Enhanced n-3 phospholipid content reduces inflammatory responses in bovine endothelial cells

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ABSTRACT

Uncontrolled inflammation contributes to the increased incidence and severity of infectious diseases in periparturient dairy cattle. The objective of this study was to determine if increasing n-3 fatty acid (FA) content and altering the profile of vasoactive eicosanoids could attenuate endothelial cell inflammatory responses. Bovine aortic endothelial cells (BAEC) were cultured with free FA mixtures that mimic the plasma NEFA composition during the first week of lactation of dairy cows or with a free FA mixture supplemented with a higher proportion of n-3 FA, including eicosapentaenoic and docosahexaenoic acids. The effects of increasing the docosahexaenoic and eicosapentaenoic acid content of BAEC on the expression of proinflammatory mediators and eicosanoid biosynthesis was assessed. Culturing BAEC with enriched concentrations of n-3 FA decreased the expression of proinflammatory cytokines, adhesion molecules, and reactive oxygen species with a concomitant increase in the biosynthesis of proresolving eicosanoids, including resolvins, protectins, and lipoxins. This study showed for the first time that increasing the n-3 FA content of endothelial cell phospholipids could alter the expression of eicosanoids and control the magnitude of inflammatory responses. Future studies are necessary to elucidate the mechanisms by which resolvins, protectins, and lipoxins may modify endothelial inflammatory pathways necessary to reduce the severity and duration of disease in periparturient cows. **Key words:** n-3 fatty acid, endothelial cell, inflammation, periparturient

INTRODUCTION

Dairy cows experience an increased incidence and severity of infectious diseases during the periparturient period that coincides with dysfunctional immune responses. Uncontrolled acute and chronic inflammation

is recognized as a major contributing factor involved in the pathology of several economically important diseases such as metritis and mastitis (Sordillo et al., 2009). An uncontrolled inflammatory reaction can occur when bacterial virulence factors such as LPS induce the synthesis of eicosanoids, cytokines, and other proinflammatory molecules at the foci of infection. These potent mediators regulate local inflammatory reactions, but also can gain access to the circulation and induce systemic responses (Aitken et al., 2011). Several studies showed that increased concentrations of tumor necrosis factor α , IL-1, and IL-6 in periparturient cows are associated with the morbidity and mortality of inflammatory-based diseases such as mastitis (Sordillo and Peel, 1992; Shuster et al., 1996). Therefore, strategies that can optimize the initiation and resolution of inflammatory responses may reduce disease severity associated with the periparturient period.

The vascular endothelium plays a critical role in regulating inflammatory responses due to their strategic location between the circulating blood and the underlying infected tissues. Endothelial cells produce vasoactive eicosanoids that are needed to regulate important inflammatory processes such as maintaining the delicate balance between vasodilation and vasoconstriction essential for optimal blood flow (Harizi et al., 2008). Eicosanoids also can control the production of tumor necrosis factor α , IL-1 β , and IL-6 within infected tissues that act as chemoattractants to facilitate neutrophil recruitment. Acting in a paracrine manner, eicosanoids enhance the expression of adhesion molecules on endothelial cells, including intercellular adhesion molecules 1 (ICAM1) and vascular adhesion molecule 1 (VCAM1; Harizi et al., 2008). Both ICAM1 and VCAM1 are involved in enhancing the adhesive nature of leukocyte-endothelial cell interactions and regulate the extravasation into affected tissues. Depending on the timing and magnitude of expression, certain eicosanoids can either enhance or resolve inflammatory responses by regulating endothelial cell functions (Serhan, 2009). The ability to regulate the expression profiles of vascular-derived eicosanoids may be beneficial in optimizing inflammatory responses to infectious pathogens.

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The expression profile of either proinflammatory or proresolving eicosanoids depends, in part, on the composition of membrane phospholipids that serve as substrates for lipid mediator biosynthesis. Oxygenation of arachidonic acid (*cis* C20:4n-6) through the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, for example, produces several proinflammatory prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), and hydroxyl derivatives such as hydroxyeicosatetraenoic acid (HETE). Increased expression of PGE₂, PGF_{2α}, and TXB₂ derived from the COX2 pathway can be detected in the sera and tissues of cows during the pathogenesis of mastitis (Atroshi et al., 1989; Boutet et al., 2003). Alternatively, metabolism of n-3 FA including eicosapentaenoic acid (EPA, *cis* C20:5n-3) and docosahexaenoic acid (DHA, *cis* C22:6n-3) through the COX and LOX pathways leads to the production of proresolving eicosanoids called resolvins (RD) and protectins (PD; Serhan, 2009). Several previous studies investigated the possibility of supplementing the diets of dairy cattle with n-3 FA with the intent of altering eicosanoid biosynthesis and immune responses. Increasing the dietary intake of fish oil, which is rich in both EPA and DHA, was able to reduce uterine concentrations of PGF_{2α} in periparturient cows and thereby mitigate pregnancy losses (Mattos et al., 2004; Bilby et al., 2006). Supplementation of n-3 FA, given as both linseed and fish oil, were noted to attenuate the alterations of lymphocyte proliferation in dairy cattle during negative energy balance and enhanced immune responses of dairy cows exposed to high ambient temperatures (Lacetera et al., 2007; Caroprese et al., 2009). Research in humans and rodents supports the concept that increased consumption of DHA and EPA improves endothelial function by modulating inflammatory responses and reducing the pathology of inflammatory-based diseases (Calder, 2008). No information, however, is available of how DHA and EPA supplementation can directly influence endothelial cell phospholipid composition and subsequent vascular inflammatory responses in dairy cattle. The hypothesis of this study was that increasing cellular n-3 FA content and altering the profile of vasoactive eicosanoid biosynthesis would attenuate endothelial cell inflammatory responses.

MATERIALS AND METHODS

Chemicals and Reagents

Antibiotics and antimycotics, trypsin-EDTA, HEPES buffer, and Ham's F-12K (Kaighn's Nutrient Mixture F-12) were purchased from Cellgro (Herndon, VA). HyClone Laboratories Inc. (Logan, UT) supplied fetal bovine serum. The RNeasy Mini Kit for isolation of

RNA was purchased from Qiagen Inc. (Valencia, CA). Reagents for real-time quantitative PCR (qPCR) were purchased from Applied Biosystems (Foster City, CA) and Qiagen Inc. All other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture and FFA Treatments

Bovine aortic endothelial cells (BAEC) were isolated using collagenase digest and cloned as single cells by the limiting dilution as previously described (Sordillo, 1998; Sordillo et al., 2008). Confluent BAEC were cultured for 24 h with 2 different treatments. The 0.75 mM FFA treatment was designed to emulate plasma NEFA content during intense lipid mobilization during the first week of lactation as described previously (Contreras et al., 2010). The FFA mixture included myristic (C14:0, 3%), palmitic (C16:0, 30%), stearic acid (C18:0, 45%), oleic (*cis* C18:1n-9, 16%), linoleic (*cis* C18:2n-6, 5%), and DHA (1%). The second treatment consisted of the 0.75 mM FFA mixture supplemented with EPA and DHA to increase the n-3 FA content (0.75 mM n-3 FFA). For the 0.75 mM n-3 FFA treatment, the mixture included myristic (3%), palmitic (30%), stearic acid (45%), oleic (9%), linoleic (5%), EPA (4%), and DHA (4%). The amounts of EPA and DHA added to FFA mixtures were based on previous research where different types of fish oils were fed to ruminants (Castañeda-Gutiérrez et al., 2007).

All FA were saponified before addition to cell cultures. In addition, FA were bound to albumin, which is their natural transporter in plasma, to mimic in vivo physiological conditions (Kim et al., 2005). Briefly, palmitic and stearic acids were dissolved in ethanol at 90°C solution and further sonicated with a Branson Sonifier 250 (Branson Ultrasonics Corp., Danbury, CT) for 10 s at an output of 10%. Once readily dissolved, the FA mixture was dried under nitrogen, and then resuspended in NaOH (0.1 mol/L) at 90°C. Oleic acid, linoleic acid, DHA, and EPA were dissolved in NaOH (0.1 mol/L) at 70°C and then mixed with palmitic and stearic acid NaOH solution. The FFA mixture was complexed with 10% BSA (FA-free) at 55°C for 10 min. The FFA mixtures were then filter sterilized (0.44 μM) and stored under argon atmosphere at -20°C for future use. The final concentration of the mixture was 100 μM of FFA, with an albumin molar ratio of 7:1.

In addition to the 2 different FFA mixtures, BAEC also were exposed to culture medium without addition of FFA mixture (0 mM) as a negative control, culture medium plus albumin without any FFA bound as the FFA mixture control, and culture medium plus 50 ng of LPS/mL as a positive inflammatory control. The

BAEC were used at passage 9 or less for all experiments.

Cell Viability Assay

Cell death was evaluated following FFA exposure using trypan blue exclusion. Cell viability was measured using Promega CellTiter-Glo Viability Assay (Promega Corp., Madison, WI), which quantifies the amount of ATP as an indicator of metabolically active cells. Both assays were performed as previously described (Contreras et al., 2012).

Lipid Extractions

Total lipids from media and BAEC were extracted using a hexane:ethanol (1:1) solvent mix as previously described (Contreras et al., 2010). Endothelial cells were collected by gentle scraping, washed in Hanks' balanced salt solution (HBSS) and centrifuged at $233 \times g$ for 20 min. Prior to lipid extraction, BAEC were resuspended in PBS to a minimum concentration of 5.5×10^6 cells/mL. To obtain total lipids, media alone or BAEC cell pellets were combined with the solvent mix and vortexed for 10 min, and then centrifuged at $2,095 \times g$ for 10 min at room temperature. The hexane layer was collected for total FA 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 aminopropyl (Strata NH₂) sorbent cartridges (Phenomenex Inc., Torrance, CA) as described by (Kaluzny et al., 1985). Briefly, after conditioning of the cartridges with hexane and adding the sample to the cartridge, 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 acid fractions 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 (100 m $\times$ 25 mm with a 0.2- μ m film thickness). Analyte elution times were identified using a mix of pure methyl ester standards (Nu-Chek Prep Inc., Elysian, MN).

Gene Transcript Expression

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 previously described (Corl et al., 2008). Briefly, total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen Inc.). Deoxyribonuclease digest was conducted during the RNA extraction using the RNase-Free DNase Set (Qiagen Inc.). Purified RNA was converted to cDNA using the Applied Biosystems High-Capacity cDNA Archive Kit (Applied Biosystems). All qPCR assays were conducted using TaqMan gene expression assays from Applied Biosystems. TaqMan primer and probe sets (Table 1) 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 μ L of TaqMan Fast Universal PCR Master Mix (2 $\times$), and 1 μ L of the appropriate TaqMan Gene Expression Assay Mix (20 $\times$) on the Applied Biosystems 7500 Fast Real-Time PCR System. A non-reverse-transcriptase control was run to ensure that genomic DNA was not being amplified. The relative quantification of each gene was calculated using the 7500 Fast SDS Software (version 1.3.1; Livak and Schmittgen, 2001). Data were calculated based on the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta C_t}$) of relative quantification, using β -actin as the control gene as previously described (Steibel et al., 2009). The calibrator was set as the control BAEC cultured in the 0 mM FFA mixture.

Protein Blot Analyses

Protein quantification was performed by protein blot analysis as previously described (Contreras et al., 2012). 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 μ g) were electrophoresed on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The Millipore Snap i.d. Protein Detection System (Millipore Corp., Billerica, MA) was used 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 PLC, Cambridge, MA), anti-human COX2 (1:200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-rabbit 15LOX1 (1:2000; Cayman Chemical Co., Ann Arbor, MI), and anti-human ICAM1 (1:100 dilution; Invitrogen Corp., Carlsbad, CA) in 1% BSA 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%

Table 1. Gene targets and primer sequences for quantitative PCR

Target ¹	Accession no.	Direction	Sequence (5' to 3')
ICAM1	NM-174348	Forward	GCAGGTGGTCCACAAACAC
		Reverse	GCAATCCCGCTGGTCTAGTC
		Probe	ATGTCTGTACGGCCCC
VCAM1	NM-174484	Forward	ACAAAGGCAGAGTACACAAACACTT
		Reverse	GAGGAGGGACTGACCAAGATG
		Probe	CCTGGGAGCAACATTA
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	CAGAACTGCTCGTAGCTGTAGTC
		Probe	CATGCCCCGACTCCTTC
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

¹ICAM1 = intercellular adhesion molecule 1; VCAM1 = vascular adhesion molecule 1; COX1 and COX2 = cyclooxygenase 1 and 2, respectively; 15LOX1 = 15 lipoxygenase.

dry milk; Pierce Biotechnology Inc., Rockford, IL) for 10 min at 21°C, washed 3 times with TBS, exposed to HRP substrate (Pierce Biotechnology Inc.), and visualized by chemiluminescence using the ChemiDoc XRS (Bio-Rad Laboratories Inc., Hercules, CA) and Quantity One software (Bio-Rad Laboratories Inc.). Anti-human actin (1:3,000 dilution; Millipore Corp.) served as the loading control. The 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 FFA-supplemented BAEC.

Reactive Oxygen Species Determination

The generation of reactive oxygen species (ROS) in BAEC cultures was determined by flow cytometry using the intracellular fluorescent probe 6-carboxy-2',7'-dichlorodihydrofluorescein (DCF) diacetate as previously described (Sordillo, 1998). Briefly, after treatments, BAEC were washed with HBSS and collected from culture dishes by trypsinization. Cells were resuspended in 350 μ L of HBSS before the addition of 100 μ M carboxy-DCF diacetate (Molecular Probes; Invitrogen Corp.). Following 30-min incubation at 21°C, the samples were analyzed with the FACS-Vantage flow cytometer (Becton, Dickinson and Co., Franklin Lakes, NJ). The fluorescence intensity was evaluated for at least 3 independent experiments.

Eicosanoid Extraction and Quantification

Media were aspirated and BAEC were collected by gentle scraping. To all samples, methanol was added to a 10% solution by volume, flash frozen, and stored frozen at -80°C until analyzed. A mixture of antioxidant reducing agents consisting of 2.4 mg of butyl hydroxy toluene dissolved in ethanol, 2.4 mg of EDTA dissolved in water, 24 mg of triphenylphosphine dissolved in methanol, and 24 mg of indomethacin dissolved in ethanol was prepared fresh for each extraction. Internal deuterated eicosanoid standards were mixed and added before each extraction (0.1 ng/ μ L concentration) and included LTB_{4-d4}, TXB_{2-d4}, PGF_{2 α -d4}, PGE_{2-d4}, PGD_{2-d4}, 13(S)-hydroxyoctadecadienoic acid (HODE)_{-d4}, 6-keto PGF_{1 α -d4}, 9(S)-HODE_{-d4}, 12(S)-(HETE)_{-d8}, and 15(S)-HETE_{-d8}.

At the times of analyses, BAEC samples were thawed slowly on ice and resuspended in 3,300 μ L with cold PBS and then 12 μ L of antioxidant reducing agent, 100 μ L of internal standard mixture, and 3 μ L of formic acid were added. To a 10-mL aliquot of culture media, 40 μ L of antioxidant reducing agent, 100 μ L of internal standard mixture, and 10 μ L of formic acid were added. The BAEC samples were sonicated 4°C for 3 min at output 10% using a Misonix S-400 sonicator. The BAEC and media samples were then centrifuged at 3500 $\times g$ for 10 min at 4°C. Lipid mediators were isolated from the samples by solid-phase extraction using

an Oasis HLB 12-cc LP extraction cartridge (Waters Corp., Milford MA). Columns were first conditioned with 5 mL of methanol, followed by 5 mL of water, and then 5 mL of a 40% methanol rinse at 1 drop/s. The cartridges were dried under vacuum for 4 min and samples were eluted in 5 mL of methanol-acetonitrile (50:50, vol/vol), dried in a Savant SVD121P SpeedVac (Thermo Scientific, Waltham, MA), and suspended in 100 μ L of acetonitrile-water-formic acid (37:63:0.02, vol/vol/vol). An aliquot of sample (18 μ L) was collected for protein quantification by Bradford analysis.

Eicosanoids were isolated using 2 distinct ultra-high pressure liquid chromatography (LC) and mass spectrometry methods. Both used reverse-phase LC on a Waters ACQUITY ultra-performance LC (UPLC) ethylene bridged hybrid (BEH) C18 1.7- μ m column (2.1 $\times$ 100 mm) at a flow rate of 0.6 mL/min at 35°C and a single quadrupole mass spectrometer (Waters ACQUITY SQD H-Class) in electrospray negative ionization mode. The electrospray voltage was -3 kV and the turbo ion spray source temperature was 450°C. Nitrogen was used as the drying gas. For each method, 10- μ L samples were injected in triplicate. An isocratic mobile phase consisting of acetonitrile:water:0.1% formic acid (45:55:10; vol/vol/vol) with an analysis time of 15 min was used to analyze LTB₄, PGE₂, PGD₂, 5(S),6(R)-lipoxin (LX) A₄, PGF_{2 α} , TxB₂, 6-keto PGF_{1 α} , RD₁, and RD₂. The second method used an isocratic mobile phase of acetonitrile:methanol:water:0.1% formic acid (47.4:15.8:26.8:10; vol/vol/vol) and an analysis time of 10 min to analyze 9(S)-HODE, 13(S)-HODE, 15-deoxy- Δ 12,14-PGJ₂, 5(S)-HETE, 12(S)-HETE, 15(S)-HETE, 7(S)-maresin 1, 7(S),17(S)-dihydroxy-8(E),10(Z),13(Z),15(E), 19(Z)-docosapentaenoic acid, and LTD₄. Eicosanoids were identified in samples by matching their deprotonated (i.e., [M-H]⁻) *m/z* values and LC retention times with those of a pure standard. Extraction and ionization efficiencies were measured for the internal standards by comparing the intensity of the samples with the ion intensity of the extraction controls. The efficiency range was between 17 and 80%.

Quantitation of eicosanoid production was performed with Waters Empower 2 software (Waters Corp.). A linear calibration curve with 5 points ($R^2 > 0.99$) was generated for each eicosanoid with standards and internal standards purchased from Cayman Chemical Co. The curves ranged from 0.00013 to 2.68 ng/ μ L. Empower 2 (Waters Corp.) identified the sample peak by matching its retention time with the standard. A response was calculated for each matched peak by dividing the sample peak response by its internal standard response. Results were expressed as picogram of eicosanoid per microgram of protein.

Statistical Analyses

Statistical evaluation was conducted using Proc Mixed procedure (SAS v9.1; SAS Institute Inc., Cary, NC) and Prism 4 (GraphPad Software, La Jolla, CA). The linear mixed model included the fixed effect of treatment and the random effect of BAEC clone passage number. Comparisons of the means were made by ANOVA followed by Tukey-Kramer's honestly significant difference (HSD) post hoc test. Data are shown as least squares means $\pm$ standard error of the mean. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

FFA Treatment Altered BAEC Phospholipid Profiles

Cell viability was initially assessed at 24 h of incubation when BAEC were exposed to 0 mM FFA, albumin, 0.75 mM FFA, and 0.75 mM n-3 FFA. Cell death counts were below 10% for all treatment groups (data not shown). The FFA treatments induced significant changes in the phospholipid composition of BAEC (Table 2). A significant increase in the concentrations of stearic acid occurred when cells were supplemented with both 0.75 mM and 0.75 mM n-3 FFA compared with the 0 mM control. As expected, changes in the concentrations of PUFA were observed after exposure to both treatments. Palmitoleic acid (*cis* C16:1n-7) and vaccenic acid (*cis* C18:1n-7) content was reduced significantly in BAEC when supplemented with 0.75 mM FFA and 0.75 mM n-3 FFA compared with the 0 mM control. Similarly, arachidonic acid was reduced in 0.75 mM FFA and 0.75 mM n-3 FFA treatments when compared with albumin or 0 mM treatment. Addition of EPA and DHA in the 0.75 mM n-3 FFA treatment significantly increased the concentration of these 2 PUFA compared with the 0 mM control, albumin, and 0.75 mM FFA. In contrast, the concentration of EPA was reduced significantly in the 0.75 mM treatment compared with the 0 mM and albumin treatments. The concentration of docosapentaenoic acid (*cis* C22:5n-3) was significantly decreased by exposure to the 0.75 mM n-3 treatment compared with the 0.75 mM, 0 mM, and albumin treatments.

n-3 Supplementation Reduced Cytokine and Adhesion Molecules Expression

Gene expression of IL-6 and IL-8 was affected by exposure to high FFA treatments (Figure 1A and 1B). Bovine aortic endothelial cells exposed to the 0.75 mM FFA mixture had a statistically higher IL-6 gene expres-

Table 2. Fatty acid composition of bovine aortic endothelial cell phospholipid fractions after culture with different concentrations of an FFA mixture mimicking plasma FFA in periparturient dairy cows or with albumin as a carrier control¹

FA, g/100 g	FFA supplementation				P-value
	0 mM	Albumin	0.75 mM	0.75 mM n-3	
C14:0	2.642 ± 0.26	2.59 ± 0.22	2.37 ± 0.18	2.69 ± 0.09	0.63
<i>cis</i> C14:1n-5	2.264 ± 0.50	2.55 ± 0.52	2.6 ± 0.66	3.99 ± 2.33	0.90
<i>trans</i> C14:1n-5	1.479 ± 0.33	1.34 ± 0.24	1.37 ± 0.31	1.47 ± 0.23	0.60
C15:0	1.419 ± 0.25	1.2 ± 0.25	1.83 ± 0.30	1.3 ± 0.34	0.33
C16:0	24.9 ± 0.87	25.3 ± 1.56	25.3 ± 2.00	30.6 ± 2.92	0.070
<i>cis</i> C16:1n-7	0.786 ± 0.10 ^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
<i>cis</i> C18:1n-7	1.031 ± 0.36 ^a	1.16 ± 0.32 ^{ab}	0.39 ± 0.13 ^c	0.16 ± 0.06 ^{bc}	0.030
<i>cis</i> C18:1n-9	4.202 ± 1.51	5.72 ± 1.54	4.04 ± 1.45	1.01 ± 0.20	0.49
<i>trans</i> C18:1n-9	1.353 ± 0.41	1.55 ± 0.35	1.24 ± 0.34	0.37 ± 0.11	0.12
<i>cis</i> C18:2n-6	0.714 ± 0.24	0.73 ± 0.23	1.14 ± 0.56	0.16 ± 0.03	0.40
<i>cis</i> C18:3n-3	0.333 ± 0.06	0.33 ± 0.08	0.27 ± 0.07	0.27 ± 0.05	0.76
<i>cis</i> C18:3n-6	0.453 ± 0.05	0.48 ± 0.04	0.45 ± 0.04	0.48 ± 0.05	0.52
<i>cis</i> C20:4n-6	2.882 ± 0.40 ^a	2.51 ± 0.58 ^a	0.83 ± 0.25 ^b	0.49 ± 0.19 ^b	0.006
<i>cis</i> C20:5n-3	0.318 ± 0.01 ^a	0.33 ± 0.05 ^a	0.13 ± 0.00 ^b	1.17 ± 0.04 ^c	0.001
<i>cis</i> C22:1n-9	0.526 ± 0.12	0.51 ± 0.13	0.36 ± 0.05	0.59 ± 0.26	0.08
<i>cis</i> C22:3n-3	0.88 ± 0.10	0.97 ± 0.11	0.54 ± 0.19	0.47 ± 0.09	0.11
<i>cis</i> C22:5n-3	0.509 ± 0.21 ^a	0.62 ± 0.28 ^a	0.5 ± 0.32 ^a	0.2 ± 0.09 ^c	0.009
<i>cis</i> C22:6n-3	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	0.17

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹Values are means ± SEM ($n = 6$). The 0.75 mM n-3 mixture included eicosapentaenoic acid (EPA, *cis* C20:5n-3) and docosahexaenoic acid (DHA, *cis* C22:6n-3) at nutritionally achievable concentrations.

sion than control, albumin-, or 0.75 mM n-3 FFA-supplemented cells. The gene expression for IL-8 increased in the 0.75 mM FFA mixture treatment group when compared with the control group. Stimulation with LPS was used as a positive inflammatory control and induced a strong upregulation of proinflammatory cytokine (IL-6 and IL-8) gene expression that was statistically higher than any of the treatment or control groups.

The *ICAM1* gene expression was enhanced by supplementation with 0.75 mM FFA mixture when compared with 0 mM; however, no differences were observed between 0.75 mM FFA and 0.75 mM n-3 FFA treatment groups (Figure 2A). The gene expression for VCAM1 was statistically increased by treatment with 0.75 mM FFA and 0.75 mM n-3 FFA mixtures compared with the 0 mM FFA control group. Treatment of BAEC with the 0.75 mM n-3 FFA treatment; however, statistically decreased VCAM1 expression when compared with the 0.75 mM FFA treatment group (Figure 2B). As expected, the LPS-positive control induced a statistically higher gene and cell surface expression of both adhesion molecules compared with any of the treatment and control groups.

***n*-3 Supplementation Decreased ROS Concentrations**

The amount of DCF increased statistically in BAEC cultured with 0.75 mM FFA and in those stimulated

with the positive inflammatory control LPS. Exposure to 0.75 mM n-3 FFA reduced the production of ROS to concentrations comparable to 0 mM FFA and albumin treatments (Figure 3).

***n*-3 Supplementation Altered Eicosanoid Biosynthesis**

Gene and protein expression of COX1 was not altered by any of the treatments when compared with the 0 mM FFA treatment or albumin controls (results not shown). In contrast, COX2 gene expression was increased statistically when BAEC were exposed to 0.75 mM FFA and 0.75 mM n-3 FFA mixture treatments compared with the 0 mM FFA control group (Figure 4A); however, exposure to the 0.75 mM n-3 FFA mixture statistically decreased gene transcription when compared with the 0.75 mM FFA treatment group. Protein expression of COX2 statistically increased in the 0.75 mM FFA treatment group when compared with the 0.75 mM n-3 FFA, 0 mM FFA, and albumin control groups (Figure 4B). Addition of any FFA supplementation or LPS had a tendency to increase 15LOX expression ($P = 0.08$) in BAEC when compared with the 0 mM FFA control group (Figure 5).

The LPS stimulation group statistically enhanced the production of PGF_{1α}, PGF_{2α}, and TXB (Figure 6), but no differences were observed among other treat-

Figure 1. Supplementation with mixtures of FFA reflecting plasma concentrations in periparturient cows alone (0.75 mM) or added with eicosapentaenoic acid (EPA, *cis* C20:5n-3) and docosahexaenoic acid (DHA, *cis* C22:6n-3; 0.75 mM n-3) for 24 h induced significant changes in the gene expression of (A) IL-6 and (B) IL-8. Values are shown as $2^{-\Delta\Delta CT}$ (where CT = cycle threshold), with 0 mM FFA set as calibrator. Results represent means $\pm$ SEM from triplicate experiments and bars with different letters (a–c) are significantly different ($P < 0.05$).

ments. No significant differences in PGE_2 biosynthesis were observed between treatment groups, although there tended to be an increase in BAEC exposed to the 0.75 mM n-3 FFA treatment ($P = 0.07$; Figure 6). Biosynthesis of PD_1 and RD_1 , oxygenation products of DHA, were statistically enhanced by supplementation with 0.75 mM n-3 FFA compared with 0 mM FFA, albumin, and LPS (Figure 7A and 7B). The production of LXA_4 , another antiinflammatory mediator, was increased statistically in BAEC exposed to 0.75 mM n-3 FFA (Figure 7C). The COX-derived PGD_2 was sta-

Figure 2. Supplementation with mixtures of FFA reflecting plasma concentrations in periparturient cows alone (0.75 mM) or added with eicosapentaenoic acid (EPA, *cis* C20:5n-3) and docosahexaenoic acid (DHA, *cis* C22:6n-3; 0.75 mM n-3) for 24 h induced significant changes in the gene expression of (A) ICAM1 and (B) VCAM1. Values are shown as $2^{-\Delta\Delta CT}$ (where CT = cycle threshold), with 0 mM FFA set as calibrator. Results represent means $\pm$ SEM from triplicate experiments and bars with different letters (a–d) are significantly different ($P < 0.05$). ICAM1 = intercellular adhesion molecule 1; VCAM1 = vascular adhesion molecule 1.

tistically enhanced by supplementation with 0.75 n-3 FFA compared with 0 mM FFA, albumin, and LPS. Production of the linoleic acid-derived lipid mediators (9- and 13-HODE) were statistically increased when BAEC were exposed to either 0.75 mM FFA or 0.75 mM n-3 FFA compared with all controls (Figure 8A and 8B). In contrast, arachidonic acid-derived 12- and 15-HETE biosynthesis by BAEC was not affected by supplementation with FFA mixtures (Figure 8C and

Figure 3. Effect of 0.75 mM FFA or 0.75 mM FFA mixture added with eicosapentaenoic acid (EPA, *cis* C20:5n-3) and docosahexaenoic acid (DHA, *cis* C22:6n-3; 0.75 mM n-3) supplementation for 24 h on generation of reactive oxygen species using an intracellular fluorescent probe. Reactive oxygen species values are expressed as means $\pm$ SEM ($n = 5$) of relative fluorescence units fold change over the 0 mM FFA-supplemented bovine aortic endothelial cells. Bars with different letters (a and b) are significantly different ($P < 0.05$). DCF = 6-carboxy-2',7'-dichlorodihydrofluorescein.

8D). Selective inhibitors of COX1, COX2, and 15-LOX were used to further evaluate the contribution of these enzymes on HODE and HETE biosynthesis. Production of 9-HODE was decreased by inhibitor addition in the albumin group. Bovine aortic endothelial cells supplemented with albumin and indomethacin plus PD146176 had a lower production of 9-HODE and 13-HODE than the albumin control without inhibitors (Table 3).

DISCUSSION

Increasing dietary intake of n-3 FA can alter plasma FFA composition, change the FA content of immune cells, and reduce inflammatory responses in periparturient cows (Lacetera et al., 2007; Silvestre et al., 2011). The mechanism by which n-3 FA may alter host inflammatory responses, however, was not clearly defined. This study tested the hypothesis that increasing the content of n-3 FA in bovine endothelial cells can alter eicosanoid biosynthesis and decrease vascular proinflammatory responses.

The FA content of BAEC was altered by supplementing cultures with FFA mixtures that mimic the composition of plasma NEFA fractions previously reported in periparturient cows (Contreras et al., 2010). Both palmitic and stearic acids were the most abundant FA incorporated into BAEC phospholipids and these find-

Figure 4. Supplementation with 0.75 mM FFA (0.75 mM) or 0.75 mM FFA mixture added with eicosapentaenoic acid (EPA, *cis* C20:5n-3) and docosahexaenoic acid (DHA, *cis* C22:6n-3; 0.75 mM n-3) for 24 h induced significant changes in the gene and protein expression of cyclooxygenase (COX). (A) Relative gene expression of COX2 in bovine aortic endothelial cells (BAEC) for different treatments; values are shown as means $\pm$ SEM of $2^{-\Delta\Delta CT}$ (where CT = cycle threshold), with 0 mM FFA set as calibrator ($n = 4$). (B) Protein expression of COX2 in BAEC for different treatments; values are expressed as means $\pm$ SEM of the ratio of COX1 or COX2 to actin as a fold change over the 0 mM FFA-supplemented BAEC ($n = 4$). Bars with different letters (a–d) are significantly different ($P < 0.05$).

ings are consistent with those reported in peripheral blood mononuclear cells and neutrophils isolated from periparturient dairy cows (Contreras et al., 2010; Silvestre et al., 2011). The significant increase in stearic acid following FFA supplementation also was accompanied by a concomitant change in the concentrations of certain PUFA. The significant decrease in arachidonic acid and EPA in the 0.75 mM FFA treatment group, for example, is consistent with previous studies that reported a decrease in PUFA in human endothelial

Figure 5. Effect of 0.75 mM FFA (0.75 mM) or 0.75 mM FFA mixture added with eicosapentaenoic acid (EPA, *cis* C20:5n-3) and docosahexaenoic acid (DHA, *cis* C22:6n-3; 0.75 mM n-3) supplementation for 24 h in the relative gene expression of 15 lipoxygenase (15LOX1). Values are shown as means $\pm$ SEM of $2^{-\Delta\Delta CT}$ with 0 mM FFA set as calibrator (n = 4).

cell phospholipids as the proportion of saturated FA increased (Héliès-Toussaint et al., 2006).

Conversely, the FFA treatments enriched with n-3 FA were effective at increasing the amount of both DHA and EPA in BAEC phospholipids even in the presence of the larger proportions of SFA. Phospholipid incorporation of DHA was more effective compared with EPA when these n-3 FA were added to BAEC cultures. As observed in plasma lipid fraction distribution, supplementing only DHA to cultured endothelial cells resulted in the preferential incorporation into the phospholipid fraction and specifically into phosphatidylcholine (Chen et al., 2007). In contrast, EPA was preferentially incorporated into neutral lipids including the cholesterol esters. This study only assessed how the composition of the NEFA lipid fraction can alter BAEC phospholipid content. The possibility does exist, however, that the FA content of endothelial cell phospholipids in vivo may reflect the FA content of other lipid fractions such as neutral lipids in addition to plasma NEFA. The FA composition of these lipid fractions is partly determined by the activity of lipoprotein lipases and this can vary depending on the specific organ and metabolic state (Davies et al., 2010). The results from this study, however, demonstrate that the FA content of endothelial cells can be modified in vitro to reflect the changes in NEFA profiles observed in plasma, leukocytes, milk, and various tissues in periparturient dairy cows (Mattos et al., 2004; Contreras et al., 2010; Silvestre et al., 2011).

Changing the FA composition of BAEC by mimicking plasma NEFA profiles resulted in a greater expression of proinflammatory markers, including IL-6, IL-8, ICAM1, VCAM1, and ROS production. These findings are consistent with previous studies that showed that relatively high concentrations of SFA, including palmitate and stearate, can increase endothelial cell inflammatory responses, as evident by increased cytokines and vascular adhesion molecules in bovine and human models (Contreras et al., 2010; Harvey et al., 2010). In contrast, enrichment of BAEC phospholipids with EPA and DHA decreased the inflammatory response, as indicated by reduced expression of IL-6, VCAM1, and ROS production. Previous studies showed that reductions in the expression of endothelial adhesion molecules and proinflammatory cytokines in response to DHA supplementation are dependent on the degree of incorporation of this n-3 acid into the phospholipids. For example, DHA reduces *VCAM1* gene and protein surface expression in venous endothelial cells after exposure to proinflammatory cytokines and lipopolysaccharide (Weber et al., 1995). Similarly, individual supplementation of EPA and DHA was reported to diminish human aortic and saphenous endothelial cell expression of IL-6 in a dose-dependent manner (Khalfoun et al., 1997). Several mechanisms were proposed for the reduction of both VCAM1 and IL-6 in response to EPA and DHA supplementation. First, DHA reduces the expression of protein kinase B, a pathway directly related to the development to lesions in human atherosclerosis (Chen et al., 2003). A second possible mechanism is the reduction in activity of NF κ B by diminishing inhibitor κ B (I κ B) phosphorylation, thus reducing transcription of both VCAM1 and IL-6 (Goua et al., 2008). A third mechanism, involves alterations in the production of eicosanoids with proinflammatory or antiinflammatory properties that directly affect the expression of VCAM1 and IL-6 and other vascular adhesion molecules and proinflammatory cytokines (Calder, 2008).

The expression profile of eicosanoids depends, in part, on the oxygenation of certain FA through the COX and LOX enzymatic pathways. Linoleate, arachidonate, DHA, and EPA all can compete for these enzymatic pathways to generate either proinflammatory or pro-resolving eicosanoids. Oxygenation of arachidonic acid through the COX2 pathway, for example, can lead to the biosynthesis of proinflammatory eicosanoids such as PGE₂, PGF_{1 α} , PGF_{2 α} , and TXB₂. In this study, neither of the FFA mixtures altered the expression of these proinflammatory eicosanoids. This was surprising, as the 0.75 mM FFA mixture did significantly increase the expression of COX2. A possible explanation may be the significant reduction in arachidonic acid content of BAEC resulting from the FFA supplementation

Figure 6. Changes in bovine aortic endothelial cells proinflammatory eicosanoid biosynthesis following in vitro culture with FFA mixtures. Effect of 0.75 mM FFA or 0.75 mM FFA mixture added with eicosapentaenoic acid (EPA, *cis* C20:5n-3) and docosahexaenoic acid (DHA, *cis* C22:6n-3; 0.75 mM n-3) supplementation for 24 h on eicosanoid biosynthesis by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. Values are means $\pm$ SEM expressed in nanograms of the specific eicosanoid per microgram of total protein (n = 8). TXB = thromboxane B. Bars with an asterisk are significantly different from the nonsupplemented control ($P < 0.05$).

that would be available for metabolism through the COX2 pathway. Alternatively, BAEC supplemented with the n-3 FFA mixture did have significantly higher concentrations of LXA₄ and PGD₂. These eicosanoids also are derived from the metabolism of arachidonic acid through the COX pathway, but have known anti-inflammatory activities. A possible explanation for the selective synthesis of LXA₄ and PGD₂ over the other proinflammatory PG maybe due to the activity of other downstream enzymes involved in their biosynthesis. Future studies should profile the expression and activity of other enzymes downstream of COX and LOX to gain a better understanding of selective eicosanoid biosynthesis and the possible diversion of FA substrates through different enzymatic pathways.

Interestingly, PGD₂ is the precursor of 15d-PGJ₂, an antiinflammatory lipid mediator that is capable of directly activating peroxisome proliferator-activated receptors (PPAR), thereby suppressing inflammatory re-

sponses in endothelial cells (Marx and Walcher, 2007). Higher abundance of PGD₂ and possibly increased activity of these nuclear receptors in BAEC supplemented with 0.75 mM n-3 FFA may explain, in part, the reductions in the gene expression of IL-6 and IL-8 (Marx et al., 2004). The 15d-PGJ₂ was not evaluated in the present study; therefore, it is unknown if higher availability of PGD₂ could have increased substrate availability for PGJ₂ synthesis. Future studies should evaluate 15d-PGJ₂ and PPAR receptor activity during FFA supplementation with or without EPA and DHA to elucidate mechanisms of action.

Lipoxin A₄ is generated by an initial oxidation of arachidonic acid by 15LOX followed by metabolism through the 5LOX pathway, usually involving cell-to-cell interactions (Serhan, 2009). Bovine aortic endothelial cells are known to express very low amounts of 5LOX; however, studies in other arterial endothelial cells such as human pulmonary demonstrated that these

Figure 7. Changes in bovine aortic endothelial cells antiinflammatory eicosanoid biosynthesis following in vitro culture with FFA mixtures. Effect of 0.75 mM FFA or 0.75 mM FFA mixture added with eicosapentaenoic acid (EPA, *cis* C20:5n-3) and docosahexaenoic acid (DHA, *cis* C22:6n-3; 0.75 mM n-3) supplementation for 24 h on eicosanoid biosynthesis by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. Values are means $\pm$ SEM expressed in nanograms of the specific eicosanoid per microgram of total protein ($n = 8$). RD₁ = resolvin. Bars with different letters (a and b) are significantly different ($P < 0.05$). Bars with an asterisk are significantly different from the 0.75 mM treatment group ($P < 0.05$).

vascular cells enhance the expression of 5LOX during inflammatory responses (Cao et al., 2000; Zhang et al., 2002). Increased biosynthesis of LXA₄ in the 0.75 mM n-3 FFA treatment group may be partially responsible for the reduced expression of cytokines and adhesion molecules. Lipoxin A₄ is an antiinflammatory eicosanoid that can reduce the production of IL-6 and IL-8 in endothelial cells alone or in co-culture systems with leukocytes (Serhan, 2009). Moreover, lipoxin A₄ can decrease the expression of adhesion molecules including L-selectin and ICAM1 in endothelial cells, thereby reducing the attachment of neutrophils and lymphocytes (Chinthamani et al., 2012).

Supplementation of BAEC with the 0.75 mM n-3 FFA treatment also lead to the enhanced expression of RD₁ and PD₁. These antiinflammatory eicosanoids are part of a newly discovered family of lipid mediators in which EPA and DHA, rather than arachidonic acid, serve as substrate through the LOX pathways. Both RD₁ and PD₁ can downregulate adhesion molecule expression

in aortic endothelial cells and play a significant role in preventing inflammatory-based diseases in humans (Merched et al., 2008; Serhan, 2009). Here, we demonstrate a significant increase in RD₁ and PD₁ in the n-3 FA supplemented group that coincided with a reduction in the expression of proinflammatory cytokines and adhesion molecules. Future studies should focus on the specific role these novel lipid mediators may have in resolving inflammatory responses that may lead to increased disease severity in periparturient cows.

The biosynthesis of 9- and 13-HODE can occur through the enzymatic oxidation of linoleic acid through either the COX or LOX pathways (Hamberg, 1998; Ringbom et al., 2001). Alternatively, nonenzymatic oxidization can occur where free radicals, such as superoxide anion or singlet oxygen, react with linoleic acid within the cell (Niki, 2009). Nonenzymatic oxidation is exacerbated during times of ROS accumulation and oxidative stress, a condition frequently observed in periparturient dairy cows (Sordillo et al., 2007; Sordillo

Table 3. Eicosanoid biosynthesis by bovine aortic endothelial cells following in vitro culture with FFA mixtures¹

Lipid mediator ²	Treatment	Inhibitor				P-value
		Control	Indomethacin	PD146176	Indomethacin PD146176	
9-HODE	0 mM	0.07 ± 0.03	0.04 ± 0.03	0.05 ± 0.04	0.04 ± 0.04	NS
	Albumin	0.19 ± 0.14 ^b	0.03 ± 0.02 ^a	0.04 ± 0.02 ^a	0.04 ± 0.03 ^a	<0.05
	0.75 mM	2.35 ± 0.44	2.76 ± 0.85	3.49 ± 1.16	1.73 ± 0.76	NS
	0.75 mM n-3	1.53 ± 0.30 ^b	1.27 ± 0.52 ^{ab}	1.44 ± 0.38 ^b	0.51 ± 0.30 ^a	<0.05
	LPS	0.07 ± 0.05	0.21 ± 0.16	0.05 ± 0.03	0.05 ± 0.04	NS
13-HODE	0 mM	0.06 ± 0.03	0.05 ± 0.03	0.04 ± 0.02	0.04 ± 0.03	NS
	Albumin	0.07 ± 0.04	0.07 ± 0.05	0.05 ± 0.02	0.08 ± 0.06	NS
	0.75 mM	2.98 ± 0.73	3.39 ± 1.21	4.19 ± 1.35	2.00 ± 0.9	NS
	0.75 mM n-3	1.84 ± 0.48 ^b	1.68 ± 0.72 ^b	2.07 ± 0.71 ^b	0.62 ± 0.52 ^a	<0.05
	LPS	0.06 ± 0.03	0.30 ± 0.23	0.06 ± 0.02	0.07 ± 0.05	NS

^{a,b}Values within a row with different letters are significantly different ($P < 0.05$).

¹Results show the effect of 0.75 mM FFA or 0.75 mM FFA mixture supplemented with (EPA, *cis* C20:5n-3) and (DHA, *cis* C22:6n-3; 0.75 mM n-3) for 24 h on the production of eicosanoids from linoleic acid. To further evaluate the effect of enzyme activity in the synthesis of these products, specific inhibitors of cyclooxygenase (COX) 1 and 2 (indomethacin) and 15 lipoxygenase (15LOX) 1 and 2 (PD146176) were added to media 1 h before and during the supplementation with FFA mixtures. Values are means ± SEM (n = 4) expressed in micrograms per milliliter of the specific linoleic acid product per microgram of total protein.

²HODE = hydroxyoctadecadienoic.

Figure 8. Effect of 0.75 mM FFA or 0.75 mM FFA mixture added with eicosapentaenoic acid (EPA, *cis* C20:5n-3) and docosahexaenoic acid (DHA, *cis* C22:6n-3; 0.75 mM n-3) supplementation for 24 h in biosynthesis of (A) 9-hydroxyoctadecadienoic acid (HODE), (B) 13-HODE, (C) 12-hydroxyeicosatetraenoic acid (HETE) and (D) 15-HETE. Values are means ± SEM expressed in nanograms of the specific arachidonic acid product per microgram of total protein (n = 5). Bars with different letters (a and b) are significantly different ($P < 0.05$).

and Aitken, 2009). Supplementation of BAEC with both FFA mixtures resulted in a significant increase in 9- and 13-HODE when compared with control cultures. Bovine aortic endothelial cells cultured with 0.75 mM FFA mixture had a greater content of ROS and thus a higher possibility of augmented nonenzymatic oxidation of linoleic acid. This assertion is supported by that fact that addition of COX and LOX inhibitors to BAEC supplemented with 0.75 mM FFA did not reduce the production of 9-HODE and 13-HODE. In contrast, adding COX and LOX inhibitors to BAEC cultured with 0.75 n-3 FFA mixture did reduce the production of both 9- and 13-HODE, suggesting that enzymatic oxidation of linoleic acid is more active relative to nonenzymatic oxidation with increased n-3 FA exposure. In support of this conclusion, ROS did not increase when BAEC were supplemented with the n-3 FA. The ability to reduce the production of these metabolites, especially during times of oxidative stress, also may reduce the severity of inflammatory diseases in periparturient cows.

This study demonstrated that supplementation of EPA and DHA reduces ROS production in endothelial cells exposed to high FFA concentrations. Interestingly, lipid mediators that were increased in 0.75 n-3 FFA-treated cells such as RD and LX are capable of reducing the production of free radicals. In the case of RD, these compounds reduced the production of superoxide anion in human leukocytes (Spite et al., 2009). The antioxidant action of LXA₄ in vascular cells is based on the induction of heme oxygenase-1 (Nascimento-Silva et al., 2005). This study did not evaluate directly the antioxidant capacity of lipid mediators derived from n-3 EPA and DHA, therefore, future studies are needed to explore the specific mechanisms by which LX, RD, and possibly PD may control oxidative stress in endothelial cells during exposure to high FFA.

Uncontrolled inflammation contributes to the pathology of several economically important diseases of transition dairy cattle including mastitis and metritis. Intense lipid mobilization in transition cows may alter vascular inflammatory responses and contribute to disease severity (Sordillo et al., 2009). This study showed for the first time that increasing the n-3 FA content of vascular phospholipids could mitigate endothelial cell proinflammatory responses to high NEFA concentrations that models those found during the periparturient period. A novel finding from this study was that shifts in specific eicosanoid profiles may be partially responsible for controlling the magnitude of vascular inflammatory responses. Nutritional strategies that can effectively enhance the biosynthesis of n-3 FA derived antiinflammatory eicosanoids may limit uncontrolled

inflammatory responses and improve the outcome of disease in early lactation dairy cows.

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