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Oxidized ω–3 Fatty Acids Inhibit Pro-Inflammatory Responses in Glomerular Endothelial Cells

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Key Words

Leukocytes · Endothelial interactions · MCP-1 · Oxidized EPA · NF-κB

Abstract

Background: ω–3 fatty acids have beneficial effects in chronic inflammatory diseases that are characterized by accumulation of leukocytes and leukocyte-mediated tissue injury. Accumulation of leukocytes occurs, in part, due to pro-inflammatory responses in endothelial cells, such as increase in expression of leukocyte adhesion receptors and chemokines, such as MCP-1 and IL-8. ***Methods:*** ω–3 fatty acids, such as EPA, are highly polyunsaturated and readily undergo auto-oxidation. We studied the effect of oxidized EPA and unoxidized (native) EPA on leukocyte-glomerular endothelial cell interactions using adhesion assays, ELISA assays and transmigration assays. We used electrophoresis mobility shift assays to determine the effect of oxidized and unoxidized EPA on cytokine-induced nuclear factor-κB (NF-κB) activation. ***Results:*** Oxidized EPA but not unoxidized EPA dose-dependently inhibits cytokine-induced leukocyte adhesion receptors on glomerular endothelial cells, which correlates with inhibition of leukocyte-glomerular endothelial cell interactions. Oxidized EPA but not unoxidized EPA inhibits cytokine-induced glomerular endothelial and mesangial cell expression of MCP-1, and to a lesser extent IL-8. Transmigration assays show that oxidized EPA but not unoxidized EPA inhibits leukocyte transmigration across glomerular endothelial cells. Oxidized EPA but not unoxidized EPA potently inhibited cytokine-induced activation of NF-κB in glomerular endothelial and mesangial cells. ***Conclusions:*** These studies show that the beneficial effects of fish oil in chronic inflammatory diseases, including IgA nephropathy, may result from the inhibitory effects of oxidized ω–3 fatty acids on pro-inflammatory events in endothelial cells via inhibition of NF-κB activation.

Consumption of marine fish oil has been reported to improve the prognosis of several chronic inflammatory diseases characterized by leukocyte accumulation and leukocyte-mediated tissue injury, including atherosclerosis, inflammatory bowel disease, rheumatoid arthritis, etc. [1–3]. Fish oil is also recommended for treatment of IgA nephropathy [4]. Several studies suggest that ω–3 fatty acid supplementation may reduce the inflammatory response by attenuating leukocyte adhesion to the vessel wall [5–7]. However, the primary mechanism for the anti-inflammatory effects of fish oil remains unclear [8].

The beneficial effects of fish oil have been associated with the ω–3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), that are abundant in marine fish oil. EPA and DHA are highly polyunsaturated and easily undergo auto-oxidation [9, 10]. This suggests the possibility that oxidized ω–3 fatty acids may be an important component of the observed anti-inflammatory effects of fish oil. Indeed,

A.C. and A.M. contributed equally to this work.

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our previous studies have shown that oxidized EPA and not unoxidized EPA, potently inhibits leukocyte-endothelial interactions, both in vitro and in vivo [11, 12].

One of the early events in inflammation is the increased expression of leukocyte adhesion receptors and chemokines by endothelial cells in response to pro-inflammatory cytokines such as TNFα and interleukin (IL)-1. Chemokines such as monocyte chemoattractant protein-1 (MCP-1) and IL-8 in turn promote leukocyte chemotaxis, adhesion and transendothelial migration [13, 14].

Activation and cytosol-nuclear translocation of transcription factor nuclear factor-κB (NF-κB) is central to the cellular response to cytokines, LPS, mitogens, and agents invoking oxidative stresses. NF-κB activation appears to be necessary for the induction of adhesion molecule and chemokine genes and deletion of NF-κB binding sites results in an inability to induce these genes [15–19]. NF-κB is normally bound to an inhibitory protein IκB and maintained as inactive NF-κB/IκB complex. TNFα, IL-1, LPS and other inflammatory mediators phosphorylate IκB, which results in the polyubiquitination of the phosphorylated IκB and degradation by the 26S proteosome. The free NF-κB subunits, p65 and p50, translocate to the nucleus where they bind target genes, including genes for cell adhesion molecules and chemokines and initiate transcription [20–22].

The role of ω–3 fatty acids in (i) leukocyte interaction with glomerular endothelial cells (GEC), (ii) chemokine expression by mesangial cells and GEC, and (iii) leukocyte transmigration across endothelial cells has not been studied. Here we show that oxidized EPA, but not unoxidized EPA, in a dose-dependent manner inhibits these pro-inflammatory responses. Furthermore, the inhibitory effects are likely due to the oxidized EPA-mediated inhibition of cytokine-induced NF-κB activation.

Methods

Preparation of Fatty Acids

EPA was purchased from Cayman Chemicals. It was relatively unoxidized as assessed by thiobarbituric acid-reactive substances (TBARS) assay using malondialdehyde as a standard; this assay is a measure of fatty acid oxidation [9]. EPA was stored as a 100-m*M* stock in 100% ethanol under nitrogen to ensure minimal oxidation. For oxidation, 3.3 m*M* EPA was made up in phosphate-buffered saline (pH 5.0) and the sample was incubated at 37°C for 16 h. Gas chromatography mass spectrometry of the samples revealed a single peak in the unoxidized (native) EPA sample which was consistent with EPA while oxidized EPA had less than 5% of the unoxidized EPA and a number of additional peaks that likely correspond to EPA oxidation products (data not shown). Using TBARS assay, the aldehyde content of oxidized EPA was 1.78 μ*M* malondialdehdye in 100 μ*M* EPA, which is in the range seen in plasma of humans (1.52–3.45 μ*M*) and animals (1.93 μ*M*) fed fish oil diets [23–27]. For treatment of cells, oxidized EPA or unoxidized EPA was diluted in medium containing 20% fetal calf serum to final concentrations between 10 and 100 μ*M*. The vehicle control was media containing PBS.

Cells

Human GEC were obtained from Cell Systems (Kirkland, Wash., USA). Human coronary artery and human microvessel endothelial cells were obtained from Clonetics (Walkersville, Md., USA). Human umbilical endothelial cells (HUVEC) were obtained from the core culture laboratory in Division of Cardiovascular Medicine at the University of Iowa. The endothelial cells were maintained in endothelial cell medium (M-199, 20% heat-inactivated fetal bovine serum, 25 μg/ml gentamycin, 1 μg/10 μl endothelial mitogen, 50 μg/ml heparin, and 2 m*M L*-glutamine). Endothelial cells from passage 3–5 were used in adhesion assays, ELISA assays, transmigration assays and electrophoresis mobility shift assays. Mesangial cells were obtained from Clonetics and maintained in mesangial cell basal medium (Clonetics) and 5% FBS. Cells were used in experiments from passage 3–5. The effect of oxidized EPA on the viability of the cells was determined by MTT assays; supplementation of endothelial and mesangial cell medium with varying concentrations of oxidized and unoxidized EPA had no effect on the viability of cells (data not shown).

Adhesion Assay

Endothelial monolayers in 96-well plates were pretreated for 1 h with vehicle control (PBS), 100 μ*M* unoxidized EPA or 10, 25, 50,75, or 100 μ*M* oxidized EPA in endothelial growth medium. After 1 h, the endothelial cells were washed, the medium was replaced and the cells were stimulated with hTNFα (10 ng/ml) for 5 h. U937 cells were fluorescently labeled with the fluorescent probe bis-carboxyethylcarboxyfluorescein acetoxymethyl ester (BCECF-AM) (Molecular Probes, Eugene, Oreg., USA), added to wells (6×10^4/well) and incubated for 10 min at 37°C. The wells were washed, filled with M-199 with 5% FBS, plates were sealed, inverted and spun at 100 *g* for 5 min to remove non-adherent leukocytes. Adherence of labeled U937 cells was determined in a plate reader. Control studies indicated that fluorescence was a linear function of leukocytes in the range of 1,500–60,0000 cells/microplate well. Based on the standard curve obtained, the results are reported as the number of U937 cells adherent to the underlying endothelial cells (n = 3).

ELISA Assay for Leukocyte Adhesion Receptors on Endothelial Cells

Endothelial cells were plated and grown to confluence in 96-well plates and incubated with or without treatments as for adhesion assays. Cell surface expression of ICAM-1, VCAM-1, E-selectin and von Willebrand factor was determined by ELISA methods using primary binding by specific goat antibodies (R&D Systems Inc., Minneapolis, Minn., USA). After washing once with Hanks' balanced solution, primary antibodies in M-199 medium with 5% FBS (concentrations as per manufacturer) were added to each well and incubated at 37°C for 30 min. The cells were then washed twice with Hanks' solution and incubated at 37°C for 1 h with horseradish peroxidase-conjugated anti-goat IgG (Santa-Cruz Biotech, Calif., USA) diluted 1:500 in M-199 medium with 5% FBS. After washing 4 times

with Hanks' solution, antibody binding was detected by adding 100 µl of 0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine (Sigma Chemical Co., St. Louis, Mo., USA) with 0.003% hydrogen peroxide. 100 µl of 8 *N* sulfuric acid was added to stop the reaction. Plates were read on an ELISA plate reader at OD 450 nm and the results are expressed as average of the readout (± SEM) (n = 3).

ELISA Assays for MCP-1 and IL-8

Confluent human GEC and mesangial cells (Clonetics) in 96-well plates were incubated with vehicle, unoxidized EPA or oxidized EPA at varying concentrations of 10, 25, 50, 75 and 100 µ*M* for 1 h prior to stimulation with hTNFα (10 ng/ml) for 5 h. Aliquots of the medium were removed for analysis of MCP-1 and IL-8 by ELISA using matched antibodies as previously described [28, 29]. Antibody binding was visualized with horseradish peroxidase-conjugated streptavidin and TMB liquid substrate system. Results are expressed as MCP-1 and IL-8 (pg/ml) in the culture medium using values determined on a standard curve (± SEM) (n = 3).

Transmigration Assay

Transmigration assays were done using modified Boyden chambers (Costar). GEC and HUVEC were seeded on transwell inserts (3 µ*M* pore size) coated with human fibronectin. Differentiation of the U937 cells (3 × 105 cells/ml) was induced by treatment with 1 m*M* dibutyryl cAMP (Sigma) for 24 h. Confluent endothelial cells (day 6) were treated with vehicle (PBS), 100 µ*M* unoxidized EPA or oxidized EPA for 1 h prior to stimulation with TNFα (10 ng/ml) for 5 h. FMLP (10–8 *M*) (closed bars) was added to the lower chamber 45 min prior to addition of U937 (200 µl, 5 × 10^6/ml) to the top chamber. U937 transmigration was allowed to proceed for 3 h at 37°C, following which U937 cells from the lower chamber were collected, centrifuged, resuspended in 200 µl PBS and counted. Results are given as transmigration index, which is the number of cells that have migrated following treatment of the endothelial cells divided by the number of cells that migrated after vehicle control alone (n = 3).

Electrophoresis Mobility Gel Shift Assays

GEC and mesangial cells were grown to confluence in gelatin-coated 100-mm dishes. The cells were pretreated for 1 h with vehicle control, 100 µ*M* unoxidized EPA or oxidized EPA in standard growth medium prior to stimulation with TNFα (10 ng/ml) for 2 h. Nuclear extracts were prepared [30]. Protein concentration was determined by Bio-Rad DC protein assay. The double-stranded oligonucleotide containing the consensus sequence for the NF-κB was end labeled by incubating the oligonucleotide with γ^{32}P-labeled ATP and T_4 polynucleotide kinase at 37°C for 10 min according to standard protocols (Promega). For binding reactions, nuclear extracts (10 µg) were incubated in 10–15 µl of total reaction volume with 20 µl of binding buffer and ^{32}P-labeled NF-κB oligonucleotides for 20 min at room temperature. Samples were electrophoresed on a 5% nondenaturing acrylamide gel. The gels were dried and autoradiographed. Polyclonal antiserum against the p65 subunit of NF-κB was used to determine the subunit composition of gel-shifted NF-κB complexes (supershift assays). 1 µg of antibody was added to the binding reaction 20 min prior to addition of labeled oligonucleotide probe (n = 3).

Statistical Analysis

Data are presented as average ± SEM. Statistical significance was assessed by unpaired Student's t test.

Results

Oxidized EPA Inhibits U937 Adhesion to Cytokine-Activated GEC

Using an adhesion assay, we determined whether pretreatment of GEC with unoxidized EPA or oxidized EPA had an effect on leukocyte:endothelial interactions. We found that oxidized EPA pretreatment of GEC significantly inhibited U937 adhesion to cytokine-stimulated GEC, while unoxidized EPA had a minimal effect (fig. 1A). The inhibition of TNFα-induced adhesion was dose-dependent; 75 and 100 µ*M* of oxidized EPA reduced U937 adhesion to levels close to those seen for unstimulated GEC. We also compared the effect of oxidized EPA on GEC with endothelial cells derived from human umbilical vein, microvessels and coronary arteries. We found that there was a dose-dependent inhibition of U937 adhesion to cytokine-stimulated human microvessel, coronary artery and umbilical vein endothelial cells (fig. 1B–D) that are pretreated with oxidized EPA, while unoxidized EPA has a minimal effect.

Oxidized EPA Inhibits Cytokine-Induced Expression of Leukocyte Adhesion Receptors on GEC

Cytokine stimulation of endothelial cells induces expression of adhesion receptors, such as E-selectin, and VCAM-1 and ICAM-1, which support leukocyte rolling and adhesion, respectively. Treatment with oxidized EPA resulted in a dose-dependent reduction in TNFα-stimulated surface expression of ICAM-1, VCAM-1, and E-selectin in both human umbilical vein endothelial cells and GEC, whereas treatment with unoxidized EPA had no significant effect (table 1). Oxidized EPA had no significant effect on the expression of von Willebrand protein, which is constitutively expressed.

Oxidized EPA Inhibits Cytokine-Induced Mesangial and Glomerular Endothelial Cell Expression of MCP-1

We used ELISA methods to determine the effect of oxidized and unoxidized EPA on cytokine-induced endothelial expression of MCP-1 and IL-8. As expected, treatment of GEC (fig. 2A) and mesangial cells (fig. 2B) with TNFα results in a 2- to 4-fold increase in MCP-1 expression. Pretreatment of GEC and mesangial cells with oxidized EPA for 1 h significantly inhibited TNFα-induced expression of MCP-1, whereas incubation with unoxidized EPA had little effect. The inhibition was dose-dependent; 75 and 100 µ*M* oxidized EPA reduced MCP-1 expression to levels close to those seen for unstimulated GEC and mesangial

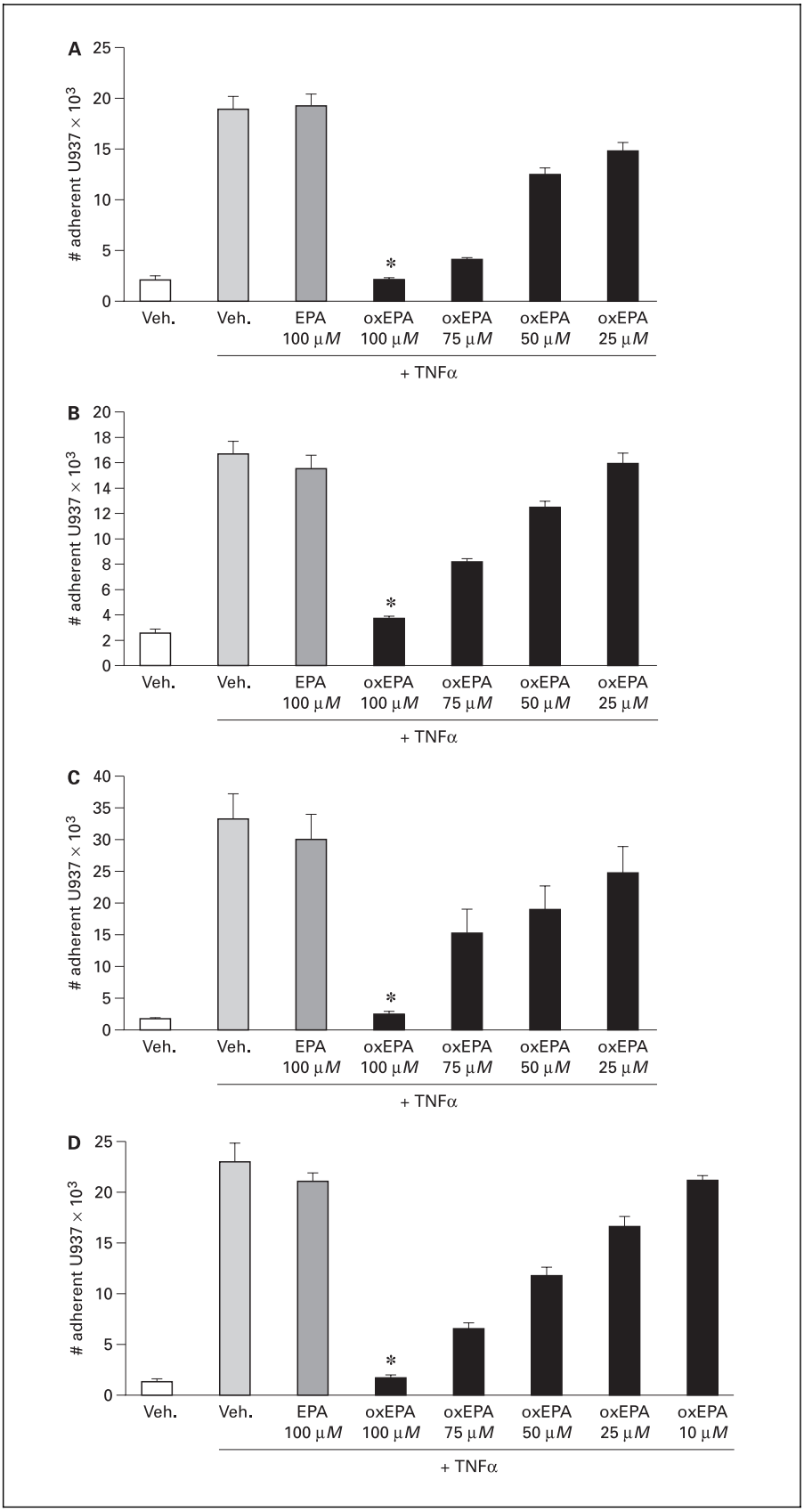

Fig. 1. Oxidized EPA inhibits U937 adhesion to cytokine-stimulated endothelial cells. GEC (**A**), human microvessel endothelial cells (**B**), human coronary artery endothelial cells (**C**), and human umbilical vein endothelial cells (**D**) were pretreated for 1 h with vehicle alone (Veh.), native unoxidized EPA (EPA) or oxidized EPA (oxEPA) at the indicated concentrations. The endothelial cells were then stimulated with TNFα for 5 h and assayed for U937 adhesion. Results are expressed as number of U937 attached to underlying endothelial cells (± SEM). * $p < 0.00001$ compared to EPA/+TNFα and Veh/+TNFα (n = 3).

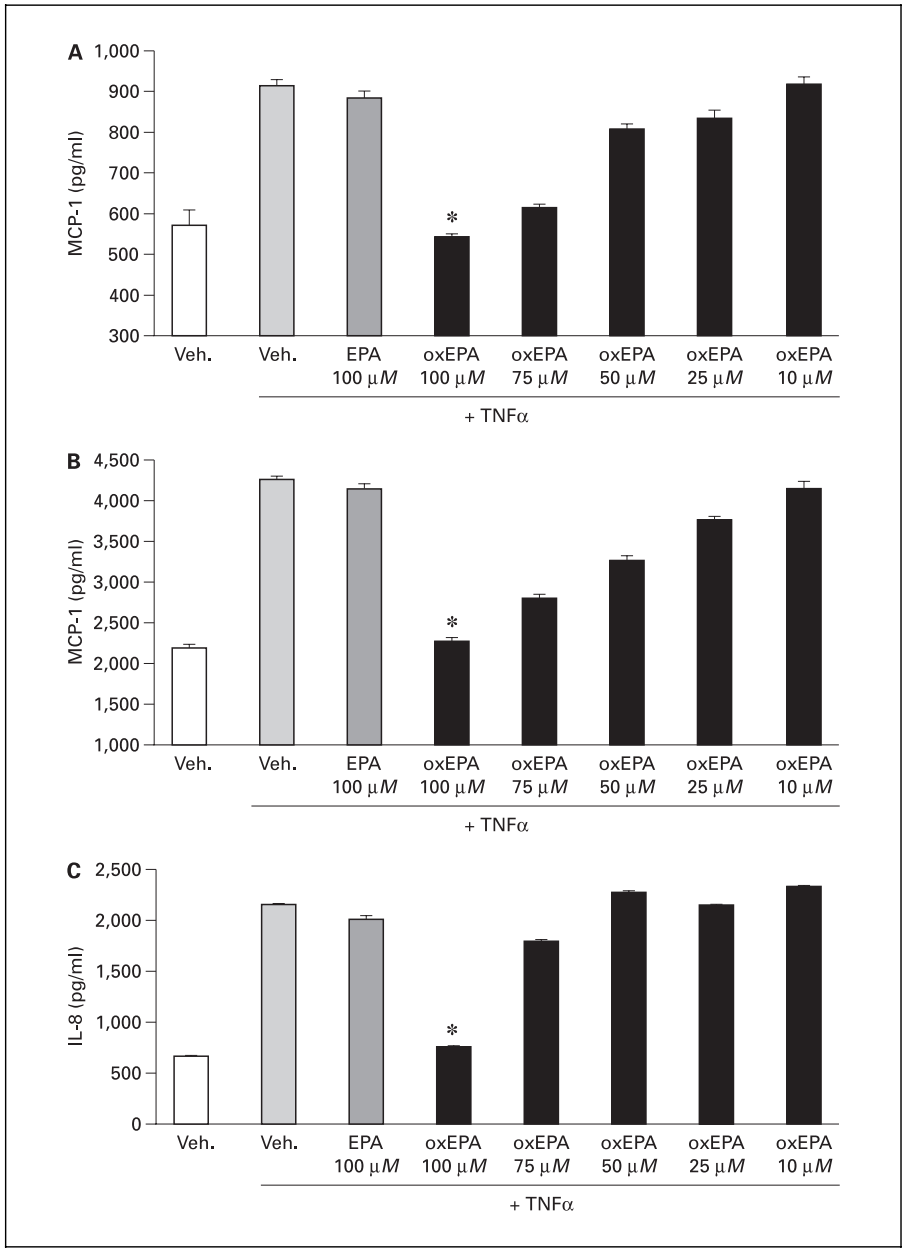

Fig. 2. Oxidized EPA inhibits cytokine-induced endothelial and mesangial chemokine expression. GEC (**A**, **C**) and mesangial cells (**B**) were pretreated for 1 h with vehicle (Veh.), 100 μ*M* unoxidized (EPA) or oxidized EPA (oxEPA) at indicated concentrations prior to stimulation with TNFα for 5 h. Aliquots of the culture medium were analyzed for MCP-1 (**A**, **B**) and IL-8 (**C**) by ELISA techniques. * $p < 0.000001$ compared to EPA/TNFα and Veh/TNFα.

cells. The effect of oxidized EPA on cytokine-induced IL-8 expression was less marked. There was a modest but significant decrease in TNFα-induced endothelial expression of IL-8 when GEC were pretreated with oxidized EPA (100 and 75 μ*M*), whereas incubation with unoxidized EPA had little effect. At concentrations of 50 μ*M* and lower, oxidized EPA had no significant effect in inhibiting TNFα-induced IL-8 expression in GEC (fig. 2C). Oxidized EPA had no significant effect in decreasing IL-8 expression in mesangial cells (data not shown).

Oxidized EPA Inhibits U937 Transmigration across Endothelial Cells

We studied the effect of oxidized EPA on transmigration of U937 across human umbilical vein endothelial cells (fig. 3A) and glomerular endothelial cell monolayers (fig. 3B) using modified Boyden chambers. Differentiation of the U937 cells (3×10^5 cells/ml) was induced by treatment with 1 m*M* dibutyryl cAMP for 24 h. We found that oxidized EPA pretreatment of human umbilical vein endothelial cells and GEC for 1 h prior to stimulation

with TNFα for 5 h results in substantial inhibition of transmigration of U937 cells from the upper chamber to the lower chamber, while unoxidized EPA had a minimal effect. The difference was even more marked when FMLP was added to the lower chamber.

Oxidized EPA Inhibits Cytokine-Induced NF-κB Activation in Glomerular Mesangial and Endothelial Cells

The expression of leukocyte adhesion receptors, and chemokines MCP-1 and IL-8, is regulated through transcription factor NF-κB [19, 20]. We studied the effect of oxidized and unoxidized EPA on cytokine-induced acti-

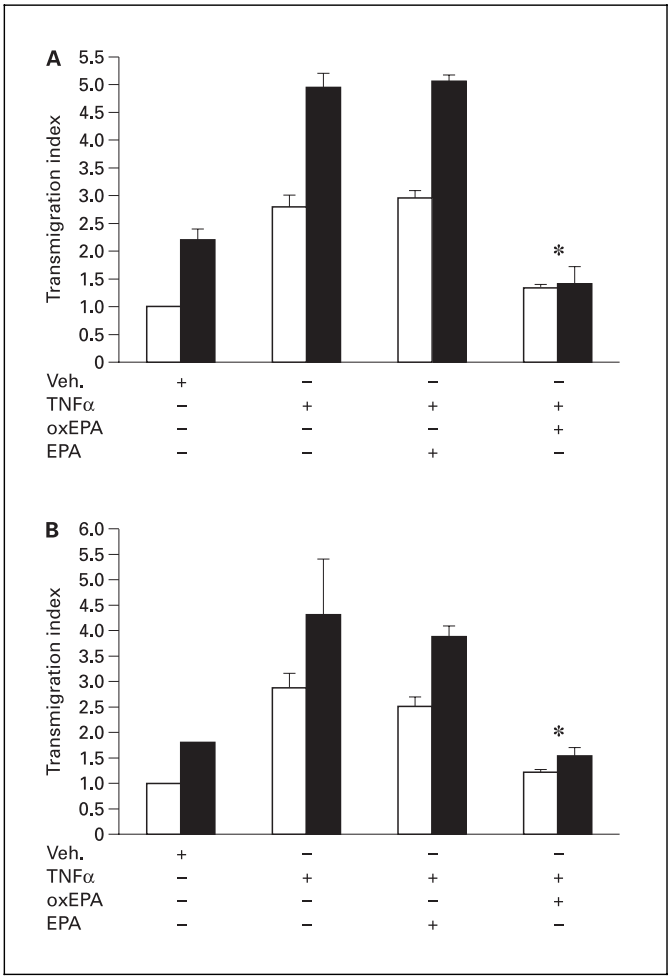

Fig. 3. Oxidized EPA inhibits transmigration of U937 across endothelial cells. Confluent GEC (**A**) and human umbilical vein endothelial cells (**B**) on transwell inserts were pretreated for 1 h with vehicle (Veh.), 100 μ*M* unoxidized EPA (EPA) or oxidized EPA (oxEPA) for 1 h prior to stimulation with 25 TNFα (10 ng/ml) for 5 h. FMLP (10–8 *M*) (closed bars) was added to the lower chamber 45 min prior to addition of U937 (200 μl, 5 × 10^6/ml) to the top chamber. U937 transmigration was allowed to proceed for 3 h at 37°C, following which U937 cells from the lower chamber were collected, centrifuged and counted. Results are given as transmigration index, which is the number of cells that have migrated following treatment of the endothelial cells divided by the number of cells that migrated after vehicle control alone (n = 3), * $p < 0.0005$ compared to EPA/TNFα and Veh/TNFα.

Table 1. Effect of oxidized EPA on expression of endothelial adhesion molecules: GEC (**A**) and human umbilical vein endothelial cells (**B**) were pretreated for 1 h with vehicle, native unoxidized EPA (EPA) or oxidized EPA (oxEPA) at indicated concentrations prior to stimulation with TNFα for 5 h

		Vehicle	Vehicle + TNFα	EPA (100 μ*M*) + TNFα	oxEPA (100 μ*M*) + TNFα	oxEPA (75 μ*M*) + TNFα	oxEPA (50 μ*M*) + TNFα	ox EPA (25 μ*M*) + TNFα	ox EPA (10 μ*M*) + TNFα
A	ICAM-1	0.226 (0.01)	1.097 (0.05)	1.052 (0.04)	0.385 (0.02)*	0.792 (0.05)	0.967 (0.03)	1.044 (0.05)	1.3 (0.03)
	VCAM-1	0.115 (0.002)	0.546 (0.002)	0.554 (0.04)	0.141 (0.002)*	0.233 (0.015)	0.381 (0.01)	0.408 (0.04)	0.517 (0.001)
	E-selectin	0.12 (0.004)	0.470 (0.009)	0.470 (0.01)	0.189 (0.004)*	0.266 (0.008)	0.309 (0.01)	0.357 (0.01)	0.434 (0.005)
	vWF	1.643 (0.06)	1.591 (0.06)	1.455 (0.02)	1.76 (0.04)	1.51 (0.02)	1.461 (0.02)	1.394 (0.07)	1.612 (0.15)
B	ICAM-1	0.143 (0.01)	1.002 (0.03)	1.005 (0.07)	0.153 (0.01)*	0.215 (0.03)	0.432 (0.05)	0.708 (0.08)	0.899 (0.07)
	VCAM-1	0.132 (0.01)	0.687 (0.13)	0.593 (0.06)	0.127 (0.01)*	0.113 (0.03)	0.188 (0.01)	0.294 (0.07)	0.386 (0.04)
	E-selectin	0.107 (0.01)	0.598 (0.03)	0.665 (0.08)	0.099 (0.01)*	0.141 (0.02)	0.313 (0.02)	0.413 (0.03)	0.518 (0.01
	vWF	4.0 (0.22)	3.9 (0.12)	3.56 (0.29)	4.0 (0.0)	3.69 (0.18)	3.56 (0.05)	3.53 (0.06)	3.57 (0.06)

Surface expression of adhesion molecules and von Willebrand factor (vWF) was quantitated by ELISA assay using appropriate monoclonal antibodies. ± SEM values are shown in parenthesis. * $p < 0.0001$ compared to vehicle + TNFα and unox. EPA + TNFα (n = 3).

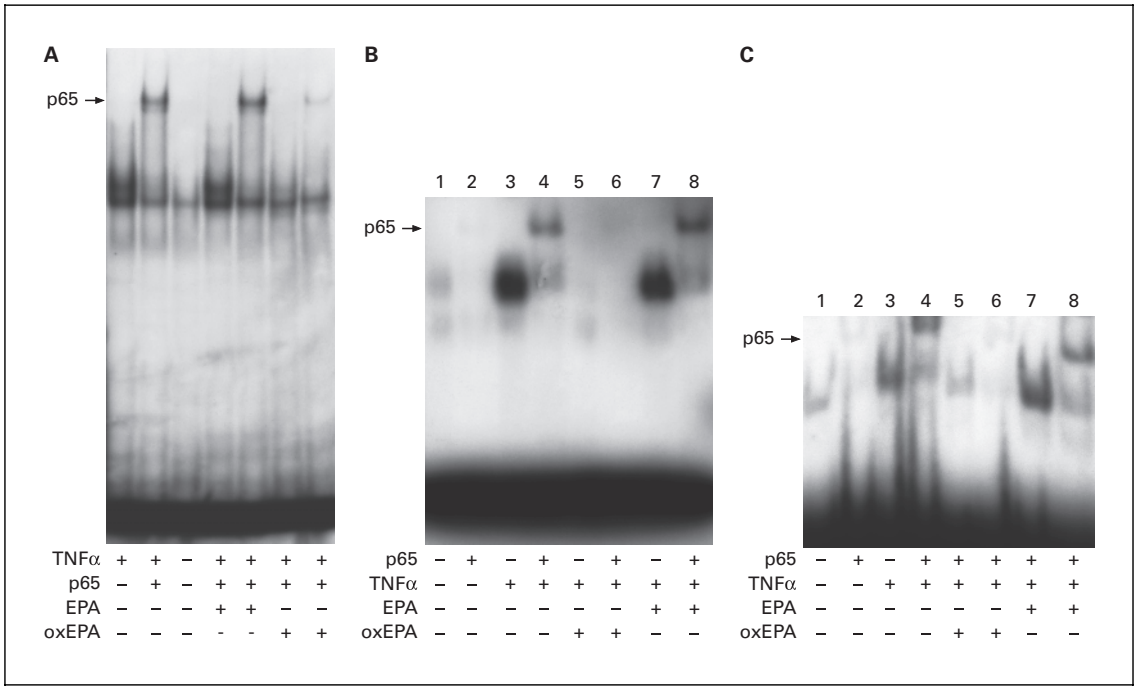

Fig. 4. Oxidized EPA inhibits TNFα-induced activation of NF-κB in GEC and mesangial cells. Nuclear extracts were prepared from human umbilical vein endothelial cells (**A**), glomerular endothelial cells (**B**) and mesangial cells (**C**), treated with vehicle, 100 μ*M* unoxidized EPA (EPA) or oxidized EPA (oxEPA) prior to stimulation with TNFα for 2 h. The nuclear extracts were co-incubated with ^{32}P-labeled NF-κB oligonucleotide and electrophoresis mobility shift assays were done. Nuclear extracts were preincubated with antibodies to p65 as indicated (n = 3).

vation of NF-κB by performing gel shift assays on nuclear extracts of human umbilical vein endothelial cells (fig. 4A), GEC (fig. 4B) and mesangial cells (fig. 4C). Treatment of endothelial and mesangial cells with TNFα for 2 h results in activation of NF-κB as indicated by the gel shifts. Pretreatment of endothelial and mesangial cells with oxidized EPA for 1 h almost completely inhibited TNFα-induced activation of NF-κB, whereas incubation with unoxidized EPA had little effect. The p65 component of NF-κB was confirmed in supershift assays using antibodies to the p65 subunit of NF-κB.

Discussion

Consumption of ω–3 fatty acids in fish oil has been reported to improve the prognosis of several chronic inflammatory diseases characterized by leukocyte accumulation, including atherosclerosis, inflammatory bowel disease, rheumatoid arthritis, psoriasis, etc. [1–3]. Fish oil is also recommended for treatment of IgA nephropathy and the beneficial effects have been noted in both native and recurrent IgA nephropathy in transplanted kidneys [4, 31]. However, the exact mechanism for their beneficial effects is not known, prompting an editorial comment – 'Dietary fish oil supplementation in IgA nephropathy: A therapy in search of a mechanism?' [32].

Our present studies indicate that the beneficial effects of fish oil in chronic inflammatory diseases, including IgA nephropathy, may be due to the oxidative modification of ω–3 fatty acids and their inhibition of cytokine-induced pro-inflammatory events in endothelial cells, such as increase in adhesion receptor and chemokine expression.

Several studies point to an important role of enhanced expression of chemokines and surface adhesion receptors on endothelial cells in the pathogenesis of inflammatory diseases including glomerulonephritis [33]. The severity of glomerulonephritis is altered in mice deficient in leukocyte adhesion receptors [33] and neutralization of MCP-1 has been shown to attenuate in vivo injury arising from inflammatory mechanisms [34].

NF-κB plays an important role in the transcription of adhesion receptors and chemokines and is involved in inflammatory and immune responses through induction of various cytokines and growth factors. NF-κB activation appears to be necessary for the induction of adhesion molecule and chemokine genes and deletion of NF-κB binding sites results in an inability to induce these genes. With regard to IgA nephropathy, NF-κB is expressed in mesangial and GEC in tissue samples obtained from patients with IgA nephropathy. The expression of NF-κB in both glomeruli and interstitium correlates with progression of renal tissue injury. Furthermore, glomerular and interstitial cells in samples from IgA nephropathy showed increased expression of MCP-1 and GM-CSF, suggesting that NF-κB is involved in the progression of tissue injury in IgA nephropathy through the induction of these genes [35]. NF-κB inhibitors have been shown to attenuate the severity of glomerular lesions in immune-complex-mediated experimental glomerulonephritis [36].

ω–3 fatty acids are easily oxidized. ω–3 fatty acids, such as EPA and DHA, are highly polyunsaturated and readily undergo oxidation at ambient and subambient temperatures, even in the absence of exogenous oxidizing reagents [9, 10]. In fact, it is very difficult to avoid the oxidation of these very labile PUFAs. More importantly, in vivo, a large increase in tissue and plasma accumulation of fatty acid oxidation products is noted in subjects consuming fish oil even after addition of antioxidant supplements to the diet, which suggests extensive oxidation of ω–3 fatty acids in vivo [25–27]. Unfortunately, very few studies have taken into account the auto-oxidation of ω–3 fatty acids when studying the anti-inflammatory properties of fish oil.

The protective effects of the ω–3 fatty acids seems somewhat paradoxical considering the current belief that oxidation of LDL and LDL-borne fatty acids is an important precedent to atherogenesis. However, studies have shown that fish oil consumption does not lead to an increase in oxidation of plasma proteins [27], which is important since protein oxidation can contribute to the development of diseases such as atherosclerosis.

In view of the ease with which ω–3 PUFAs spontaneously oxidize, and in vivo data suggesting extensive accumulation of oxidation products following fish oil consumption, we investigated the possibility that oxidized ω–3 fatty acids may be an important component of the observed anti-inflammatory effects of fish oil. In our previous studies we showed that oxidized EPA and not unoxidized EPA pretreatment of HUVEC inhibits leukocyte adhesion to cytokine-stimulated HUVEC [11, 12]. In our current studies we extend these findings to GEC and endothelial cells derived from human microvessels and coronary arteries. We show that oxidized EPA and not unoxidized EPA pretreatment of the endothelial cells results in almost similar degree of inhibition of leukocyte adhesion to cytokine-induced glomerular, microvessel and coronary artery endothelial cells. We also show that oxidized ω–3 fatty acids inhibit leukocyte transmigration across endothelial cells, thus limiting leukocyte accumulation at sites of tissue injury. Furthermore, oxidized EPA pretreatment of GEC results in a significant decrease in the cytokine-induced endothelial expression of chemokine MCP-1 and IL-8. Oxidized EPA also inhibited MCP-1 expression in human mesangial cells. Lastly, we show that the mechanism of inhibitory effects of oxidized ω–3 fatty acids on adhesion receptor and chemokine expression is through inhibition of NF-κB activation.

What is the mechanism for the oxidized EPA-mediated inhibition of cytokine-induced NF-κB activation? In our previous studies we noted that oxidized EPA is a potent activator of peroxisome proliferator-activated receptor α (PPARα) and that PPARα is needed for the inhibitory effects of oxidized EPA on leukocyte-endothelial interactions [12], which lead us to hypothesize that oxidized EPA might inhibit cytokine-induced NF-κB activation through a PPARα-dependent pathway. In our more recent studies, we have found that oxidized EPA inhibits cytokine-induced NF-κB activation in wild-type endothelial cells, while it has minimal inhibitory effects in PPARα-deficient endothelial cells suggesting that oxidized EPA mediates its inhibitory effects on NF-κB through PPARα, and that oxidation of EPA appears to be necessary for this effect.

Bochkov et al. [37] recently showed that oxidized phospholipids inhibit LPS-induced expression of cell adhesion molecules and other NF-κB-mediated upregulation of inflammatory genes indicating that oxidized fatty acids in the phospholipids might be responsible for protective effect against endotoxin-induced tissue damage. Subbanagounder et al. [38] and Letinger et al. [39] have also recently shown that oxidized phospholipids regulate MCP-1 and IL-8 expression and endothelial binding of monocytes and neutrophils, through their effects on PPARα and NF-κB, respectively, whereas native phospholipids are ineffective. It is important to note that these studies have used oxidized phospholipids derived from arachidonic acid. Unlike oxidized phospholipids which are much larger lipids that probably act though surface receptors or need to be endocytosed, oxidized fatty acids are smaller molecules that likely enter the cell directly

through the cell membrane (across a concentration gradient) and can then potentially act directly on intracellular signaling pathways.

Oxidized EPA did not have any effect on the viability of endothelial cells for the following reasons: Viability assays like MTT assays did not reveal any cytotoxic effect of oxidized and unoxidized EPA on endothelial and mesangial cells. The inhibition of leukocyte-endothelial interactions is reversible: after 48–60 h of oxidized EPA pretreatment the endothelial cells react normally to cytokine activation and there is no inhibition of leukocyte adhesion to activated endothelial cells (data not shown). Also, oxidized EPA did not have any effect on the constitutively expressed surface proteins such as von Willebrand factor, endoglin, and HLA class I molecules.

Nohe et al. [40] have recently found that prolonged incubation of endothelial cells with ω–3 fatty acids (20 h or more) prior to TNFα stimulation results in inhibition of adhesion molecule expression, while shorter incubation periods (6 h) has no effect. This suggests that oxidation of ω–3 fatty acids takes place during the long incubation period, and that the oxidation product(s) and not the native ω–3 fatty acids are most likely responsible for the inhibition of leukocyte-endothelial interactions (in our studies, oxidation of EPA is achieved by incubating the fatty acid in PBS for 16 h at 37°C).

Preliminary separation of oxidation products of EPA by column chromatography and high-performance liquid chromatography results in the generation of compounds with retention times in the range of isoprostanes, hydroperoxyeicosapentaenoic acid (HpEPEs), hydroxyeicosapentaenoic acid (HEPEs), and epoxyeicosquatrienoic acids (EEQs), as well as a large amount of secondary oxidation products (data not shown). It is likely that the secondary oxidation products are probably responsible for the observed inhibition of the pro-inflammatory responses because oxidation product(s) of EPA such as isoprostane 8-iso-PGF3α (Cayman Chemicals), HpEPEs (Cayman Chemicals), HEPEs (Cayman Chemicals), and EEQs (gift of Dr. Mike VanRollins, University of Iowa) had no inhibitory effects on the pro-inflammatory responses even in high doses (data not shown). However, incubation of the EEQs in PBS for 12 h did inhibit leukocyte-endothelial interaction suggesting that further oxidation and possibly the generation of specific secondary oxidation product(s) is required for inhibitory effects on leukocyte-endothelial interactions. Furthermore, it is likely that only a small number of these are actually responsible for the anti-inflammatory effects conferred by oxidized EPA and hence are likely to effective at much lower concentrations than used in these studies. The identification of these products could result in a new class of potent, low-toxicity, pro-inflammatory response inhibitors with potent anti-NF-κB properties for the treatment of inflammatory diseases.

Finally, we speculate that these studies may have relevance to the normal biological functioning of the endothelium. The oxidation of PUFAs is likely to occur in areas of inflammation due to the increased expression of oxidative enzymes (e.g. NADPH oxidase, myeloperoxidase, cyclooxygenase, lipoxygenase) and generation of reactive oxygen species that are capable of oxidizing highly unsaturated fatty acids such as ω–3 fatty acids. These endogenously produced oxidized fatty acids could then act locally to suppress pro-inflammatory responses on adjacent endothelium and mesangial cells (in inflammatory lesions of the kidney). This might serve as a natural inbuilt mechanism to control the ingress of leukocytes and thus limit the inflammatory response and the consequences of uncontrolled inflammatory reactions.

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