Oleic acid inhibits stearic acid-induced inhibition of cell growth and pro-inflammatory responses in human aortic endothelial cells

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Abstract **Saturated fatty acids (SFAs), significant components of both enteral/parenteral nutritional formulations (including diet), are linked to cardiovascular disease complications, such as atherosclerosis. We investigated whether oleic acid (C18:1n-9) reduces the growth inhibitory and pro-inflammatory effects of the stearic acid (C18:0) in human aortic endothelial cells (HAEC). Stearic acid induced growth inhibition at concentrations less than 50 μM, whereas higher concentrations invoked cytotoxicity. Stearic acid-induced growth inhibition and cytotoxic effects were eradicated upon cosupplementation with oleic acid (25 μM). Oleic acid (as low as 5 μM) also inhibited the stearic acid-induced increase in intercellular adhesion molecule-1 (ICAM-1) expression. Stearic acid-induced phosphorylation of nuclear factor-kappa B (NF-κB), a transcriptional regulator of ICAM-1, was also reduced by oleic acid. HAECs supplemented with either stearic or oleic acid resulted in cellular incorporation of C18:0 and C18:1n-9, respectively. Stearic acid primarily incorporated into phospholipids without increasing the total fatty acid content in HAECs. In contrast, oleic acid, with or without stearic acid, incorporated into both phospholipids and triglycerides, with a significant increase in total fatty acid amounts in triglycerides. Our data suggest that oleic acid has the ability to reduce the inflammatory effects of long-chain SFAs in HAECs through reducing cellular stearic acid incorporation and NF-κB activation.**—Harvey, K. A., C. L. Walker, Z. Xu, P. Whitley, T. M. Pavlina, M. Hise, G. P. Zaloga, and R. A. Siddiqui. **Oleic acid inhibits stearic acid-induced inhibition of cell growth and pro-inflammatory responses in human aortic endothelial cells.** ***J. Lipid Res.*** 51: **3470–3480.**

Supplementary key words apoptosis • fatty acid • dietary lipid • inflammation • lipid droplet • triglyceride

Saturated fatty acids (SFAs) represent endogenously synthesized and dietary compounds that substantially contribute to the development of coronary artery disease (CAD) (1). Current scientific evidence suggests that an accumulation of long-chain SFAs in vascular cells leads to lipid-mediated vascular cell dysfunction (2–5). SFAs vary in their carbon chain lengths from 2 to 24 carbons, and the different chain lengths impart various physiological activities to the fatty acids (6–16). For example, the long-chain SFAs myristate and palmitate acetylate cellular proteins that regulate various cellular functions (12–16). In a recent study, we demonstrated that the long-chain SFAs palmitic and stearic acids induce pro-inflammatory responses and initiate the onset of apoptosis in human aortic endothelial cells (HAEC) (17). Other investigators have also demonstrated that long-chain SFAs, specifically palmitic and stearic acids, induce apoptosis within a variety of vascular cell types (18–22). Endothelial inflammation and apoptosis play important roles in the initiation and progression of atherosclerosis (23–26).

Under pathophysiological conditions, fatty acid concentrations are capable of reaching into the millimolar range (20, 27–29). Furthermore, during lipolysis of lipoprotein triglycerides by the vasculature, fatty acid levels may increase substantially at the endothelial cell surface. Significant increases in plasma free fatty acid levels have also been reported in individuals infused with lipid emulsions during parenteral nutrition (30). As long-chain SFAs represent major components of both parenteral and enteral

The work was supported by a grant from Baxter Healthcare Corporation, Deerfield, IL. The sponsor helped design the experiments and was responsible for the decision to submit the article for publication, but it played no part in the analysis or interpretation of data.

Manuscript received 6 August 2010 and in revised form 14 September 2010.

Published, JLR Papers in Press, September 17, 2010
DOI 10.1194/jlr.M010371

Abbreviations: BCA, bicinchoninic acid; CAD, coronary artery disease; EBM-2, endothelial basal medium-2; EGM-2MV, endothelial growth medium-2 microvascular; HUVEC, human umbilical vein endothelial cell; HAEC, human aortic endothelial cell; ICAM-1, intercellular adhesion molecule-1; NF-κB, nuclear factor-kappa B; PE, phycoerythrin; SFA, saturated fatty acid.

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nutritional formulations/diets, the potential for adverse vascular effects initiated by SFAs are cause for clinical concern. These fatty acids may contribute to inflammation and vascular dysfunction in patients with both acute and chronic diseases.

The Mediterranean diet and olive oil have been associated with a reduced risk of cardiovascular disease (31, 32). The reduction in cardiovascular risk with olive oil occurs despite maintenance of SFA intake (33). One of the primary fatty acid components of the Mediterranean diet and olive oil is oleic acid, a monounsaturated fatty acid. Carluccio et al. hypothesized that the cardioprotective effects of the Mediterranean diet were a result of oleic acid's ability to inhibit endothelial activation (34). Their findings provide evidence for oleic acid's ability to diminish endothelial cell activation, irrespective of the stimulus utilized, by suppressing the upregulation of adhesion molecules. In a randomized trial, an oleic acid-rich diet was found to improve endothelial-dependent vasodilation (35). It has also been reported that oleic acid can protect Chinese hamster ovary cells from palmitate-induced lipotoxicity by promoting triglyceride synthesis within the cells (36). Gao et al. investigated the protective effect of oleate against palmitate-induced insulin resistance in L6 myotubes and reported that the protective effect was dependent upon a phosphoinositide 3-kinase mechanism (37). In MDA-MB-231 breast cancer cells, oleate counteracted the ability of SFAs to induce apoptosis (38). Other investigators have reported the beneficial effects of cosupplementing SFAs with oleic acid in other cell types (36, 37, 39, 40).

Because nutritional formulations (both parenteral and enteral) and diets contain significant amounts of long-chain SFAs that are potentially harmful to the vasculature of patients with both acute and chronic inflammatory diseases, the present investigation was undertaken to determine if oleic acid cosupplementation could reduce SFA-induced pro-inflammatory responses and cytotoxicity. This study characterizes oleic acid's ability to prevent SFA-induced endothelial cell growth inhibition and cytotoxicity, as well as its ability to diminish SFA-induced endothelial cell pro-inflammatory phenotypes. Furthermore, this study evaluates fatty acid incorporation and compartmentalized within the cell. Our results suggest that oleic acid neutralizes the negative impact of SFAs on endothelial cell functions by displacing stearic acid from the phospholipid bilayer, thereby preventing stearic acid-induced signaling cascades that promote inflammation and/or induce apoptosis.

MATERIALS AND METHODS

Materials

HAEC and the EGM-2MV Bullet kits (endothelial growth medium-2 microvascular) were purchased from Lonza Incorporation (Walkersville, MD). Chemicals and reagents were acquired from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted. Consumable tissue culture materials were obtained from Fisher Scientific (Pittsburgh, PA). The cell proliferation reagent WST-1 and the Annexin V FLUOS staining kits were purchased from Roche Applied Science (Indianapolis, IN). All fatty acids were acquired from Nu-Chek Prep, Inc. (Elysian, MN). Tritricosanoin and 1,2 ditricosanoyl-glycero-3-phosphocholine were purchased from Avanti Polar Lipids (Alabaster, AL). Oil Red O staining kit was obtained from American MasterTech (Lodi, CA).

HAEC culture

A primary cell line derived from HAEC was maintained in endothelial basal medium-2 (EBM-2) containing 5% FBS and the bullet kit materials as specified by the manufacturer. Cells were maintained at 37°C in a humidified atmosphere in the presence of 5% CO_2. Only endothelial cell cultures of less than 10 passages and 80–90% confluence were used in this study.

Growth inhibition and cytotoxicity assay

Endothelial cells (5000 cells/well) were cultured for 6 h in EBM-2 complete medium in 96-well, flat bottom tissue culture plates to establish a linear growth rate. At this stage, a subset of established wells were used to determine cellular growth (X) (baseline at day 0) by administering WST-1 reagent (10 μl/well), whereas medium in other wells was replaced with EBM-2 complete medium supplemented with treatment conditions (100 μl total volume/well) for growth inhibition assessment. Cell cultures were maintained for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. WST-1 reagent was added, and readings were used to calculate growth inhibition or cell loss based on baseline readings. Vehicle controls (EBM-2 supplemented with 2.2% fatty acid BSA) were used to determine total cell growth (Y). The cell growth (Z) was normalized to 100% using the formula $[(Y - X / X) \times 100]$. Effects of fatty acid supplementation were determined compared to Z values. Values from 100 to 0 indicate cell growth inhibition, whereas values from 0 to −100 indicate cell death. Results are expressed as mean ± SD for at least four determinations.

Apoptosis detection

Cells (1.0×10^5) were plated in EBM-2 complete medium in 6-well tissue culture-treated plates overnight to initiate a linear growth rate. Spent media was replaced with 2 ml of complete EBM-2 supplemented with varying combinations of albumin-bound stearic and oleic fatty acids. Stock solutions (1 mM) of stearic and oleic fatty acids were prepared by complexing with BSA as previously described (41). At the conclusion of the treatments, the cells were rinsed in PBS and trypsinized from the wells. All spent medium, PBS washes, and trypsinized cells were collected and combined to harvest both adherent and nonadherent cells for analysis. Cell pellets were resuspended in Annexin V FLUOS/propidium iodide labeling solution, which was prepared as described by the manufacturer. Cell suspensions were labeled in the dark at room temperature for 20 min. Analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15 mW air-cooled argon-ion laser emitting at a 488 nm wavelength. Annexin V FLUOS was detected through a 530 nm band pass filter, while propidium iodide was detected through a 650 nm long pass filter. Data were quantified using CellQuest software (Becton Dickinson).

Flow cytometric analysis of adhesion molecule expression

Trypsinized endothelial cells (1×10^5/sample) were washed in PBS containing 0.5% BSA and resuspended into a volume of 100 μl of this labeling buffer. Cells were labeled with 0.25 μg of phycoerythrin (PE)-conjugated intercellular adhesion molecule-1 (ICAM-1), otherwise known as CD54, antibody for 20 min. Subsequently, the cells were washed twice in PBS containing 0.5% BSA and resuspended into 250 μl of the wash buffer. An isotype control was established for each sample set to ensure specificity of

the antibody binding. Analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson) equipped with a 15 mW air-cooled argon-ion laser emitting at a 488 nm wavelength. Fluorescence was detected through a 585 nm band pass filter and quantified using CellQuest Software (Becton Dickinson). Results indicate the mean fluorescent intensity of gated endothelial cells, which excluded cellular debris and particles.

Western blot analysis

HAEC cultures were supplemented with albumin-bound fatty acids for 24 h in 2 ml of EBM-2 complete medium in 6-well tissue culture-treated plates. Treated cells were solubilized with 250 μl of lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 100 mM NaF, 2 mM Na_3VO_4, 10% glycerol, 1% Nonidet P-40, 2 mM PMSF, 1 μg/ml leupeptin, 0.15 units/ml aprotinin, and 2.5 mM diisopropyl fluorophosphate) for 10 min on ice. Detergent-solubilized extracts were centrifuged to remove insoluble matter, and the protein content of each sample was determined using a BCA (bicinchoninic acid) Protein Assay Kit (Pierce, Rockford, IL). Subsequently, 10 μg of protein in Laemmli sample loading buffer was loaded onto each lane and separated on a 4–12% polyacrylamide gradient gel. Resolved proteins were electrophoretically transferred onto nitrocellulose membranes (Millipore Corporation, Bedford, MA) for immuno-Western blot analysis. Blots were then incubated in specific primary antibodies according to the manufacturer's specifications, and proteins were detected using a peroxidase-conjugated secondary antibody (1:2500 dilution in TBS containing 1% Tween 20 and 1% BSA) with an ECL system (Amersham Pharmacia Biotechnology, Piscataway, NJ) as specified by the manufacturer. For reprobing, membranes were stripped in buffer consisting of 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50°C, followed by six washes in TBS containing 1% Tween 20. Stripped blots were blocked and reprobed with an alternative antibody.

Fatty acid incorporation into the endothelial cells

Subconfluent endothelial cells were cultured for 24 h in EBM-2 complete media either in the presence or absence of fatty acids. After incubation, the cells were trypsinized and washed three times in PBS (calcium- and magnesium-free) containing 0.5% fatty acid-free BSA to ensure removal of free fatty acids. Cell pellets were resuspended in PBS and sonicated to lyse the cells. An internal standard (C23:0) was added at a known volume to the cell lysates. A portion of the remaining lysates was used to determine the protein concentration to quantify fatty acid content in relationship to the protein content.

Lipids were extracted with chloroform-methanol (2:1) using the Folch method (42). Using a modified procedure from Lepage and Roy, the fatty acids were transesterified in 2 ml of methanol-benzene (4:1, v/v) after cooling the samples in a dry ice bath for 10 min and adding acetyl chloride to each sample (43). Transesterification occurred at room temperature over a 24-h incubation period. The reaction was neutralized with the addition of a 6% K_2CO_3 solution. The samples were vortexed and centrifuged. The benzene layer was recovered and separated on a GC system (Shimadzu GC2010) equipped with a Zebron ZB-WAX plus column (100 m, 0.25 mm ID, 0.25 μm film thickness). The oven temperature was programmed from 30°C (2 min hold) to 180°C at 20°C/min (2 min hold), then to 207°C at 4°C/min (3 min hold), then to 220°C at 2°C/min (2 min hold), and finally to 240°C at 2°C/min (2 min hold). Detection was performed with a flame ionization detector at 250°C to resolve fatty acids peaks, which were identified using authentic standards (Restek Corp., Bellefonte, PA). Data was analyzed with Shimadzu's GC solutions software.

Fatty acid analysis of the phospholipid and triglyceride fractions was performed by extracting and preparing cell lysates as follows. Internal standards for phospholipids (1,2 ditricosanoyl-glycero-3-phosphocholine; C23:0-PL) and triglycerides (tritricosanoin; C23:0-TG) were added to a known volume of cell lysates. Lipids were extracted using the Folch method. The lipids were resolved on silica gel G-coated plates via thin layer chromatography using a benzene/diethyl ether/ethyl-acetate/acetic acid (80:10:10:0.2 v/v) solvent system. Using standards, the phospholipid and triglyceride fractions were identified by spraying the plates with a primulin (0.01% w/v) solution in acetone-water (40:40 v/v) and exposing to an UV light source. The identified bands were scraped from the silica gels, and 2 ml of methanol: benzene (4:1 v/v) was added to each sample tube. The tubes were gently vortexed and sonicated for 5 min in a water bath at room temperature. Tubes were then placed on a dry ice bath for 10 min before the addition of 200 μl of acetyl chloride. Samples were purged with nitrogen gas and permitted to acclimate to room temperature. Triglyceride samples were placed in a heating block for 10 min. at 70°C to facilitate methanolysis of the internal standard. Transesterification of all samples occurred at room temperature over a 24-h incubation period. This reaction was neutralized and recovered as previously described. The GC column and conditions are described above.

Oil Red O staining of lipid droplets

Triglycerides and cholesterol esters can be packaged intracellularly as lipid droplets. HAECs were plated on 4-well Permanox chamber slides and supplemented with albumin-bound fatty acids for 24 h. The neutral lipids packaged within the HAECs were stained using an Oil Red O staining kit procedure described by the manufacturer. Cell nuclei were counterstained with modified Mayer's hematoxylin.

Statistical analysis

Data represent the mean ± SD of at least three determinations. Statistical significance between groups of data was determined using Student's *t*-test. When a calculated *P* value of less than 0.05 is reported, statistical significance is indicated with an asterisk.

RESULTS

Neutralizing effect of oleic acid on stearic acid-induced HAEC growth inhibition and cytotoxicity

Endothelial cells were treated with varying concentrations of stearic and oleic acid for 48 h under standard culture conditions. **Fig. 1** depicts the attenuating effect of oleic acid on stearic acid-induced growth inhibition and cytotoxicity. Stearic acid inhibited growth of HAECs at concentrations up to 50 μM. At higher concentrations, stearic acid induced cytotoxicity (i.e., cell death). Cosupplementation of stearic acid with oleic acid, at concentrations as low as 5 μM, significantly improved the negative impact of stearic acid. Oleic acid (25 μM) cosupplementation negated the growth inhibitory effects of up to 50 μM stearic acid. Furthermore, oleic cosupplementation prevented stearic-induced cytotoxicity at higher concentrations. Although oleic cosupplementation prevented the stearic acid-induced cytotoxicity at the 100 μM dose, the HAECs were still significantly growth inhibited.

Effect of oleic acid supplementation on stearic acid-induced apoptosis/necrosis

HAECs were treated with stearic and oleic acid for 24 h prior to labeling with Annexin V FLUOS and propidium

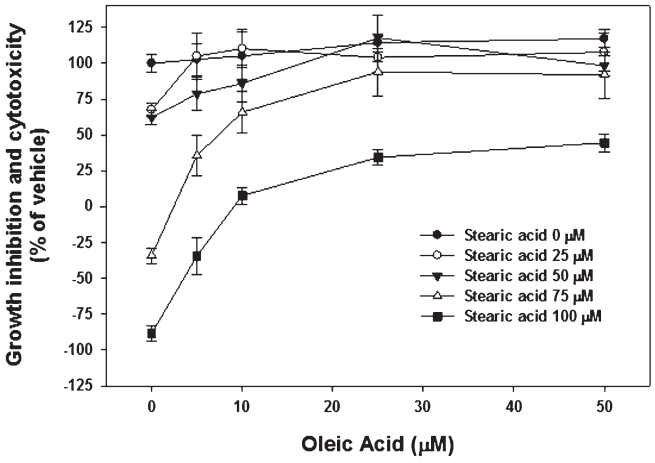

Fig. 1. Effect of oleic acid cosupplementation on stearic acid-induced HAEC growth inhibition and cytotoxicity. HAECs were supplemented with albumin-bound stearic and oleic acids at varying concentrations for 48 h at 37°C in 5% CO_2. Cells were administered the WST-1 assay reagent to determine the metabolic activity of the cells. Values between 0 and 100 represent growth inhibition, whereas values below 0 indicate cell loss compared to the day 0 starting population. Calculations of these values are described in Materials and Methods. Data represent the mean ± SD of at least four determinations. HAEC, human aortic endothelial cell.

iodide to quantify the extent of the induction of apoptosis and/or necrosis. **Table 1** categorically summarizes the results of the fatty acid-supplemented endothelial cells. The vast majority of vehicle-treated cells were viable while a subset of both apoptotic and necrotic cell populations were observed, which denotes the natural turnover of primary cultured cells. Stearic acid treatment significantly increased HAEC apoptosis and necrosis in a dose-dependent manner. Incubation with oleic acid (10 μM) prevented the majority of the cells from developing apoptosis and necrosis. When HAECs were cosupplemented with 25 μM of oleic acid, virtually all indications of the stearic acid-induced apoptosis and necrosis were eradicated.

Effect of oleic acid supplementation on stearic acid-induced activation of caspase-3

Caspase-3 is a key executioner enzyme associated with programmed cell death (i.e., apoptosis). Activation of caspase-3 requires proteolytic cleavage into fragments, which can target the nuclear enzyme poly ADP-ribose polymerase (PARP). Stearic acid supplementation (75 μM) instigated an accumulation of cleaved caspase-3 within 24 h; however, this pro-apoptotic response was prevented with oleic acid cosupplementation (**Fig. 2**). The counteractive effects of oleic acid cosupplementation were observed at the lowest concentration assessed. These data are consistent with the apoptosis and necrosis findings in that cosupplementation of stearic acid with oleic acid (>25 μM) eliminated the initiation and progression of programmed cell death. In parallel, these data support the findings of oleic acid's ability to attenuate the stearic acid-induced cytotoxicity in HAECs.

Effect of oleic acid supplementation on stearic acid-induced ICAM-1 surface expression

Increased surface expression of ICAM-1 in endothelial cells is indicative of a pro-inflammatory phenotype; therefore, we ascertained the effect of stearic acid in combination with oleic acid supplementation on the relative abundance of ICAM-1 expression. HAECs were treated with varying concentrations of oleic acid with or without stearic acid (50 μM) for 24 h under standard culture conditions. Our investigation previously demonstrated that 50 μM of stearic acid is growth inhibitory but not apoptotic or necrotic (17). Stearic acid supplementation at levels that do not induce cytotoxicity increased ICAM-1 surface expression on HAECs in a dose-dependent manner. **Fig. 3** shows stearic acid (50 μM) induced nearly a 2-fold increase in basal ICAM-1

TABLE 1. Effect of oleic acid supplementation on stearic acid-induced apoptosis and necrosis

		Apoptosis/Necrosis (% ± SD)		
		Viable	Apoptotic	Necrotic
Vehicle		78.9 ± 1.9	7.8 ± 1.1	13.2 ± 2.0
Oleic 0 μM + Stearic	25 μM	76.5 ± 0.2	10.0 ± 0.7[a]	13.5 ± 1.0
	50 μM	65.7 ± 1.2[a]	15.2 ± 1.3[a]	19.1 ± 1.4[a]
	75 μM	28.1 ± 2.9[a]	39.0 ± 2.0[a]	32.9 ± 1.2[a]
	100 μM	9.2 ± 1.9	46.4 ± 3.1[a]	44.4 ± 2.0[a]
Oleic 10 μM + Stearic	0 μM	79.5 ± 0.6	7.4 ± 0.5	13.1 ± 0.1
	25 μM	78.9 ± 2.6	9.0 ± 1.7	12.0 ± 0.9
	50 μM	71.8 ± 0.7[a]	10.9 ± 0.6[a]	17.3 ± 1.2[a]
	75 μM	74.5 ± 1.9[a]	9.8 ± 1.5	15.7 ± 0.6
	100 μM	43.0 ± 3.8[a]	32.1 ± 2.0[a]	25.4 ± 1.1[a]
Oleic 25 μM + Stearic	0 μM	73.5 ± 4.1[a]	13.3 ± 3.7[a]	13.2 ± 2.1
	25 μM	82.3 ± 3.3	7.6 ± 1.3	10.1 ± 2.1
	50 μM	76.9 ± 2.6	8.3 ± 0.4	14.8 ± 2.5
	75 μM	77.9 ± 1.8	8.5 ± 0.6	14.0 ± 0.8
	100 μM	78.3 ± 0.6	8.1 ± 1.0	13.7 ± 0.4

Before apoptosis/necrosis analysis using flow cytometry, endothelial cells were supplemented for 24 h with oleic and stearic acids, alone or in combination. The data reflects the percentage of cells observed in each classification. Results are expressed as the mean ± SD of three determinations.

[a]Significant differences ($P < 0.05$) using Students' *t*-test compared to the vehicle control.

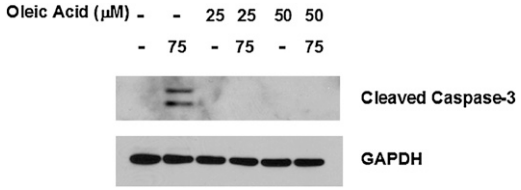

Fig. 2. Effect of oleic acid on stearic acid-induced activation of caspase-3. HAECs were treated with stearic and oleic acids, alone and in combination, for 24 h at 37°C in 5% CO_2. HAEC lysates (15 μg each) were electrophoretically separated in a 4–12% polyacrylamide gradient gel and transferred onto a nitrocellulose membrane. The blot was probed with an antibody that recognizes the cleaved active form of caspase-3, which is present as doublet bands. GAPDH served as the protein loading control. This experiment was repeated to confirm the appearance of cleaved caspase-3 in HAECs supplemented with stearic acid alone. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HAEC, human aortic endothelial cell.

surface expression in HAECs. When HAECs were incubated with oleic acid in conjunction with stearic acid (50 μM), the stearic acid-induced upregulation of ICAM-1 was completely suppressed. The inhibition of the stearic acid-induced increase in ICAM-1 expression was eliminated at even the lowest amount of oleic acid supplementation (5 μM). Notably, ICAM-1 surface expression levels were not altered upon HAEC supplementation with oleic acid alone.

Effect of oleic acid supplementation on stearic acid-induced phosphorylation of NF-κB

Stearic acid treatment significantly increased cell surface expression levels of ICAM-1; subsequently, this proinflammatory response was prevented upon oleic acid cosupplementation (Fig. 3). Activation of NF-kB by phosphorylation of the p65 subunit can lead to increased expression of ICAM-1 among many other proteins (44). Our previous investigation reported that stearic acid induced phosphorylation of IκB-α and NF-κB, the transcriptional regulators of ICAM-1 (17). HAECs supplemented with stearic acid (50 μM) induced phosphorylation of the p65 subunit of NF-κB (**Fig. 4**). Oleic acid supplementation did not alter the baseline phosphorylation status; however, when stearic acid (50 μM) was cosupplemented with oleic acid, the phosphorylation state of NF-κB remained at or below baseline activation. The counteractive effects of oleic acid cosupplementation were observed at the lowest concentration assessed.

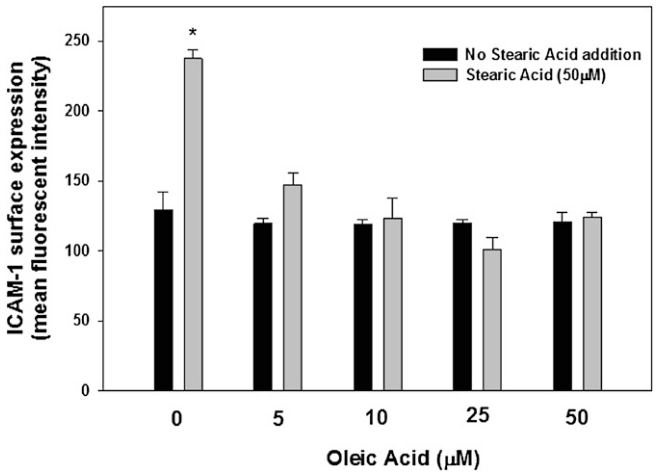

Fig. 3. Effect of oleic acid on stearic acid-induced HAEC upregulation of ICAM-1. Endothelial cells were supplemented for 24 h with oleic and stearic acids, alone or in combination. Relative ICAM-1 membrane expression levels were quantified by flow cytometry and presented as the mean fluorescent intensity ± SD of three determinations. *Statistically significant differences ($P < 0.05$) from vehicle using Student's *t*-test. HAEC, human aortic endothelial cell; ICAM-1, intercellular adhesion molecule-1.

Cellular incorporation of stearic and oleic acid

HAECs were supplemented with an equivalent quantity of stearic and oleic acids alone and in combination for 24 h prior to fatty acid extraction and GC analysis. **Table 2** summarizes the results of the targeted fatty acid analysis. Nonsupplemented vehicle cells consistently contain similar quantities of stearic and oleic acid in their composi-

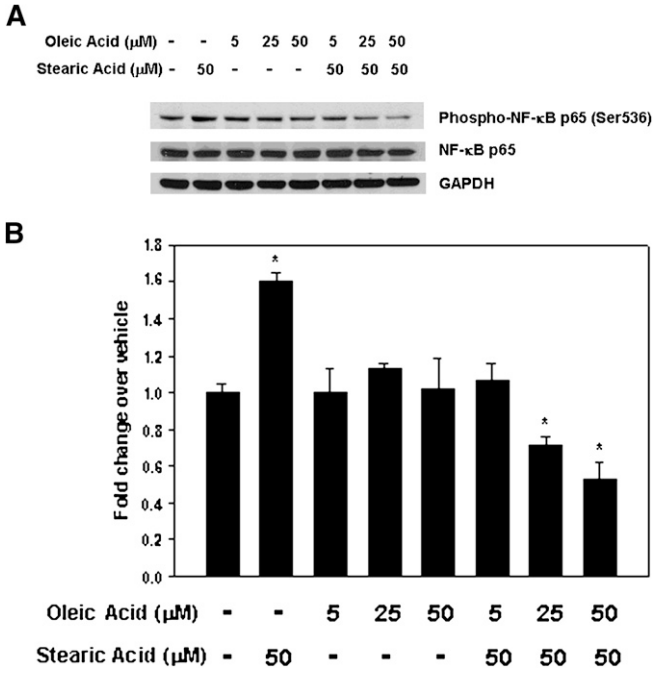

Fig. 4. Effect of oleic acid on stearic acid-induced phosphorylation of NF-κB, the transcriptional regulator of ICAM-1. A: HAECs were treated with stearic and oleic acids, alone and in combination at varying doses, for 24 h at 37°C in 5% CO_2. HAEC lysates (15 μg each) were electrophoretically separated in a 4–12% polyacrylamide gradient gel and transferred onto a nitrocellulose membrane. A phospho-specific antibody that recognizes the phosphorylation of the serine 536 residue on the p65 regulatory subunit of NF-κB determined the relative activation state of the enzyme. A nonphospho-specific antibody to NF-κB p65 and GAPDH served as protein loading controls. B: Quantification of the phosphorylated NF-κB p65 in relationship to the loading controls was determined by densitometry analysis. Data represent the mean values of three independent blots ± SD. *Statistically significant differences ($P < 0.05$) from vehicle using Student's *t*-test. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HAEC, human aortic endothelial cell; ICAM-1, intercellular adhesion molecule-1; NF-κB, nuclear factor-kappa B.

tion. In HAECs supplemented with 25 μM stearic acid, a notable increase in C18:0 was observed. Interestingly, these cells had a corresponding increase in C18:1n-9, which can be attributed to conversion from C18:0. Cells supplemented with 25 μM oleic acid more than doubled the C18:1n-9 cellular content. This increase was largely at the expense of the SFAs, specifically C16:0 and C18:0, and arachidonic acid to a lesser extent. When both fatty acids were equally cosupplemented, analogous increases were observed, albeit the levels were considerably lower than either fatty acid supplemented individually.

On the basis of our previous experimental data, it is apparent that HAECs supplemented with greater than 50 μM stearic acid initiate detrimental effects (17). We have shown in this study that these stearic acid-induced negative cellular effects can be essentially eliminated with oleic acid. Therefore, we investigated the impact of oleic acid upon stearic acid cellular incorporation. **Table 3** summarizes the effect of oleic acid dose on the fatty acid profile of endothelial cells supplemented with stearic acid (50 μM). Stearic acid supplementation alone increased the overall cellular quantity of stearic acid (C18:0) to one-half of the total fatty acid content, which is nearly triple the amount present in control cells. Interestingly, cosupplementation of stearic acid with as little as 5 μM oleic acid dramatically reduced the percentage of C18:0 in the cells (**Fig. 5** and Table 3). At concentrations greater than 10 μM oleic acid, C18:0 was overtaken by C18:1n-9 in terms of percentage of the fatty acid composition, and the differences are exaggerated at even higher concentrations. When equimolar concentrations of both stearic and oleic acids are presented to endothelial cells, oleic acid is preferentially incorporated (Table 3).

TABLE 2. Fatty acid composition of human aortic endothelial cells supplemented with stearic and oleic acids

Fatty Acid	Vehicle (% ± SD)	Stearic 25 μM (% ± SD)	Oleic 25 μM (% ± SD)	Stearic and Oleic 25 μM (% ± SD)
C8:0	0.32 ± 0.11	0.25 ± 0.03	0.25 ± 0.03	0.24 ± 0.05
C10:0	0.28 ± 0.08	0.23 ± 0.05	0.19 ± 0.02	0.25 ± 0.02
C12:0	0.24 ± 0.09	0.11 ± 0.02	0.13 ± 0.01	0.20 ± 0.04
C14:0	3.24 ± 0.13	1.40 ± 0.14	1.68 ± 0.05	1.24 ± 0.08
C16:0	27.45 ± 1.37	14.99 ± 1.47	15.15 ± 0.85	13.05 ± 0.54
C16:1n-7	3.18 ± 0.17	1.62 ± 0.08	1.38 ± 0.15	1.14 ± 0.06
C18:0	18.10 ± 0.66	31.46 ± 0.95	13.35 ± 1.48	26.31 ± 1.05
C18:1n-9	19.02 ± 0.97	26.03 ± 0.87	50.22 ± 5.84	38.79 ± 1.64
C18:1n-7	7.90 ± 0.79	4.60 ± 0.42	5.06 ± 0.89	4.41 ± 0.23
C18:2n-6	1.61 ± 0.07	1.06 ± 0.04	0.71 ± 0.07	0.64 ± 0.03
C18:3n-6	0.19 ± 0.01	0.12 ± 0.05	0.11 ± 0.02	0.08 ± 0.02
C18:3n-3	0.10 ± 0.03	0.13 ± 0.04	0.10 ± 0.05	0.11 ± 0.03
C20:0	0.17 ± 0.02	0.37 ± 0.04	0.13 ± 0.02	0.30 ± 0.03
C20:4n-6	11.45 ± 0.54	12.25 ± 0.39	6.91 ± 0.87	8.81 ± 0.35
C22:5n-3	0.23 ± 0.08	0.27 ± 0.03	0.15 ± 0.04	0.16 ± 0.02
C22:0	0.23 ± 0.04	0.21 ± 0.01	0.15 ± 0.03	0.19 ± 0.01
C22:5n-6	1.93 ± 0.14	1.16 ± 0.01	1.14 ± 0.12	0.97 ± 0.06
C22:5n-3	2.08 ± 0.17	1.86 ± 0.06	1.68 ± 0.18	1.64 ± 0.09
C24:0	0.31 ± 0.04	0.26 ± 0.01	0.22 ± 0.02	0.21 ± 0.01
C22:6n-3	1.96 ± 0.20	1.62 ± 0.12	1.32 ± 0.13	1.26 ± 0.05

HAECs were treated with oleic and stearic acids, alone or in combinations, for 24 h. Lipid extracts were analyzed by GC to determine the fatty acid profiles for each treatment Results were calculated based on the addition of an internal standard and normalized to protein content. Data represent the mean ± SD of three independent treatments as the percentage of fatty acid composition.

HAEC, human aortic endothelial cell.

Phospholipid and triglyceride fatty acid characterization

Clearly, HAECs supplemented with stearic and oleic acids dramatically altered the fatty acid profile; therefore, we set forth to determine where these fatty acids were incorporated. Phospholipid and triglyceride fractions were obtained from HAECs supplemented with either 50 μM stearic acid, oleic acid, or a combination of both. The fatty acid composition of the phospholipid components are summarized in **Table 4**. Stearic acid-supplemented cells significantly increased their C18:0 content. A slight increase in C18:1n-9 was also observed, while palmitic acid assumed the greatest loss of any individual fatty acid. HAECs supplemented with 50 μM oleic acid substantially increased the amount of oleic acid present in the phospholipid component. Moreover, the fatty acid combination resulted in a fatty acid pattern that was intermediate between the stearic acid- and oleic acid-supplemented cells.

The triglyceride component (**Table 5**) was relatively small compared to the fatty acid content present in phospholipids (Table 4). The amount and composition of the triglycerides in stearic acid-supplemented HAECs did not substantially differ from vehicle controls; however, a small increase in C18:0 was observed. HAECs supplemented with oleic acid generated a notable increase in the amount of fatty acids stored in the form of triglycerides, which predominantly consisted of oleic acid. Furthermore, the stearic and oleic acid combination resulted in an increase in triglyceride content but at levels that fell short of oleic acid supplementation alone. Oleic acid was a major contributor to the fatty acid triglyceride composition, followed by stearic and palmitic SFAs.

Accumulation of fatty acids in the form of triglycerides

To confirm the triglyceride accumulation in endothelial cells supplemented with oleic acid, we utilized an Oil red O staining kit to visualize the lipid droplets. **Fig. 6** represents HAECs supplemented with 50 μM of oleic acid, stearic acid, or a combination of both compared to vehicle-treated cells. Excess triglycerides packaged in the form of lipid droplets were not readily apparent in vehicle and stearic acid-supplemented cells. However, an accumulation of lipid droplets were prominently displayed in endothelial cells supplemented with oleic acid, either in the presence or absence of stearic acid.

DISCUSSION

Parenteral and enteral nutrition (including oral diets) is utilized to provide healthy and ill patients with an energy source in the form of lipids. The lipids also provide essential fatty acids to these individuals. Commercially available lipids are derived from various oils that often vary between plant and animal origins. As a result, the fatty acid content of the lipids differs significantly. SFAs represent a sizable component (15–55%) of the percentage of fatty acids present in most diets used for either parenteral or enteral administration. Our previous investigation evaluated the impact of individual

TABLE 3. Effect of oleic acid cosupplementation on stearic acid-treated HAECs

Fatty Acid	Vehicle % ± SD	Stearic 50 μM % ± SD	Stearic 50 μM + Oleic 5 μM % ± SD	Stearic 50 μM + Oleic 10 μM % ± SD	Stearic 50 μM + Oleic 25 μM % ± SD	Stearic 50 μM + Oleic 50 μM % ± SD
C8:0	0.28 ± 0.06	0.32 ± 0.06	0.18 ± 0.03	0.17 ± 0.02	0.15 ± 0.02	0.15 ± 0.01
C10:0	0.26 ± 0.03	0.34 ± 0.06	0.19 ± 0.03	0.33 ± 0.27	0.14 ± 0.03	0.18 ± 0.02
C12:0	0.14 ± 0.01	0.20 ± 0.12	0.11 ± 0.02	0.07 ± 0.02	0.08 ± 0.05	0.10 ± 0.03
C14:0	3.44 ± 0.21	1.52 ± 0.17	1.13 ± 0.12	1.03 ± 0.02	0.97 ± 0.06	0.87 ± 0.03
C16:0	27.30 ± 3.12	12.02 ± 1.30	9.57 ± 1.07	8.41 ± 0.81	9.46 ± 0.80	9.85 ± 1.32
C16:1n-7	3.41 ± 0.21	1.50 ± 0.71	1.39 ± 0.13	1.23 ± 0.06	1.14 ± 0.07	1.01 ± 0.07
C18:0	17.23 ± 1.02	50.72 ± 1.51	38.38 ± 2.20	36.02 ± 1.23	31.04 ± 0.42	27.63 ± 1.56
C18:1n-9	20.33 ± 1.02	19.22 ± 2.36	31.30 ± 2.76	34.54 ± 1.28	39.04 ± 1.11	43.81 ± 1.26
C18:1n-7	8.36 ± 1.04	2.14 ± 0.16	3.79 ± 0.38	3.45 ± 0.73	4.03 ± 0.25	4.02 ± 0.80
C18:2n-6	1.66 ± 0.12	0.99 ± 0.05	1.07 ± 0.21	0.99 ± 0.07	1.00 ± 0.41	0.78 ± 0.15
C18:3n-6	0.16 ± 0.05	0.13 ± 0.01	0.10 ± 0.01	0.09 ± 0.07	0.07 ± 0.06	0.01 ± 0.01
C18:3n-3	0.05 ± 0.03	0.02 ± 0.00	0.01 ± 0.01	0.01 ± 0.0	0.01 ± 0.01	0.01 ± 0.00
C20:0	0.16 ± 0.03	0.50 ± 0.05	0.44 ± 0.02	0.43 ± 0.03	0.40 ± 0.02	0.39 ± 0.03
C20:4n-6	10.85 ± 1.67	7.27 ± 0.69	8.60 ± 0.57	9.13 ± 0.20	8.80 ± 0.61	7.62 ± 0.55
C20:5n-3	0.27 ± 0.04	0.24 ± 0.03	0.18 ± 0.05	0.26 ± 0.04	0.28 ± 0.17	0.29 ± 0.14
C22:0	0.22 ± 0.07	0.22 ± 0.04	0.12 ± 0.07	0.19 ± 0.01	0.17 ± 0.05	0.16 ± 0.04
C22:5n-6	1.79 ± 0.39	0.59 ± 0.06	0.74 ± 0.02	0.70 ± 0.09	0.72 ± 0.05	1.06 ± 0.11
C22:5n-3	1.72 ± 0.60	0.90 ± 0.14	1.12 ± 0.09	1.17 ± 0.08	1.10 ± 0.10	1.06 ± 0.11
C24:0	0.38 ± 0.02	0.31 ± 0.05	0.22 ± 0.02	0.42 ± 0.26	0.22 ± 0.03	0.24 ± 0.02
C22:6n-3	1.98 ± 0.32	0.89 ± 0.00	1.36 ± 0.06	1.35 ± 0.13	1.19 ± 0.04	1.13 ± 0.20

Stearic acid-treated (50 μM) HAECs were cosupplemented with varying doses of oleic acid (5–50 μM) for 24 h. Albumin-treated cells served as the vehicle for a baseline comparison. Lipid extracts were analyzed by GC to determine the fatty acid profiles for each treatment. Results were calculated as described. Data represent the ± SD of three independent treatments as the percentage of fatty acid composition.

HAEC, human aortic endothelial cell.

SFAs upon endothelial cell fatty acid composition, growth, and functional responses (17). Long-chain SFAs, stearic acid in particular, evoked pro-inflammatory responses and elevated quantities of stearic acid in the cells, resulting in endothelial cell apoptosis and necrosis (17). Long-chain SFAs are believed to be substantial contributors to atherosclerosis and coronary heart disease (1). Diets rich in oleic acid (i.e., Mediterranean diets) have been reported to reduce the risk of atherosclerotic disease, such as CAD (33). Therefore, we hypothesized that oleic acid antagonizes the effects of long-chain SFAs, such as stearic acid, on vascular cells. The purpose of this investigation was to determine the effects of oleic acid and stearic acid on endothelial cell integrity, function, and lipid incorporation. Our results indicate that oleic acid inhibits the toxic effects of stearic acid on endothelial cells in culture.

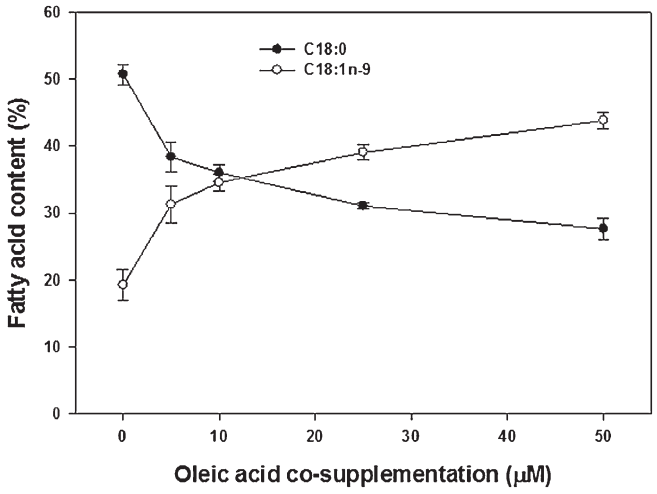

Fig. 5. Effect of oleic acid cosupplementation on the fatty acid profile of stearic acid-supplemented HAECs. HAECs were cultured with stearic acid (50 μM) in combination with varying amounts of oleic acid (0–50 μM) for 24 h. Lipid extracts were analyzed by GC to determine the fatty acid profiles for each treatment. Based on data presented in Table 3, a comparison of C18:0 and C18:1n-9 incorporation is presented as percentage of total fatty acid cellular content. Data represent a mean ± SD of three determinations. HAEC, human aortic endothelial cell.

Initially, our studies focused on the effect of oleic acid cosupplementation on stearic acid-induced growth inhibition and cytotoxicity. HAECs treated with stearic acid are growth-inhibited at lower concentrations (0–50 μM); however, when exogenously added stearic acid surpassed 50 μM, significant endothelial cell loss from both apoptosis and necrosis was observed. In our study, both the growth inhibition and cell death induced by stearic acid was inhibited upon cosupplementation with oleic acid.

We substantiated these findings using a flow cytometry-based apoptosis and necrosis detection assay. Stearic acid-induced apoptosis and necrosis was circumvented upon cosupplementation with 25 μM oleic acid. SFAs are known to induce apoptosis in a variety of cell types, including, but not limited to, breast cancer cell lines, hematopoietic cell lines, and primary cell types (19, 38, 45–47). Artwohl et al. published observations of stearic acid-induced apoptosis in HAECs and human umbilical vein endothelial cells (HUVEC) (20, 46). In the current study, we report the generation of cleaved caspase-3 in cells treated with 75 μM stearic acid. Consistent with our apoptosis and necrosis data, the appearance of cleaved caspase-3 was inhibited with oleic acid. Cleaved caspase-3 is well known to induce apoptosis (48).

TABLE 4. Analysis of the phospholipid fatty acid profile

	Vehicle	Stearic 50 μM	Oleic 50 μM	Stearic 50 μM + Oleic 50 μM
Fatty Acid	*μg/mg protein ± SD*	*μg/mg protein ± SD*	*μg/mg protein ± SD*	*μg/mg protein ± SD*
C8:0	0.31 ± 0.10	0.43 ± 0.24	0.32 ± 0.15	0.23 ± 0.01
C10:0	0.16 ± 0.06	0.19 ± 0.06	0.18 ± 0.10	0.14 ± 0.04
C12:0	0.29 ± 0.05	0.24 ± 0.06	0.19 ± 0.06	0.19 ± 0.07
C14:0	4.69 ± 0.37	1.79 ± 0.10	2.17 ± 0.65	1.60 ± 0.74
C16:0	38.22 ± 2.62	17.40 ± 0.36	21.86 ± 6.24	16.65 ± 5.22
C16:1n-7	5.05 ± 0.33	2.36 ± 0.29	2.13 ± 0.30	1.74 ± 0.22
C18:0	22.98 ± 1.91	70.88 ± 4.43	20.76 ± 6.63	39.46 ± 6.04
C18:1n-9	28.01 ± 2.04	33.47 ± 2.25	78.94 ± 8.77	57.11 ± 4.13
C18:1n-7	11.99 ± 0.76	5.51 ± 0.30	6.47 ± 0.96	5.11 ± 0.79
C18:2n-6	2.24 ± 0.32	2.55 ± 1.14	2.16 ± 1.65	1.19 ± 0.19
C20:0	0.19 ± 0.04	0.63 ± 0.06	0.16 ± 0.07	0.40 ± 0.05
C20:4n-6	16.00 ± 1.27	14.49 ± 0.81	8.17 ± 0.45	13.32 ± 0.48
C20:5n-3	0.24 ± 0.09	0.31 ± 0.05	0.20 ± 0.08	0.19 ± 0.05
C22:0	0.31 ± 0.02	0.40 ± 0.02	0.25 ± 0.04	0.32 ± 0.04
C22:5n-6	2.69 ± 0.12	1.39 ± 0.14	1.50 ± 0.13	1.50 ± 0.05
C22:5n-3	2.50 ± 0.15	1.92 ± 0.02	2.04 ± 0.22	2.17 ± 0.18
C24:0	0.51 ± 0.21	0.28 ± 0.05	0.22 ± 0.05	0.22 ± 0.04
C22:6n-3	3.09 ± 0.21	1.92 ± 0.25	1.87 ± 0.14	2.07 ± 0.03

Endothelial cells were treated with stearic and oleic acids (50 μM), alone or in combination, for 24 h before analysis. Phospholipids were resolved using thin layer chromatography and extracted as described in Materials and Methods. Fatty acid quantification was based on an internal standard and normalized to protein content. Results are expressed as the mean ± SD of three determinations.

Our laboratory previously reported the pro-inflammatory effects of long-chain SFAs on endothelial cells (17). We reported that stearic acid-supplemented HAECs significantly upregulated ICAM-1 surface expression when exposed to concentrations of stearic acid as low as 10 μM. In the current study, cells treated with 50 μM stearic acid increased ICAM-1 expression 2-fold. Furthermore, this statistically significant increase in the pro-inflammatory marker was diminished to basal levels with as little as 5 μM oleic acid cosupplementation. Stearic acid-supplemented HAECs also demonstrated an increase in serine phosphorylation of the p65 subunit of NF-κB. HAECs supplemented with oleic acid alone did not alter the baseline phosphorylation status of NF-κB. However, cosupplementation of stearic with oleic acid diminished the activation of NF-κB, which is consistent with the reduction in ICAM-1 surface expression levels.

To discern how these individual and combinations of fatty acids altered the fatty acid composition of cells, HAECs were treated with fatty acids for 24 h under standard tissue culture conditions, and GC assessment of the HAEC fatty acid content was determined. The per-

TABLE 5. Analysis of the triglyceride fatty acid profile

	Vehicle	Stearic 50 μM	Oleic 50 μM	Stearic 50 μM + Oleic 50 μM
Fatty Acid	*μg/mg protein ± SD*	*μg/mg protein ± SD*	*μg/mg protein ± SD*	*μg/mg protein ± SD*
C8:0	0.44 ± 0.05	0.30 ± 0.05	0.26 ± 0.05	0.34 ± 0.14
C10:0	0.43 ± 0.03	0.36 ± 0.09	0.28 ± 0.09	0.34 ± 0.13
C12:0	0.46 ± 0.03	0.36 ± 0.07	0.36 ± 0.24	0.37 ± 0.02
C14:0	0.66 ± 0.19	0.75 ± 0.34	0.94 ± 0.57	0.93 ± 0.75
C16:0	4.01 ± 1.31	4.88 ± 2.81	4.96 ± 2.96	6.65 ± 2.30
C16:1n-7	0.22 ± 0.16	0.27 ± 0.10	0.37 ± 0.20	0.32 ± 0.16
C18:0	6.41 ± 0.96	8.25 ± 2.87	5.12 ± 2.39	6.81 ± 0.69
C18:1n-9	3.62 ± 1.02	2.88 ± 1.25	20.77 ± 4.37	12.62 ± 3.46
C18:1n-7	0.33 ± 0.18	0.21 ± 0.07	0.74 ± 0.31	0.65 ± 0.42
C18:2n-6	0.53 ± 0.16	0.22 ± 0.11	0.47 ± 0.28	0.44 ± 0.33
C20:0	ND	ND	ND	0.17 ± 0.10
C20:4n-6	0.21 ± 0.12	ND	0.81 ± 0.22	0.23 ± 0.04
C20:5n-3	ND	ND	ND	ND
C22:0	0.17 ± 0.01	0.20 ± 0.01	0.19 ± 0.01	0.20 ± 0.02
C22:5n-6	ND	ND	0.12 ± 0.01	ND
C22:5n-3	0.46 ± 0.07	0.32 ± 0.09	0.81 ± 0.31	0.32 ± 0.15
C24:0	ND	0.16 ± 0.11	ND	ND
C22:6n-3	ND	ND	ND	ND
Sum	17.95	19.16	36.20	30.39

Endothelial cells were treated with stearic and oleic acids (50 μM), alone or in combination, for 24 h before analysis. Triglycerides were acquired simultaneously with the phospholipid component as described. Fatty acid quantification was based on an internal standard and normalized to protein content. Results are expressed as the mean ± SD of three determinations. "ND" indicates fatty acids that were not detected in the triglyceride profile.

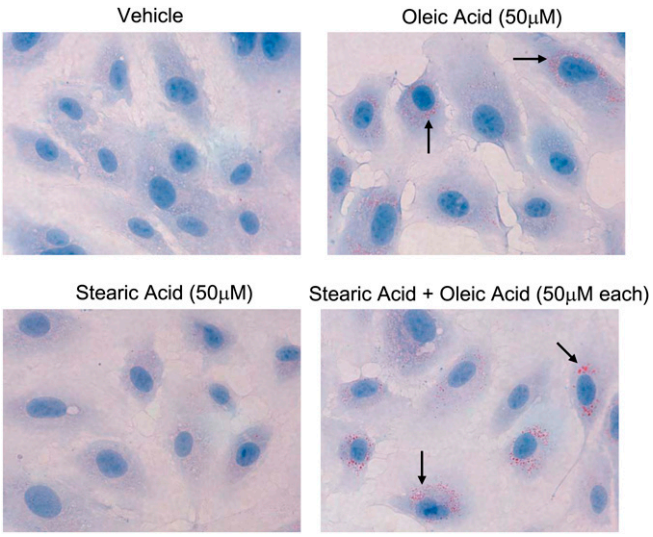

Fig. 6. Effect of oleic and stearic acids on the accumulation of neutral lipid storage. HAECs were supplemented with 50 μM of stearic acid, oleic acid, or both in combination for 24 h at 37°C in 5% CO_2. As described in Materials and Methods, lipid droplets containing triglycerides were stained with Oil Red O, and nuclei were counterstained with hematoxylin for cellular definition. Only in cells supplemented with oleic acid, in the presence or absence of stearic acid, were lipid droplets readily visible as indicated by the arrows. No excess accumulation of lipid droplets was observed when HAECs were cosupplemented with both fatty acids. HAEC, human aortic endothelial cell.

centage of C18:0 and C18:1n-9 fatty acids in the vehicle-treated cells were remarkably similar; however, when HAECs were supplemented with either stearic or oleic fatty acids at equivalent concentrations, the fatty acid profile was dramatically altered. The 25 μM stearic acid-treated HAECs revealed an increase in the proportion of C18:0. Interestingly, there was a corresponding elevation in the proportion of C18:1n-9 in the stearic acid-supplemented HAECs, which was likely due to the conversion of C18:0 into C18:1n-9 by stearyol CoA desaturase. Incorporation of oleic acid resulted in a significant increase in C18:1n-9, but this increase was primarily at the expense of stearic and palmitic acids and, to a lesser extent, arachidonic acid. When stearic and oleic acids were cosupplemented at equimolar concentrations, increases in both were readily apparent, albeit at more modest levels than in either one individually. The conversion of C18:0 into C18:1n-9 could be one mechanism by which endothelial cells resist the negative impact of incorporation of stearic acid.

The results of the growth inhibition and ICAM-1 expression assays indicated that only a minimal quantity of oleic acid was required to offset the pro-inflammatory and growth inhibitory effects induced by stearic acid. We treated HAECs with 50 μM stearic acid in combination with varying amounts of oleic acid to determine alterations in the fatty acid profile. Interestingly, cosupplementation of stearic acid with as little as 5 μM of oleic acid dramatically decreased the percentage of C18:0 in the cells. Thus, one possible mechanism for the antagonistic effects of oleic acid may be its ability to lower stearic acid levels in cells.

Listenberger et al. suggested that oleic acid prevents palmitic acid-induced apoptosis in Chinese hamster ovary cells by channeling palmitic acid into neutral lipids in the form of triglycerides (36). In our study, HAECs were treated with either stearic acid, oleic acid, or a combination of both; subsequently, the fatty acid profiles of the phospholipids and triglycerides were determined. Similar changes in the fatty acid profile of the phospholipids were observed compared to the whole cell lipid extracts. Of particular interest, 50 μM stearic acid treatment did not markedly increase the quantity of triglyceride cellular fatty acid; however, both oleic acid and the combination of stearic and oleic acids demonstrated an accumulation of triglycerides. This observation was confirmed by staining fatty acid-supplemented endothelial cells with Oil Red O to visualize the cells' neutral lipid storage. In stark contrast to Listenberger's findings, cosupplementation with stearic and oleic acids did not cause an increase in the quantity of C18:0 packaged into triglycerides (36). The Listenberger study suggests that the protective effect of oleic acid on palmitic acid-induced apoptosis is not a result of a decrease in palmitic acid uptake in the presence of oleic acid. In contrast, our results suggest that oleic acid diminishes uptake of stearic acid in endothelial cells.

In conclusion, our results are consistent with the hypothesis that the pro-inflammation and cytotoxicity generated by an accumulation of stearic acid in cells can be diminished by relatively low amounts of oleic acid cosupplementation. Our evidence suggests that endothelial cells attempt to convert excessively incorporated stearic acid into less harmful oleic acid as a means of cell preservation. However, this process can lead to an abundance of C18:0 in the phospholipids, which can evolve into a pro-inflammatory state. We hypothesize that too much C18:0 accumulates into the phospholipids, thereby creating an extremely rigid structure due to the tightly packed SFA content in the membrane, which results in lipotoxicity. These results indicate that long-chain SFAs are potentially damaging to endothelial cells; however, if SFAs are presented to endothelial cells with at least a moderate quantity of oleic acid, the SFA-induced pro-inflammatory and eventual cytotoxic effects can be averted. Our results provide mechanistic support to the benefit of diets containing elevated levels of oleic acid (i.e., Mediterranean diet) on the development of inflammatory and cardiovascular diseases.

REFERENCES

1. Jakobsen, M. U., E. J. O'Reilly, B. L. Heitmann, M. A. Pereira, K. Balter, G. E. Fraser, U. Goldbourt, G. Hallmans, P. Knekt, S. Liu, et al. 2009. Major types of dietary fat and risk of coronary heart disease: a pooled analysis of 11 cohort studies. *Am. J. Clin. Nutr.* **89:** 1425–1432.
2. Eckel, R. H., W. W. Barouch, and A. G. Ershow. 2002. Report of the National Heart, Lung, and Blood Institute-National Institute of Diabetes and Digestive and Kidney Diseases Working Group on

the pathophysiology of obesity-associated cardiovascular disease. *Circulation*. **105:** 2923–2928.
3. Sheehan, M. T., and M. D. Jensen. 2000. Metabolic complications of obesity. Pathophysiologic considerations. *Med. Clin. North Am.* **84:** 363–385 (vi.).
4. Unger, R. H. 2002. Lipotoxic diseases. *Annu. Rev. Med.* **53:** 319–336.
5. Schaffer, J. E. 2003. Lipotoxicity: when tissues overeat. *Curr. Opin. Lipidol.* **14:** 281–287.
6. Rioux, V., and P. Legrand. 2007. Saturated fatty acids: simple molecular structures with complex cellular functions. *Curr. Opin. Clin. Nutr. Metab. Care.* **10:** 752–758.
7. Mensink, R. P., P. L. Zock, A. D. Kester, and M. B. Katan. 2003. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *Am. J. Clin. Nutr.* **77:** 1146–1155.
8. Lundblad, J. L., and R. L. Seng. 1991. Inactivation of lipid-enveloped viruses in proteins by caprylate. *Vox Sang.* **60:** 75–81.
9. Dichtelmuller, H., D. Rudnick, and M. Kloft. 2002. Inactivation of lipid enveloped viruses by octanoic Acid treatment of immunoglobulin solution. *Biologicals.* **30:** 135–142.
10. Takeuchi, H., S. Sekine, K. Kojima, and T. Aoyama. 2008. The application of medium-chain fatty acids: edible oil with a suppressing effect on body fat accumulation. *Asia Pac. J. Clin. Nutr.* **17(Suppl 1):** 320–323.
11. St-Onge, M. P., and P. J. Jones. 2002. Physiological effects of medium-chain triglycerides: potential agents in the prevention of obesity. *J. Nutr.* **132:** 329–332.
12. Rioux, V., D. Catheline, M. Bouriel, and P. Legrand. 2005. Dietary myristic acid at physiologically relevant levels increases the tissue content of C20:5 n-3 and C20:3 n-6 in the rat. *Reprod. Nutr. Dev.* **45:** 599–612.
13. Beauchamp, E., D. Goenaga, J. Le Bloc'h, D. Catheline, P. Legrand, and V. Rioux. 2007. Myristic acid increases the activity of dihydroceramide Delta4-desaturase 1 through its N-terminal myristoylation. *Biochimie.* **89:** 1553–1561.
14. Lockridge, J. B., M. L. Sailors, D. J. Durgan, O. Egbejimi, W. J. Jeong, M. S. Bray, W. C. Stanley, and M. E. Young. 2008. Bioinformatic profiling of the transcriptional response of adult rat cardiomyocytes to distinct fatty acids. *J. Lipid Res.* **49:** 1395–1408.
15. Resh, M. D. 1999. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim. Biophys. Acta.* **1451:** 1–16.
16. Smotrys, J. E., and M. E. Linder. 2004. Palmitoylation of intracellular signaling proteins: regulation and function. *Annu. Rev. Biochem.* **73:** 559–587.
17. Harvey, K. A., C. L. Walker, T. M. Pavlina, Z. Xu, G. P. Zaloga, and R. A. Siddiqui. 2010. Long-chain saturated fatty acids induce pro-inflammatory responses and impact endothelial cell growth. *Clin. Nutr.* **29:** 492–500.
18. Rho, M. C., K. Ah Lee, S. Mi Kim, C. Sik Lee, M. Jeong Jang, Y. Kook Kim, H. Sun Lee, Y. Hyun Choi, B. Yong Rhim, and K. Kim. 2007. Sensitization of vascular smooth muscle cell to TNF-alpha-mediated death in the presence of palmitate. *Toxicol. Appl. Pharmacol.* **220:** 311–319.
19. Hickson-Bick, D. L., G. C. Sparagna, L. M. Buja, and J. B. McMillin. 2002. Palmitate-induced apoptosis in neonatal cardiomyocytes is not dependent on the generation of ROS. *Am. J. Physiol. Heart Circ. Physiol.* **282:** H656–H664.
20. Artwohl, M., M. Roden, W. Waldhausl, A. Freudenthaler, and S. M. Baumgartner-Parzer. 2004. Free fatty acids trigger apoptosis and inhibit cell cycle progression in human vascular endothelial cells. *FASEB J.* **18:** 146–148.
21. Staiger, K., H. Staiger, C. Weigert, C. Haas, H. U. Haring, and M. Kellerer. 2006. Saturated, but not unsaturated, fatty acids induce apoptosis of human coronary artery endothelial cells via nuclear factor-kappaB activation. *Diabetes.* **55:** 3121–3126.
22. de Vries, J. E., M. M. Vork, T. H. Roemen, Y. F. de Jong, J. P. Cleutjens, G. J. van der Vusse, and M. van Bilsen. 1997. Saturated but not mono-unsaturated fatty acids induce apoptotic cell death in neonatal rat ventricular myocytes. *J. Lipid Res.* **38:** 1384–1394.
23. Hansson, G. K. 2005. Inflammation, atherosclerosis, and coronary artery disease. *N. Engl. J. Med.* **352:** 1685–1695.
24. Shimokawa, H. 1999. Primary endothelial dysfunction: atherosclerosis. *J. Mol. Cell. Cardiol.* **31:** 23–37.
25. Davignon, J., and P. Ganz. 2004. Role of endothelial dysfunction in atherosclerosis. *Circulation.* **109:** III27–III32.
26. Blann, A. D., and C. N. McCollum. 1994. Circulating endothelial cell/leukocyte adhesion molecules in atherosclerosis. *Thromb. Haemost.* **72:** 151–154.
27. Roden, M., T. B. Price, G. Perseghin, K. F. Petersen, D. L. Rothman, G. W. Cline, and G. I. Shulman. 1996. Mechanism of free fatty acid-induced insulin resistance in humans. *J. Clin. Invest.* **97:** 2859–2865.
28. Reaven, G. M., C. Hollenbeck, C. Y. Jeng, M. S. Wu, and Y. D. Chen. 1988. Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. *Diabetes.* **37:** 1020–1024.
29. Fagot-Campagna, A., B. Balkau, D. Simon, J. M. Warnet, J. R. Claude, P. Ducimetiere, and E. Eschwege. 1998. High free fatty acid concentration: an independent risk factor for hypertension in the Paris Prospective Study. *Int. J. Epidemiol.* **27:** 808–813.
30. Stingl, H., W. Raffesberg, P. Nowotny, W. Waldhausl, and M. Roden. 2002. Reduction of plasma leptin concentrations by arginine but not lipid infusion in humans. *Obes. Res.* **10:** 1111–1119.
31. Diet, nutrition, and the prevention of chronic diseases. Report of a WHO Study Group. 1990. WHO technical report series, No. 797-TRS 797. World Health Organization, Geneva.
32. Keys, A., A. Menotti, M. J. Karvonen, C. Aravanis, H. Blackburn, R. Buzina, B. S. Djordjevic, A. S. Dontas, F. Fidanza, M. H. Keys, et al. 1986. The diet and 15-year death rate in the seven countries study. *Am. J. Epidemiol.* **124:** 903–915.
33. Fernandez-Jarne, E., E. Martinez-Losa, M. Prado-Santamaria, C. Brugarolas-Brufau, M. Serrano-Martinez, and M. A. Martinez-Gonzalez. 2002. Risk of first non-fatal myocardial infarction negatively associated with olive oil consumption: a case-control study in Spain. *Int. J. Epidemiol.* **31:** 474–480.
34. Carluccio, M. A., M. Massaro, C. Bonfrate, L. Siculella, M. Maffia, G. Nicolardi, A. Distante, C. Storelli, and R. De Caterina. 1999. Oleic acid inhibits endothelial activation: a direct vascular antiatherogenic mechanism of a nutritional component in the Mediterranean diet. *Arterioscler. Thromb. Vasc. Biol.* **19:** 220–228.
35. Fuentes, F., J. Lopez-Miranda, P. Perez-Martinez, Y. Jimenez, C. Marin, P. Gomez, J. M. Fernandez, J. Caballero, J. Delgado-Lista, and F. Perez-Jimenez. 2008. Chronic effects of a high-fat diet enriched with virgin olive oil and a low-fat diet enriched with alpha-linolenic acid on postprandial endothelial function in healthy men. *Br. J. Nutr.* **100:** 159–165.
36. Listenberger, L. L., X. Han, S. E. Lewis, S. Cases, R. V. Farese, Jr., D. S. Ory, and J. E. Schaffer. 2003. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc. Natl. Acad. Sci. USA.* **100:** 3077–3082.
37. Gao, D., H. R. Griffiths, and C. J. Bailey. 2009. Oleate protects against palmitate-induced insulin resistance in L6 myotubes. *Br. J. Nutr.* **102:** 1557–1563.
38. Hardy, S., W. El-Assaad, E. Przybytkowski, E. Joly, M. Prentki, and Y. Langelier. 2003. Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells. A role for cardiolipin. *J. Biol. Chem.* **278:** 31861–31870.
39. Coll, T., E. Eyre, R. Rodriguez-Calvo, X. Palomer, R. M. Sanchez, M. Merlos, J. C. Laguna, and M. Vazquez-Carrera. 2008. Oleate reverses palmitate-induced insulin resistance and inflammation in skeletal muscle cells. *J. Biol. Chem.* **283:** 11107–11116.
40. Mishra, R., and M. S. Simonson. 2005. Saturated free fatty acids and apoptosis in microvascular mesangial cells: palmitate activates pro-apoptotic signaling involving caspase 9 and mitochondrial release of endonuclease G. *Cardiovasc. Diabetol.* **4:** 2.
41. van Greevenbroek, M. M., W. F. Voorhout, D. W. Erkelens, G. van Meer, and T. W. de Bruin. 1995. Palmitic acid and linoleic acid metabolism in Caco-2 cells: different triglyceride synthesis and lipoprotein secretion. *J. Lipid Res.* **36:** 13–24.
42. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.
43. Lepage, G., and C. C. Roy. 1986. Direct transesterification of all classes of lipids in a one-step reaction. *J. Lipid Res.* **27:** 114–120.
44. Wang, T., X. Zhang, and J. J. Li. 2002. The role of NF-kappaB in the regulation of cell stress responses. *Int. Immunopharmacol.* **2:** 1509–1520.
45. Paumen, M. B., Y. Ishida, M. Muramatsu, M. Yamamoto, and T. Honjo. 1997. Inhibition of carnitine palmitoyltransferase

I augments sphingolipid synthesis and palmitate-induced apoptosis. *J. Biol. Chem.* **272:** 3324–3329.
46. Artwohl, M., A. Lindenmair, V. Sexl, C. Maier, G. Rainer, A. Freudenthaler, N. Huttary, M. Wolzt, P. Nowotny, A. Luger, et al. 2008. Different mechanisms of saturated versus polyunsaturated FFA-induced apoptosis in human endothelial cells. *J. Lipid Res.* **49:** 2627–2640.
47. Lu, Z. H., Y. M. Mu, B. A. Wang, X. L. Li, J. M. Lu, J. Y. Li, C. Y. Pan, T. Yanase, and H. Nawata. 2003. Saturated free fatty acids, palmitic acid and stearic acid, induce apoptosis by stimulation of ceramide generation in rat testicular Leydig cell. *Biochem. Biophys. Res. Commun.* **303:** 1002–1007.
48. Porter, A. G., and R. U. Janicke. 1999. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ.* **6:** 99–104.