Nonesterified fatty acids modify inflammatory response and eicosanoid biosynthesis in bovine endothelial cells

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ABSTRACT

Intense lipid mobilization during the transition period in dairy cows is associated with increased disease susceptibility. The potential impact of altered plasma nonesterified fatty acids (NEFA) concentrations and composition on host inflammatory responses that may contribute to disease incidence and severity are not known. The objective of this study was to evaluate if increased NEFA concentrations could modify vascular inflammatory responses *in vitro* by changing the expression of important inflammatory mediators that are important in the pathogenesis of infectious diseases of transition cows such as mastitis and metritis. Bovine aortic endothelial cells (BAEC) were cultured with different concentrations of a NEFA mixture that reflected the plasma NEFA composition during different stages of lactation. The expression of cytokines, adhesion molecules, and eicosanoids were measured to assess changes in BAEC inflammatory phenotype. Addition of NEFA mixtures 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. A significant increase also occurred in mRNA expression of cytokine and adhesion molecules that are associated with increased inflammatory responses during the transition period. Expression of cyclooxygenase 2, an important enzyme associated with eicosanoid biosynthesis, was increased in a NEFA concentration-dependent manner. The production of linoleic acid-derived eicosanoids 9- and 13-hydroxyoctadecadienoic acids also was increased significantly after treatment with NEFA mixtures. This research described for the first time specific changes in vascular inflammatory response during *in vitro* exposure to NEFA mixtures that mimic the composition and concentration found in cows during the transition period. These findings could explain, in part, alterations in inflammatory responses observed during intense lipid mobilization

stages such as in the transition period of dairy cows. Future studies should analyze specific mechanisms by which high NEFA concentrations induce a vascular proinflammatory phenotype including the effect of 9 and 13-hydroxyoctadecadienoic acids and other lipid mediators.

Key words: nonesterified fatty acid, endothelial cell, transition period, lipid mobilization

INTRODUCTION

A hallmark of the transition period in dairy cows is intense lipid mobilization. This mechanism of metabolic 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 NEFA into the bloodstream (Herd, 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 FA composition of different organs and cell populations, including blood, liver, adipose tissue, and peripheral blood mononuclear cells (Douglas et al., 2007; Contreras et al., 2010). A common change was an increase in the concentrations of SFA, including palmitic (C16:0) and stearic (C18:0) acids. Previous studies in dairy cows associated high plasma NEFA concentrations with impaired host defense mechanisms, with a concomitant increase in disease susceptibility (Lacetera et al., 2004; Scalia et al., 2006; Sordillo et al., 2009). The ways in which increased sera NEFA concentrations may initiate and promote dysfunctional immune and inflammatory responses in transition cows, however, are not fully understood.

Interactions between leukocytes and the vascular endothelium determine efficiency of host inflammatory responses during infectious diseases. Indeed, endothelial cells regulate the trafficking of leukocyte migration to infected tissues (Ley et al., 2007). During the early stages of inflammation, the vasculature expresses sev-

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eral chemotactic proinflammatory cytokines, including IL-6 and IL-8, and different adhesion molecules, including intercellular adhesion molecule (**ICAM1**) and vascular adhesion molecule (**VCAM1**), which facilitate circulating leukocyte transmigration into underlying tissues (Sprague and Khalil, 2009). Once the source of inflammation is eliminated, antiinflammatory mediators are produced and adhesion molecule expression is inhibited in vascular endothelium to reduce leukocyte migration and promote tissue remodeling and healing (Frangogiannis, 2008). In humans, the development of certain chronic inflammatory diseases is linked directly to alterations in the resolution mechanisms of inflammation. For example, overexpression of both adhesion molecules (ICAM1 and VCAM1), and certain cytokines (IL-6 and IL-8) is part of the pathophysiology of atherosclerosis and peripheral arterial disease (Weber et al., 1995; Signorelli et al., 2003). Previous studies in dairy cows also showed increase expression of these proinflammatory cytokines and adhesion molecules in tissues during the periparturient period and during the pathogenesis of inflammatory-based diseases such as mastitis (Aitken et al., 2009; Aitken et al., 2011).

Another component of the onset and resolution of inflammation is the biosynthesis of eicosanoids. These lipid molecules are produced by endothelial cells and leukocytes following the oxidation of phospholipid-derived PUFA. The main enzymatic pathways for eicosanoid biosynthesis are cyclooxygenases (**COX**) and lipoxygenases (**LOX**). Certain COX and LOX metabolites, such as prostaglandins (**PG**), thromboxanes (**TX**), and leukotrienes, are potent vasoactive mediators that modulate directly endothelial cell inflammatory responses (Bogatcheva et al., 2005). At the same time, other COX and LOX metabolites, such as lipoxins, resolvins, and certain prostaglandins, have antiinflammatory and proresolving functions that promote the resolution of inflammatory processes (Serhan et al., 2008). Imbalances between the biosynthesis of pro- and antiinflammatory eicosanoids during inflammatory processes are part of the pathogenesis of vascular diseases in humans (Wittwer and Hersberger, 2007).

The distribution of eicosanoids within an inflammatory foci are determined, in part, by the concentration of certain FA that serve as precursors for their biosynthesis (Calder, 2006). The way that shifts in plasma NEFA concentration and composition during the periparturient period may affect the expression of vasoactive eicosanoids, however, is unknown. This information may provide new insights of how increased lipid mobilization is linked with increased disease susceptibility and severity in periparturient cows. The objective of this study was to evaluate how increasing NEFA concentrations could modify vascular inflammatory responses in vitro

by changing the expression of cytokines, eicosanoids, and adhesion molecules. The hypothesis of this study was that high NEFA concentrations, such as those seen during the transition period in dairy cows, can increase the proinflammatory phenotype of bovine endothelial cells.

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 (**FBS**). 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 Inc. (Foster City, CA) and Qiagen Inc. All other chemicals were purchased from Sigma (St. Louis, MO).

Vascular Cell Culture

Bovine aortic endothelial cells (**BAEC**) were isolated from the aorta of 6 lactating dairy cows by collagenase digestion as described previously (Sordillo et al., 1998). Bovine aortic endothelial cells were cloned to a single-cell level by the limiting dilution method and maintained until confluent as previously described (Sordillo et al., 2008). Ham's F-12K containing 10% FBS, 2 mM L-glutamine, 1% (vol/vol) antibiotic-antimycotic, and 100 µg of heparin/mL, but without phenol red, was used as the base culture medium. Bovine aortic endothelial cells were grown in 225-mm plastic tissue-culture flasks until confluence and then passed to tissue culture dishes at a 5.5×10^6 cells/mL concentration. Bovine aortic endothelial cells were exposed for 4 or 24 h to 3 different NEFA mixture concentrations emulating different degrees of lipid mobilization as observed during the transition period: 0.25 mM for low, 0.5 mM for intermediate, and 0.75 mM for high mobilization intensity. Bovine aortic endothelial cells were exposed to culture media without addition of NEFA mixture (0 mM) as a negative control. Culture media plus albumin at equivalent concentration to the 0.75mM NEFA treatment as the NEFA vehicle control and media plus LPS (50 ng/mL) served as positive inflammation control. Treatments were applied by replacing the base media with the treatments or controls. Three independent rounds of experiments were performed for (1) qPCR, Western blots, and FA analyses; (2) flow cytometry; and (3) eicosanoid detection and quantification. Bovine aortic endothelial cell maximum passage was 9, and

clones obtained from 3 different cows were used for each experiment.

Cell Viability Assay

Trypan blue exclusion was used to evaluate cell death. Following treatment of BAEC with the different controls and NEFA mixture concentrations for 4 and 24 h, cells were collected using trypsin, diluted 1:10 in trypan blue, and counted on a hemocytometer. Viable and nonviable cells were counted using a hemocytometer to obtain the percentage of viable cells. Metabolic activity, and thereby cell viability, was measured using the Promega CellTiter-Glo Viability Assay (Promega Corp., Madison, WI), which quantifies the amount of ATP, an indicator of metabolically active cells. Endothelial growth factor (30 $\mu\text{g/mL}$) was used as a positive control for this assay, as it sustains cell growth. A 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 directly reflects the number of viable cells. Luminescence was measured using a Wallac Victor3 1420 Multilabel Plate Reader (PerkinElmer Inc., Waltham, MA).

NEFA-Albumin Mixture Solution

The composition of the NEFA mixture was calculated based upon a previous study that characterized the FA profiles of plasma NEFA in periparturient cows (Contreras et al., 2010). The mixture resembled the FA profile in the NEFA fraction during the first week of lactation when the greatest lipid mobilization intensity was observed 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 (C22:6n3c; 1%) FA. Nonesterified FA were bound to albumin, which is their natural transporter in plasma, to mimic in vivo physiological conditions. The NEFA-albumin mixtures were solubilized 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 (Branson Ultrasonics Corp., Danbury, CT) for 10 s at an output of 10%. Once readily dissolved, the solution was dried under nitrogen, and then resuspended in 0.1 M NaOH at 90°C. Oleic (C18:1n9c), linoleic (C18:2n6c), and docosahexaenoic acid (C22:6n3c) acids were dissolved in 0.1 M NaOH at 70°C and then mixed with the palmitic (C16:0) and stearic (C18:0) acid NaOH solution. The NEFA mixture solution was complexed with 10% BSA (FA-free) at 55°C for 10 min. The NEFA-albumin 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 with a NEFA:albumin molar ratio of 7:1.

Lipid Extractions

Total lipids from media and BAEC were extracted using a hexane:ethanol (1:1) solvent mix as previously described (Contreras et al., 2010). Bovine aortic endothelial cells were harvested by gentle scraping, washed in Hanks' buffered salt solution (HBSS), and centrifuged at $233 \times g$. Prior to lipid extraction, BAEC were resuspended in HBSS to a minimum concentration of 5.5×10^6 cells/mL. To obtain total lipids, media or BAEC were combined with the solvent mix (1:2) and vortexed for 10 min, and then centrifuged at $2,095 \times g$ for 10 min at 25°C. 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 lipid, NEFA, and phospholipid 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, the neutral lipid fraction was eluted with chloroform:2-propanol (2:1), the NEFA with 2% acetic acid in diethyl ether, and the phospholipid fraction with methanol. 1,2-diheneicosanoyl-*sn*-glycero-3-phosphocholine (21:0 phosphatidylcholine) was used as the 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 PerkinElmer Clarus 500 gas chromatography apparatus (PerkinElmer Inc.) with a Supelco SP-2560 column (100 m $\times$ 25 mm with a 0.2- μm film thickness; Supelco Inc., Bellefonte, PA). Analyte elution times were identified using a mix of pure methyl ester standards (NuChek Prep Inc., Elysian, MN).

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., 2008). Briefly, total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen Inc.). A DNase digest was performed 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 Inc.). All qPCR assays were conducted using TaqMan gene expression assays from Applied Biosystems Inc. The TaqMan

primer and probe sets (Table 1) were designed from bovine sequences with the Applied Biosystems Pipeline software and synthesized by Applied Biosystems Inc. 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 nonreverse transcriptase control was run to ensure that genomic DNA was not being amplified. The relative quantification of each gene was calculated utilizing the 7500 Fast SDS software (version 1.3.1; Gallagher et al., 2003). 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 (Steibel et al., 2009). The calibrator was the 0 mM NEFA culture media control cells.

Western Blot Analyses

Protein quantification was performed by protein blot analysis. Bovine aortic endothelial cells were harvested by gentle scraping. Whole cell lysates were harvested in M-Per reagent (Pierce Biotechnology Inc., Rockford, IL) and centrifuged at 10,000 $\times g$ for 10 min at 4°C to remove membrane fractions. Supernatants 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 (1:2,000; Cayman Chemical Co., Ann Arbor, MI), and anti-human ICAM1 (1:100 dilution; Invitrogen Corp., Carlsbad, CA) in 1% BSA in TBS and 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 Biotechnology Inc.) for 10 min at 25°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. 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 culture media control BAEC.

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	ATGTCCTGTACGGCCCC
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	CATGCCCCGACTCCCTC
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	AACTGGTTGCGGTGTCTGA
		Probe	CCTTCGCCGCTCCGC

¹ICAM = intercellular adhesion molecule; VCAM = vascular adhesion molecule; COX = cyclooxygenase; LOX = lipoxygenase.

Reactive Oxygen Species Determination by Flow Cytometry

Reactive oxygen species (ROS) generation in BAEC cultures was determined by flow cytometry using the intracellular fluorescent probe carboxy-2'-7'-dichlorofluorescein (DCF) as previously described (Eruslanov and Kusmartsev, 2010). 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 room temperature, the samples were analyzed with the FACSVantage flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The fluorescence intensity was evaluated for at least 3 independent experiments.

Eicosanoid Detection and Quantification

Bovine aortic endothelial cells and media were harvested by gentle scraping and addition of ethanol to a 10% solution by volume. Specimens were then sonicated in a water bath at 4°C for 3 min at output 10% using a Misonix S-400 sonicator (Qsonica LLC, Newton, CT). Samples were then centrifuged at 4°C for 5 min at 3,000 $\times g$ and the supernatant was used for extraction. Lipid mediators were isolated from specimens by solid-phase extraction using Strata X SPE columns (Phenomenex Inc.). Cartridges were first conditioned with 2 mL of methanol (MeOH) and then washed with 2 mL of H₂O. Samples were loaded into the cartridge, washed with 2 mL of MeOH (40% vol/vol), and finally eluted with MeOH:acetonitrile (50:50 vol/vol). Eluates were dried using a Savant SpeedVac concentrator (Thermo Electron Corp., West Palm Beach, FL) and then redissolved in acetonitrile:water:formic acid (36:64:0.2 vol/vol/vol).

Eicosanoids were isolated using 2 distinct ultra-high pressure liquid chromatography (UPLC) and mass spectrometry (UPLC-MS) methods. Both used reverse-phase liquid chromatography on a Waters ACQUITY UPLC 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 quadrupole mass spectrometer Waters H-class Acquity UPLC in electrospray negative ionization mode (Waters Corp., Milford, MA). The electrospray voltage was -3 kV and the turbo ion spray source temperature was 450°C. Collisional activation used nitrogen as the collision gas. For each method, a 10- μ L sample was injected 3 consecutive times using a 10- μ L injection loop. An isocratic mobile phase consisting of acetonitrile:water:formic acid (45:55:0.01; vol/vol/vol) with an analysis time of 15 min was used to measure leukotriene B₄, PGE₂,

6-keto F_{1 α} (PGF_{1 α}), PGF_{2 α} , 6(R)-lipoxin A₄ (LXA₄), TXB₂, resolvin D₁ (RD₁), and D₂ (RD₂). The second method used an isocratic mobile phase of acetonitrile:methanol:water:formic acid (47.4:15.8:26.8:0.01; vol/vol/vol/vol) and an analysis time of 10 min to analyze 9(S)-hydroxyoctadecadienoic acid (HODE), 13(S)-HODE, 5(S)-hydroxyeicosatetraenoic acid (HETE), 12(S)-HETE, and 15(S)-HETE. Eicosanoids were identified in samples by matching their mass - 1 and LC retention time with those of a pure standard. Internal deuterated standards were added before harvesting cells and supernatants to control for sample extraction, injection, and ionization variability. Standards included PG D₂-d₄ (50 ng/ μ L), E₂-d₄ (50 ng/ μ L), F_{2 α} -d₄ (50 ng/ μ L), 5(S)-HETE-d₈ (50 ng/ μ L), 13(S)-HODE-d₄ (50 ng/ μ L), 9(S)-HODE-d₄ (50 ng/ μ L), leukotriene D₄-d₅ (50 ng/ μ L), 15(S)-HETE-d₈ (50 ng/ μ L), 12(S)-HETE-d₈ (50 ng/ μ L), leukotriene B₄-d₄ (50 ng/ μ L), thromboxane B₂-d₄ (50 ng/ μ L), 6-keto PGF_{1 α} -d₄ (50 ng/ μ L), and 5(S), 6(R)-lipoxin A₄-d₅ (50 ng/ μ L). 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.

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 range from 0.013 to 22.68 ng/ μ L. Empower 2 identified the sample peak by matching its retention time with the standard. A response is calculated for each matched peak by dividing the sample peak's response by its internal standard's response. This response was multiplied by the concentration of the internal standard for each analyte. The concentration of each analyte was calculated using the response peak, injection volume, and protein concentration of the sample, and concentrations are reported as picograms of eicosanoid per microgram of protein.

Statistical Analyses

Statistical evaluation was conducted using the MIXED procedure (SAS v9.1; SAS Institute Inc., Cary, NC). Comparisons of the means were made by ANOVA followed by the Tukey-Kramer honestly significant difference (HSD) post hoc test as necessary. Specific contrasts were performed to evaluate (1) linear and quadratic relationships of the NEFA mixture concentration effect and (2) the presence of albumin as FA transporter in comparison to 0 mM control and the highest NEFA concentration (0.75 mM). Data are shown as means $\pm$ standard errors of the means. Differences at $P < 0.05$ were considered statistically significant.

Table 2. Fatty acid composition of the bovine aortic endothelial cell phospholipid fraction after 24 h of culture with different concentrations of a NEFA mixture or albumin as a loading control¹

FA, ² g/100 g	FFA complex supplementation					<i>P</i> -value	Aorta lipid content
	0 mM	Albumin	0.25 mM	0.5 mM	0.75 mM		
C14:0	2.402 ± 0.3	2.59 ± 0.2	2.02 ± 0.16	1.627 ± 0.3	2.145 ± 0.2	0.06	1.05
C15:0	2.239 ± 0.7	1.57 ± 0.5	3.263 ± 0.92	3.52 ± 0.96	2.315 ± 0.3	0.26	0.67
C16:0	26.27 ± 1.9	28.4 ± 1.7	22.93 ± 1.98	21.96 ± 2.44	25.36 ± 1.6	0.07	21.36
C16:1n7c	0.957 ± 0.1	0.89 ± 0.1	0.813 ± 0.07	0.631 ± 0.18	0.988 ± 0.2	0.10	0.44
C18:0	27.98 ± 2.2 ^a	30.2 ± 2.6 ^a	32.98 ± 2.41 ^{ab}	34.99 ± 2.58 ^b	34.99 ± 1.8 ^b	0.01	32.71
C18:1n7c	1.537 ± 0.3 ^b	1.43 ± 0.4 ^b	0.901 ± 0.08 ^a	0.561 ± 0.11 ^a	0.434 ± 0.1 ^a	<0.01	1.24
C18:1n9c	8.463 ± 1.3	8.19 ± 1.8	7.312 ± 0.35	5.611 ± 1.15	5.88 ± 1.9	0.15	11.57
C18:1n9t	1.674 ± 0.3	1.92 ± 0.4	1.649 ± 0.58	1.211 ± 0.3	1.29 ± 0.2	0.23	1.99
C18:2n6c	0.815 ± 0.2	0.75 ± 0.3	1.663 ± 0.5	1.83 ± 0.63	1.392 ± 0.5	0.13	3.04
C18:3n6c	0.535 ± 0.1	0.57 ± 0.1	0.615 ± 0.05	0.789 ± 0.15	0.523 ± 0.1	0.15	0.52
C20:4n6c	3.433 ± 0.8 ^c	2.49 ± 0.8 ^{bc}	1.871 ± 0.44 ^{ab}	1.648 ± 0.37 ^{ab}	1.34 ± 0.3 ^a	<0.01	5.53
C22:6n3c	4.547 ± 1.3	5.04 ± 2.3	2.182 ± 1.04	2.893 ± 1.22	3.488 ± 1.1	0.14	2.74

^{a-c}Means within a column with different superscript letters differ ($P < 0.05$).

¹Values are expressed as means ± SEM ($n = 6$). The FA composition of phospholipids in aorta intima from an early-lactation cow ($n = 1$) is described in the far-right column.

²c = *cis*; t = *trans*.

RESULTS

Cell Viability

No significant changes in viability were observed by trypan blue exclusion at 4 and 24 h of incubation when BAEC were exposed to 0 mM (no NEFA addition), albumin, or 0.25, 0.5, or 0.75 mM of the NEFA mixture, with cell death counts below 10%. Similarly, no differences in ATP production, an indirect measure of cell viability, were detected among treatments after 4 and 24 h of culture (data not shown).

NEFA Supplementation Altered BAEC Phospholipid FA Profiles

Addition of NEFA mixtures to culture media did not significantly alter the FA composition of the phospholipid membrane of BAEC at 4 h (results not shown). After 24 h in culture with the NEFA mixture, however, significant compositional changes were observed (Table 2). A significant increase in the concentrations of stearic acid (C18:0) occurred when cells were supplemented with the 0.5 and 0.75 mM NEFA mixtures when compared with 0 mM or albumin control treatments. A linear and a quadratic effect ($P < 0.01$) on the phospholipid content of stearic acid (C18:0) by NEFA mixture supplementation was demonstrated by contrasts. In addition, a significant decrease was observed in the concentration of *cis*-vaccenic acid (C18:1n7c) with 0.25, 0.5, and 0.75 mM NEFA mixtures when compared with albumin and 0 mM treatments and this effect was linear and quadratic ($P < 0.01$). Similarly, arachidonic acid (C20:4n6c) content decreased significantly with 0.25, 0.5, and 0.75 mM NEFA mixtures when compared

with the 0 mM treatment. Contrast analysis demonstrated a linear and quadratic effect on arachidonic acid (C20:4n6c) content ($P < 0.01$) in response to NEFA supplementation.

NEFA Supplementation Altered Expression of Cytokine and Adhesion Molecules

After 24 h of culture, the gene expression of IL-6 and IL-8 increased linearly in response to the addition of NEFA mixtures at different concentrations (Table 3). Interleukin-6 and IL-8 expressions were significantly lower in BAEC exposed to albumin compared with the 0.75 mM NEFA group (Table 3). Also, a linear effect was observed in the gene expression of adhesion molecules ICAM1 and VCAM1 in response to NEFA supplementation (Table 3). This effect was reflected in the protein expression of ICAM1 that was enhanced in BAEC exposed to 0.5 and 0.75 mM NEFA compared with 0 mM treatment (Figure 1). Lipopolysaccharide exposure was used as a positive inflammatory control and, as expected, induced a strong endothelial cell response by upregulating the gene expression of proinflammatory cytokines (IL-6 and IL-8) and adhesion molecules (ICAM1 and VCAM1). At the protein level, LPS stimulation induced a significant increase in ICAM1 expression (Figure 1).

NEFA Supplementation Increased ROS Concentration in Cultured BAEC

The BAEC content of ROS was measured by quantifying the cellular concentration of DCF using flow cytometry. This highly fluorescent compound is produced

Table 3. Effect of NEFA mixture supplementation on gene expression of proinflammatory cytokines and eicosanoid enzymes, generation of reactive oxygen species (ROS), and production of eicosanoids

Item	NEFA mixture supplementation ¹						SEM	Contrast			
	0 mM	0.25 mM	0.5 mM	0.75 mM	Albumin	LPS		0.75 mM vs. albumin	0 mM vs. albumin	Linear	Quadratic
Gene ²											
<i>IL-6</i>	20.131	19.019	17.694	15.939	19.169	9.378	1.023	0.007	NS	<0.001	NS ⁵
<i>IL-8</i>	9.961	8.875	7.617	7.010	9.069	4.464	0.643	0.009	NS	<0.001	NS
<i>VCAM1</i>	13.440	12.057	9.836	9.638	12.028	6.444	0.739	0.003	NS	<0.001	NS
<i>ICAM1</i>	9.532	9.119	8.034	7.125	9.088	3.643	0.598	<0.001	NS	<0.001	NS
<i>COX2</i>	17.009	16.039	15.260	12.786	17.254	7.185	1.147	0.011	NS	0.003	NS
<i>15LOX1</i>	21.686	21.559	21.194	20.817	21.280	19.969	0.766	NS	NS	0.027	NS
ROS ³	164.210	184.190	265.690	292.280	182.160	262.470	26.256	0.007	NS	<0.001	NS
Eicosanoid ⁴											
PGE ₂	0.010	0.016	0.009	0.009	0.012	0.061	0.012	NS	NS	NS	NS
PGF ₁	0.071	0.068	0.074	0.048	0.012	1.809	0.420	0.045	0.002	NS	NS
PGF ₂	0.016	0.009	0.010	0.008	0.007	0.029	0.004	NS	0.001	0.014	NS
TXB ₂	0.453	0.359	0.221	0.221	0.215	0.543	0.052	NS	<0.001	<0.001	NS
9-HODE	0.047	1.372	2.299	3.248	0.032	0.051	0.265	<0.001	NS	<0.001	NS
13-HODE	0.061	2.088	3.306	3.982	0.044	0.067	0.276	<0.001	NS	<0.001	<0.0001
12-HETE	0.900	0.689	0.579	0.266	0.390	0.867	0.219	NS	NS	NS	NS
15-HETE	0.380	0.376	0.322	0.107	0.148	0.559	0.252	NS	NS	NS	NS

¹Bovine aortic endothelial cells (BAEC) were supplemented with a NEFA mixture or vehicle control (albumin) at different concentrations for 24 h. As a positive proinflammatory control, BAEC were stimulated with LPS for 12 h. Values are LSM for different treatments and controls.

²Values for gene expression are shown as Δ cycle threshold (Δ Ct). ICAM1 = Interleukin, intercellular adhesion molecule 1; VCAM1 = vascular adhesion molecule 1; COX = cyclooxygenase; LOX = lipoxygenase.

³Reactive oxygen species values are in arbitrary units of fluorescence intensity of 2'-7'-dichlorofluorescein (DCF).

⁴Values for eicosanoids are expressed in picograms per microgram of total protein. PG = prostaglandin, TX = thromboxane; HODE = hydroxyoctadecadienoic acid; HETE = hydroxyeicosatetraenoic acid.

⁵ $P > 0.05$.

Figure 1. Bovine aortic endothelial cells (BAEC) were supplemented with an albumin (vehicle control) or NEFA mixture at 3 concentrations for 24 h. As a positive proinflammatory control, BAEC were stimulated with LPS for 12 h. The effect of NEFA treatment on BAEC protein expression of intercellular adhesion molecule 1 (ICAM1) was assessed by protein blot. Values are expressed as means $\pm$ SEM of the ratio of ICAM1 to actin as a fold change over the 0 mM NEFA-supplemented BAEC. Bars with different letters (a–c) are significantly different ($P < 0.05$).

by the oxidation of carboxy-DCF diacetate by hydrogen peroxide or superoxide anion. As summarized in Table 3, DCF concentrations increased linearly in response to NEFA mixture exposure. Albumin-supplemented cells had lower ROS content than BAEC exposed to 0.75 mM NEFA (Table 3). Stimulation with the positive inflammatory control (LPS) also enhanced the concentration of DCF in BAEC.

NEFA Supplementation Altered Lipid Mediator Biosynthesis in BAEC

Eicosanoid Enzymes. Changes in NEFA mixture concentrations in cultured media did not induce significant changes in the gene expression of COX1 (data not shown). Changes also were not detected when BAEC were supplemented with albumin or stimulated with LPS (positive inflammatory control). Similarly, no changes occurred in the protein expression of COX1 of BAEC cells when cultured for 24 h with different NEFA mixture concentrations or both controls albumin and LPS (Figure 2a). In contrast to COX1, COX2 mRNA expression increased linearly in a NEFA mixture concentration-dependent manner (Table 3). At the same time, BAEC exposed to albumin had significantly

lower COX2 transcription compared with 0.75 mM NEFA-supplemented cells (Table 3). The expression of COX2 protein was significantly augmented when BAEC were exposed to 0.75 mM NEFA (Figure 2b). The gene expression of 15LOX1 was increased linearly in response to NEFA supplementation (Table 3).

Lipid Mediator Biosynthesis. The concentrations of COX- and 15LOX-derived metabolites were determined by UPLC-MS. At 24 h of supplementation, PGE₂ concentration did not differ among treatments or control groups (Table 3). Concentrations of PGF₁ were significantly higher in LPS-stimulated cells compared with other treatment groups ($P < 0.05$). Albumin controls had a significantly lower PGF₁ production compared with 0 and 0.75 mM NEFA groups (Table 3). Similar to PGF₁, PGF₂ and thromboxane B₂ biosynthesis was higher in the LPS group compared with those supplemented with albumin or any NEFA concentration ($P < 0.05$). Supplementation of albumin reduced the biosynthesis of both arachidonic acid (C20:4n6c) metabolites compared with 0 mM treatment (Table 3). Interestingly, a linear reduction in the production of PGF₂ and TXB₂ occurred in response to NEFA supplementation (Table 3).

Changes were observed in the BAEC production of linoleic acid (C18:2n6c)-derived eicosanoids after exposure to the treatments. Both 9-HODE and 13-HODE concentrations increased linearly after NEFA mixture supplementation (Table 3). Furthermore, a quadratic effect was also found on the production of 13-HODE in response to the addition of NEFA mixtures at different concentrations (Table 3). No changes in the production of these metabolites were observed when BAEC were exposed to the positive inflammatory control (LPS) compared with both 0 mM and albumin treatment groups. As for 15LOX-derived arachidonic acid (C20:4n6c) metabolites, including 12 and 15 HETE, no significant changes in their biosynthesis occurred as a result of exposure to different concentrations of NEFA mixtures (Table 3).

DISCUSSION

Intense lipid mobilization during the transition period of dairy cows releases abundant NEFA from adipose tissue, inducing alterations in plasma NEFA composition and concentrations (Contreras et al., 2010). Previous work reported changes in leukocyte function with exposure to 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 after supplementation with a NEFA mixture that mimic transition cow plasma NEFA concentration and composition. These

Figure 2. Bovine aortic endothelial cell (BAEC) cyclooxygenase (COX) protein expression in response to supplementation with an albumin or NEFA mixture at different concentrations for 24 h or stimulation with LPS for 12 h. Values are expressed as means $\pm$ SEM of the ratio of (a) COX1 or (b) COX2 to actin as a fold change over the 0 mM NEFA-supplemented BAEC. Bars with different letters (a and b) are significantly different ($P < 0.05$). Protein bands in the picture were derived from different experiments and subsequently compiled for the figure.

changes included alterations in BAEC phospholipid FA composition, increased gene and protein expression of adhesion molecules and proinflammatory cytokines, enhanced generation of ROS, and alterations in the biosynthesis of eicosanoids from the COX2 and 15LOX enzymatic pathways.

Significant changes in BAEC phospholipid FA content were observed only after exposing BAEC for 24 h to different NEFA mixture 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. (2006) reported that supplementation with SFA increased their concentrations in endothelial phospholipids and, at the same time, this addition was balanced by a decrease in the concentrations of PUFA of both n-3 and n-6 series. Similarly, our study showed a significant increase in the concentration of stearic acid (C18:0) and a significant reduction in PUFA, such as vaccenic (C18:1n7c) and arachidonic (C20:4n6c) acids. Endothelial cells have a reduced ability to store FA as triglycerides and it was expected that the majority of FA would either be directed to phospholipid biosynthesis or become β -oxidized in the mitochondria (Héliès-Toussaint et al., 2006). Interestingly, of the supplied SFA [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 concentration 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 β -oxidation than stearic acid (C18:0) due to its shorter chain length (Crunkhorn et al., 2007). Therefore, it may be expected that palmitic acid (C16:0) would be used mainly as energy substrate over stearic acid (C18:0). Results from this study only reflect the influence of plasma NEFA to endothelial cell FA composition and do not take into account the contribution of other plasma lipid fractions usually transported by lipoproteins such as cholesterol esters, triglycerides, and phospholipids. The contribution of esterified FA on phospholipid composition is tissue specific and is largely determined by the activity of lipoprotein lipases, produced by parenchymal cells, monocytes, adipocyte, and myocytes (Engler et al., 1999; Davies et al., 2010). The experiments in this study, however, demonstrated the possibility of manipulating the FA content of endothelial cells by supplementing NEFA mixtures to reflect specific vascular FA profiles found in vivo.

After 24 h of supplementation with NEFA mixtures, the phospholipid FA content of BAEC in our study

partially resembled the phospholipid content of aorta intima phospholipids in vivo (Table 2). Concentrations of oleic and arachidonic acids were lower in cultured BAEC when compared with the content in the aorta. This may be reflecting the low concentrations of oleic (17.2%) and arachidonic (0.4%) acid in the FBS used in the present study for media preparation. Previously, Stoll and Spector (1984) reported that the FA content of serum greatly influenced the FA profile of phospholipids in primary cells or cell lines. Future studies with cultured bovine cells should closely evaluate the FA content of the media supplemented, as this fact could influence the phospholipid composition of cells. In addition, currently, no published studies are available that cite the dynamics of FA composition of vascular endothelial cells during the periparturient period. Based on our results, it is likely that higher concentrations of SFA and reduced content of PUFA, such as those observed in the plasma of transition cows, induce changes in the phospholipid content of endothelium. In humans, high concentrations of palmitate and stearate have detrimental effects on endothelial function, including a decrease in cellular membrane fluidity and induction in the expression of vascular adhesion molecules (Harvey et al., 2010). In dairy cows, trafficking processes are altered during the transition period as indicated 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 induced by high concentrations of plasma NEFA may affect leukocyte migration patterns.

After exposing BAEC to NEFA concentrations over 0.5 mM for 24 h, both ICAM1 and VCAM1 increased their gene and protein expression. Previous studies in endothelial cells reported enhanced expression of both adhesion molecules during supplementation of palmitic and stearic acids, the main FA in the NEFA mixture used in this report. Harvey et al. (2010) exposed human aortic endothelial cells to BSA bound myristic, stearic, and palmitic acids for 24 h reporting enhanced surface expression of ICAM1 as measured by flow cytometry. They demonstrated that during supplementations of stearic acid at 50 to 100 μ M, ICAM1 expression was induced in response to nuclear factor κ B (NF- κ B) activation. Similar to SFA, linoleic acid- (C18:2n6c) derived eicosanoids 9- and 13-HODE also are capable of inducing the expression of ICAM1 and VCAM1 in endothelial cells in a concentration-dependent manner by phosphorylation of inhibitor of κ B (κ B- α) and NF- κ B (Friedrichs et al., 1999; Viita et al., 1999). Both 9- and 13-HODE were increased significantly in our experiments when BAEC were cultured with 0.5 mM and 0.75 mM NEFA mixtures. The enhanced expression of

vascular adhesion molecules are implicated in the pathology of several inflammatory-based vascular diseases in humans (Weber et al., 1995; Signorelli et al., 2003). The increased expression of ICAM1 and VCAM1 in response to high concentrations of NEFA may contribute to dysfunctional inflammatory responses in periparturient dairy cattle (Aitken et al., 2011) and merits further investigation.

Interleukin-6 and IL-8 transcription also was up-regulated after 24 h of NEFA exposure. Interleukin-6-enhanced expression was previously linked to NF- κ B activation by palmitic (C16:0), stearic (C18:0), and linoleic (C18:2n6c) acids, as demonstrated in pulmonary artery and coronary artery endothelial cells (Toborek et al., 1996; Staiger et al., 2006). Furthermore, palmitic acid (C16:0) plasma levels in humans were directly correlated to IL-6 content in blood (Staiger et al., 2006). Similarly, IL-8 transcription enhancement in human aortic cells is also mediated by palmitic (C16:0) and oleic (C18:1n9c) acids (Packard and Libby, 2008). Although an induction mechanism for 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). Interleukin-6 and IL-8 are reported as inflammatory markers in human diseases characterized with high concentrations of plasma NEFA, such as obesity and diabetes (Dandona et al., 2004). Dairy cows also are characterized by high plasma NEFA concentrations during exacerbated lipid mobilization and this is directly associated with the incidence of inflammatory diseases such as clinical mastitis and metritis (Melendez et al., 2009). In the case of clinical mastitis, enhanced expression of IL-6 and IL-8 is associated with the pathogenesis of this disease when caused by coliform bacteria (Aitken et al., 2011). Currently, the mechanisms by which IL-6 and IL-8 expression is induced by high plasma NEFA concentrations and how this affects susceptibility to inflammatory based diseases remains unknown.

Nonesterified FA supplementation at 0.5 mM and 0.75 mM concentrations enhanced gene and protein expression of COX2. Although not demonstrated in endothelial cells, previous research in monocytes concluded that SFA, especially laurate and palmitate, enhance the expression of this eicosanoid enzyme through toll-like receptor 4 signaling (Lee et al., 2001). The activation of this receptor is sufficient to induce NF- κ B inflammatory pathway and, therefore, enhance the gene and protein expression of COX2. Interestingly, at the time of sample collection (24 h after NEFA supplementation), only LPS-stimulated cells had a significant elevation in the gene expression of 15LOX compared

with nonsupplemented controls. Possibly, transcriptional activity at this time is diminished due to the observed increased concentrations of linoleic acid products, including 13-HODE, which may act as negative feedback signal for 15LOX expression (Lee et al., 2001). A better characterization of the interactions of SFA, such as palmitic and stearic acids, and the linoleic acid products on COX2 and 15LOX activity in endothelial cells is needed. The effect of transient and continuous elevations in NEFA concentrations during the transition period should also be characterized.

Previously published reports in transition cows documented increased SFA content with a concomitant decrease in PUFA within mononuclear cells obtained during times of intense lipid mobilization (Contreras et al., 2010). A possible consequence of these changes is the alteration of substrate availability for eicosanoid biosynthetic pathways, including COX and 15LOX. Bovine aortic endothelial cell supplementation with higher concentrations of NEFA induced a reduction in the phospholipid content of arachidonic acid (C20:4n6c) and other PUFA of 20 or more carbon structure. Decreased availability of this group of PUFA may have induced the use of alternate substrates such as 18-carbon-chain PUFA. In fact, we obtained a significantly higher production of linoleic acid (C18:2n6c) metabolites of the 15LOX pathway and 9- and 13-HODE and observed no changes in 5-, 12-, and 15-HETE, which are arachidonic (C20:4n6c) acid derivatives. These shifts in the 15LOX pathway final products could be explained by either short supply of arachidonic acid in our culture media or the higher affinity of 15-LOX for linoleic acid (C18:2n6c) over arachidonic acid and other FA of 20 carbons (Brash et al., 1997). A possible consequence of higher concentration of linoleic acid (C18:2n6c) metabolites is an alteration of inflammation resolution processes. Although not demonstrated in endothelial cells, 9- and 13-HODE are known to interfere with the antiinflammatory actions of peroxisome proliferator-activated receptors (PPAR). For example, 13-HODE downregulates PPAR γ activity (Hsi et al., 2002). Furthermore, high levels of these 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 and other factors that determine PUFA availability to eicosanoid enzymes, including phospholipase activity and specific availability of selected FA.

Enhanced concentrations of 9- and 13-HODE may not be completely explained by 15LOX activity. Non-enzymatic lipid peroxidation is an alternate source of

these linoleic acid metabolites. This type of oxidation is initiated by either free radicals such as superoxide anion (O_2^-) or by singlet oxygen or ozone (Niki, 2009). Free radical oxidation is exacerbated during oxidative stress states such as those observed in obese individuals and periparturient dairy cows (Furukawa et al., 2004; Sordillo and Aitken, 2009). In the case of linoleic acid, 9- and 13-hydroxyperoxyoctadecadienoic acid (HPODE) are the initial products of nonenzymatic peroxidation that are further oxidized to 9- and 13-HODE. Interestingly, supplementation with 0.5 and 0.75 mM NEFA mixtures increased the concentration of ROS as measured by fluorescence intensity. These results confirm that high NEFA concentrations may induce oxidative stress and could, as a result, promote nonenzymatic oxidation of phospholipids in the cellular membrane. In the future, it will be necessary to determine the nonenzymatic-dependent generation of lipid mediators of inflammation as a consequence of high NEFA concentration exposure by inhibiting specific eicosanoid enzymes, such as COX and LOX.

Supplementation of different concentrations of the proposed NEFA mixtures containing albumin as transporter significantly reduced the biosynthesis of key pro-inflammatory COX derivatives. Consequently, it is not possible to determine the specific effects of the NEFA from the potential confounding effects of the albumin. Linoleic acid, a component of the supplemented NEFA mixture, may have reduced the activity of COX2-catalyzed PG biosynthesis. Limited PG biosynthesis in the presence of linoleate was demonstrated previously in bovine pulmonary artery endothelial cells and placental structures (Kaduce et al., 1982; Cheng et al., 2011). The presence of albumin in the NEFA mixture also could explain reductions in PG biosynthesis because of its potent activity as a ligand binder that could have reduced the availability of PGH₂ for prostaglandin synthases (Roche et al., 2008). Furthermore, albumin is known to reduce the half-life of arachidonic acid- (C20:4n6c) derived compounds due to the strong alkalinity of the PG binding site that accelerates the catabolism of those metabolites (Fitzpatrick et al., 1984; Yang et al., 2002). These albumin properties were not taken into account when designing the experiments reported in this work and require further analysis, especially in studies evaluating lipid mediator biosynthesis that deliver FA using albumin as a transporter.

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 (Boden, 2008; Scaglione et al., 2010). Similarly, dairy cows face severe energy imbal-

ances during the transition period, the lactation stage with highest 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 concentration can enhance the proinflammatory phenotype of endothelial cells. Non-esterified FA supplementation altered the BAEC phospholipid FA profile, enhanced expression of adhesion molecules and cytokines, increased the production of ROS, and promoted the biosynthesis of specific linoleic acid derived metabolites with known proinflammatory functions, including 9- and 13-HODE. These factors could modify tissue inflammatory responses and leukocyte migration. Future studies should analyze specific mechanisms by which NEFA induce inflammatory responses. It is necessary to evaluate the activation of proinflammatory intracellular signaling pathways upon NEFA supplementation and their interaction with eicosanoid biosynthetic pathways. 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 the vascular proinflammatory phenotype.

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