixture solution was complexed with 10% bovine serum albumin (fatty

acid-free) at 55°C for 10 min. The NEFA-albumin complexes were then filter sterilized (0.44 μm) and stored under argon atmosphere at -20°C for future use. The final concentration of the complex was 100 μM of NEFA, with an albumin molar ratio of 5:1.

Lipid Extractions

Total lipids from media and BAEC were extracted using a hexane:ethanol (1:1) solvent mix as previously described (Contreras et al., 2010a). BAEC were harvested by gentle scraping, washed in Hank's balanced salt solution (HBSS) and centrifuged at 233 g. Prior to lipid extraction, BAEC were resuspended in HBSS to a minimum concentration of $5.5x10^6$ cells/mL. To obtain total lipids, media or BAEC were combined with the solvent mix and vortexed for 10 min, and then centrifuged at 2,095 g for 10 min at room temperature. The hexane layer was collected for total fatty acid composition and lipid fractioning. Nonadecanoic acid was used as internal standard.

Lipids were further separated into neutral lipids, NEFA and phospholipid fractions by solid-phase extraction using Strata cartridges containing aminopropyl (NH_2) sorbent (Phenomenex, Torrance, CA, USA) as described by (Kaluzny et al., 1985). Briefly, after conditioning of the cartridges with hexane, the neutral lipid fraction was eluted with chloroform:2-propanol (2:1), the NEFA with 2% acetic acid in diethyl ether, and the phospholipids fraction with methanol. A preparation of 1,2-Diheneicosanoyl-*sn*-Glycero-3-phosphocholine (21:0 phosphatidyl choline) was used as internal standard for lipid fractioning. Fatty acids from total extractions and each lipid fraction were transesterified and methylated using 20% methanolic hydrochloric acid for 2 h at 90°C (Browse et al., 1986). Methylated extractions were analyzed using a Perkin Elmer - Clarus 500 gas chromatography apparatus with

a Supelco SP-2560 column (100m x 25 mm with a 0.2um film thickness). Fatty acid peaks were identified by using a mix of pure methyl ester standards (Nu Check, Elysian, MN, USA).

Gene Transcript Quantification by qPCR

Total RNA was extracted from BAEC for quantification of IL-6, IL-8, ICAM1, VCAM1, COX1, COX2, 15LOX1, and β-actin mRNA transcript expression by qPCR as formerly described (Corl et al., 2008a). Briefly, total RNA was extracted utilizing the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA). DNase digest was done during the RNA extraction using the RNase-Free DNase Set (Qiagen, Valencia, CA, USA). Purified RNA was converted to cDNA using the Applied Biosystems High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). All qPCR assays were conducted utilizing TaqMan gene expression assays from Applied Biosystems. TaqMan primer and probe sets (Table 11) were designed from bovine sequences with the Applied Biosystems Pipeline software and synthesized by Applied Biosystems. Samples were assayed in triplicate with 50 ng of cDNA per reaction along with 10 μL of TaqMan Fast Universal PCR Master Mix (2X), and 1 μL of the appropriate TaqMan Gene Expression Assay Mix (20X) on the Applied Biosystems 7500 Fast Real-Time PCR System. A nonreverse transcriptase control was run to ensure genomic DNA was not being amplified. The relative quantification of each gene was calculated utilizing the 7500 Fast SDS Software (version 1.3.1) (Livak, 2001). Data were calculated based on the comparative Ct method ($2^{-\Delta\Delta Ct}$) of relative quantification using β-actin as the control gene (Steibel et al., 2009). The calibrator was set as the 0 mM NEFA complex supplementation.

Western Blot Analyses

Protein quantification was performed by protein blot analysis as previously described (Contreras et al., 2010a). In brief, BAEC cells were harvested and whole cell lysates obtained.

Supernatant were collected and total protein was quantified using the Coomassie brilliant blue method. Equal amounts of protein (30 μg) were electrophoresed on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The Millipore Snap i.d. Protein Detection System (Millipore, Billerica, MA, USA) was utilized to carry out the remaining steps. The membrane was blocked in 0.5% dry milk in Tris-buffered saline (TBS) with 0.01% Tween-20 and washed 3 times with TBS. Membranes were incubated for 10 min with anti-bovine COX1 (1:400 dilution, Abcam, Cambridge, MA, USA), anti-human COX2 (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-rabbit 15LOX1 (1:2000, Cayman, Ann Arbor, MI, USA), and anti-human ICAM1 (1:100 dilution, Invitrogen, Carlsbad, CA, USA) in 1% bovine serum albumin in TBS with 0.01% Tween-20. Following 3 washes with TBS, membranes were incubated with the correspondent anti-host IgG secondary antibody labeled with horseradish peroxidase (HRP; 1:3,000 dilution in 0.5% dry milk, Pierce, Rockford, IL, USA) for 10 min at room temperature, washed 3 times with TBS, exposed to HRP substrate (Pierce), and visualized by chemiluminescence using the ChemiDoc™ XRS (Bio-Rad, Hercules, CA, USA) and Quantity One® software (Bio-Rad, Hercules, CA, USA). Anti-human actin (1:3,000 dilution, Millipore, Billerica, MA, USA) served as the loading control. Density of the bands was quantified by the Quantity One® software. The ratio of specific antibodies:actin was calculated and the values were expressed as a fold change over the 0 mM NEFA supplemented BAEC.

Eicosanoid Detection and Quantification

BAEC eicosanoid production was detected and quantified using ultra high pressure liquid chromatography-mass spectrometry (UPLC-MS). In brief, BAEC and media were harvested by gentle scraping and addition of EtOH to a 10% solution by volume. Internal standards were added prior to harvesting cells and supernatants. Specimens were then sonicated while in water

bath at 4°C for 3 min at output 10% using a Misonix S-400 sonicator (Newton, CT, USA). Samples were then centrifuged at 4°C for 5 min at 3000 g and the supernatant was used for extraction. Lipid mediators were isolated from specimens by solid phase extraction using Strata X SPE columns (Phenomenex, Torrance, CA, USA). Cartridges were first conditioned with 2 mL of MeOH then washed with 2 mL of H_2O. Samples were loaded into the cartridge, washed with 2 mL of MeOH (20% v/v), and finally eluted with MeOH:acetonitrile (50:50 v/v). Eluates were dried using a Savant Speedvac (Thermo, West Palm Beach, FL, USA) and then redissolved in acetonitrile:water:formic acid (36:64:0.2 v/v) to perform UPLC-MS using a Waters H-class Acquity UPLC® chromatographer (Waters, Milford, MA, USA). Lipid mediators were separated using an Acquity UPLC® BEH C18 1.7µm 2.1 ^{x} 100 mm column (Waters, Milford, MA, USA). 15LOX derived eicosanoids were separated at a flow rate of 600 µL/min at 37ºC with the column equilibrated in methanol-acetonitrile- water-acetic acid (15.8:47.4:36.8:0.02; v/v/v/v) and 20 µL of sample were injected. Eicosanoids were isolated during a 15 min run using the same solvent mixture. COX eicosanoids were separated at a flow rate of 600 µL/min at 37ºC. The column was equilibrated in water-acetonitrile-acetic acid (65:35:0.02; v/v/v) and 20 µL of sample were injected. Eicosanoids were isolated by liquid chromatography during an 18 min run using acetonitrile-acetic acid (65:35:0.02; v/v/v). Eicosanoids were then analyzed using a mass spectrometer (Acquity SQD,Waters, Milford, MA, USA). UPLC-MS results were quantified using Empower-Pro 2 software (Waters, Milford, MA, USA). Measured eicosanoids included: prostaglandins (PG) D_2, E_2, F_1, $F_2\alpha$, and thromboxane B_2 (TXB_2) for the cyclooxygenases pathway. For the 15LOX1 pathway, linoleic acid (C18:2n6c) derivates: 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE) and

arachidonic acid (C20:4n6c) products; 5, 12 and 15-hydroxyeicosatetraenoic acids (5, 12, and 15-HETE). Results are expressed as ng of lipid mediator per mL of media.

Statistical Analyses

Statistical evaluation was conducted using Proc GLM procedure (SAS v9.1, SAS Institute Inc, Cary, NC, USA) and Prism 4 (GraphPad Software, La Jolla, CA, USA). Comparisons of the means were made by analysis of variance followed by Tukey–Kramer's HSD *post hoc* test as necessary. Data are shown as means ±SEM. Differences with $p < 0.05$ were considered statistically significant.

Results

Cell Viability

As assessed by trypan blue exclusion, no significant changes in viability were observed at 24 h of incubation when BAEC were exposed to 0 mM, albumin, 0.75 mM, and 0.75Ω3 of the NEFA complex with cell death counts below 10%. After 24 h of culture with either the NEFA complexes (0.75 mM and 0.75Ω3) or albumin control, BAEC were determined to be viable by measuring ATP production. No differences in cell viability were detected among treatments (data not shown).

EPA (C20:5n3c) and DHA (C22:6n3c) Supplementation Altered Fatty Acid Profiles in BAEC Phospholipids

Culture with the different treatments induced significant changes in the phospholipid composition of BAEC (Table 12). There was a significant increase in the concentrations of stearic acid (C18:0) when cells were supplemented with both 0.75 mM and 0.75Ω3 supplementations when compared to 0 mM or albumin control. Culturing BAEC for 24 h with 0.75 mM caused a decrease in the concentration of heptadecanoic acid (C17:0) when compared to albumin or 0 mM. As expected, several changes in the concentrations of polyunsaturated fatty

acids were observed after exposure to any of the treatments. Vaccenic acid (C18:1n7c) content decreased significantly in cells cultured with either 0.75 mM or 0.75Ω3 when compared to 0 mM control. The content of other polyunsaturated fatty acid arachidonic (C20:4n6c) was reduced in 0.75 mM and 0.75Ω3 treatments when compared to albumin or 0 mM. Addition of EPA (C20:5n3c) and DHA (C22:6n3c) in the 0.75Ω3 treatment significantly increased the concentration of these 2 polyunsaturated fatty acids when compared to neither the other treatment, 0.75 mM nor the controls. Furthermore, the concentration of EPA (C20:5n3c) was reduced significantly in 0.75 mM NEFA complex treatment when compared to 0 mM and albumin treatment groups. The concentration of docosapentaenoic acid (C22:5n3c) also was significantly decreased by exposure to the 0.75Ω3 treatment group when compared to the 0.75 mM NEFA treatment and each of the controls.

EPA (C20:5n3c) and DHA (C22:6n3c) Supplementation Reduced Pro-inflammatory Cytokine and Adhesion Molecules Gene and Protein Expression

Gene expression of IL-6 and IL-8 was altered by exposure to all of the treatments (Figure 9). For IL-6, the highest gene expression was observed when BAEC were exposed to 0.75 mM NEFA complex, followed by supplementation with 0.75Ω3 when compared to the 0 mM control group. As for IL-8, 0.75 mM supplementation of NEFA complex significantly increased gene expression when compared to the negative control (0 mM).

ICAM1 gene expression was enhanced by supplementation with 0.75 mM NEFA complex when compared to 0 mM, however, no differences were observed between 0.75 mM and 0.75Ω3 treatment groups (Figure 9). Protein expression of ICAM1 was augmented significantly when BAEC were exposed to both 0.75 mM and 0.75Ω3 treatment groups when compared to the 0 mM control (Figure 10). As for the other adhesion molecule evaluated

(VCAM1), gene expression was significantly increased by treatment with 0.75 mM and 0.75Ω3 NEFA complex treatments when compared to the 0 mM control group. Furthermore, the gene transcription of the adhesion molecule was significantly lower during exposure to 0.75Ω3 NEFA treatment when compared to 0.75 mM NEFA treatment group (Figure 9).

EPA (C20:5n3c) and DHA (C22:6n3c) altered Lipid Mediator Biosynthesis in BAEC

Eicosanoid Enzymes.

Gene expression of COX1 was not altered by any of the treatments when compared to the 0 mM treatment or albumin controls (Figure 11a). As with gene transcription, the enzyme protein expression remained unchanged when exposed to the different treatments (Figure 11b). COX2 gene expression, however, was altered significantly when BAEC were exposed to 0.75 mM and 0.75Ω3 NEFA complex treatments when compared to the 0 mM control (Figure 11c). Additionally, exposure to 0.75Ω3 NEFA complex significantly decreased the gene transcription when compared to 0.75 mM NEFA complex treatment group. Genomic results were reflected directly in protein expression of COX2, where supplementation of BAEC with 0.75 mM significantly increased the protein expression of COX2 when compared to 0.75Ω3, 0 mM and albumin control (Figure 11d). As for LOX enzymes, addition of any NEFA supplementation or LPS had a tendency to increase the expression of both 15LOX1 ($p=0.08$) and 15LOX2 ($p=0.073$) when compared to 0 mM control group (Figure 14).

Lipid Mediator Biosynthesis.

Exposure of BAEC to NEFA supplementation with or without EPA (C20:5n3c) and DHA (C22:6n3c) changed eicosanoid production in BAEC. Biosynthesis of COX-derived PGE_2, PGF_1, and TXB_2 were not affected by exposure to any of the treatments (Figure 12). BAEC production of PGD_2 (Figure 12) and resolvin D_1 (Figure 13), a COX2 derivative of EPA (C20:5n3c) and

DHA (C22:6n3c), were significantly enhanced by supplementation with 0.75Ω3 when compared to 0.75 mM and the controls (0 mM, albumin, and LPS).

As for 15LOX arachidonic acid (C20:4n6c) products (Figure 15), there were no significant changes in the production of 5-HETE and 12-HETE. However, 15-HETE production was significantly enhanced by exposure of BAEC to 0.75Ω3 when compared to 0 mM controls. 15LOX linoleic product biosynthesis was altered by exposure to both treatments. Both NEFA mixes (0.75 mM and 0.75Ω3) increased the production of 13-HODE when compared to any of the controls including albumin, 0 mM, and LPS. The production of 9-HODE was significantly increased with values peaked when BAEC were exposed to 0.75 mM, followed by 0.75Ω3 when compared to 0 mM.

Discussion

Excessive lipid mobilization during the transition period is associated with increased metabolic and inflammatory disease susceptibility in dairy cows (Sordillo et al., 2009). Indeed, alterations in the concentration and content of plasma NEFA induce changes in the fatty acid composition of cellular membranes of cells involved with inflammatory responses (Contreras et al., 2010b). Previously, we demonstrated that intense lipid mobilization alters cellular membrane phospholipid fatty acid profile, enhances the expression of pro-inflammatory cytokines and adhesion molecules, and modifies the biosynthesis of eicosanoids by COX and 15LOX pathways (Contreras et al., 2010a). Here we demonstrate that in vitro supplementation of EPA (C20:5n3c) and DHA (C22:6n3c) added to a NEFA complex mixture were able to reduce inflammatory markers in BAEC.

The amounts of EPA (C20:5n3c) and DHA (C22:6n3c) added to culture media were based on previous research in ruminants where different types of fish oils were fed to dairy cattle

(Kitessa et al., 2001; Castañeda-Gutiérrez et al., 2007). When dairy cattle was supplemented with fish oil, the plasma phospholipid content of both EPA (C20:5n3c) and DHA (C22:6n3c) fatty acids reached similar concentrations of between 4 and 9% of the total phospholipid fatty acids (Kitessa et al., 2001). Based on our previous in vivo study demonstrating that plasma fatty acid phospholipid content directly reflects fatty acid profiles of cellular membranes (Contreras et al., 2010b) ANEFA complex with equal amounts of both EPA (C20:5n3c) (4%) and DHA (C22:6n3c) (4%) was selected for use in the present in vitro studies.

Similar to our previous in vitro study (Contreras et al., 2010a), the only saturated fatty acid that was significantly increased with NEFA supplementation was stearic acid (C18:0) for both 0.75mM and 0.75Ω3 treatments, reflecting its higher concentration in the NEFA mixture, and possibly the preference for the usage of palmitic acid (C16:0) as energy substrate over stearate (C18:0) (Crunkhorn et al., 2007). Consequent to high saturated fatty acid content in both treatments, the concentration of specific polyunsaturated fatty acids decreased in a compensatory manner. For example, there was a significant reduction in the content of arachidonic acid (C20:4n6c) in BAEC phospholipids and this reduction was more notorious in the 0.75Ω3 treated cells. This is in agreement with a previous study (Hadjiagapiou and Spector, 1987) demonstrating that DHA (C22:6n3c) supplementation suppresses incorporation of arachidonic acid (C20:4n6c) into endothelial cells phospholipids. At the same time, 0.75Ω3 NEFA supplementation effectively increased the phospholipid content of both EPA (C20:5n3c) and DHA (C22:6n3c) in BAEC. Interestingly, phospholipid incorporation of DHA (C22:6n3c) was more effective compared to EPA (C20:5n3c). As with the plasma lipid fraction distribution, DHA (C22:6n3c) is taken preferentially by endothelial cells into the phospholipid fraction

specifically into phosphatidylcholine (Chen et al., 2007). n the other hand, EPA (C20:5n3c) is preferentially incorporated into neutral lipids including cholesterol esters.

Inclusion of EPA (C20:5n3c) and DHA (C22:6n3c) in the NEFA complex mixture reduced the expression of different inflammatory markers in BAEC when compared to 0.75 mM NEFA treatment including VCAM1 and IL-6. Previously (De Caterina et al., 1994; Weber et al., 1995) demonstrated that DHA (C22:6n3c) directly reduces VCAM1 gene and protein surface expression in endothelial cells after exposure to different pro-inflammatory cytokines and lipopolysaccharide. A first likely mechanism is the modulation by DHA of serine-threonine kinase PKB, a pathway directly related to the development to atheroma lesions in human atherosclerosis (Chen et al., 2003). A second mechanism is the reduction in the activity of NFκB by diminishing inhibitor kappa B (I*κ*B) phosphorylation thus reducing endothelial adhesion molecule expression (Goua et al., 2008). Similarly, both EPA (C20:5n3c) and DHA (C22:6n3c) diminish endothelial cell expression of IL-6 in a dose dependent manner through the inhibition of NFκB inflammatory pathway (Khalfoun et al., 1997; Lee et al., 2009). To further explore mechanisms by which EPA (C20:5n3c) and DHA (C22:6n3c) inhibit endothelial cytokine and adhesion molecule expression during lipid mobilization, future studies should evaluate NFκB and PKB activity.

The 0.75Ω3 NEFA supplementation was effective in reducing the gene and protein expression of COX2. One of the fatty acids added to the 0.75Ω3 mixture, DHA (C22:6n3c), is proven to inhibit COX2 in several ways. After incorporation into cellular membrane phospholipids, the fatty acid blocks nuclear factor kappa beta (NF-κB) mediated transcriptional regulation through activation of extracellular signal-regulated kinases (ERK), diminished production of ROS, and inhibition of protein kinase C (PKC) activation (Massaro et al., 2006). In

the present study we assured incorporation of DHA (C22:6n3c) into the phospholipid membrane by culturing BAEC for 24 h, therefore it is possible that all mechanisms previously described could have diminished COX2 gene and protein expression. Further studies are necessary to characterize the mechanisms used by EPA (C20:5n3c) and DHA (C22:6n3c) to reduce COX2 expression during intense lipid mobilization events

Although both NEFA complex treatments (0.75 mM and 0.75Ω3) increased COX2 gene transcription and protein synthesis than the negative controls, no changes in the production of known COX2 derived pro-inflammatory eicosanoids such as PGE_2, PGF_1, and TXB_2 were observed. As described in our previous in vitro study (Contreras et al., 2010a), a possible explanation for the lack of metabolite production response is the low availability of the preferential COX2 substrate, arachidonic acid (C20:4n6c). However, there are several COX2 derived metabolites that were not analyzed during this study that could have had changes in their biosynthesis. Further studies should include a complete lipidomic analysis.

Supplementation with the 0.75Ω3 NEFA complex enhanced the biosynthesis of PGD_2. It is likely that the synthesis of its natural derivate, prostaglandin J2 (15d-PGJ2) may have been increased. Together with EPA (C20:5n3c) and DHA (C22:6n3c), this PG is capable of directly activating PPAR alpha and gamma nuclear receptors therefore suppressing inflammatory responses in endothelial cells (Kliewer et al., 1995; Marx and Walcher, 2007). Higher activity of these nuclear receptors in BAEC supplemented with 0.75Ω3 may explain, in part, the reductions in the gene expression of IL-6 and IL-8 (Marx et al., 2004). Complimentary studies should evaluate PPAR receptor activity during NEFA supplementation with or without EPA (C20:5n3c) and DHA (C22:6n3c) to elucidate mechanisms of action and likely provide possible targets for pharmacological or nutraceutical interventions.

A beneficial effect of EPA (C20:5n3c) and DHA (C22:6n3c) supplementation was demonstrated in the present study with the increased biosynthesis of resolvin D_1. This anti-inflammatory molecule can reduce the production of IL-6 and IL-8 in endothelial cells alone or in co-culture systems with leukocytes (Serhan et al., 2008). Together with resolvin $E_{2,}$ both anti-inflammatory lipid mediators are able of restricting immune cell migration (Tian et al., 2009). Resolvin D_1 is synthesized by a multi-enzyme process initiated with COX2 using DHA (C22:6n3c) as substrate, followed by a series of oxidations by 15LOX and 5LOX (Seki et al., 2010). BAEC are known to express only very low amounts of 5LOX , however, studies in other arterial endothelial cells such as human pulmonary demonstrated that these vascular cells enhance the expression of 5LOX during inflammatory responses (Cao et al., 2000; Zhang et al., 2002). In the present study, we demonstrated production of resolvin D_1 by BAEC under conditions emulating intense lipid mobilization while supplemented with EPA (C20:5n3c) and DHA (C22:6n3c). Further studies are necessary to characterize resolvin D_1 biosynthesis by BAEC including the possibility of 5LOX expression by these cells during exposure to high concentrations of NEFA.

As with COX, 15LOX enzymes were affected by supplementation with 0.75 mM and 0.75Ω3. Both treatments tended to increase the 15LOX1 and 15LOX2 gene expression. As possible consequence, the production of 13- hydroxioctadecadienoic acid **(**HODE), a linoleic acid (C18:2n6c) 15LOX derivative, was increased. In contrast, inclusion of omega-3 polyunsaturated fatty acids EPA (C20:5n3c) and DHA (C22:6n3c) reduced significantly the production of 15LOX derivative 9-HODE when compared to 0.75 mM. The cause of this reduction is not clear, but competition of EPA (C20:5n3c) and DHA (C22:6n3c) with arachidonic acid (C20:4n6c) for the enzyme is a possibility, as it was previously demonstrated

with COX (Lokesh and Kinsella, 1994). Interestingly, the production of 15-hydroxieicosatetraenoic acid (HETE), an arachidonic acid (C20:4n6c) product of 15LOX metabolism, was enhanced with 0.75Ω3 when compared to other treatments. This could be partially explained by the tendency of the 0.75Ω3 NEFA treatment to enhance the gene transcription of 15LOX2. This enzyme converts arachidonic acid (C20:4n6c) exclusively to 15*S*-hydroperoxyeicosatetraenoic acid (15-HPETE), the precursor of 15-HETE (Brash et al., 1997). Increased production of this 15-HETE could add some beneficial effects to 0.75Ω3 supplementation. This 15LOX derivative is precursor to both pro-inflammatory and anti-inflammatory compounds. Among anti-inflammatory metabolites, the family of lipoxins promotes inflammatory resolution and restricts immune cell migration (Wittwer and Hersberger, 2007). Further studies including a more detailed analysis of 15LOX EPA (C20:5n3c) and DHA (C22:6n3c) derivatives are needed.

Conclusion

Elevated concentration and altered composition of NEFA as observed in transition dairy cows during intense lipid mobilization induce a pro-inflammatory state in different organs and cell populations. We demonstrated previously that these changes include enhanced endothelial adhesion molecule and pro-inflammatory cytokine expression and shifts in COX and 15LOX eicosanoid biosynthetic pathways (Contreras et al., 2010a). In the present study, in vitro supplementation of EPA (C20:5n3c) and DHA (C22:6n3c) to BAEC, while exposed to total NEFA concentrations that mimic intense lipid mobilization, restricted the activity of COX2 enzyme, diminished the expression of pro-inflammatory cytokines, and induced the biosynthesis of resolution mediators like resolvin D_1. Furthermore, there was a shift in the production 15LOX1 metabolites to anti-inflammatory mediators that may have diminished the pro-

inflammatory state during intense lipid mobilization environment. Further studies are needed to further identify lipid mediators synthesized during exposure to high NEFA concentrations. Moreover it is important to determine specific 15LOX and COX metabolite (i.e. resolvin D_1 and 15-HETE) activity and their individual mechanism of action.

Acknowledgments

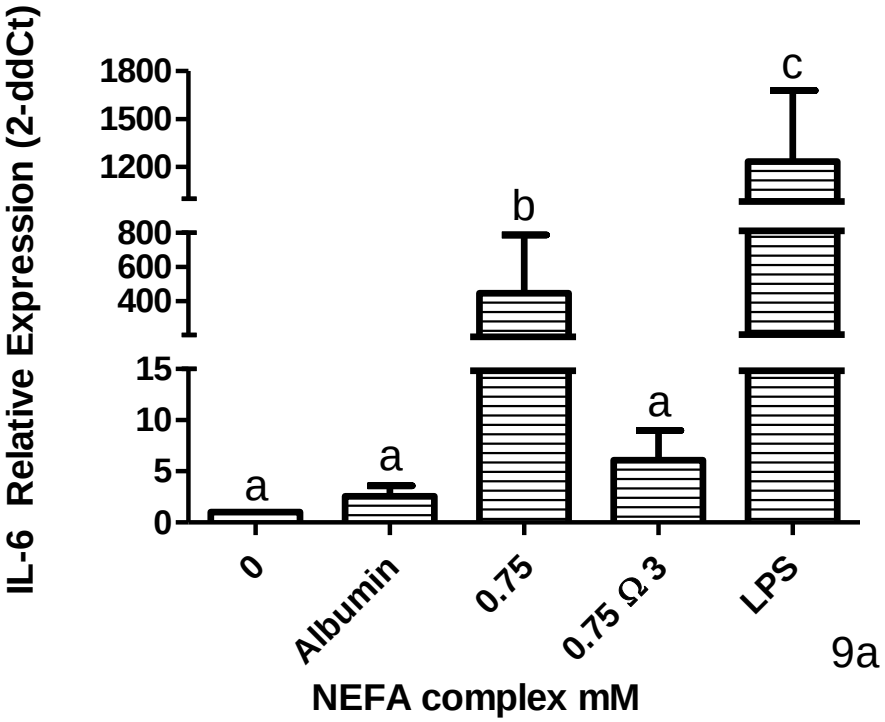
Authors acknowledge the technical assistance of Justin Zyskowski, Chris Corl, and Jeffery Gandy for laboratory analyses and Juan David Munoz for statistical analysis. This project was supported in part by Agriculture and Food Research Initiative Competitive Grants Programs 2007-35204-18463 and 2010-03506 from the USDA National Institute for Food and Agriculture and by an endowment from the Matilda R. Wilson Fund (Detroit, MI).

Table 11. Gene targets and primer sequences for qPCR

Target	Accession#		Sequence (5' to 3')
ICAM1	NM-174348	Forward	GCAGGTGGTCCACAAACAC
		Reverse	GCAATCCCGCTGGTCTAGTC
		Probe	ATGTCCTGTACGGCCCC
VCAM1	NM-174484	Forward	ACAAAGGCAGAGTACACAAACACTT
Il-6	X57317	Forward	AGGACGGATGCTTCCAATCTG
		Reverse	GAAGACCAGCAGTGGTTCTGAT
		Probe	CAATCAGGCGATTTGC
Il-8	NM-173925	Forward	GCTCTCTTGGCAGCTTTCCT
		Reverse	GGCATCGAAGTTCTGTACTCATTCT
		Probe	CAGAACTGCAGCTTCAC
COX1	NM-001105323.1	Forward	ATGGAGTTCAACCAGCTTTACCA
		Reverse	CAGAAACTGCTCGTAGCTGTAGTC
		Probe	CATGCCCGACTCCTTC
COX2	NM-174445.2	Forward	GGCGATGAGCAGTTGTTCCA
		Reverse	TGCTGTACGTAGTCTTCAATCACAAT
		Probe	CAAGCAGGCTAATCCT
15LOX1	NM-174501	Forward	GTGCCTTCCGTCTATACATCCTATG
		Reverse	CCCGGATGTTAATTTCCATGGTGTA
		Probe	CCCGGATGTTAATTTCCATGGTGTA
β-actin	NM-173979	Forward	CCGCCCCGCTAGCA
		Reverse	AACTGGTTGCGGTGTCGA
		Probe	CCTTCGCCGCTCCGC

Table 12. Fatty acid composition of bovine aortic endothelial cells (BAEC) phospholipid fraction after culture with a NEFA complex emulating fatty acid contents and concentration of plasma NEFA in transition cows, the same composition and content with EPA (C20:5n3c) and DHA (C22:6n3c) added, or albumin.

Fatty acid, g/100 g	NEFA complex supplementation				
	0mM	Albumin	0.75mM	0.75Ω3	P value
C14:0	2.642 ± 0.26	2.59 ± 0.22	2.37 ± 0.18	2.69 ± 0.09	0.630
C14:1n5c	2.264 ± 0.5	2.55 ± 0.52	2.6 ± 0.66	3.99 ± 2.33	0.895
C14:1n5t	1.479 ± 0.33	1.34 ± 0.24	1.37 ± 0.31	1.47 ± 0.23	0.599
C15:0	1.419 ± 0.25	1.2 ± 0.25	1.83 ± 0.3	1.3 ± 0.34	0.332
C16:0	24.9 ± 0.87	25.3 ± 1.56	25.3 ± 2	30.6 ± 2.92	0.070
C16:1n7c	0.786 ± 0.1 [a]	0.83 ± 0.03 [a]	0.59 ± 0.15 [b]	0.69 ± 0.08 [b]	0.009
C17:0	1.531 ± 0.16 [a]	1.42 ± 0.17 [ab]	1.12 ± 0.19 [c]	1.23 ± 0.18 [bc]	0.040
C18:0	25.83 ± 1.12 [a]	26.7 ± 1.89 [ab]	30.9 ± 1.58 [bc]	33.2 ± 1.58 [c]	0.012
C18:1n7c	1.031 ± 0.36 [a]	1.16 ± 0.32 [ab]	0.39 ± 0.13 [c]	0.16 ± 0.06 [bc]	0.030
C18:1n9c	4.202 ± 1.51	5.72 ± 1.54	4.04 ± 1.45	1.01 ± 0.2	0.490
C18:1n9t	1.353 ± 0.41	1.55 ± 0.35	1.24 ± 0.34	0.37 ± 0.11	0.120
C18:2n6c	0.714 ± 0.24	0.73 ± 0.23	1.14 ± 0.56	0.16 ± 0.03	0.398
C18:3n3c	0.333 ± 0.06	0.33 ± 0.08	0.27 ± 0.07	0.27 ± 0.05	0.755
C18:3n6c	0.453 ± 0.05	0.48 ± 0.04	0.45 ± 0.04	0.48 ± 0.05	0.518
C20:4n6c	2.882 ± 0.4 [a]	2.51 ± 0.58 [a]	0.83 ± 0.25 [b]	0.49 ± 0.19 [b]	0.006
C20:5n3c	0.318 ± 0.01 [a]	0.33 ± 0.05 [a]	0.13 ± 0 [b]	1.17 ± 0.04 [c]	0.001
C22:1n9c	0.526 ± 0.12	0.51 ± 0.13	0.36 ± 0.05	0.59 ± 0.26	0.08
C22:3n3c	0.88 ± 0.1	0.97 ± 0.11	0.54 ± 0.19	0.47 ± 0.09	0.11
C22:5n3c	0.509 ± 0.21 [a]	0.62 ± 0.28 [a]	0.5 ± 0.32 [a]	0.2 ± 0.09 [c]	0.009
C22:6n3c	2.45 ± 0.45 [a]	2.65 ± 0.98 [a]	2.65 ± 0.47 [a]	5.41 ± 0.44 [b]	0.013
Unidentified	12.45 ± 0.88	11 ± 0.31	11.2 ± 0.94	10.7 ± 0.96	0.174



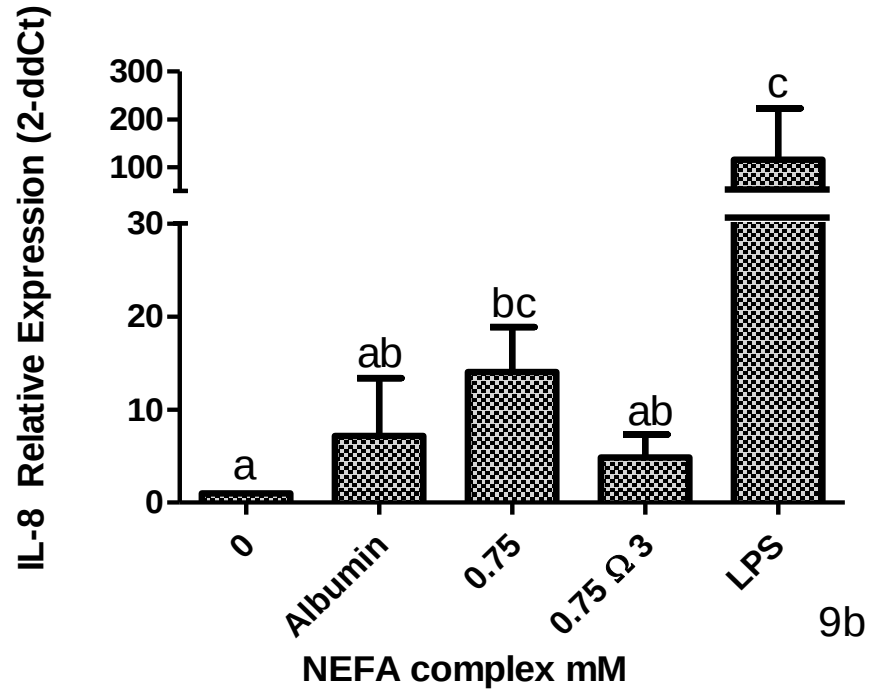
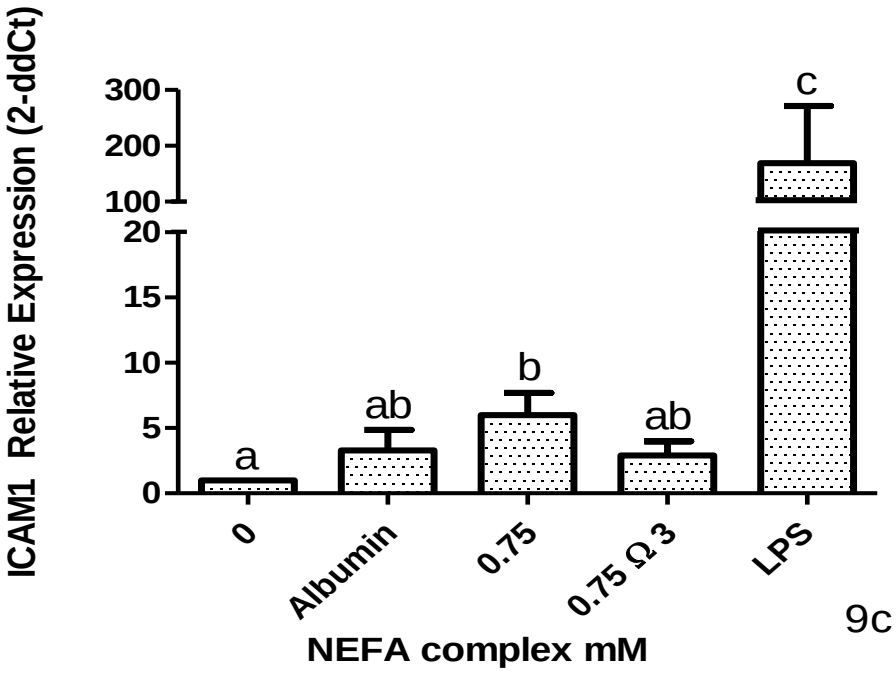
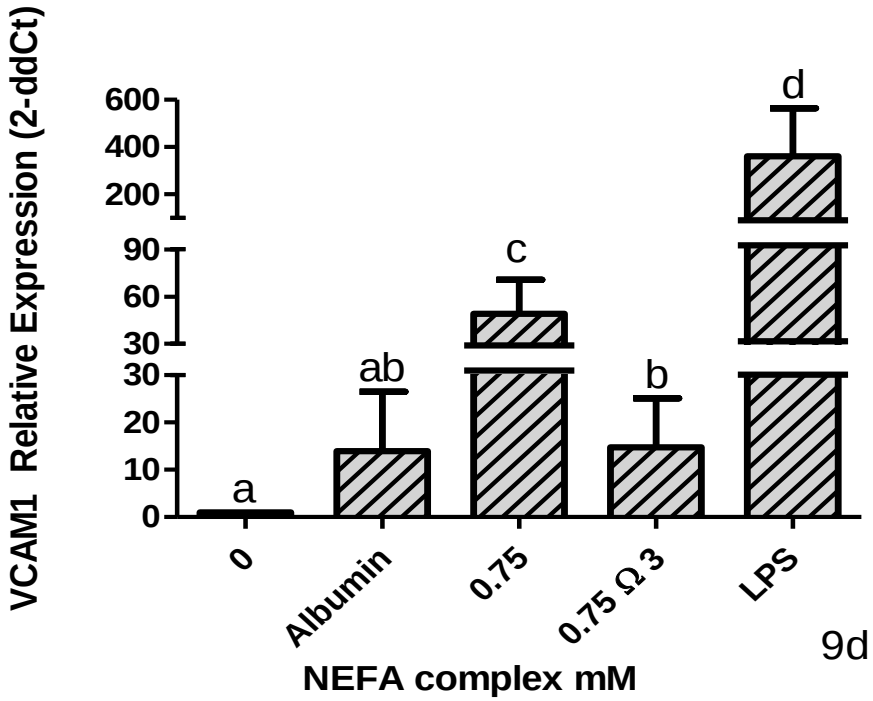


Figure 9. Supplementation with 0.75 mM NEFA or 0.75 mM NEFA mixture added with EPA (C20:5n3c) and DHA (C22:6n3c) (0.75Ω3) for 24 h induced significant changes in the gene expression of (a) IL-6 , (b) IL-8, (c) ICAM1, and (d) VCAM1.

Values are shown as $2^{-\Delta\Delta CT}$ with 0 mM set as calibrator.

Figure 9. (cont'd)





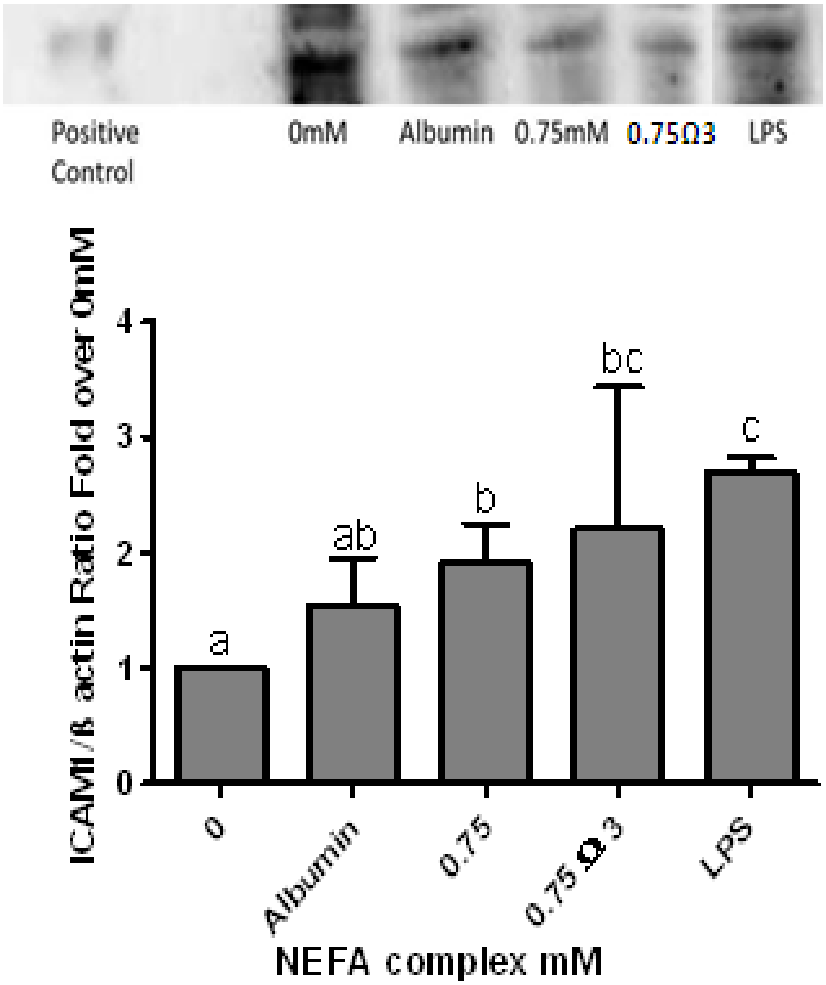


Figure 10. Supplementation with 0.75 mM NEFA or 0.75 mM NEFA mixture added with EPA (C20:5n3c) and DHA (C22:6n3c) (0.75Ω3) for 24 h induced significant changes in the protein expression of ICAM1.

Values are expressed as the ratio of ICAM1 to actin as a fold change over the 0 mM NEFA supplemented BAEC.

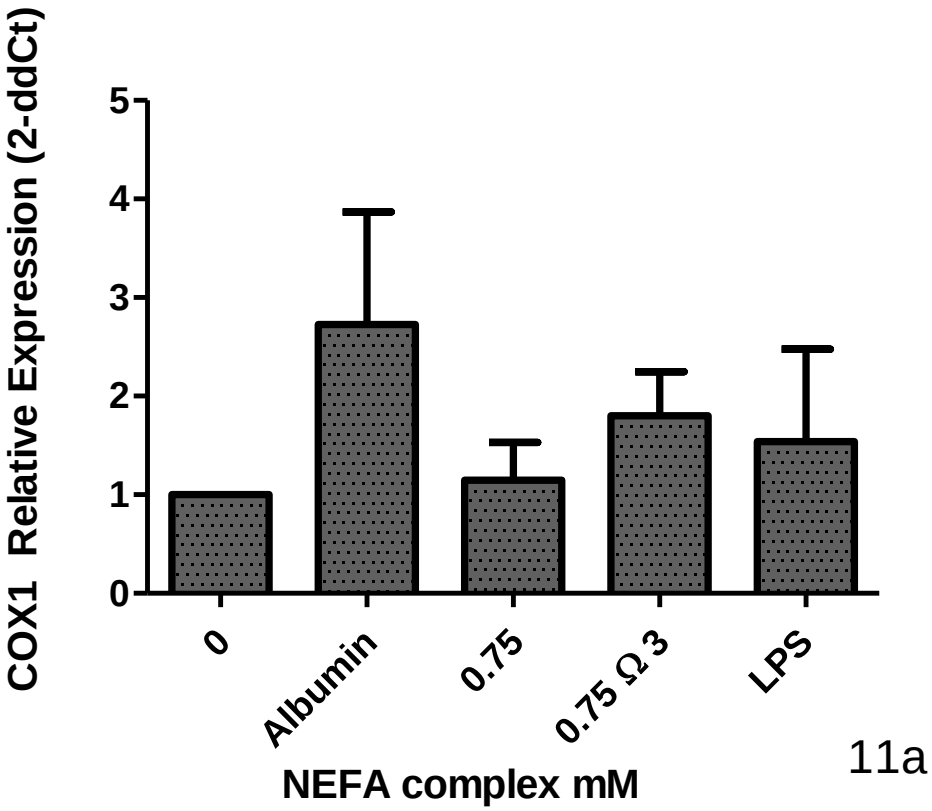
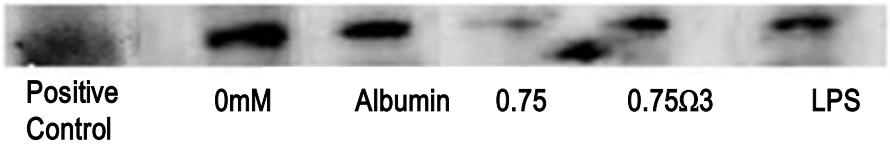
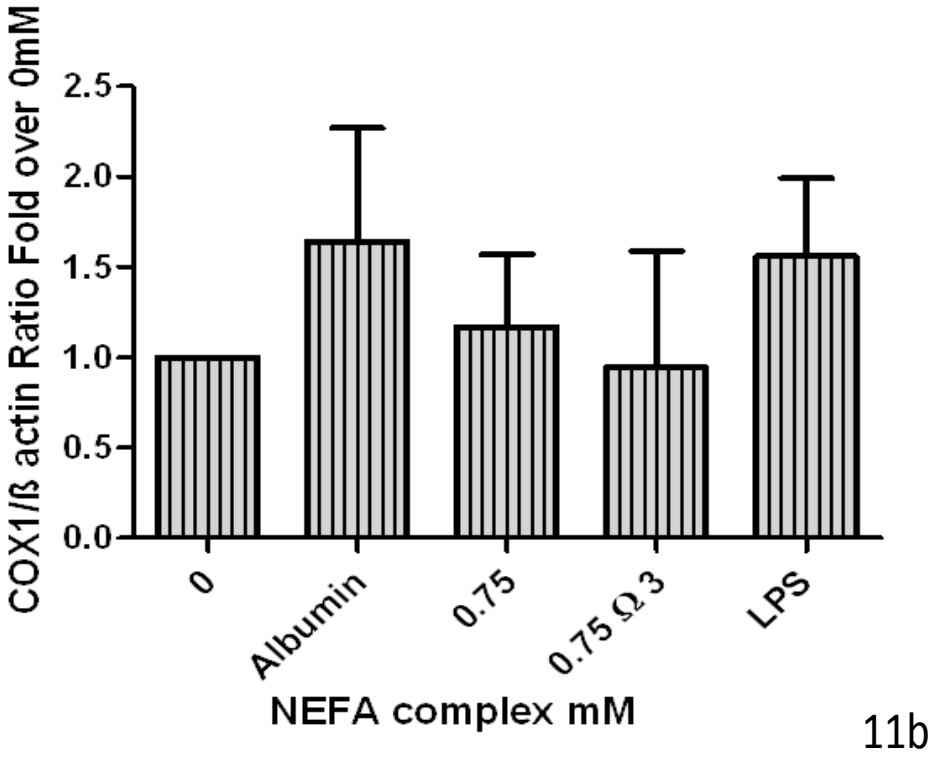


Figure 11. Supplementation with 0.75 mM NEFA or 0.75 mM NEFA mixture added with EPA (C20:5n3c) and DHA (C22:6n3c) (0.75Ω3) for 24 h induced significant changes in the gene and protein expression of COX.

Relative gene expression of (a) COX1 and (c) COX2 in BAEC for different treatments, values are shown as $2^{-\Delta\Delta CT}$ with 0 mM set as calibrator. Protein expression of (b) COX1 and (d) COX2 in BAEC for different treatments. Values are expressed as the ratio of COX1 or COX2 to actin as a fold change over the 0 mM NEFA supplemented BAEC

Figure 11. (cont'd)





11b

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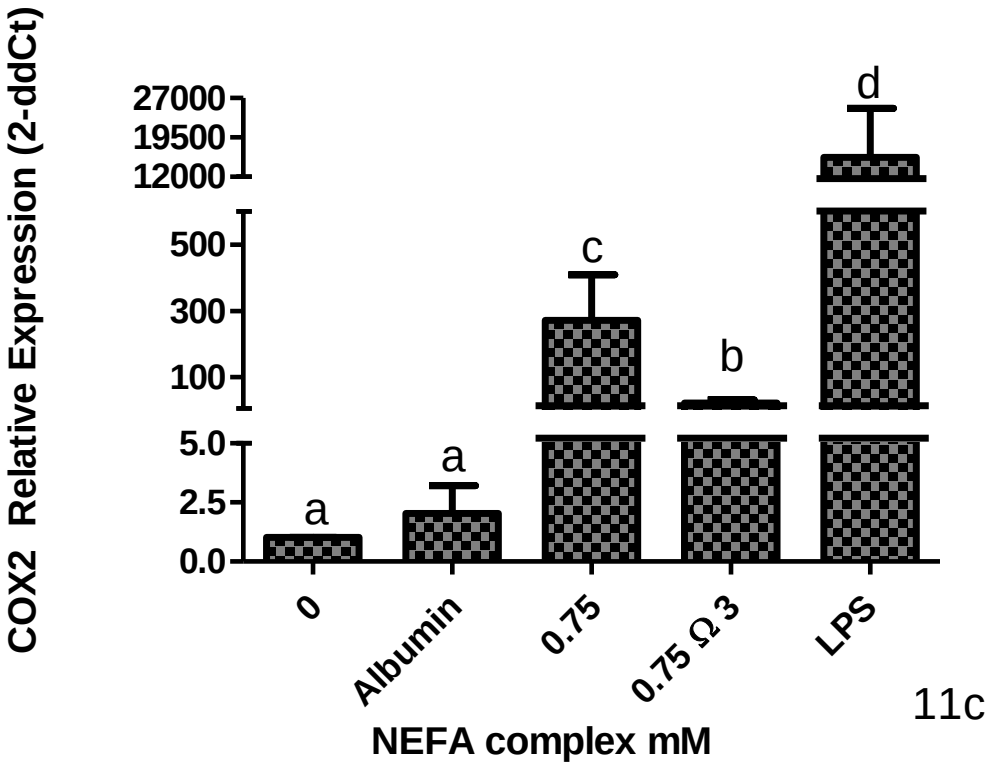

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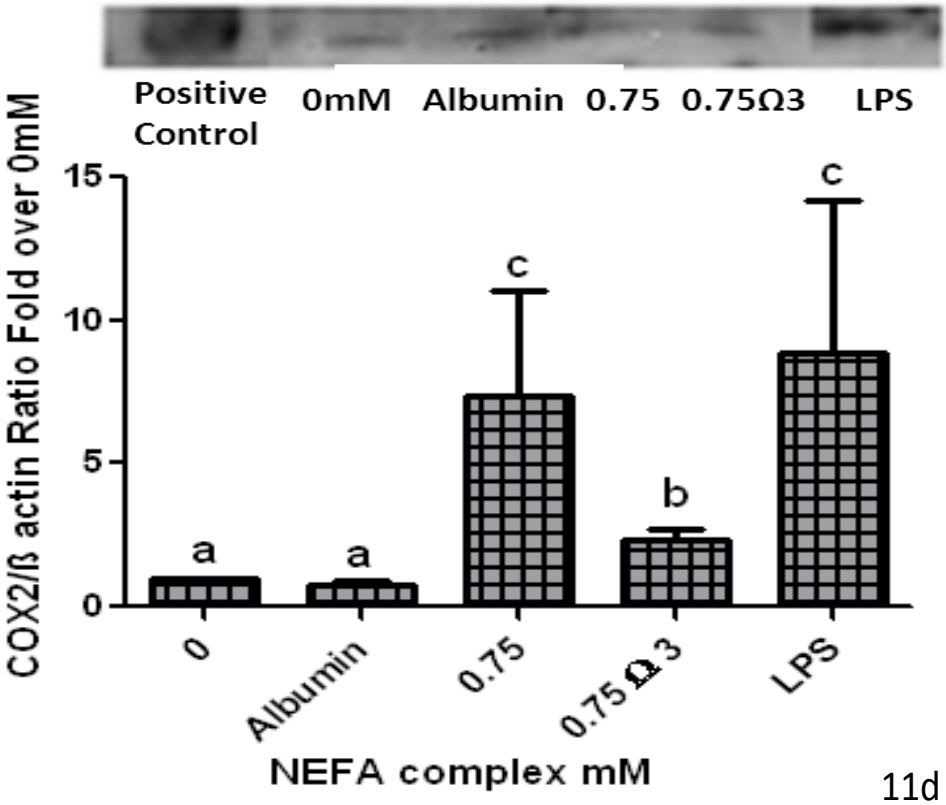
Figure 11. (cont'd)



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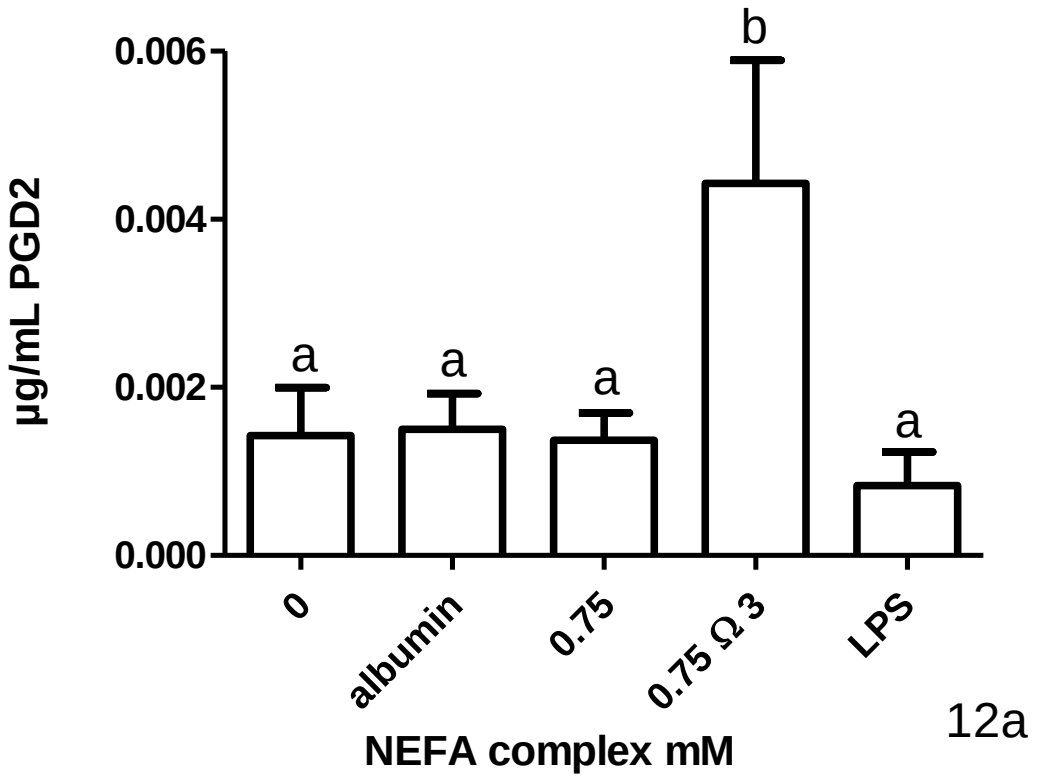
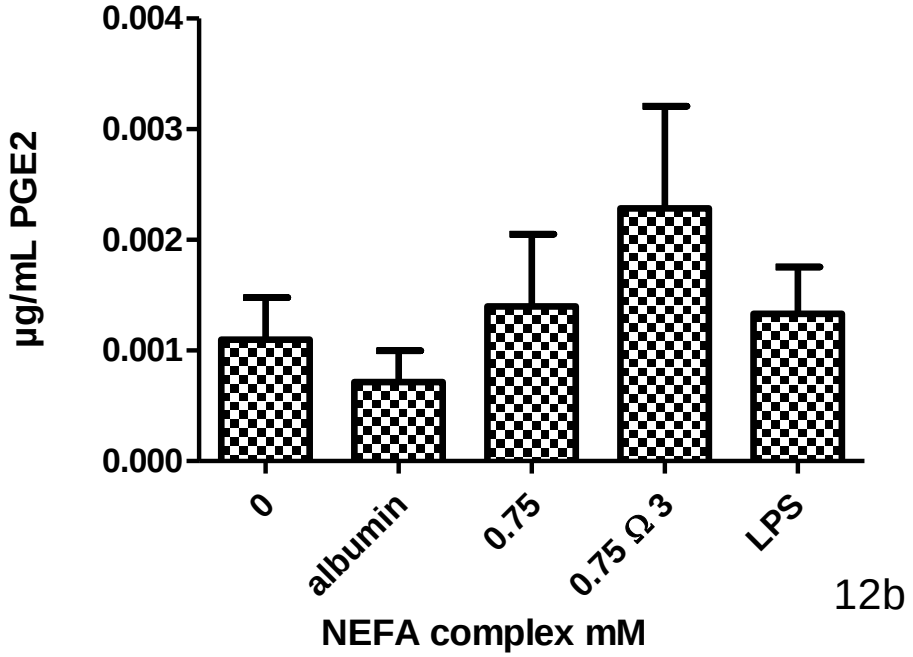


Figure 12. Effect of 0.75 mM NEFA or 0.75 mM NEFA mixture added with EPA (C20:5n3c) and DHA (C22:6n3c) (0.75Ω3) supplementation for 24 h on eicosanoid biosynthesis by COX enzymes.

Values are expressed in µg/mL. Prostaglandins (PG) (a) D_2, (b) E_2, (c) F_1, and (d) thromboxane B_2 (TXB_2) were isolated, identified and quantified by ultrahigh pressure liquid chromatography-mass spectrometry.

Figure 12. (cont'd)



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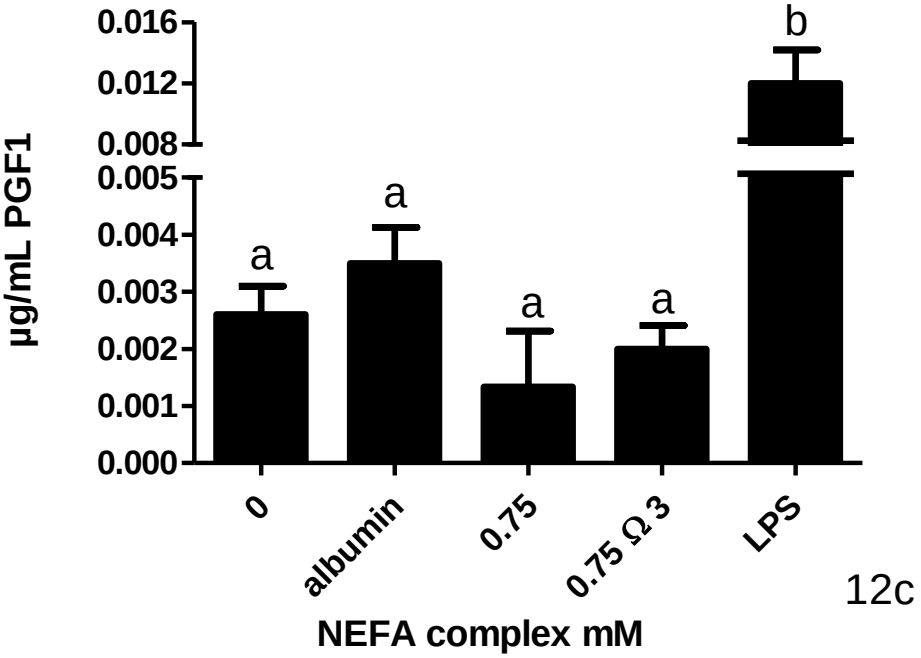

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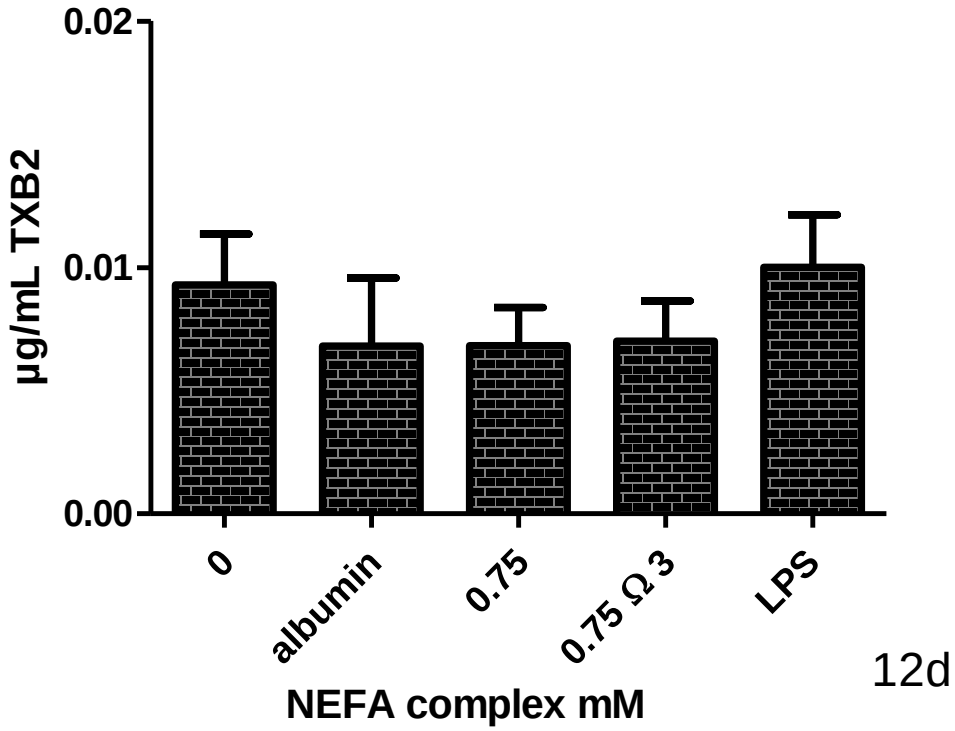


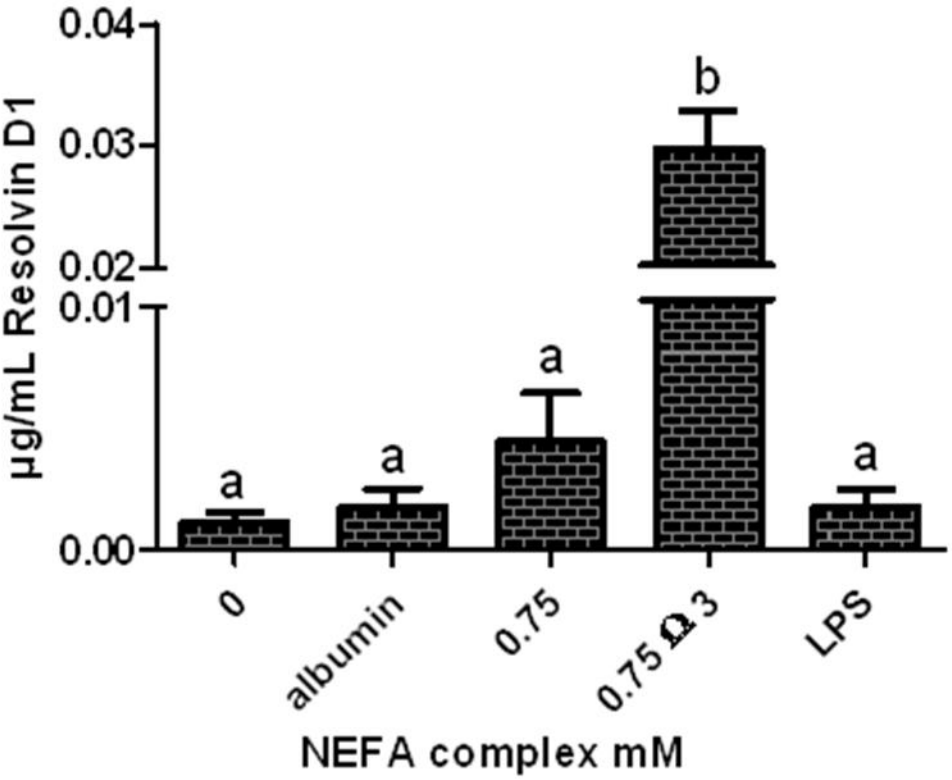


Figure 13. Effect of 0.75 mM NEFA or 0.75 mM NEFA mixture added with EPA (C20:5n3c) and DHA (C22:6n3c) (0.75Ω3) supplementation for 24 h on biosynthesis of COX2 derivate resolvin D_1.

Values are expressed in µg/mL. Metabolites was isolated, identified and quantified by UPLC-MS.

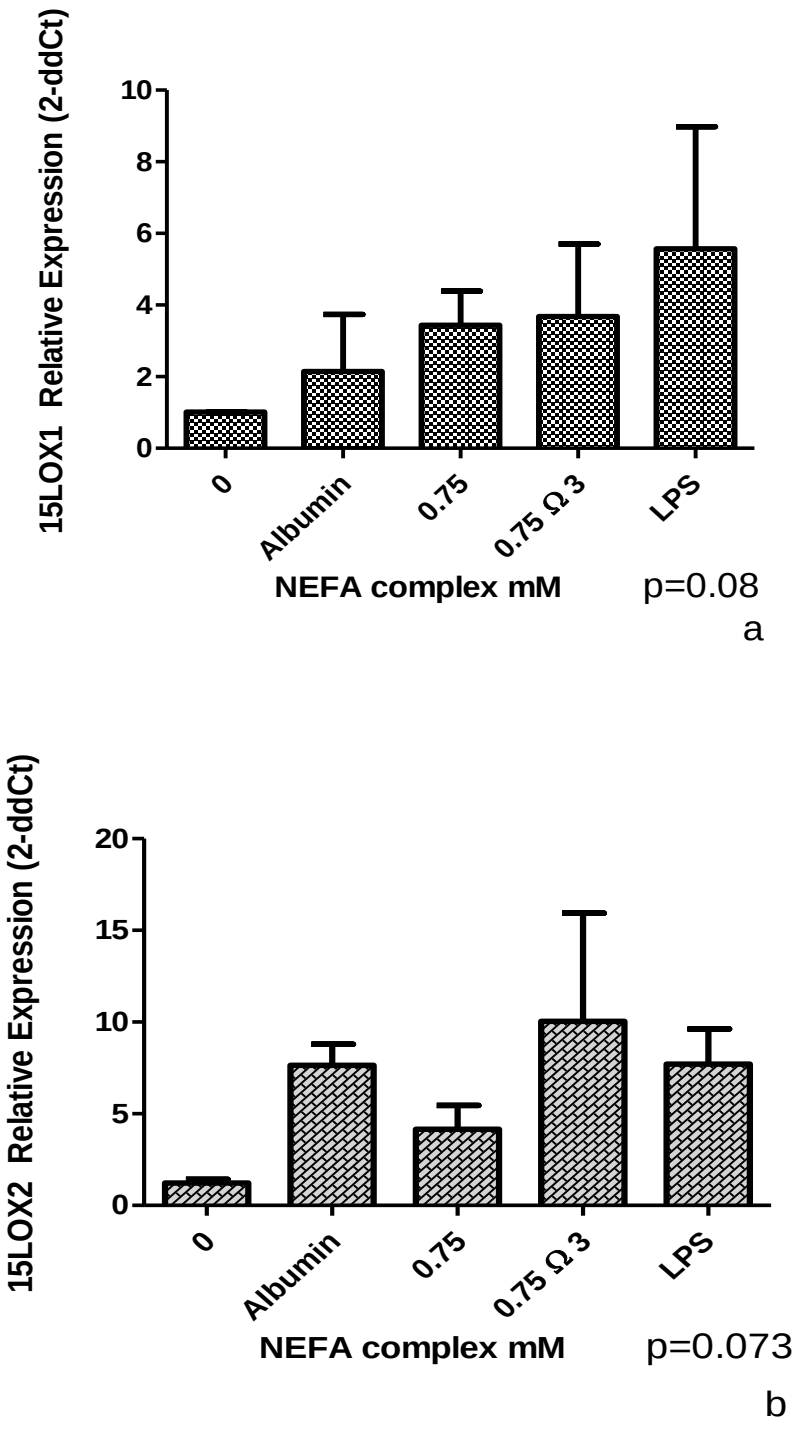


Figure 14. Supplementation with 0.75 mM NEFA or 0.75 mM NEFA mixture added with EPA (C20:5n3c) and DHA (C22:6n3c) (0.75Ω3) for 24 h induced significant changes in 15LOX1 (a) and 15LOX2 (b) gene expression.

Values are shown as $2^{-\Delta\Delta CT}$ with 0 mM set as calibrator.

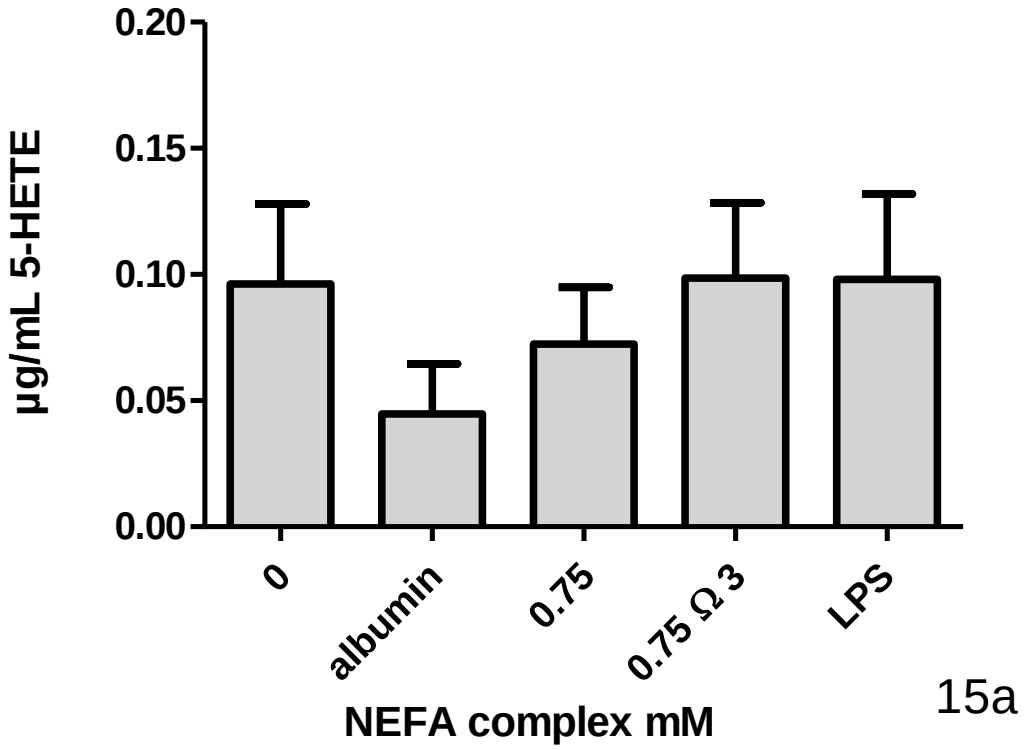
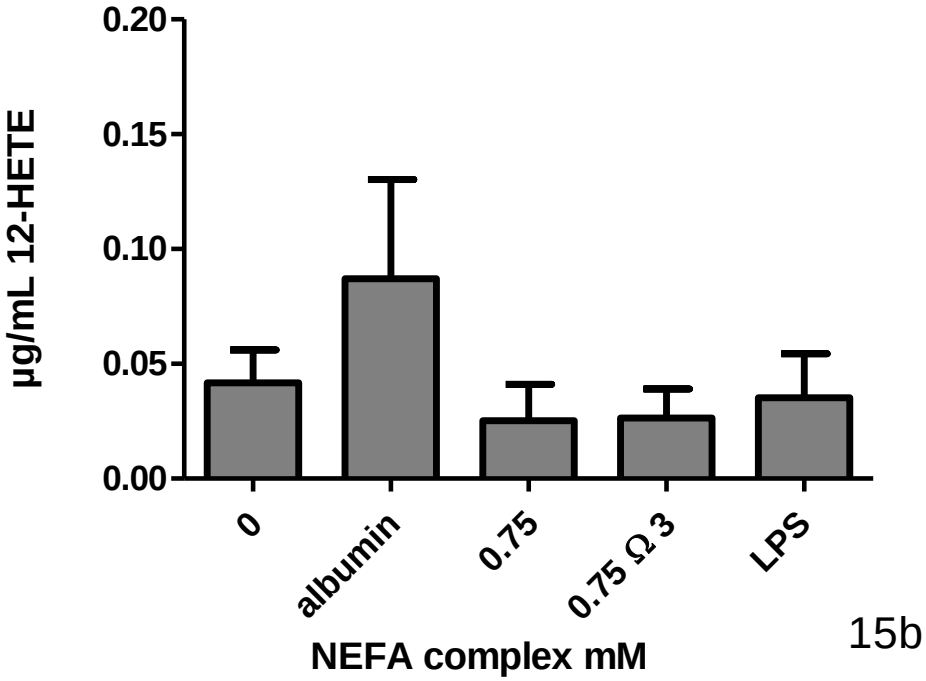


Figure 15. Effect of 0.75 mM NEFA or 0.75 mM NEFA mixture added with EPA (C20:5n3c) and DHA (C22:6n3c) (0.75Ω3) supplementation for 24 h in eicosanoid biosynthesis by 15LOX.

Values are expressed in µg/mL. Arachidonic acid metabolites included: (a) 5-HETE, (b) 12-HETE, (c) 15-HETE. Linoleic acid (C18:2n6c) metabolites included (d) 9-HODE and (e) 13-HODE. Hydroxieicosatetraenoic acid (HETE), hydroxioctadecadienoic acid (HODE).

Figure 15. (cont'd)



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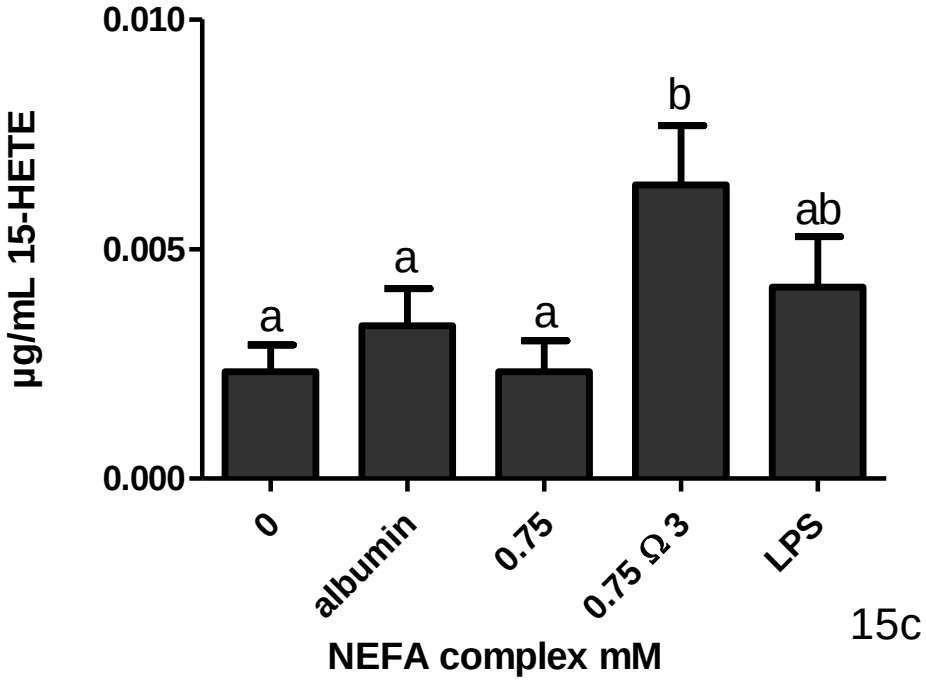

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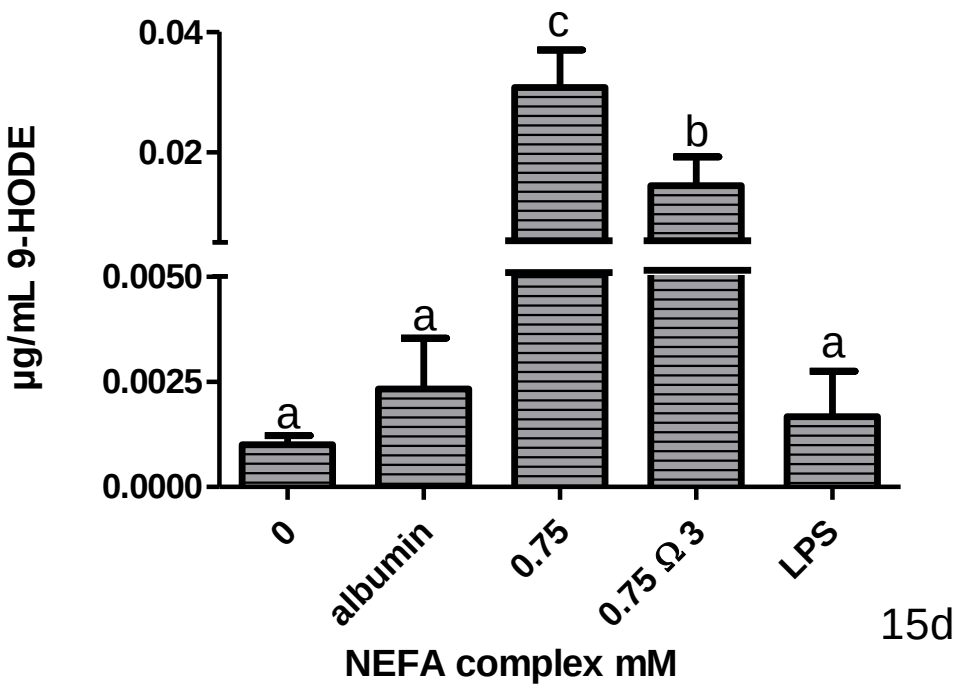

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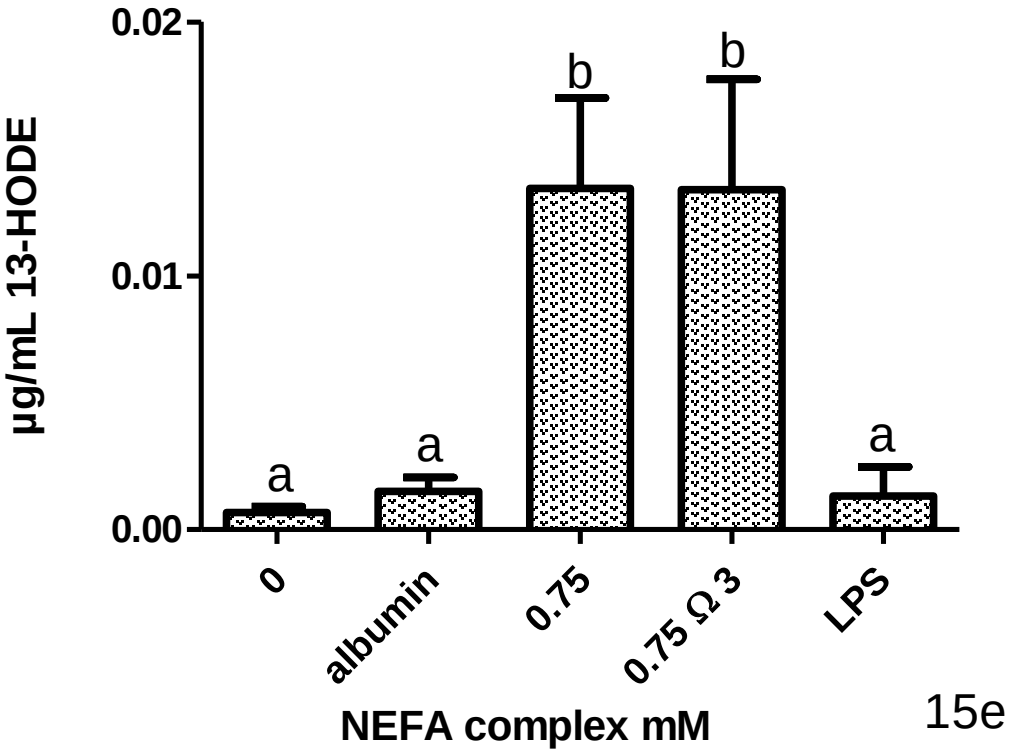

15e

CHAPTER 5
Conclusion

The transition period is still recognized as the time during lactation with the highest incidence of diseases (LeBlanc et al., 2006). Higher susceptibility to disease is now recognized to be induced, in part, by changes in both metabolic and immune functions (Sordillo et al., 2009). Metabolically, tremendous energy requirements driven by both fetal needs and lactogenesis accompanied by an intense drop in dry matter intake bring the transition cow to a negative energy balance state (Allen et al., 2005; Leroy and Vanholder, 2008). To supply energy deficits, dairy cows mobilize lipid reserves from adipose tissue releasing non-esterified fatty acids (NEFA) into circulation therefore altering systemic lipid homeostasis. In humans, disturbances in plasma lipids have been termed dyslipidemias and they are highly correlated with the pathogenesis of metabolic and chronic inflammatory diseases (Ginsberg and MacCallum, 2009) .

Among human dyslipidemias, elevated plasma NEFA is commonly observed in metabolic syndrome, diabetes, and obesity. In fact, research supports the concept that high concentrations of plasma NEFA directly induce insulin resistance and alter immune and inflammatory functions (Egan et al., 1999; Montecucco and Mach, 2009). Some of the mechanisms include, modification of intracellular signaling, controlling gene expression, induction of lipotoxicity and apoptosis, and the modification lipid mediator biosynthesis (Calder and Yaqoob, 2007). In dairy cows, previous research linked dyslipidemia during the transition period with increased susceptibility to disease, (Drackley, 1999); however, underlying mechanisms were either unknown or highly dependent on research from species with major physiological differences from ruminants such as humans and/or rodents. The present dissertation expanded our knowledge of lipid mobilization in the transition dairy cow and

elucidated some of the mechanisms through which this adaptation process causes an increased susceptibility to disease impacting the dairy industry worldwide.

Our first step was to characterize lipid mobilization in the transition cow. Current literature was scarce in the description of how lipolytic processes during negative energy balance stages modify plasma lipids in cows. Although plasma NEFA quantification is a standard practice during nutritional evaluations of dairy herds, changes in their fatty acid composition were unknown. We characterized for the first time, the composition of different plasma lipid fractions including NEFA and phospholipids during the transition period. Furthermore, we elucidated the influences of such plasma profiles in the fatty acid composition of phospholipid membranes of peripheral blood mononuclear cells (PBMC). The main finding was that there was an increase in the concentrations of saturated fatty acids and diminished amounts of polyunsaturated fatty acids in the plasma NEFA and phospholipid fractions and these profiles were directly reflected in the fatty acid content of cellular membranes of PBMC. Characterization of cellular membrane lipid profiles is critical, since such composition greatly influences cellular lipid mediator biosynthesis and therefore inflammation processes. Further studies are necessary to elucidate the mechanistic processes of lipolysis in ruminants at the adipose tissue level. This study provided essential information to further analyze the impact of altered plasma NEFA content and concentration in different cell populations including endothelial cells. Clearly, future studies looking at possible pharmacological or nutraceutical targets should be tested under conditions emulating intense lipid mobilization that were characterized in this research.

Immune and inflammatory responses are highly dependent on the interactions between immune cells with the vasculature. Endothelial cells are trafficking regulators of leukocyte

migration to and from tissues. Until now, effects of altered lipid homeostasis specific to the transition period on endothelial function were unidentified. In the third chapter, we supplemented bovine aortic endothelial cells (BAEC) with a fatty acid mixture that mimicked the plasma NEFA fraction fatty acid profile during the first week of lactation as assessed in the second chapter. We also altered the NEFA mixture concentration to emulate different degrees of lipid mobilization, including transition period (0.75 mM), early lactation (0.5 mM), and late lactation (0.25 mM). This study demonstrated for the first time that in dairy cows, lipid mobilization could induce a state of low grade inflammation that has been associated with the development of endothelial dysfunction in humans. Addition of NEFA to culture media that mimicked the concentration and composition observed in transition cows was able to alter the fatty acid profile of BAEC phospholipids by increasing the concentration of stearic acid (C18:0) and decreasing the content of arachidonic acid (C20:4n6c) and other long chain polyunsaturated fatty acids. Besides altering fatty acid profiles, NEFA supplementation enhanced the expression of endothelial adhesion molecules and pro-inflammatory cytokines. NEFA supplementation also could have modified inflammatory lipid mediator biosynthetic pathways at the substrate level by altering phospholipid fatty acid profile, at the enzyme level by enhancing the expression of COX2 and 15LOX, and at the metabolite level by enhancing the biosynthesis of specific COX and 15LOX metabolites including PGE_2, 9- and 13-HODE. Although this study characterized the consequences of altered NEFA content and composition on the inflammatory responses of BAEC, further studies are needed to individually evaluate the participation of each fatty acid within the NEFA mixture in the development of endothelial dysfunction. Moreover, specific mechanisms by which fatty acids modify inflammatory responses were not analyzed. A complete lipidomic analysis is required to characterize lipid mediator pathways response to enhanced lipid

mobilization. To complement lipidomic profiling, it is necessary to elucidate mechanism of action of specific lipid mediators on cellular inflammatory responses, some examples include the activation or inhibition of nuclear receptors (peroxisome proliferator-activated receptors), the molecular components of nuclear factor kappa B (NFκB) that were activated or restricted by the different fatty acids, and direct modifications to eicosanoid biosynthetic pathways COX and 15LOX. However, this study created new perspectives in the use of specific NEFA supplementation that emulates lipid mobilization in dairy cows, by characterizing specific alterations in endothelial inflammatory responses including the development of endothelial dysfunction and alterations in eicosanoid biosynthesis.

Research in humans and rodents supports consumption of omega-3 fatty acids. These long chain fatty acids improve endothelial function and cardiovascular health by attenuation of inflammatory responses and enhancement of its resolution (Calder, 2004). A next logical step was to assess the efficacy of including specific omega-3 polyunsaturated fatty acids in the NEFA complex supplemented to endothelial cells. The last chapter assessed in vitro changes of inflammatory responses in bovine endothelial cells induced by EPA (C20:5n3c) and DHA (C22:6n3c) while exposed to an environment mimicking intense lipid mobilization. Supplementation of both fatty acids successfully increased their concentrations in cellular phospholipids. Furthermore, addition of EPA (C20:5n3c) and DHA (C22:6n3c) restricted the activity of the COX2 enzyme, diminished the expression of adhesion molecules and pro-inflammatory cytokines, and enhanced biosynthesis of some lipid resolution mediators including PGD_2 and resolvin D_1. Since many of the transition cow diseases are characterized by alterations in inflammatory responses in their pathophysiology (i.e. mastitis and metritis), this study provided scientific ground for the possible use of EPA (C20:5n3c) and DHA (C22:6n3c) as

nutraceuticals during the periparturient period. This study, however, did not address any of the several possible mechanisms by which omega-3 modulate endothelial inflammatory responses. Further studies are necessary to elucidate specific interactions between different fatty acids and their effect on inflammatory responses by bovine endothelial cells while exposed to different degrees of lipid mobilization. It is important to evaluate cause-effect relationship between individual fatty acids including saturated and polyunsaturated and lipid mediator biosynthesis by specific enzymes such as COX2 and 15LOX1 and 2. Determining such interactions will direct the development of novel interventions including pharmaceuticals and nutraceuticals to restrict inflammatory responses in acute and chronic diseases

Constant genetic selection for higher milk production with specific quality standards will continue to put the modern dairy cow into constant metabolic challenges. Mobilization of lipid reserves is a biological adaptation mechanism that has helped producers to meet cow's energy requirements. Understanding the dynamics of lipid mobilization during the transition period could lead to novel nutraceutical or pharmacological interventions that modulate inflammatory responses, potentially improving not only the wellbeing of dairy cows, but also human health where many alterations in lipid metabolism are cause of chronic and inflammatory diseases.

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