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Differential Effect of 14 Free Fatty Acids in the Expression of Inflammation Markers on Human Arterial Coronary Cells

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ABSTRACT: Cardiovascular disease is the leading cause of death in the United States, and circulating free fatty acids (FFAs) are known risk factors associated with cardiovascular inflammation. The influence of 14 dietary FFAs (including saturated, mono- and polyunsaturated, and *trans*) on the expression of inflammatory markers in human coronary arterial smooth muscle (HCASM) and endothelial (HCEC) cells using a cell culture model was investigated. HCASM and HCEC cell cultures were incubated with 200 μM of each FFA for 8 or 24 h, respectively, at 37 °C in a 5% CO_2 humidified incubator. Inflammatory markers were assessed by ELISA or Western blot in the supernatant or cell lysates respectively. Results showed significant differences in the expression of inflammatory markers among the fatty acid treatments and the control, with myristic and palmitic acids being identified as the most and linoleic acid as the least pro-inflammatory. This suggests that FFAs may induce low-grade inflammation in human coronary arterial cells and provides more information on mode of action.

KEYWORDS: *free fatty acids, inflammation, human coronary arterial cells, IL-6, IL-8, MCP-1, COX-2*

INTRODUCTION

Digestion and absorption of fat primarily involve emulsification and hydrolysis in the gastrointestinal tract, absorption as free fatty acids or monoglycerides, and resynthesis of new triglycerides in the liver that are carried as lipoproteins (mainly VLDL and chylomicrons) in the blood.[1] Free fatty acids (FFAs) are released into the bloodstream by the action of hormone-sensitive lipase on the adipose tissue or endothelium lipoprotein lipase on circulating lipoproteins. The FFAs are bound to serum albumin and transported to peripheral tissues.[2−5] Postprandial spikes in dietary fatty acids can occur from 4 to 8 h after eating occasions. FFA levels and the time curves in serum are dependent on the fatty acid profile of the meal and the individual's daily diet.[6,7] FFAs in plasma in healthy adults (fasting−postprandial) range from 350 to 550 μmol/L but are elevated due to several conditions including obesity (410−730 μmol/L), insulin resistance (560−570 μmol/L), and type 2 diabetes mellitus (690−770 μmol/L) and can increase up to 1 mmol/L after a high-fat meal.[2,8−12] Plasma FFAs are elevated in obese individuals due to the release of FFAs by the enlarged adipose tissue and consequent reduction of FFA clearance from the blood; FFA elevation further results in the inhibition of the antilipolytic action of insulin, which increases FFA release into the bloodstream from chylomicrons and adipose tissue.[13] Elevation of circulating FFAs has been shown to induce pro-inflammatory changes and oxidative stress,[14] impair endothelial and vascular function,[12] inhibit immune response,[4] and even promote insulin resistance.[10] Elevated FFAs are thus now considered an indicator of cardiometabolic stress and a risk factor for cardiovascular disease (CVD)[15] including ischemic heart disease[16] and sudden cardiovascular death.[17,18]

It has been suggested that fatty acid composition of the diet influences the overall inflammatory state, endothelial function, and risk of type 2 diabetes and cardiovascular disease. Studies have mainly focused on *trans* fatty acids and indicate that they are directly related to increased health risks.[19−22] However, the mechanism by which not only *trans* but overall increased free fatty acids induce inflammation is not well understood. Furthermore, an assessment of inflammatory response to individual fatty acids has not yet been performed.

The purpose of this study was to evaluate the effect of 14 dietary relevant FFAs, including saturated, mono- and polyunsaturated, and *trans*, on the expression of inflammation markers in human coronary arterial smooth muscle (HCASM) and endothelial (HCEC) cells using a cell culture model. Evaluation of inflammatory markers in cell culture supernatant as well as in the cell cytoplasm will allow for a better understanding of the mechanism by which FFAs promote inflammation and resulting undesirable cardiovascular risks.

MATERIALS AND METHODS

Materials. Fatty acids certified >99% purity (butyric, lauric, myristic, palmitic, stearic, oleic, linoleic, linolenic, arachidonic, EPA, DHA, elaidic, *trans* vaccenic, and conjugated linoleic acid) were purchased from Nu-Chek Prep (Elysian, MN, USA). Sterile Dulbecco's PBS was purchased from Invitrogen (Grand Island, NY, USA). Fatty acid free bovine serum albumin (BSA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Human primary coronary artery endothelial (HCAE) and smooth muscle (HCASM) cell cultures were purchased from ATCC (Manassas, VA, USA). IL-6, IL-8, and MCP-1 ELISA kits were purchased from PeproTech (Rocky Hill, NJ, USA). Goat anti-COX-2 polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Electrophoresis gels and Western blot kits (WesternBreeze) were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were of analytical grade and obtained from Fisher Scientific (Waltham, MA, USA). Water was autoclaved Milli-Q Type 1 ultrapure (Millipore Corp., Bedford, MA,

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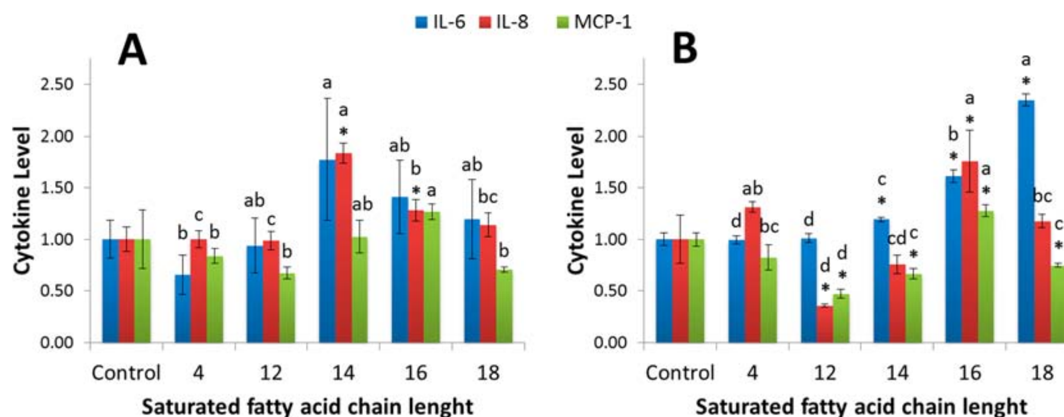

Figure 1. Effect of saturated fatty acids on levels of IL-6, IL-8, and MCP-1 in HCAE (A) and HCASM (B) cells. * Treatment is different from control. Different letters indicate differences between treatments.

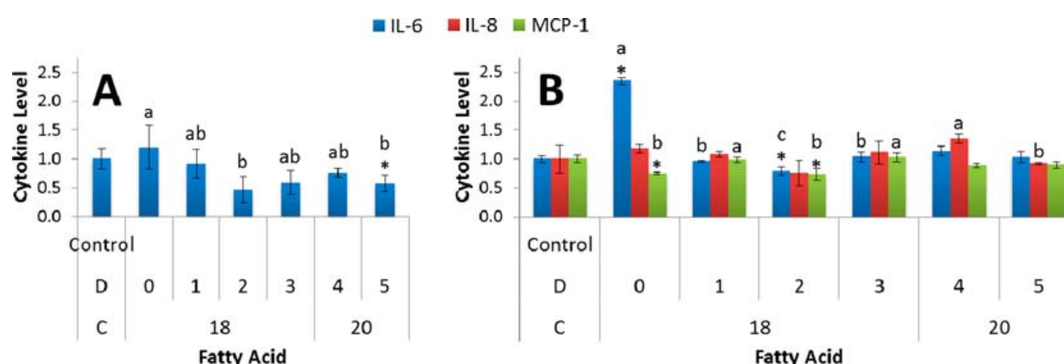

Figure 2. Effect of *all-cis* unsaturated fatty acids on levels of IL-6, IL-8, and MCP-1 in HCAE (A) and HCASM (B) cells. * Treatment is different from control. Different letters indicate differences between treatments involving fatty acids of the same carbon chain length. C, fatty acid carbon chain length; D, degree of unsaturation.

USA) for all of the studies (quality at least equivalent to DEPC-treated water).

Preparation of Fatty Acid Solutions. Fatty acids were conjugated to BSA according to the methods developed by McIntosh, Toborek, and Henning, and van Greevenbroek et al.[23−26] Briefly, for each 1 mL of 10 mM solution, 10 μmol of fatty acid was aseptically weighed in a sterile tube and dissolved in 90 μL of hexane in a laminar flow hood. A 1 N KOH solution was then added equimolar to the fatty acid, and the mixture was vortexed for 10 s. The salt was dried under nitrogen until it had a white, chalky appearance. The salt was immediately diluted in 90 μL of warm (<50 °C) distilled deionized water and mixed with 910 μL of a 25% BSA solution (25% BSA in DPBS + HEPES, pH 7.2, sterile filtered). The pH was checked and adjusted as necessary with 1 N KOH. The conjugated fatty acid solutions were aliquoted, flushed with argon, and frozen at −80 °C. Aliquots of the BSA solution were stored similarly to be used as control. Conjugation of all mono- or polyunsaturated fatty acids was carried out under argon flow, using a sterile technique and disposable sterile vials and pipets. For oxidized solutions the same procedure was followed except that fatty acid dilution in hexane was followed by a 30 min incubation at 80 °C under oxygen.

Cell Culture. HCASM and HCAE cells were grown using Cascade Medium 231 or 200 (Gibco, Portland, OR, USA), respectively. Cells were fed every other day until 80% confluence and split at 1 to 3 or 1 to 2. Trypsin solution from Cascade was applied to lift the cells; trypsin was then neutralized with CMF-PBS. Cells were spun down and resuspended at a density of 10500 cells/cm^2 in their corresponding media using 6-well plates. For the different treatments, cells were incubated at 37 °C and 5% CO_2 in a humidified incubator (Sanyo Biomedical, Wood Dale, IL, USA).

Induction of Inflammation. Cells were treated with fatty acids at a final concentration of 200 μM in the media. A BSA control was included in each experiment. BSA never exceeded 0.5% in the media. Cells were incubated for 8 h (HCAE) or 20 h (HCASM). Following incubation, cell culture supernatant was removed and analyzed by ELISA for IL-6, IL-8, and MCP-1. Cells were washed once with PBS, scraped, and treated with RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor mixture, 1 mM PMSF, and 2 mM sodium orthovanadate, followed by incubation at 4 °C for 30 min. The cell lysates were then centrifuged at 14000*g* and 4 °C for 30 min to remove impurities. Protein concentration in the cytoplasmic extracts was determined with the Bio-Rad DC Protein Assay Kit (Hercules, CA, USA). Supernatants and lysates were analyzed immediately or aliquoted and stored at −80 °C until further analyses. Cell viability was assayed using the MTT CellTiter 96 Non-Radioactive Cell Proliferation Assay from Promega (Madison, WI, USA).

COX-2 was analyzed in selected treatments by Western blot. Briefly, equal protein levels (50 μg) of the cytoplasmic extracts were separated using electrophoresis with 12% Bis-Tris gels and transferring to PVDF membranes. Membranes were blocked with 5% BSA in TPBS (PBS + Tween 20 at 0.5%), followed by an incubation with primary goat anti-COX-2 antibody 1:200 with 5% BSA in TTBS. The bound antibody was visualized by probing with horseradish peroxidase-conjugated secondary antibody (rabbit anti-goat 1:2000) followed by exposure to chemiluminescent substrate (SuperSignal West Pico, Thermo Scientific, Rockford, IL, USA) with X-ray film (Kodak X-omat 1000A processor). Densitometry of Western blot bands was performed with Quantity One 1-D analysis software version 4.6.5 (Bio-Rad Laboratories).

Statistical Analysis. Inflammation marker levels were normalized on the basis of the nontreated control and adjusted for cell viability, where marker levels for BSA control were set to 1.0. All results are

presented as the mean ± SD of three separate experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA). Separation of means was performed by the Tukey's Studentized test (SAS, version 9.2). Differences were considered statistically significant at $P < 0.05$.

■ RESULTS AND DISCUSSION

The effect of carbon chain length on levels of inflammatory markers was evident in both endothelial and smooth muscle cells. As seen in Figure 1, levels of IL-6 were directly related with carbon chain length for fully saturated fatty acids of 4−14 carbons on HCAE and all fatty acids on HCASM cells. The longest saturated fatty acids tested on HCASM cells, myristic (14:0), palmitic (16:0), and stearic (18:0), induced IL-6 levels significantly higher than the control. A similar chain length effect was observed in the levels of IL-8 for fatty acids of 4−14 carbons on HCAE and fatty acids of 12−16 carbons on HCASM cells. Palmitic acid (16:0) was the only fatty acid that increased IL-8 levels higher than control on both cell lines. MCP-1 levels also increased with increasing fatty acid chain length up to 16 carbons; however, significant differences from control were observed only for palmitic acid on HCASM cells. Interestingly, MCP-1 levels decreased in cells treated with stearic (18:0) acid, and levels were significantly lower than control in HCASM cells treated with saturated fatty acids with 12, 14, and 18 carbons in the main chain. Overall, myristic and palmitic acids were the most pro-inflammatory of the fully saturated fatty acids.

The effect of *all-cis* unsaturated fatty acids on inflammation marker levels was evaluated in both cell lines (Figure 2). When treated with 18-carbon fatty acids, levels of IL-6 in both cell lines decreased as the amount of double bonds increased from zero to two and then slightly increased with three double bonds. This slight increase may have been due to oxidation during sample preparation. Levels of IL-6 decreased in cells treated with 20-carbon fatty acids. These longer unsaturated fatty acids also exhibited decreases in inflammatory markers with increasing unsaturation from four to five double bonds, but the effect was evident only in HCAE (Figure 2A). Similarly, IL-8 levels in HCASM cells decreased with increasing unsaturation only for 20-carbon chain fatty acids (Figure 2B). MCP-1 levels in HCASM cells (Figure 2B) were equal to or lower (18:0, 18:2) than control for all treatments, with no apparent relation to degree of unsaturation. IL-8 and MCP-1 levels remained unchanged for all treatments in HCAE (not shown).

The effect of *trans* unsaturation was evaluated using the 18-carbon fatty acids oleic (18:1), elaidic (18:1 *trans* 9), and *trans*-vaccenic (18:1 *trans* 11). Significant increases in IL-6 and MCP-1 levels were observed only in HCASM cells for *trans*-vaccenic and elaidic acids, respectively (Figure 3B). All other treatments were not different from the control or from each other (not shown).

Linoleic acid (18:2) conjugation did not have a clear effect as compared to its nonconjugated counterpart (Figure 3). Levels of IL-6 did not differ among both forms of linoleic acid, but were equal to or lower than control in both cell lines (Figure 3). MCP-1 levels marginally increased with conjugation in HCASM cells, where MCP-1 for linoleic acid was lower than control. IL-8 levels remained unchanged for both treatments and cell lines (not shown). Overall, linoleic acid proved to be the least pro-inflammatory fatty acid on both cell lines.

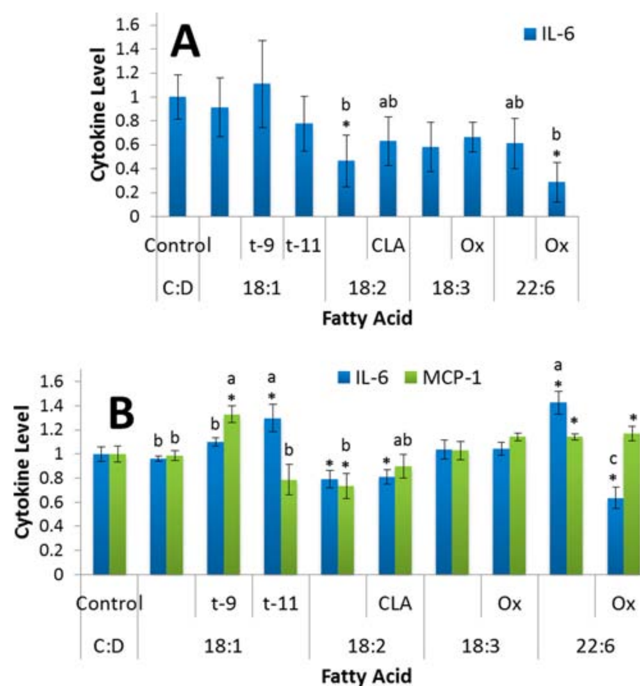

Figure 3. Effect of *trans* (t), conjugated (CLA), and oxidized (Ox) fatty acids on levels of IL-6 and MCP-1 in HCAE (A) and HCASM (B) cells. * Treatment is different from control. Different letters indicate differences between the treatment and its corresponding *all-cis* or nonoxidized counterpart. C, fatty acid carbon chain length; D, degree of unsaturation.

The effect of fatty acid oxidation on inflammation marker levels was evaluated using linolenic acid (18:3) and DHA (22:6), both omega-3 fatty acids (Figure 3). Treatment with oxidized linolenic acid did not result in changes of inflammatory markers on either cell line, as all levels did not differ from control or from the nonoxidized treatment. In contrast, oxidized DHA resulted in a reduction of IL-6 for both cell lines to levels lower than control. Notably, IL-6 levels for HCASM treated with nonoxidized DHA were significantly higher than control. Levels of MCP-1 in HCASM did not differ for cells treated with oxidized DHA, as compared to its nonoxidized counterpart; however, the levels were higher than control in both cases. IL-8 levels in both cell lines treated with DHA and oxidized DHA did not differ from control or from each other (not shown).

COX-2 analysis (Figure 4) revealed a stimulation of this marker by oxidized linolenic acid and DHA in HCAE and by palmitic acid in HCASM cells. No major differences were observed between oxidized and nonoxidized omega-3 fatty acid treatments, except for HCAE cells treated with nonoxidized DHA (22:6), which yielded COX-2 levels higher than control. In contrast, COX-2 levels in HCASM cells treated with DHA or oxidized DHA were consistently lower than control.

Increases in levels of secreted cytokines may be due to the production of mitochondrial reactive oxygen species (ROS) deriving from fatty acid β-oxidation in the cells, a phenomenon previously observed in kidney damage in diabetes.[27] Studies have also shown that increased oxidation of FFAs in aortic endothelial cells without added insulin results in an increase in production of superoxide in the mitochondria, which in turn activates the pro-inflammatory signal cascade and inactivates

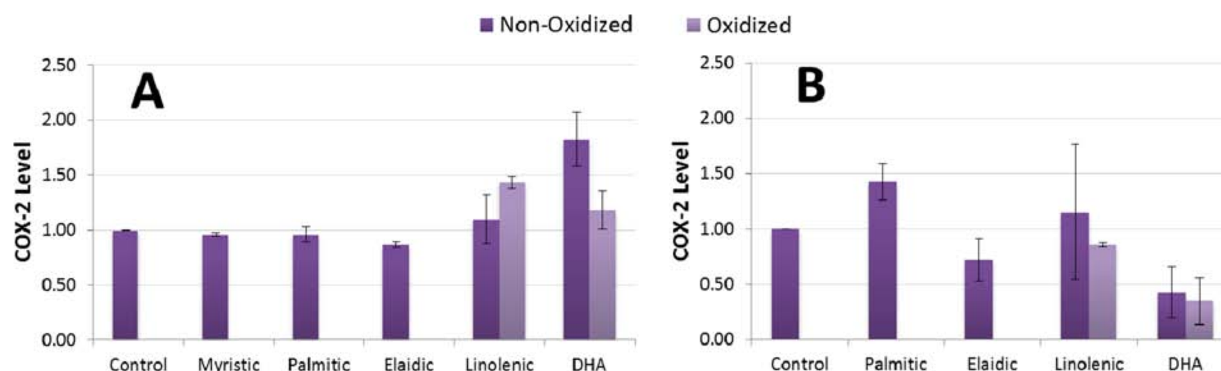

Figure 4. Changes in levels of COX-2 in the cytoplasm of HCAE (A) and HCASM (B) cells as influenced by treatment with selected fatty acids.

Table 1. Fatty Acid Composition of Common Fats and Oils (Adapted from ISEO, 2006)[41]

oil or fat	fatty acid %								
	butyric 4:0	lauric 12:0	myristic 14:0	palmitic 16:0	stearic 18:0	oleic 18:1	linoleic 18:2	linolenic 18:3	trans FAs
beef tallow			3	24	19	43	3	1	1−8
butterfat	4	3	11	27	12	29	2	1	1−8
canola				4	2	62	22	10	
cocoa butter				26	34	34	3		
coconut		47	18	9	3	6	2		
corn				11	2	28	58	1	
cottonseed			1	22	3	19	54	1	
lard			2	26	14	44	10		
olive				13	3	71	10	1	
palm kernel		48	16	8	3	15	2		
palm			1	45	4	40	10		
peanut				11	2	48	32		
safflower				7	2	13	78		
soybean				11	4	24	54	7	
sunflower				7	5	19	68	1	

prostacyclin synthase and eNOS, important antiatherogenic enzymes.[28]

Reductions in IL-6 and MCP-1 observed with increasing double bonds in endothelial and smooth muscle cells (Figure 2) may be due to reduction in cell activation, as previously observed in endothelial cells, where a greater number of double bonds (not necessarily the position of the last double bond) was critical for the greater activity of omega-3 as compared to omega-6 fatty acids in the inhibition of endothelial activation. This activation refers to the ability of the cell to promote monocyte migration, a process in which both IL-6 and MCP-1 are important signaling proteins.[29]

Table 1 illustrates the fatty acid composition of common fats and oils. Although a breakdown of *trans*-fatty acids is not shown, naturally occurring *trans*-fatty acids are present in small amounts in ruminant fats and include *trans*-vaccenic acid and conjugated linoleic acid (CLA).[30] Partially hydrogenated vegetable oils may contain *trans*-fatty acids, mainly elaidic acid, at levels ranging from 10 to 40%.[31] Major dietary sources of arachidonic acid (20:4) are meat, poultry, and game followed by fish and seafood. Dietary sources of EPA (20:5) and DHA (22:6) are mainly fish and seafood.[32] Shellfish fat may contain up to 4.5% arachidonic acid (sea scallops), 21.5% EPA (Pacific oyster), and 22.6% DHA (sea scallops). Marine and fresh water fish oils may also contain up to 3.9% ARA, 13.5% EPA, and 21.9% DHA.[33]

Several studies have shown that plasma fatty acid composition is a good indicator of and can be modified by the fatty acid composition of the diet.[34−36] On the basis of the results of our study, the fatty acids with higher pro-inflammatory potential through the production of inflammation markers are myristic and palmitic acids. Important sources of these fatty acids include coconut and palm kernel oil (myristic) and palm oil (palmitic). In contrast, the least pro-inflammatory fatty acid, and perhaps even with anti-inflammatory potential, was linoleic acid, an omega-3 fatty acid that can be found prominently in safflower, sunflower, corn, cottonseed, and soybean oils. Increased consumption of such oils could potentially result in reduced cardiovascular risk due to a reduction in the production of inflammatory markers by coronary arterial cells. Studies have also identified hyper-cholesterolemic effects of myristic, palmitic, and lauric acid and cholesterol-lowering effects of linoleic acid.[37−39] Changes in plasma cholesterol in addition to inflammatory effects may further explain how increased FFAs in plasma contribute to an elevated risk of CVD. Although the results of our study do not suggest a clear anti-inflammatory effect of the omega-3 fatty acids EPA and DHA, consumption of foods and oils rich in EPA and DHA should not be discouraged due to other well-documented beneficial actions that play an important role in cardiovascular health, such as cholesterol-lowering effects.[40]

Results of our study also suggest that palmitic and myristic acids may be utilized in an in vitro system to stimulate the production of markers of inflammation from human arterial coronary cells. Further studies should focus on evaluating fatty

acid mixtures simulating the composition of dietary relevant fats and oils.

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Notes

The authors declare no competing financial interest.

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