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The role of fatty acids and caveolin-1 in tumor necrosis factor α–induced endothelial cell activation

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Abstract

Hypertriglyceridemia and associated high circulating free fatty acids are important risk factors for atherosclerosis. In contrast to omega-3 fatty acids, linoleic acid, the major omega-6 unsaturated fatty acid in the American diet, may be atherogenic by amplifying an endothelial inflammatory response. We hypothesize that omega-6 and omega-3 fatty acids can differentially modulate tumor necrosis factor α (TNF-α)–induced endothelial cell activation and that functional plasma membrane microdomains called *caveolae* are required for endothelial cell activation. Caveolae are particularly abundant in endothelial cells and play a major role in endothelial trafficking and the regulation of signaling pathways associated with the pathology of vascular diseases. To test our hypothesis, endothelial cells were preenriched with either linoleic acid or α-linolenic acid before TNF-α–induced endothelial activation. Measurements included oxidative stress and nuclear factor κB–dependent induction of cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) under experimental conditions with intact caveolae and with cells in which caveolin-1 was silenced by small interfering RNA. Exposure to TNF-α induced oxidative stress and inflammatory mediators, such as p38 mitogen-activated protein kinase (MAPK), nuclear factor κB, COX-2, and PGE_2, which were all amplified by preenrichment with linoleic acid but blocked or reduced by α-linolenic acid. The p38 MAPK inhibitor SB203580 blocked TNF-α–mediated induction of COX-2 protein expression, suggesting a regulatory mechanism through p38 MAPK signaling. Image overlay demonstrated TNF-α–induced colocalization of TNF receptor type 1 with caveolin-1. Caveolin-1 was significantly induced by TNF-α, which was further amplified by linoleic acid and blocked by α-linolenic acid. Furthermore, silencing of the caveolin-1 gene completely blocked TNF-α–induced production of COX-2 and PGE_2 and significantly reduced the amplified response of linoleic acid plus TNF-α. These data suggest that omega-6 and omega-3 fatty acids can differentially modulate TNF-α–induced inflammatory stimuli and that caveolae and its fatty acid composition play a regulatory role during TNF-α–induced endothelial cell activation and inflammation.

1. Introduction

Severe endothelial cell activation and injury can lead to necrotic and apoptotic cytotoxicity and ultimately to disruption of endothelial integrity. Dysfunction of endothelial cells and associated inflammatory events are critical underlying causes of the initiation of cardiovascular diseases such as atherosclerosis [1,2]. The mechanisms by which selected fatty acids induce endothelial cell activation, oxidative stress, and inflammation are not fully understood. Oxidative stress–induced transcription factors, which regulate inflammatory cytokine and adhesion molecule production, are important regulatory elements in the induction of inflammatory responses. One of these transcription factors, nuclear factor κB (NF-κB), plays a significant role in these regulatory processes [3]. Binding sites for NF-κB and related transcription factors were identified in the promoter regions of a variety of inflammatory genes [4,5] such as interleukin-6, vascular cell adhesion molecule–1, or cyclooxygenase-2 (COX-2), all of which are up-regulated by tumor necrosis factor α (TNF-α) [6,7].

Numerous risk factors for the development of atherosclerosis have been identified, including obesity [8] and hypertriglyceridemia [9]. Increased circulating free fatty acid

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levels are associated with hypertriglyceridemia and obesity [10], and high plasma free fatty acids may contribute to an environment of increased oxidative stress and inflammation in the vasculature and especially in vascular endothelial cells [11,12]. Dietary balance of long-chain fatty acids may influence processes involving leukocyte-endothelium interactions, such as atherogenesis and inflammation [13]. Although diets high in omega-6 fatty acids may lead to a decrease in serum cholesterol [14], replacing saturated with unsaturated omega-6–rich lipids may not be desirable because of their ability to easily oxidize. High intake of linoleic acid–rich oils or fats will lead to an increase in cellular oxidative stress and can elicit an inflammatory response [15], events that have been implicated in most chronic diseases. Omega-6 fatty acids and especially linoleic acid can cause endothelial cell dysfunction as well as potentiate TNF-α–mediated endothelial injury [16]. We have recently demonstrated that both the extracellular signal regulated kinase (ERK1/2) and phosphoinositide-3 kinase/amino kinase terminal signaling pathways can contribute to the effect of linoleic acid on NF-κB–dependent transcription and endothelial cell activation [17].

In contrast to omega-6 fatty acids, omega-3 fatty acids can influence cardiovascular disease pathology by beneficially modulating inflammation. Epidemiologic and interventional studies have shown a dose-dependent decrease in risk of cardiovascular disease end points with increased dietary consumption of moderate amounts of omega-3 fatty acids, either plant or marine derived [14]. Current estimates indicate that more than 90% of the omega-3 consumed by US citizens is in the form of α-linolenic acid, not the longer-chain omega-3 fatty acids found in fish oils [18]. Independent of their dietary source, omega-3 fatty acids contribute to cardioprotective properties, which include down-regulation of proinflammatory and proatherogenic genes, including adhesion molecules and cytokines, during early atherogenesis and possibly also during later stages of plaque development and plaque rupture [19]. For example, an α-linolenic acid–rich oil decreased oxidative stress and CD40 ligand in patients with mild hypercholesterolemia [20], and reduced levels of soluble cell adhesion molecules in plasma [21] and recurrence of coronary heart disease [22]. In addition, by partially replacing omega-6 analogues in membrane phospholipids with omega-3 fatty acids, it is possible to decrease the transcriptional activation of inflammatory and proatherogenic genes involved in endothelial cell activation and atherosclerosis [23].

There is increasing evidence that lipid raft proteins and lipids play an important role in health and disease [24]. A major subclass of lipid rafts is caveolae, that is, membrane domains that have been implicated in the pathology of atherosclerosis [25]. The lack of the caveolin-1 gene may provide protection against the development of atherosclerosis [26]. This may be important in understanding mechanisms of atherosclerosis because caveolae are particularly abundant in endothelial cells, where they are believed to play a major role in the regulation of endothelial vesicular trafficking as well as the uptake of lipids and related lipophilic compounds [27]. There is evidence that fatty acids can alter localization and function of caveolae-associated signaling proteins in mouse colonic mucosa [28]. Besides their role in cellular uptake of lipophilic substances, including fatty acids [29], caveolae house an array of cell signaling molecules; and numerous genes involved in endothelial cell dysfunction and inflammation are associated with caveolae [25]. Examples of caveolae-facilitated targeting of proteins involved in proinflammatory signal transduction include activators of p44/p42 (ERK) mitogen-activated protein kinase (MAPK) pathway, H-Ras [30,31], nonreceptor tyrosine kinase c-Src [31], and the upstream regulator of NF-κB, IκB kinase [32]. Furthermore, caveolins have been reported to colocalize with cyclooxygenase, suggesting that caveolins play a role in regulating the function of this enzyme [33,34].

A major objective of the current study was to explore specific mechanisms involved in fatty acid–mediated activation of endothelial cells. Our current data support our hypothesis that omega-6 and omega-3 fatty acids can differentially modulate TNF-α–induced endothelial cell activation and that regulatory mechanisms are associated within caveolae and linked to caveolae function and associated gene inductions.

2. Materials and methods

2.1. Materials

Linoleic acid and α-linolenic acid (>99% pure by gas-liquid chromatography) were obtained from Nu-Chek Prep (Elysian, MN). Human TNF-α was purchased from Sigma (St Louis, MO). The p38 MAPK inhibitor SB203580 was purchased from Calbiochem (EMD Biosciences, San Diego, CA).

2.2. Cell culture and experimental media

Endothelial cells were isolated from porcine pulmonary arteries and cultured as previously described [35]. Arteries obtained during routine slaughter were donated by the College of Agriculture, University of Kentucky. The basic culture medium consisted of medium 199 (GIBCO Laboratories, Grand Island, NY) containing 10% (vol/vol) fetal bovine serum (HyClone Laboratories, Logan, UT). The experimental media were composed of medium 199 enriched with 5% (vol/vol) fetal bovine serum and enriched with 20 μmol/L linoleic acid or α-linolenic acid. Preparation of experimental media with fatty acids was performed as described earlier [36].

2.3. Measurement of oxidative stress

Cellular oxidation was determined by 2′,7′-dichlorofluorescein fluorescence as described earlier [37]. This method is based on the conversion of 2′,7′-dichlorofluorescin into fluorescent 2′,7′-dichlorofluorescein by oxygen reactive species, primarily peroxyl radicals and peroxides. Cells

were first pretreated with different fatty acids followed by TNF-α treatment. Afterward, cells were incubated with 100 μmol/L 2,7-dichlorofluorescin diacetate (Molecular Probes, Eugene, OR) for 30 minutes. A multiwell fluorescent plate reader (Molecular Devices, Sunnyvale, CA) was used for the imaging study. Excitation and emission wavelengths were 490 and 520 nm, respectively.

2.4. Transcription factor NF-κB activation studies: electrophoretic mobility shift assay

Nuclear extracts containing active proteins were prepared from cells according to the method of Beg et al [38], with minor modifications. Binding reactions were performed in a 20-μL volume containing 7 μg of nuclear protein extracts. Nuclear extracts were incubated for 25 minutes with ^{32}P-end–labeled oligonucleotide probes containing enhancer DNA element NF-κB (5′-AGTTGAGGGGACTTTCC-CAGGC-3′) (Santa Cruz Biotechnology, Santa Cruz, CA). After binding, the protein-DNA complex and uncomplexed DNA in the mixture were resolved on native 5% polyacrylamide gels using 0.5× TBE buffer (50 mmol/L Tris-Cl, 45 mmol/L boric acid, 0.5 mmol/L EDTA, pH 8.4) and visualized by autoradiography. Control reactions using supershift assay were performed to demonstrate the specificity of the shifted DNA-protein complexes for NF-κB.

2.5. Measurement of caveolin-1 protein expression, p38 MAPK phosphorylation, and COX-2 protein levels by Western blotting

Cells were harvested using cell lysis buffer as previously described [17]. Protein samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 12% gradient gels for caveolin-1 and p38 MAPK and 10% gradient gels for COX-2 and then transferred to nitrocellulose membranes using a Bio-Rad immunoblot transfer apparatus (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The nonspecific sites on the membrane were blocked for 1 hour at room temperature with 5% nonfat dry milk in Tris-buffered saline with Tween-20 followed by incubation with caveolin-1 (Affinity BioReagents, Golden, CO), p38 MAPK (total and phosphorylation forms, respectively), or COX-2 (Santa Cruz Biotechnology) primary antibodies overnight at 4°C. β-Actin (Sigma) was used as a housekeeping gene in caveolin-1 and COX-2 protein measurements. Bands were visualized using the appropriate horseradish peroxidase–conjugated secondary antibody followed by electrochemiluminescence immunoblotting detection reagents (Amersham Biosciences, Little Chalfont, United Kingdom). Detection and quantitative analysis were performed using a digitizing system (UN-SCAN-IT; Silk Scientific, Orem, UT).

2.6. Immunofluorescence microscopy

This technique was adapted from previous protocols [39]. Cells were plated on LAB-TEKII chamber slides (Nalge Nunc International, Naperville, IL) and grown to confluence. After experimental treatments, cells were fixed in 4% (vol/vol) paraformaldehyde (in phosphate-buffered saline [PBS]) for 1 hour at room temperature. After permeabilization with 0.1% (vol/vol) Triton X-100 in PBS for 5 minutes, cells were washed 3 times with PBS. Nonspecific binding sites were blocked with 5% (vol/vol) donkey serum in PBS. Cells were then incubated with anti–caveolin-1 antibody (Affinity BioReagents) and anti–TNF receptor type 1 (TNFR-1) antibody (R&D Systems, Minneapolis, MN), followed by incubation with Alexa Fluor 488–labeled donkey anti-mouse immunoglobulin G antibody and Alexa Fluor 546–labeled donkey anti-goat immunoglobulin G antibody, respectively

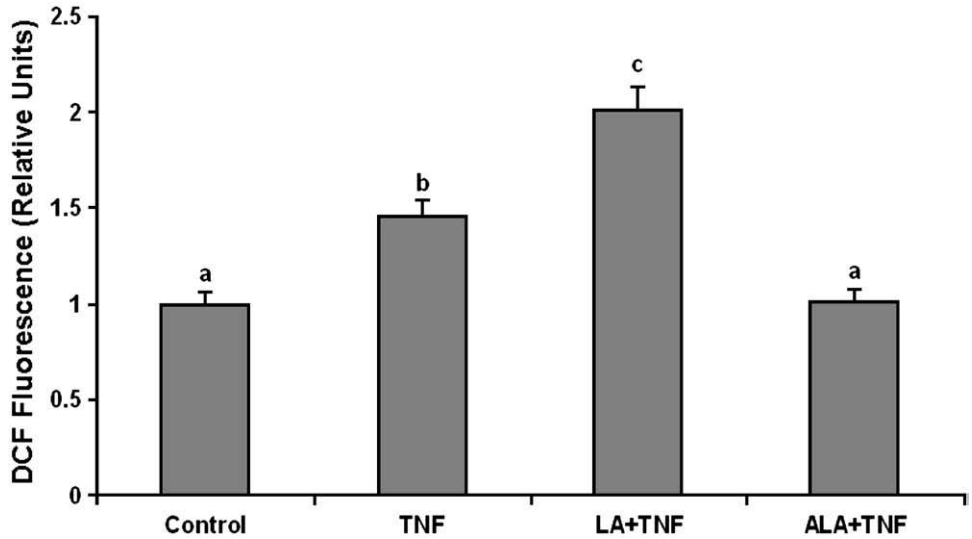


Fig. 1. Effect of linoleic acid (LA) and α-linolenic acid (ALA) on TNF-α–induced oxidative stress. Cultures were pretreated in media supplemented with 20 μmol/L LA or ALA for 24 hours followed by exposure to 0.5 ng/mL TNF-α for an additional 3 hours. Values are means ± SEM (n = 3). Different letters represent significant differences among the treatment groups.

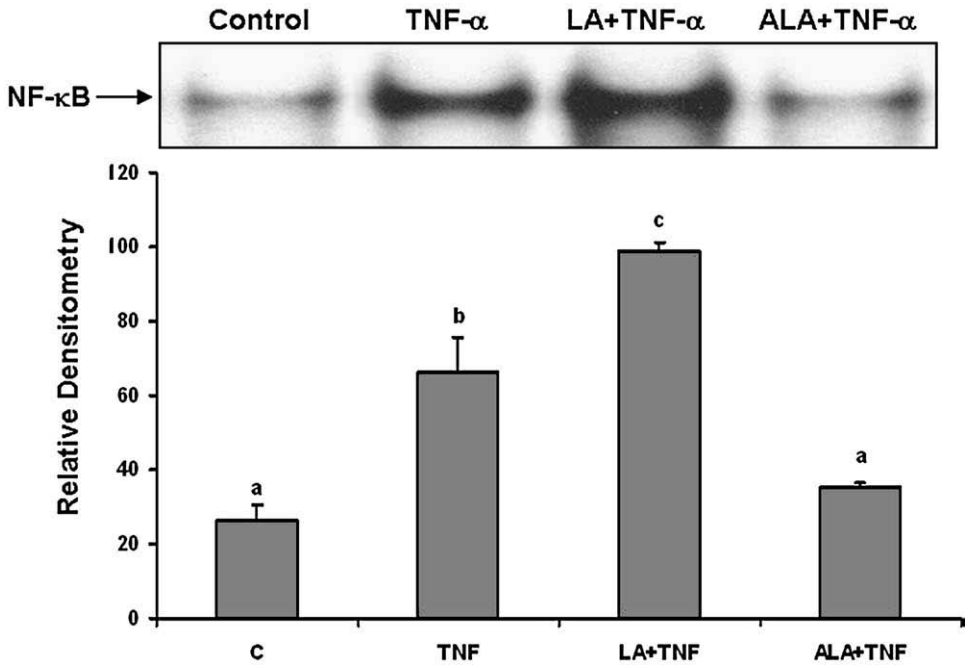


Fig. 2. Effect of LA and ALA on TNF-α–induced activation of NF-κB. Cells were treated with 20 μmol/L of LA or ALA for 24 hours before exposure to 0.5 ng/mL TNF-α for an additional 6 hours. Experiments were repeated 3 times, and the blots shown are a representative of one of the experiments. The bar graph shows the corresponding densitometric analysis of the blots. Values are means ± SEM. Different letters represent significant differences among treatment groups.

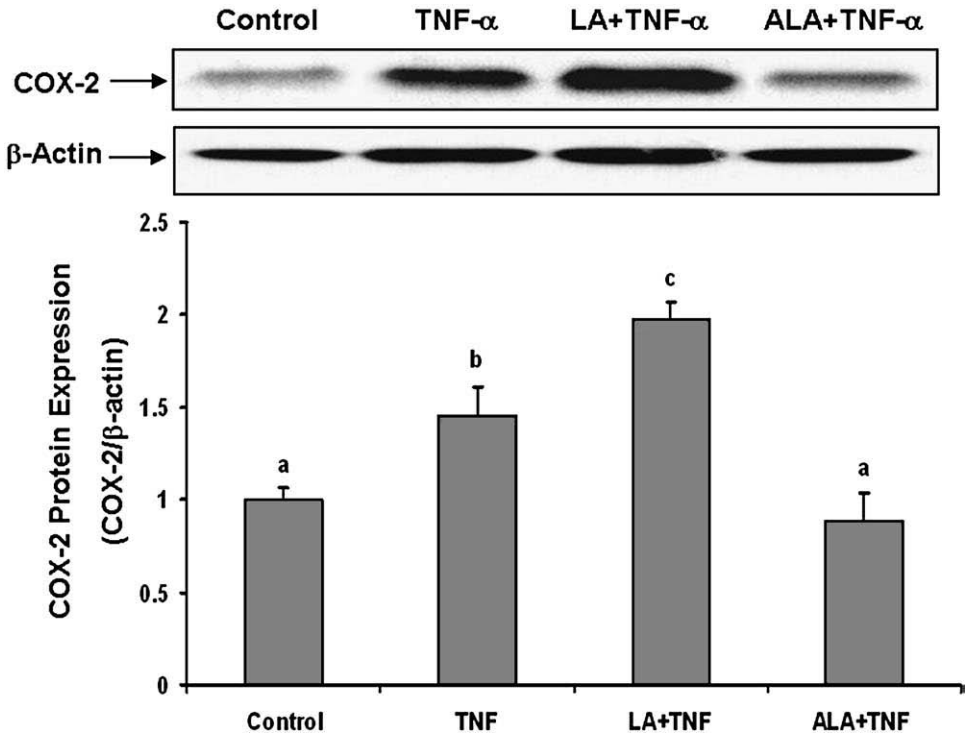


Fig. 3. Effect of LA and ALA on TNF-α–induced expression of COX-2. Cells were pretreated with 20 μmol/L of LA or ALA for 24 hours and then exposed to 0.5 ng/mL TNF-α for an additional 8 hours. The COX-2 protein levels were measured by Western blotting and normalized according to β-actin expression. Experiments were repeated 3 times, and the blots shown are representative of one of the experiments. The bar graph shows the corresponding densitometric analysis of the blots. Values are means ± SEM. Different letters represent significant difference among treatment groups.

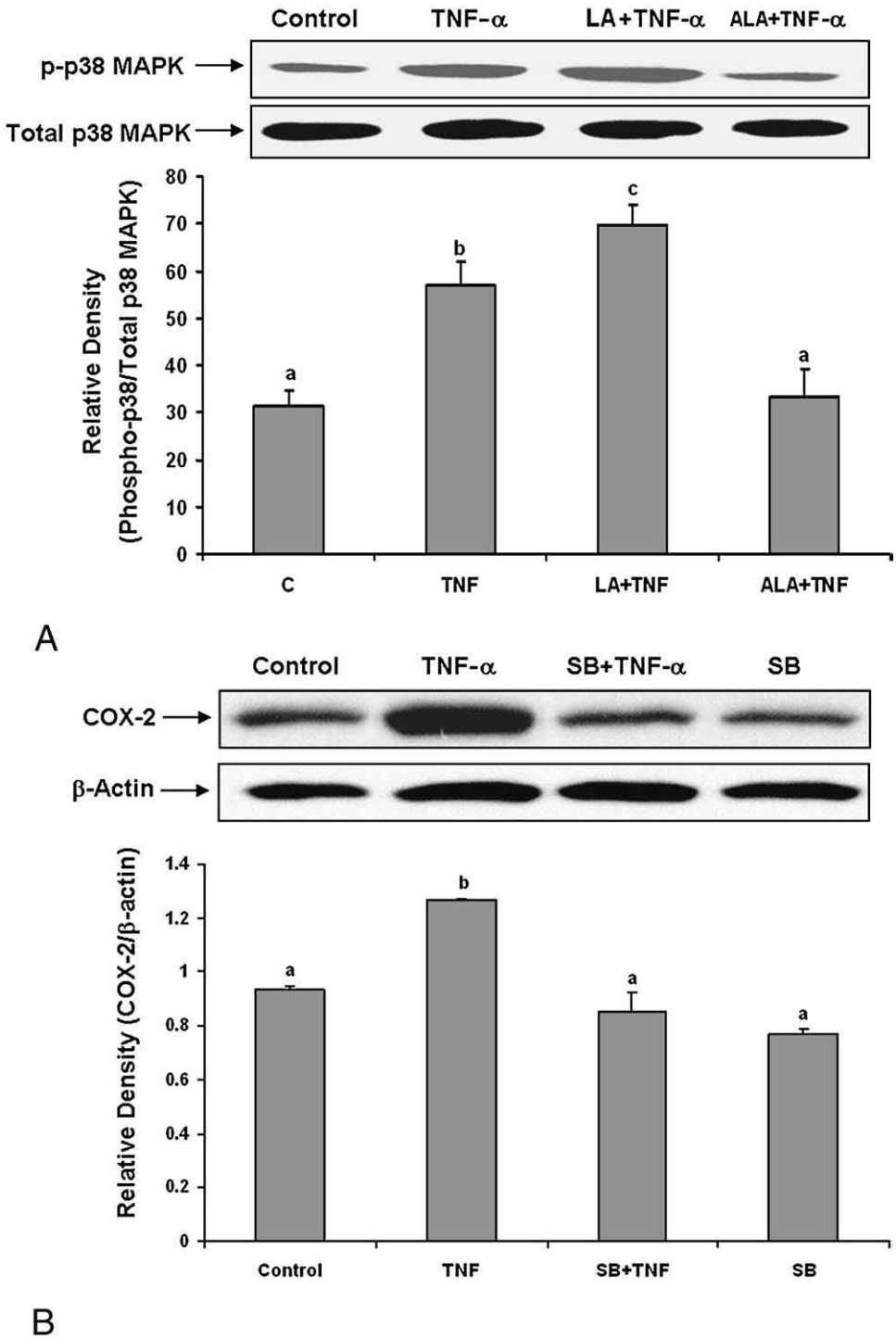


Fig. 4. Effect of LA and ALA on TNF-α–induced p38-MAPK activation and the effect of p38-MAPK inhibitor on TNF-α–induced COX-2 expression. A, Endothelial cells were pretreated with 20 μmol/L of LA or ALA for 24 hours and then exposed to 0.5 ng/mL TNF-α for 10 minutes. Phosphorylated p38 MAPK and total p38 MAPK were detected by Western blotting using specific dually phosphorylated p38 MAPK antibody (Thr 180/Tyr 182) or anti-total p38 MAPK antibody, respectively. B, Cells were exposed to 0.5 ng/mL TNF-α for 8 hours or first preenriched with 10 μmol/L SB203508 for 30 minutes followed by coexposure to TNF-α for an additional 8 hours. The COX-2 protein levels were measured by Western blotting and normalized according to β-actin expression. Experiments were repeated 3 times, and the blots shown are a representative of one of the experiments. The bar graph shows the corresponding densitometric analysis of the blots. Values are means ± SEM. Different letters represent significant difference among treatment groups.

(Invitrogen-Molecular Probes, Carlsbad, CA). Cell nuclei were stained using Hoechst (AnaSpec, San Jose, CA). Slides were mounted in aqueous mounting medium with antifading agents (Biomeda, Foster City, CA) and covered with coverslips. Images were captured digitally by Fluoview 300 Confocal Microscopy (Olympus America, Center Valley, PA).

2.7. Prostaglandin E_2 determination

Cells were seeded in 60-mm culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ) and grown to confluence. After pretreatment with fatty acids, cells were exposed to 3,3′,4,4′-tetrachlorobiphenyl (PCB77). Subsequently, supernatants of cell cultures were collected into microcentrifuge tubes (ISC BioExpress, Kaysville, UT), centrifuged at 4°C to remove cellular debris, and stored at −80°C. Prostaglandin E_2 (PGE_2) levels were assessed using a PGE_2-specific enzyme immunoassay (EIA) (Cayman Chemicals, Ann Arbor, MI) following the manufacturer's protocol. Absorbance at 405 nm was detected using a microplate spectrophotometer SpectraMaxPro M2 (Molecular Devices).

2.8. Caveolin-1 small interfering RNA and transfection

Caveolin-1 gene silencer was designed as previously described [40]. Cells were transfected with control small

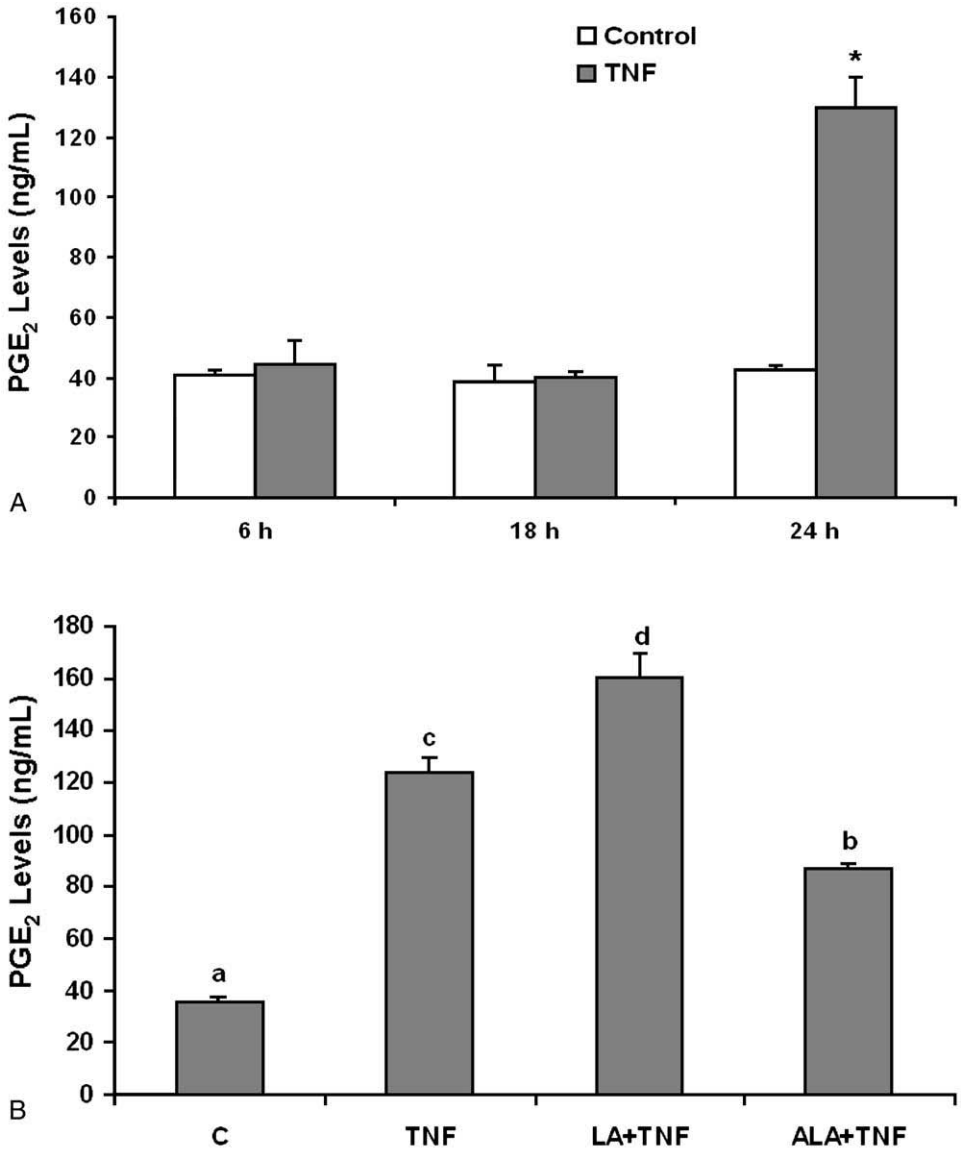
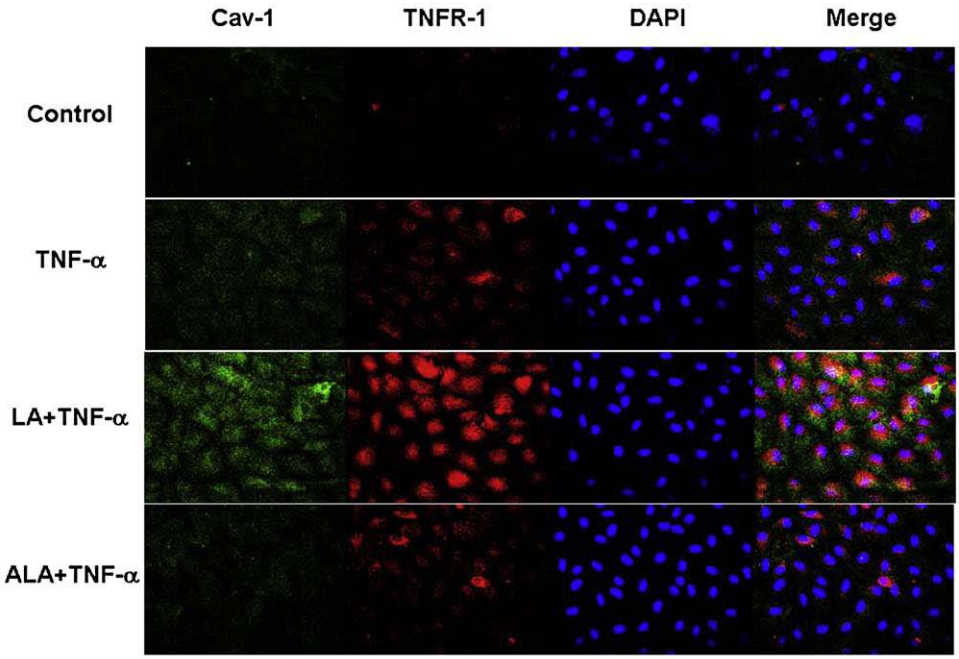
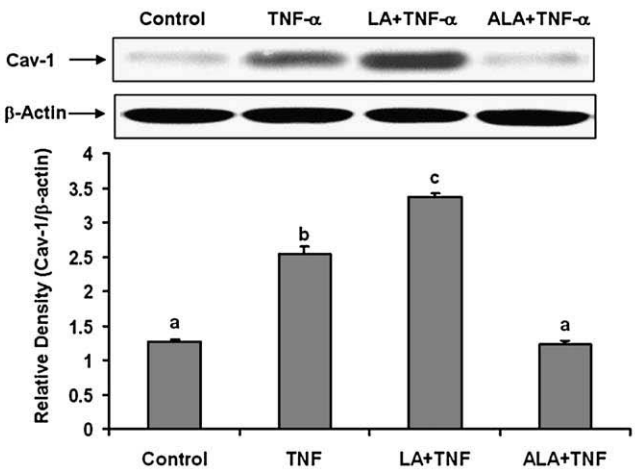


Fig. 5. Effect of LA and ALA on TNF-α–stimulated release of PGE_2 from endothelial cells. A, Cells were exposed to 0.5 ng/mL TNF-α for 6, 18, and 24 hours. Supernatants of cell cultures were collected, and PGE_2 levels were measured by EIA. Bars represent means ± SEM from 3 independent experiments. ∗Significant difference compared with the control treatment. B, Cells were pretreated with 20 μmol/L of LA or ALA for 24 hours and then exposed to 0.5 ng/mL TNF-α for an additional 24 hours. Supernatants of cell cultures were collected, and PGE_2 levels were measured by EIA. Values are means ± SEM (n =3). Different letters represent significant difference among treatment groups.



A

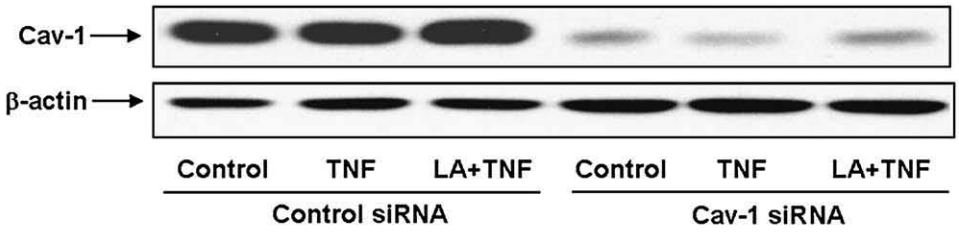


B

Fig. 6. Effect of LA and ALA on TNF-α–induced up-regulation of caveolin-1 and colocalization of TNFR-1 and caveolin-1. Cells were treated with 0.5 ng/mL TNF-α alone for 4 hours or pretreated with 20 μmol/L LA or ALA for 24 hours followed by exposure to TNF-α for 4 hours. A, Immunofluorescence staining for caveolin-1 (fluoroisothiocyanate green fluorescence), TNFR-1 (Texas red fluorescence), and nuclei (4′,6-diamidino-2-2phenylindole [DAPI] blue fluorescence). Regions of colocalization of caveolin-1 and TNFR-1 are depicted in yellow in the merged images. Experiments were repeated 3 times, and the epifluorescence micrographs shown are representative fields of one of the experiments (original magnification, ×400). B, Caveolin-1 protein levels were detected by Western blotting and normalized according to β-actin expression. Experiments were repeated 3 times, and the blots shown are a representative of one of the experiments. The bar graph shows the corresponding densitometric analysis of the blots. Values are means ± SEM. Different letters represent significant differences among treatment groups.

interfering RNA (siRNA) or caveolin-1 siRNA at a final concentration of 40 nmol/L using GeneSilencer (Genlantis, San Diego, CA) in Optimem I medium (Invitrogen). Cells were incubated with transfection mixtures for 4 hours and then replaced with regular medium. Forty-eight hours after transfection, cells were

Fig. 7. Effect of caveolin-1 silencing on TNF-α– and LA-induced COX-2 expression and PGE_2 synthesis. Cells were transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with scrambled control siRNA. Afterward, cells were treated with 0.5 ng/mL TNF-α alone for 24 hours or pretreated with 20 μmol/L LA for 24 hours followed by exposure to TNF-α for an additional 24 hours. A, Caveolin-1 protein levels were measured by Western blotting and normalized according to β-actin expression. B, The COX-2 protein levels were measured by Western blotting under the same experimental conditions as in panel A. C, Supernatants of cell cultures were collected, and PGE_2 levels were measured by EIA. Experiments were repeated 3 times, and the blots shown are a representative of one of the experiments. Values are means ± SEM. Different letters represent significant difference among treatment groups.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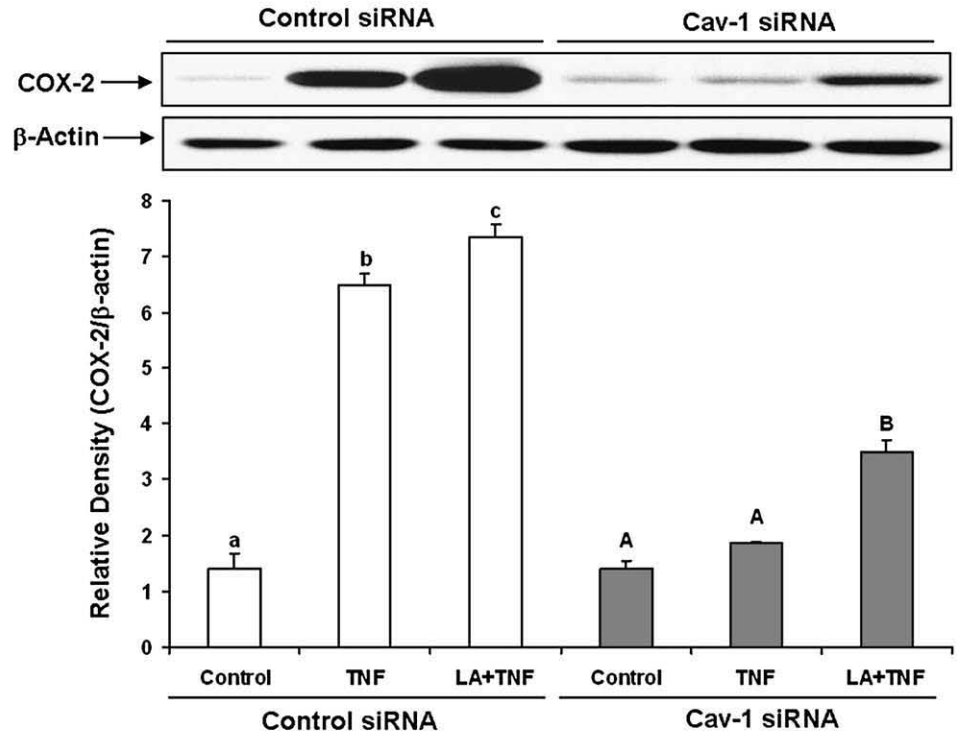
Control siRNA
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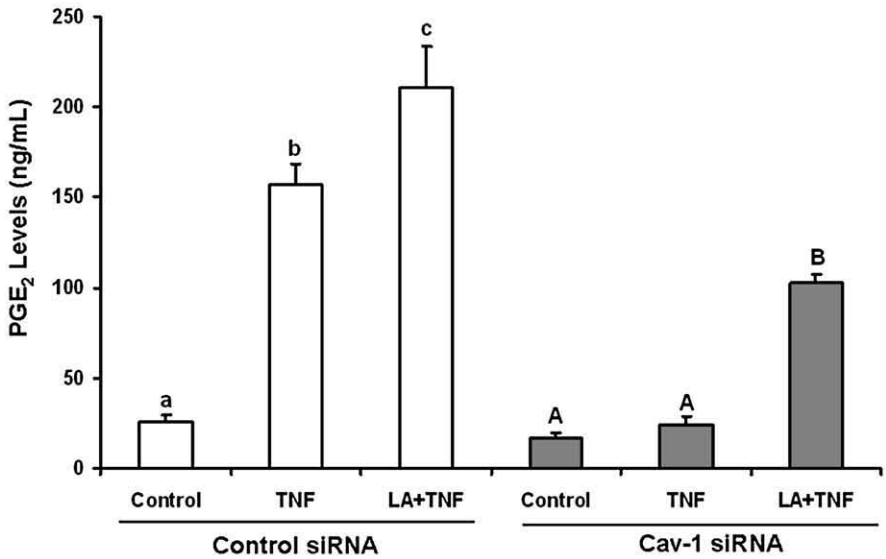
PGE2 Levels (ng/mL)
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100
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a
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Control
TNF
LA+TNF
Control
TNF
LA+TNF
Control siRNA
Cav-1 siRNA

C

treated with TNF-α in the presence or absence of linoleic acid.

2.9. Statistical analysis

Values are reported as mean ± standard error of the mean (SEM) of at least 3 independent experiments. Data were analyzed using Sigma Stat software (Jandel, San Rafael, CA). One-way analysis of variance was followed by post hoc comparisons using the least significant difference method. A statistical probability of $P < .05$ was considered significant.

3. Results

3.1. Linoleic acid and α-linolenic acid modulate cellular oxidative stress, NF-κB activity, and COX-2 expression induced by TNF-α

To examine the effects of omega-6 and omega-3 fatty acids on TNF-α–induced endothelial activation, cells were pretreated with either linoleic acid or α-linolenic acid, followed by exposure to TNF-α. As indicated in Figs. 1 to 3 , exposure to TNF-α alone significantly increased cellular oxidative stress (reactive oxygen species [ROS]), NF-κB DNA binding activity, and COX-2 protein expression, respectively. Pretreatment with linoleic acid followed by exposure to TNF-α further induced these effects compared with cultures treated only with TNF-α (Figs. 1-3). In contrast, pretreatment with α-linolenic acid blocked the TNF-α–induced oxidative stress and subsequent induction of NF-κB and COX-2.

3.2. Linoleic acid and α-linolenic acid modulate TNF-α–induced activation of p38 MAPK

In mammalian cells, MAPKs are strongly activated by growth factors, environmental stresses, and inflammatory cytokines [41]. As shown in Fig. 4A, p38 MAPK was significantly activated by TNF-α; and pretreatment with linoleic acid followed by exposure to TNF-α further increased p38 MAPK phosphorylation. In contrast, p38 MAPK activation was blocked by preenrichment with α-linolenic acid.

To further evaluate the role of p38 MAPK in TNF-α–induced inflammatory gene expression, endothelial cells were pretreated with the specific p38 MAPK inhibitor SB203580 before exposure to TNF-α. As shown in Fig. 4B, SB203580 significantly decreased TNF-α–induced COX-2 expression.

3.3. Linoleic acid and α-linolenic acid modulate TNF-α–induced PGE_2 production

Cyclooxygenase-2 plays a key role in inflammation, and PGE_2 is a major inflammatory mediator. As shown in Fig. 5A, a 24-hour exposure to TNF-α significantly induced PGE_2 production. Compared with the TNF-α treatment group, enriching endothelial cells with linoleic acid further enhanced TNF-α–induced PGE_2 levels (Fig. 5B). In contrast, cellular exposure to α-linolenic acid markedly reduced the proinflammatory effect of TNF-α (Fig. 5B).

3.4. Linoleic acid and α-linolenic acid modulate TNF-α–induced caveolin-1 up-regulation and the co-localization of TNFR-1 and caveolin-1

The first step in the TNF-α–induced inflammatory response is the binding of the cytokine to its cognate receptor. Tumor necrosis factor type 1 has been reported to be enriched in caveolae [42]. Caveolin-1 has also been shown to form a complex with the TNF receptor [43]. To assess the effects of linoleic acid and α-linolenic acid on caveolin-1 expression and its colocalization with TNFR-1, endothelial cells were first preincubated with either fatty acid, followed by treatment with TNF-α. The immunofluorescence images in Fig. 6A demonstrate that TNF-α can increase caveolin-1 and TNFR-1 expression compared with the control group and that pretreated cells with linoleic acid followed by TNF-α can further increase these effects compared with TNF-α treatment alone. The overlapped images also indicate that the colocalization of caveolin-1 and TNFR-1 is markedly increased in cells pretreated with linoleic acid followed by TNF-α compared with TNF-α treatment alone. In contrast, cells pretreated with α-linolenic acid followed by exposure to TNF-α diminished both TNF-α–induced caveolin-1 up-regulation and the colocalization of TNFR-1 and caveolin-1. The effects of linoleic acid and α-linolenic acid on TNF-α–induced caveolin-1 expression were confirmed by measuring caveolin-1 protein levels by Western blotting (Fig. 6B).

3.5. Caveolin-1 silencing decreases TNF-α–induced COX-2 expression

To determine the role of caveolin-1 in TNF-α–induced endothelial cell activation, we used siRNA to specifically down-regulate caveolin-1. As indicated in Fig. 7A, caveolin-1 siRNA reduced caveolin-1 expression by approximately 80% as compared with control siRNA. The TNF-α–induced COX-2 expression was totally blocked by caveolin-1 silencing (Fig. 7B). Caveolin-1 silencing also diminished the combined COX-2 induction induced by both linoleic acid and TNF-α. Similar to the COX-2 data, caveolin-1 silencing blocked TNF-α–induced PGE_2 production and also diminished TNF-α–induced PGE_2 production in the presence of linoleic acid (Fig. 7C).

4. Discussion

Atherosclerosis is considered an inflammatory disease, which involves the interplay of prooxidative activities, induction of inflammatory cytokines and adhesion molecules, and activation of vascular endothelial cells, all events that promote vascular leukocyte infiltration and plaque development [1]. Inflammatory events also include the cyclooxygenase and subsequent eicosanoid pathways

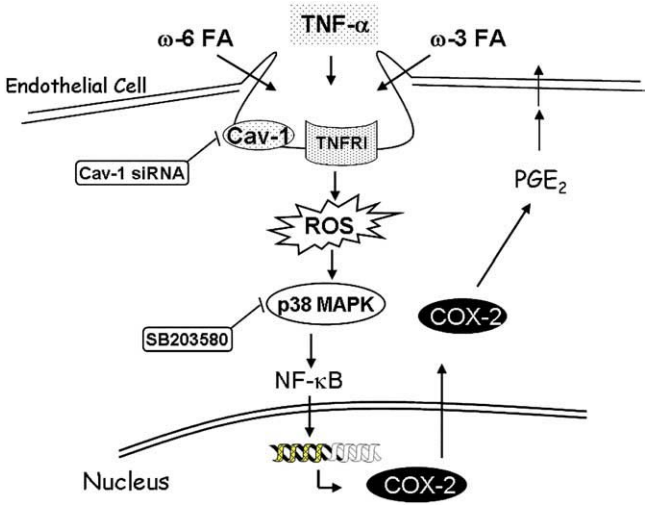

Fig. 8. Proposed mechanism for fatty acid–mediated modulation of endothelial cell activation induced by TNF-α–induced up-regulation of caveolin-1 and the activation of TNFR-1–mediated signaling pathway, which includes induction of oxidative stress (ROS), p38 MAPK, NF-κB, and COX-2. The TNF-α–induced cell signaling and PGE_2 production are further enhanced by LA but blocked by ALA. Finally, targeted knockdown of caveolin-1 completely abrogates TNF-α–induced PGE_2 production, indicating that caveolin-1 plays a mechanistic role in TNF-α–induced endothelial cell activation and modification by dietary fatty acids.

[44,45]. For example, inflammatory cytokines like interleukin-1β can induce COX-2 expression and the release of PGE_2, events that may be mediated through activation of p42/44 and p38 MAPKs, and the NF-κB pathway [46]. Our present data demonstrate that TNF-α can markedly induce oxidative stress, p38 MAPK, NF-κB, COX-2, and PGE_2 in vascular endothelial cells.

Hypertriglyceridemia has been identified as an independent risk factor for atherosclerosis, and increased circulating free fatty acid levels are associated with hypertriglyceridemia and obesity [10]. High plasma free fatty acids may contribute to an environment of increased oxidative stress and inflammation in the vasculature [11,12], and we have previously shown that linoleic acid can markedly amplify a TNF-α–mediated endothelial inflammatory response [47]. We also have recently demonstrated that both the ERK1/2 and phosphoinositide-3 kinase/amino kinase terminal signaling pathways can contribute to the stimulatory effect of linoleic acid on NF-κB–dependent transcription and endothelial cell activation [17]. In the current study, exposure to TNF-α induced oxidative stress, p38 MAPK, NF-κB, COX-2, and PGE_2, which were amplified by preenrichment with linoleic acid. This clearly supports our hypothesis that diets high in omega-6 fatty acids, and especially in linoleic acid, are proinflammatory and thus may contribute to the early pathology of atherosclerosis.

In contrast to omega-6 fatty acids, epidemiologic and interventional studies have shown a dose-dependent decrease in risk of cardiovascular disease end points with increased dietary consumption of moderate amounts of omega-3 fatty acids, either plant or marine derived [14]. Our data clearly demonstrate the anti-inflammatory properties of omega-3 fatty acids. In fact, TNF-α–induced oxidative stress, p38 MAPK, NF-κB, COX-2, and PGE_2 in endothelial cells were blocked or reduced when cells were enriched with α-linolenic acid. Mechanisms of vascular inflammation are largely regulated through p38 MAPK signaling [48-51], and our data suggest that p38 MAPK is an important player in fatty acid modification of the TNF-α–induced inflammatory signaling cascade. The p38 MAPK inhibitor SB203580 blocked TNF-α–mediated induction of COX-2 protein expression, suggesting a regulatory mechanism through p38 MAPK signaling. This was supported by evidence that p38 MAPK increases nuclear recruitment of NF-κB in cells exposed to inflammatory stimuli [52].

Mechanisms of linoleic acid–mediated induction of proinflammatory events, including enhanced expression and production of COX-2 and PGE_2, as well as the observed protective properties by linolenic acid, are not clear. One possible reason for the linoleic acid–mediated endothelial activation and dysfunction is the observation that the conversion of linoleic acid to more unsaturated metabolites is diminished because of very low δ6-desaturase activity in endothelial cells [2]. We have reported previously that, when enriching endothelial cells with linoleic acid or linolenic acid, both fatty acids can incorporate into cellular phospholipids, but only linoleic acid accumulated into cell triglycerides [53]. Furthermore, eicosapentaenic acid (the bioactive elongation and desaturation metabolite of linolenic acid) increased in cellular phospholipids after enrichment with linolenic acid, whereas arachidonic acid levels decreased after enrichment with linoleic acid [53]. It is very likely that linoleic acid can displace arachidonic acid from cell membrane phospholipids and thus make arachidonic acid more available for prostaglandin synthesis.

Although our data suggest that exposure to TNF-α can cause endothelial cell dysfunction and that omega-6 fatty acids (and in particular linoleic acid) can amplify TNF-α–induced endothelial activation [47], mechanisms of this interaction may be multilayered and are not known. We hypothesize that caveolae play a critical role in facilitating the cellular uptake and trafficking of fatty acids and trigger the initial cell signaling induced by TNF-α and lipids. Our present data suggest that functional caveolae are necessary for TNF-α– and/or linoleic acid–mediated activation of endothelial cells. Tumor necrosis factor α markedly induced both caveolin-1 and TNFR-1, the events that were further induced when cells were first enriched with linoleic acid. Tumor necrosis factor receptor type 1 contains a death domain; and this region is required for TNF-α–induced proinflammatory cellular responses, such as activation of NF-κB [54]. Furthermore, silencing caveolin-1 totally blocked both TNF-α–induced COX-2 expression and cellular release of PGE_2. We recently investigated caveolin-1–regulated mechanisms

associated with polychlorinated biphenyl (PCB)-induced markers of peroxynitrite formation and DNA binding of NF-κB [55] and demonstrated that cellular exposure to polychlorinated biphenyl (PCB77) (a persistent organic pollutant) increased endothelial nitric oxide synthase (eNOS) phosphorylation and NO production, as well as peroxynitrite levels. We also found that caveolin-1 silencing abolished the PCB-stimulated eNOS phosphorylation, suggesting a regulatory role of caveolae in PCB-induced eNOS signaling. In another study, perturbation of membrane raft structural integrity with cholesterol-sequestering compounds caused delocalization of eNOS from caveolae and inhibited TNF-α–induced ROS production and protein tyrosine nitration [56].

It is very likely that selected fatty acids either stabilize or perturb caveolae functions, thus leading to modifications of caveolae-dependent signaling. There is evidence that fatty acids can alter localization and function of caveolae-associated signaling proteins in mouse colonic mucosa [28]. Our data provide evidence that TNF-α–induced up-regulation of caveolin-1 is further enhanced when endothelial cells are first enriched with linoleic acid. In contrast, preenrichment with α-linolenic acid totally blocked TNF-α–mediated induction of caveolin-1. These data suggest that omega-3 fatty acids may in part be anti-inflammatory by decreasing caveolin-1 expression or by causing dysfunctional caveolae, leading to down-regulation of caveolin-dependent downstream signaling. Using human breast cancer cells, others have shown recently that omega-3 fatty acids can alter lipid raft composition and decrease epidermal growth factors [57], suggesting that selected fatty acids can modify caveolae-associated cell functions. There is evidence that caveolae play a role in lipid trafficking and uptake and transport of fatty acids [24]. Furthermore, preferential uptake of fatty acids via the FAT/CD36, a receptor exclusively located in lipid rafts such as caveolae, may explain the fatty acid effects we observed. In fact, working with mouse embryonic fibroblasts, it was recently demonstrated that mistargeting of FAT/CD36 in cells lacking the caveolin-1 gene resulted in reduced fatty acid uptake compared with the wild-type cells [58].

Our observations that linoleic acid can amplify the TNF-α–mediated induction of both caveolin-1 and COX-2 is significant because of the link between caveolins (caveolae) and the pathology of atherosclerosis [25]. Caveolin-1 has been reported to colocalize with interleukin-1β–induced COX-2 [59], suggesting the dependence of COX-2 induction on functional caveolae. Recent evidence suggests that high-fat diets can up-regulate caveolin-1 expression in aorta of diet-induced obese rats [60], suggesting that our fatty acid data may mimic an in vivo response by activating COX-2. High-fat diets contribute to hypertriglyceridemia, and the vascular endothelium can be exposed to significant levels of free fatty acids derived from lipoprotein lipase–mediated hydrolysis of triglyceride-rich lipoproteins [16].

In summary, we provide novel data demonstrating that omega-6 and omega-3 fatty acids can differentially modulate TNF-α–induced inflammatory stimuli and that these events require functional caveolae (Fig. 8). Furthermore, functional changes of caveolae associated with modifications by dietary fatty acids appear to affect critical phases of induction of oxidative stress–sensitive transcription factors and inducible inflammatory parameters during endothelial cell activation. Because caveolae and caveolins have been implicated in several human diseases and in particular vascular diseases, our data may have implications in understanding novel mechanisms of inflammatory diseases modulated by dietary lipids.

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