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The fatty acid oxidation product 15-A_{3t}-Isoprostane is a potent inhibitor of NFκB transcription and macrophage transformation

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Abstract

Fatty acids such as eicosapentaenoic acid (EPA) have been shown to be beneficial for neurological function and human health. It is widely thought that oxidation products of EPA are responsible for biological activity, although the specific EPA peroxidation product(s) which exert these responses have not yet been identified. In this work we provide the first evidence that the synthesized representative cyclopentenone IsoP, 15-A_{3t}-IsoP, serves as a potent inhibitor of lipopolysaccharide-stimulated macrophage activation. The anti-inflammatory activities of 15-A_{3t}-IsoP were observed in response not only to lipopolysaccharide, but also to tumor necrosis factor alpha and IL-1b stimulation. Subsequently, this response blocked the ability of these compounds to stimulate nuclear factor kappa b (NFκB) activation and production of proinflammatory cytokines. The bioactivity of 15-A_{3t}-IsoP was shown to be dependent upon an unsaturated carbonyl residue which transiently adducts to free thiols. Site directed mutagenesis of the redox sensitive C179 site of the Ikappa kinase beta subunit, blocked the biological activity of 15-A_{3t}-IsoP and NFκB activation. The vasoprotective potential of 15-A_{3t}-IsoP was underscored by the ability of this compound to block oxidized lipid accumulation, a critical step in foam cell transformation and atherosclerotic plaque formation. Taken together, these are the first data identifying the biological activity of a specific product of EPA peroxidation, which is formed in abundance *in vivo*. The clear mechanism linking 15-A_{3t}-IsoP to redox control of NFκB transcription, and the compound's ability to block foam cell transformation suggest that 15-A_{3t}-IsoP provides a unique and potent tool to provide vaso- and cytoprotection under conditions of oxidative stress.

Keywords

Alzheimer's disease; cytoprotection; eicosapentaenoic acid; inflammation; oxidative stress

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In the last three decades, we have increasingly come to recognize that 'omega' or n-3 fatty acids play essential roles in human health and development, particularly in development of the nervous system (Connor 1997; Connors *et al.* 2008). α -linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid are all polyunsaturated fatty acids which have been at the forefront of these studies and have independently displayed cytoprotective activity. One of the primary mechanisms by which these fatty acids enhance cellular function is by the reduction of inflammation *in vivo* (Calder 2006). Human epidemiological and recent clinical intervention trials suggest that consumption of fish or dietary supplementation with fish oil, which is rich in EPA, reduces the incidence of cognitive decline, atherosclerosis, diabetes, and other inflammatory disorders (Kris-Etherton *et al.* 2002; Yokoyama and Origasa 2003; Barnham *et al.* 2004; Calder 2006; Mozaffarian 2007; Yokoyama *et al.* 2007; Saito *et al.* 2008). Given the unstable nature of fatty acids, identifying the bioactive metabolites and mechanism of action of n-3 fatty acids is essential to design targeted therapeutics for disorders linked to inflammatory stress. The mechanisms through which EPA exerts its biological activity are, however, poorly understood. The complexity of this problem is exacerbated by the fact that when in the *cis* configuration, the omega-3 fatty acids readily form dozens of potentially bioactive byproducts. Transformation events are potentiated in the presence of oxidative stress and native EPA is highly susceptible to beta-oxidation particularly in the brain (Chen *et al.* 2011).

We have previously determined that oxidation of EPA yields a family of prostaglandin (PG)-like molecules termed isoprostanes (IsoPs). IsoPs are formed *in vitro* and *in vivo* in settings of oxidative stress (Gao *et al.* 2006; Brooks *et al.* 2008a). One class of IsoPs shown to form in abundance is cyclopentenone IsoPs or A_3/I_3 -IsoPs. These compounds have an analogous structure to the arachidonic acid-derived cyclopentenone prostaglandins (PGs), PGA_2 and PGJ_2 (Fig. 1). Even more recent work suggests that oxidative metabolites of EPA are biological active (Chaudhary *et al.* 2004; Mishra *et al.* 2004). These studies have not, however, determined which *specific* product(s) formed from native EPA are responsible for the biological activities reported.

Oxidative stress, neuroinflammation, and atherosclerosis are closely coupled and co-occur in many neurologic diseases, in particular stroke. Pathologic activation of macrophages in the brain and neurovasculature is a major source of cerebral oxidative damage, inflammatory damage, and atherogenesis. EPA supplementation suppresses macrophage activation and subsequent neuroinflammation and pathology in several models of neurologic disease. Given that under conditions of oxidative stress, EPA-derived IsoPs form at levels 10 times higher than do IsoPs from arachidonic acid (Gao *et al.* 2007), we sought to isolate a single bioactive compound formed in abundance by oxidation of native EPA and determine the mechanism of bioactivity.

Experimental procedures

Materials

15- A_{3I} -IsoP (Fig. 1) was obtained by total synthesis in a manner similar to the synthesis of A_2 -IsoPs (Zanoni *et al.* 2002). 15- A_{3I} -IsoP was stored in ethyl acetate at -80°C until immediately before use at which time it was dried under nitrogen and resuspended in ethanol. 15- A_{3I} -IsoP was added to culture medium immediately before its addition to cells. As compounds with α,β -unsaturated carbonyls rapidly react with albumin, serum-free medium was used for all experiments (Musiek *et al.* 2005). 15- A_{3I} -IsoP was stable in this culture media for > 24 h. The $I\kappa B$ (C179A) mutant expression vector was a gift of Dr M. Karin (University of California, San Diego). Lipopolysaccharide (LPS) (from *Salmonella* Minnesota Re 595), arabinoside cytosine and Oil Red O stain were purchased from Sigma-

Aldrich (St Louis, MO, USA). Tumor necrosis factor- α (TNF α) and interleukin-1 β (IL-1 β) were obtained from R & D Systems (Minneapolis, MN, USA). inhibitor of kappa b (I κ B α), cyclo-oxygenase 2 (COX-2), and NF κ B p65 subunit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and inducible nitric oxide synthase (iNOS), extracellular signal-related kinase, and the glyceraldehyde 3-phosphate dehydrogenase antibodies were from Cell Signaling Technologies (Danvers, MA, USA). GW9662, T00907, and G418 sulfate were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). Fetal bovine serum was purchased from Hyclone (Logan, UT, USA). All cell culture media and supplies were from Invitrogen (Carlsbad, CA, USA) unless otherwise noted.

Cell culture

RAW267.4 murine macrophage cells were obtained from ATCC (Manassas, VA, USA). NF κ B reporter macrophages were a generous gift from the laboratory of Dr Timothy Blackwell. These cells were originally obtained from the bone marrow of transgenic mice expressing a reporter plasmid containing the human immunodeficiency virus-long terminal repeat 36-bp enhancer (containing a total of eight NF κ B-binding sites) upstream of the herpes simplex virus minimal thymidine kinase promoter driving expression of Photinus luciferase (Blackwell *et al.* 2000). All cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin. Cells were plated on 24- or 6-well plates at least 24 h before any experimental manipulation.

Immunofluorescence microscopy

For immunofluorescence staining, RAW264.7 cells were grown on glass coverslips. Following exposure to 15-A_{3t}-IsoP or vehicle, cultures were fixed in 10% formaldehyde for 10 min, rinsed with phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100, and blocked for 1 h with 8% bovine serum albumin diluted in PBS. Coverslips were then incubated overnight at 4°C in rabbit anti-p65 (1 : 100) primary antibody in 1% bovine serum albumin. Cells were washed in PBS for a total of 25 min and incubated in Cy-2-labeled secondary antibodies for 1 h. Cells were then washed again and stained with 1.4 μ M 4',6-diamidino-2-phenylindole (DAPI) for 10 min followed by further washes. Coverslips were mounted on microscope slides, and fluorescence was visualized with a Zeiss Axioplan microscope.

Cell counts

Cells were counted by fluorescence microscopy and the number of cells exhibiting nuclear localization of NF κ B after treatment for the indicated time with LPS and 15-A_{3t}-IsoP was obtained as a percentage of the whole DAPI positive population (~200 cells counted/variable). Three independent experiments were quantified by an investigator blinded to the experimental condition. Experiments quantifying the percentage of cells exhibiting nuclear accumulation of the NF κ B p65 subunit were performed by immunofluorescence microscopy using a 40 \times objective. Data are the mean \pm SD from at least 10 fields of view and represent a total of 500–750 cells.

NF κ B reporter assay

Primary cells stably expressing NF κ B-driven luciferase reporter were utilized to determine the direct effect on NF κ B-mediated transcription. Following exposure to 15-A_{3t}-IsoP or vehicle, cells were scraped at selected time points in Lysis Buffer (Promega, Madison, WI, USA). The cell debris was pelleted by centrifugation at 17 178 *g* for 5 min, and 20 μ L of the supernatant was used to assay luciferase activity using a luminometer. Results were later normalized to cellular protein levels.

Measurement of nitrite

Nitrite, a stable breakdown product of nitric oxide, was measured in cell medium using the Griess reaction. Briefly, 100 μ L of conditioned medium was mixed with 50 μ L each of Griess reagent R1 and R2 (Cayman Chemical) in a 96-well plate, and absorbance was measured at 540 nm. Quantitation was achieved by comparison with sodium nitrite standards in Dulbecco's modified Eagle's medium.

Measurement of PGD₂

PGD₂, the primary product of COX-2 activation in macrophages, was measured in cell media. After 24 h of LPS stimulation, PGD₂ levels were quantified in 200 μ L of cell media by stable isotope dilution assays. Briefly, [⁴H₂]-labeled PGD₂ internal standard was added to samples. Samples were then purified using C18 and silica Sep-Pack extraction methods and thin layer chromatography. Samples were then analyzed as the pentafluorobenzyl ester, methoxylamine, trimethylsilyl ether derivatives via gas chromatography-negative ion chemical ionization mass spectrometry (GC-NICI-MS) and data were normalized to protein concentrations.

I κ K transfection

I kappa kinase (I κ K), a possible target for 15-A_{3t}-IsoP interaction, was mutated at the cysteine 179 (C179) residue to determine the effect on 15-A_{3t}-IsoP bioactivity. RAW264.7 cells were transfected with NF κ B-*luc* reporter plasmid plus an empty vector, WT, or mutant (C179A) I κ K β expression vectors using the Amaxa Nucleofector System (Amaxa, Inc., Bethesda, MD, USA). Forty-eight hours after transfection, cells were pre-incubated with vehicle or 15-A_{3t}-IsoP for 30 min, stimulated with vehicle or LPS for 4 h, and then harvested and subjected to the luciferase reporter assay. RAW264.7 cells were also transfected with the pcDNA3.1 antibiotic resistance plasmid (Invitrogen) plus WT or mutant (C179A) I κ K β expression vectors as described above. Transfected cells were selected using G418 sulfate (800 μ g/mL media) for 1 month. Stably transfected cell lines were treated as described previously for PGD₂ and nitrite assays, and the media was subjected to nitrite and PGD₂ assessments.

Western blotting

Total protein extract was prepared, and a small aliquot of the protein suspension was removed and stored at -20°C for protein quantification using the bicinchoninic acid protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal protein concentrations were separated using Criterion XT 10% Tris gels (Bio-Rad). Proteins were then transferred to polyvinylidene difluoride membranes and blocked for 15 minutes using methanol. Membranes were incubated overnight in primary antibody diluted in non-fat milk containing 0.1% Tween 20. The following day, membranes were washed in PBS containing 0.1% Tween 20 and incubated in horseradish peroxidase-conjugated secondary antibodies for 1 h at 22°C. Protein bands were visualized by chemiluminescence. Western blots subject to semi-quantitative analysis using the NIH ImageJ analysis program as previously described (McLaughlin *et al.* 2003; Brown *et al.* 2010).

Sodium borohydride reduction and C18 SepPak purification of synthetic 15-A_{3t}-IsoP

15-A_{3t}-IsoP is hypothesized to exert biological activity through the α,β -unsaturated carbonyl. To test the importance of this moiety, 100 μ g of 15-A_{3t}-IsoP was adjusted to pH 3, extracted with ethyl acetate, dried, and resuspended in 1 mL of methanol. One milliliter of 12% (w/w) sodium borohydride (NaBH₄), a weak reducing agent, in water was added to the methanol and incubated on ice for 30 min. Samples were diluted in 20 mL of water and adjusted to pH 3 and then subjected to solid-phase extraction using a C18 SepPak. The

SepPak column was washed with 7 mL of methanol and 10 mL of water (pH 3), and then the sample was added, followed by washes with 10 mL of water (pH 3) and 10 mL of heptane. The samples were eluted with 10 mL of 1 : 1 ethyl acetate/heptane and dried under nitrogen and resuspended in ethanol at appropriate stock concentrations.

Conjugation of 15A_{3t}-IsoP with GSH

As another method of determining the importance of the α,β -unsaturated carbonyl of the 15-A_{3t}-IsoP activity, 100 μ g of 15-A_{3t}-IsoP was incubated in 0.1M K₃PO₄ buffer (pH 6.5) in the presence of a 10-fold excess (~1 μ g) of GSH and 1 mg of equine liver glutathione-*S*-transferase (GST) containing a mixture of GSTs at 37°C for 2 h (Chen *et al.* 1999). The incubation mixture was then acidified to pH 3 and extracted with 2 volumes of CH₂Cl₂, followed by C18 silica Sep-Pak extraction (Fam *et al.* 2002). The samples were then dried under nitrogen and resuspended in ethanol at appropriate stock concentrations.

Oil Red O staining

The accumulation of oxidized low-density lipoprotein (OxLDL) is an important step in the formation of foam cells. Lipid accumulation and foam cell formation can be visualized using Oil Red O staining (Mori *et al.* 2001). RAW264.7 cells were treated with the mitotic inhibitor cytosine arabinoside and grown on coverslips. Following cellular exposure to OxLDL (100 μ g/mL) and 15-A_{3t}-IsoP (10 μ M) for 48 h, cultures were fixed for 40 min at 22°C with 4% paraformaldehyde and stained with 0.5% Oil Red O stain for 45 min. Coverslips were subsequently washed and dehydrated with 70% EtOH. Coverslips were mounted on microscope slides, and imaging was performed with a Zeiss Axioplan microscope.

F₂-Isoprostane assay

To measure the level of 15-A_{3t}-IsoP-induced oxidative stress in cells, total F₂-IsoPs were measured in combined medium and cell pellet as described previously (Morrow and Roberts 1999). Briefly, cells were scraped directly into their conditioned medium and then collected by centrifugation. The cell pellet was resuspended in 0.5 mL of methanol containing 0.005% butylated hydroxytoluene, sonicated, and subjected to chemical saponification using 15% potassium hydroxide to hydrolyze bound F₂-IsoPs. The conditioned medium was then added back to the cell lysate and adjusted to pH 3 followed by addition of [⁴H₂]-labeled 15-F_{2t}-IsoP internal standard. Free F₂-IsoPs were then purified by C18 and silica Sep-Pak extraction and thin layer chromatography and then analyzed as the pentafluorobenzyl ester, trimethylsilyl ether derivatives via GC-NICI-MS.

Statistics

Except where otherwise noted, data were summarized and are represented as mean \pm SEM. The statistical significance of differences between means was assessed using one-way analysis of variance (ANOVA) at the 95% level ($p < 0.05$), followed by the Tukey's multiple comparison tests using GraphPad Prism software.

Results

15-A_{3t}-IsoP inhibits LPS-induced iNOS and COX-2 expression and activity in RAW264.7 macrophages

When oxidized *in vivo*, native EPA produces cyclopentenone isoprostanes which are structurally similar to anti-inflammatory cyclopentenone PGs (Fig. 1). These products are formed in abundance both *in vivo* and *in vitro* (Brooks *et al.* 2008a). We obtained a single

regio- and stereoisomer formed from the endogenous oxidation of EPA–15-A_{3t}-IsoP-and used this to study the biological activities of the cyclopentenone IsoPs as a class.

Dose–response curves using MTT assays revealed no cytotoxicity in RAW264.7 cells when incubated for 24 h in 25 μM 15-A_{3t}-IsoP or elevation in oxidative stress as measured by free and bound 15-F_{2t}-IsoP (control levels 137 ± 9 and 15-A_{3t}-IsoP 161 ± 9 pg F_{2t}-IsoP/gram protein; *n* = 5, *p* = 0.19).

We did, however, find that that 15-A_{3t}-IsoP potently suppressed LPS-induced PGD₂ and nitric oxide production as measured by accumulation of PGD₂ and nitrite in cell media and normalized to protein concentration with an approximate IC₅₀ of 20 μM (Fig. 2a and b). 15-A_{3t}-IsoP also inhibited the expression of the LPS-stimulated pro-inflammatory proteins iNOS and cyclooxygenase-2 (COX-2) (Fig. 2c and d). Time-matched incubations in 15-A_{3t}-IsoP alone did not alter the expression or activity of iNOS, COX-2 or their biochemical products (data not shown). Densitometric analysis of 15-A_{3t}-IsoP effects on LPS stimulated COX-2 expression revealed a 17 ± 4% decrease in protein levels when cells were harvested 6 h after LPS stimulation and a 93 ± 2% decrease in iNOS expression when cells were harvested 9 h after LPS stimulation.

15-A_{3t}-IsoP Acts as an Inhibitor of the NFκB pathway

As the expression of iNOS and COX-2 enzymes is known to be regulated by the transcription factor NFκB (Chawla *et al.* 2001; Chun and Surh 2004), we examined the effects of 15-A_{3t}-IsoP on NFκB signaling. NFκB transcription activity was assessed using immortalized mouse macrophage cell line expressing an NFκB-activation responsive luciferase reporter. Luciferase measurements revealed a dose-dependent decrease in NFκB-mediated transcription with 25 μM dropping LPS-stimulated activation by more than 70% (Fig. 3a). 15-A_{3t}-IsoP had no effect on NFκB transcription or the degradation of IκB even when incubations were continued for 24 h (data not shown but see Brooks *et al.* 2008b; Cox *et al.* 2009). We next sought to characterize the method by which 15-A_{3t}-IsoP inhibited NFκB signaling.

NFκB is sequestered in the cytoplasm of macrophage cells and, upon inflammatory stimulation, the p50/p65 NFκB heterodimers translocate from the cytosol to the nucleus. Immunostaining of macrophages demonstrated that the NFκB p65 subunit was confined primarily to the cytoplasm in vehicle-treated cells whereas LPS stimulation resulted in intense nuclear p65 accumulation and colocalization with the nucleic acid stain DAPI after 1 h. Pre-treatment of macrophages with 15-A_{3t}-IsoP abrogated LPS-induced p65 nuclear translocation (Fig. 3b). Cell counts demonstrated a significant change in p65-nuclear staining between LPS-stimulated and 15-A_{3t}-IsoP pre-treated cells, indicating that cyclopentenone IsoPs have the ability to prevent NFκB nuclear translocation in the presence of an inflammatory mediator. Over 90% of the 500+ cells counted in the LPS stimulated condition had colocalization of DAPI and p65 compared to less than 13% in cells exposed to 15-A_{3t}-IsoP and LPS.

Inhibition of NFκB Signaling by 15-A_{3t}-IsoP occurs at the level of IκK function

We next sought to characterize the mechanism by which 15-A_{3t}-IsoP prevented the translocation of NFκB to the nucleus. LPS-induced NFκB translocation of p65 is dependent on the phosphorylation and degradation of IκBα, the chaperone protein associating with the NFκB heterodimers which maintain the transcription factor in the cytoplasm (de Winther *et al.* 2005; Gloire *et al.* 2006).

Western blot analysis demonstrated that LPS-induced stress resulted in degradation of the vast majority of the IκBα after 15 min of stimulation (Fig. 4a) and that this decrease was

maintained for at least 30 min (Fig. 4b). 15-A₃₁-IsoP blocked the LPS-stimulated IκBα degradation both at 15 min as well as at 30 min (Fig. 4a and b).

These data suggest that either 15-A₃₁-IsoP modifies inflammatory stress signals which promote their degradation or that 15-A₃₁-IsoP might work in a manner that would solely impact the signaling of LPS-induced NFκB stabilization. To address this question, we measured the ability of 15-A₃₁-IsoP to alter the activation of other IκBα-dependent pathways including TNFα, and IL-1β induced inflammation. Each of these stimuli act via distinct receptors and signaling cascades but converge on the IκB kinase (IκK) complex to activate NFκB (Karin and Ben-Neriah 2000).

We found that 15-A₃₁-IsoP blocked the activation of NFκB triggered by all three pro-inflammatory stimuli as assessed by the NFκB-mediated reporter system (Fig. 4c). This suggests that 15-A₃₁-IsoP-mediated inhibition of NFκB signaling does not occur at a receptor level but likely at the common point in these three pathways.

Bioactivity of 15-A₃₁-IsoP is independent of PPARγ and is abrogated by chemical reduction or conjugation of the cyclopentenone ring

Several reports have indicated that either direct or indirect activation of the peroxisome proliferator activator receptor (PPAR)γ nuclear receptor by native EPA as well as cyclopentenone-containing molecules and mediate the anti-inflammatory properties of these compounds (Chawla *et al.* 2001; Chene *et al.* 2007). To assess the role of PPARγ in mediating the effects of 15-A₃₁-IsoP, we used two structurally unrelated receptor antagonists, GW9662 and T0070907. Neither compound blocked the anti-inflammatory activity measured by NFκB reporter assays 4 h after LPS stimulation. Notably, both PPARγ antagonists were used at > 10-fold higher levels than required to potentiate inflammation suggesting that modification of PPARγ is not crucially involved to the anti-inflammatory properties of 15-A₃₁-IsoP (Fig. 5a).

In evaluating structural modifications which may account for the anti-inflammatory action of 15-A₃₁-IsoP, we next evaluated thiol-rich candidate molecules whose biological activity may be compromised by Michael addition with cyclopentenone-containing compounds (Perez-Sala *et al.* 2002). In the first set of experiments, 15-A₃₁-IsoP was incubated with NaBH₄, an agent whose weak reducing properties transform the carbonyl moiety on the cyclopentenone ring to a non-reactive alcohol (Brooks *et al.* 2008a; Cox *et al.* 2008, 2009), resulting in complete inhibition of the anti-inflammatory effect of 15-A₃₁-IsoP. When 15-A₃₁-IsoP was subjected to the identical incubation and purification steps without NaBH₄, the compound maintained its biological activity as seen in NFκB-reporter assays (Fig. 5b). This suggested an essential role for the reactive cyclopentenone ring in mediating the bioactivity of 15-A₃₁-IsoP.

Cyclopentenone-containing eicosanoids can be metabolized via conjugation with GSH by GST (Atsmon *et al.* 1990; Milne *et al.* 2004). Glutathione is the most abundant cellular antioxidant. Our group has previously shown that EPA-derived A₃/J₃-IsoPs are susceptible to GSH conjugation (Brooks *et al.* 2008a) and the modification of GSH and GST by cyclopentenone isoprostanes yields GSH conjugates that no longer possess anti-inflammatory activities (Musiek *et al.* 2008). Similarly, 15-A₃₁-IsoP incubated with GSH and equine liver GST substantially inhibited its anti-inflammatory effects (Fig. 5c). It should be noted that residual GSH, GST, and NaBH₄ were removed from these experiments by C18 column purification prior to cell treatment using previously stated methods (Chen *et al.* 1999). Taken together this suggests that thiol modification of the reactive cyclopentenone ring abrogates the anti-inflammatory activity of 15-A₃₁-IsoP.

Mutation of the I κ K β cysteine 179 impairs the anti-inflammatory effects of 15-A_{3t}-IsoP

Given the importance of the cyclopentenone ring structure to the biological effects of 15-A_{3t}-IsoP, we sought to determine whether this compound may inhibit NF κ B function through adduction specific thiols within the I κ K complex. Previous studies demonstrated that the cysteine 179 (C179) residue contains a thiol group susceptible to Michael adduction. It was also shown that cyclopentenone PGs, as well as their electrophilic metabolites, are known to inhibit NF κ B signaling via adduction to this residue (Rossi *et al.* 2000). Thus, we examined the impact of a mutation of the I κ K β C179 to an alanine (C179A), which would prevent Michael adduction to that site but otherwise not affect the activity of the I κ K β . As shown in Fig. 6a, over-expression of WT or mutant C179A I κ K β caused a marked increase in NF κ B-luciferase reporter activity in transiently transfected RAW264.7 cells in the absence of LPS signaling. 15-A_{3t}-IsoP suppressed this increase significantly in WT I κ K β over-expressing cells but was unable to abrogate the inflammatory effect of LPS in the C179A mutation expressing cells. 15-A_{3t}-IsoP therefore exerts some of its anti-inflammatory activity through adduction to this particular cysteine residue. Other studies demonstrated that 15-A_{3t}-IsoP was able to significantly inhibit both PGD₂ (Fig. 6b) and nitrite (data not shown) production in cells stably transfected with the WT construct. 15-A_{3t}-IsoP, however, was less successful in inhibiting LPS-induced inflammation in cells stably transfected with the mutant C179A construct. Thus a specific residue on the I κ K complex was determined to be a primary site of 15-A_{3t}-IsoP biological activity, and, for the first time, the pathway of a specific product of non-enzymatic EPA peroxidation has been explicitly demonstrated.

15-A_{3t}-IsoP Affects lipid accumulation in macrophages in the presence of oxidized LDL

Increased activation of NF κ B signaling has been shown to correlate with atherosclerosis (Brand *et al.* 1996; Wilson *et al.* 2000). Inhibition of the NF κ B signaling pathway, however, has been offered as a possible method for prevention of foam cell formation because inhibition of this pathway decreases lipid loading in macrophages treated with OxLDL (Ferreira *et al.* 2007). To study effects that 15-A_{3t}-IsoP might exert on lipid loading, RAW264.7 macrophages were treated for 48 hours with vehicle, OxLDL (100 μ g/mL) or OxLDL and 15-A_{3t}-IsoP (10 μ M). Forty-eight hours of treatment with either OxLDL or 15-A_{3t}-IsoP did not have an effect on cellular viability (data not shown). Vehicle-treated cells demonstrated low levels of intracellular lipid accumulation as determined by Oil Red O staining. (Fig. 7a). Oil Red O stains neutral lipids, and cells demonstrating more than five intracellular Oil Red O droplets were considered to be positive for intracellular lipid accumulation. Treatment of cells with OxLDL significantly increased the number of stain-positive cells. Incubation of cells with 15-A_{3t}-IsoP along with OxLDL, however, significantly decreased lipid loading, returning to near control levels (Fig. 7b). Thus, treatment of RAW264.7 cells with 15-A_{3t}-IsoP revealed a decrease in OxLDL-induced lipid loading in cells with inhibited NF κ B signaling. These data point to an underlying mechanism for the anti-atherosclerotic effects associated with EPA supplementation.

Discussion

The CNS vasculature endures tremendous physical and biochemical stress ensuring the energetic demands of the brain are met while serving as an essential barrier to prevent the accumulation/promote the removal of toxins. Plaque formation within the vasculature is a major cause of ischemic stroke as leukocytes enter damaged tissue through blood vessel endothelium where they undergo a series of changes to become a macrophage. Neuroinflammatory stress has been implicated in acute diseases such as stroke and traumatic brain injury as well as chronic diseases including Alzheimer's, multiple sclerosis and Parkinson's disease amongst others (Bazan *et al.* 2002; Phillis *et al.* 2006; Glass *et al.* 2010).

Moreover, the use of fish oil, a rich source of the omega-3 or n-3 fatty acids has been shown to be beneficial in the prevention of important human diseases such as atherosclerosis and sudden cardiac death, and various inflammatory disorders. Although the mechanism(s) by which the n-3 fatty acids exert their biological activity is unknown, an important anti-atherogenic and anti-inflammatory mechanism of EPA action is its interference with the arachidonic acid cascade that generates pro-inflammatory eicosanoids via the cyclooxygenases and lipo-oxygenases

Oxidative stress is an essential component of macrophage transformation and it promotes the production of highly potent lipid peroxidation products formed from arachidonic acid and other polyunsaturated fatty acids. Oxidative stress and vascular dysfunction has been implicated not only in acute disorders including stroke and traumatic brain injury, but also in chronic 'peripheral' conditions which impact the CNS including diabetes, hypertension and vasculitis. Additional complications of vascular dysfunction have also been associated with neurodegenerative diseases and oncogenic transformation. A number of clinical trials have now shown improvements in learning and memory, vascular integrity, neuronal survival and human health when subjects were given dietary supplementation with the 'omega' or n-3 fatty acids docosahexaenoic acid (DHA) and EPA (De Caterina and Zampolli 2001; Simopoulos 2002; Cleland *et al.* 2006; Mozaffarian 2007; Calder 2008; Saito *et al.* 2008).

Although many studies have focused on the beneficial aspects of native EPA and its enzymatically derived metabolites, there is a growing consensus that nonenzymatic oxidation products of EPA exert potent, beneficial biological activities. Vallve *et al.* (2002) reported that non-enzymatic aldehyde oxidation products of EPA decrease the expression of the CD36 receptor in macrophages, a receptor linked to atherosclerosis. Moreover, free-radical catalyzed oxidation of EPA is a major consequence of an oxidizing environment as demonstrated by the formation of A₃/J₃-IsoPs in subjects fed an EPA-enhanced diet (Gao *et al.* 2006; Brooks *et al.* 2008a). Recent work confirmed that these EPA oxidation compounds are formed *in vivo* in micromolar quantities in settings of oxidative stress (Brooks *et al.* 2008a). We believe the current work uses EPA products likely in the pathophysiologically relevant range given that A₃/J₃-IsoPs from EPA-fed mice exposed to oxidative stress was 102 ng/g in liver. As the MW of A₃/J₃-IsoP is 353 ng/nmol, this equals 0.289 nmol/g. Assuming liver tissue has a density similar to water, then this is 0.289 nmol/mL = 289 nM.

Levels of A₃/J₃-IsoPs have not been quantified in brain tissue. However, EPA is enriched in brain tissue, suggesting that low micromolar quantities of A₃/J₃-IsoP could conceivably be achieved in brain tissue *in vivo*. Increasing concentrations of native EPA *in vivo* induces a sharp decrease in the level of arachidonic-acid derived IsoPs and a dramatic increase in EPA-derived IsoPs (Davis *et al.* 2006). EPA-derived IsoPs form at levels ten times higher in settings of oxidative stress than do IsoPs from arachidonic acid (Gao *et al.* 2007). By conducting a total synthesis of a specific regio- and stereoisomer of an endogenously formed EPA-derived cyclopentenone IsoP, 15-A₃₁-IsoP, we can begin to study activities of specific oxidation products of EPA that forms *in vivo* in micromolar quantities in settings of oxidative stress.

The enzymatic and chemical oxidation of fatty acids plays a central role in regulating inflammation. Prostaglandins, thromboxanes, IsoPs, and leukotrienes initiate and propagate signals essential for inflammatory stress via direct activation of G-protein-coupled activation, nuclear receptor activation, and in some cases by post-translational protein modification. Exposure to native EPA and DHA has been linked specifically to activation of the PPAR γ receptor (Li *et al.* 2005), inhibition of toll-like receptor activity (Lee *et al.* 2003), alterations of lipid raft composition in T-cells (Li *et al.* 2006), and inhibition of the

production of cytokines such as IL-1 β and TNF α in monocytes (Babcock *et al.* 2002) and IL-6 and IL-8 in endothelial cells (De Caterina *et al.* 1994; Khalfoun *et al.* 1997).

In evaluating the mechanism of action of 15-A_{3t}-IsoP, we found that unlike 15-A_{2t}-IsoP, that is formed by oxidation of arachidonic acid, and exacerbates oxidative stress in RAW264.7 cells (Musiek *et al.* 2005), 15-A_{3t}-IsoP was not cytotoxic and did not increase oxidative stress in macrophages. This underscores the importance of the fatty acid backbone (from which oxidized lipids are derived) in determining the biological activities of these compounds.

We found that similar to the activity of the cyclopentenone PGs, cyclopentenone IsoPs exert potent immunomodulatory effects. When administered to LPS-stimulated macrophages, 15-A_{3t}-IsoP inhibited NF κ B activation via impairment of I κ B α degradation and I κ K activity. Furthermore, 15-A_{3t}-IsoP attenuated LPS-induced expression of the NF κ B-responsive proteins iNOS and COX-2 while rapidly blocking nitric oxide and PG production. The cyclopentenone ring on these compounds of 15-A_{3t}-IsoP is necessary for their bioactivity, and their ability to undergo a Michael addition to a specific cysteine residue on the I κ K complex was important for inhibition of LPS-induced inflammation.

The proposed pathway of EPA-derived cyclopentenone IsoP anti-inflammatory action can be found in Fig. 8. These data demonstrate that the mechanism of action of A_{3t}-IsoP is similar to other α,β -unsaturated carbonyl-containing compounds such as PGA₂ and A₂/J₂-IsoPs (Rossi *et al.* 2000; Musiek *et al.* 2005). These effects were also shown to participate in a PPAR γ -independent manner. Therefore, the cytoprotective potential of EPA-derived 15-A_{3t}-IsoP lies not only in the anti-inflammatory effects we observed but also in the ability of this compound to diminish total lipid oxidation in macrophages. Based on these data, one might expect that the beneficial effects of supplementation with EPA may be closely associated with formation of A₃-IsoP levels at concentrations enabling anti-inflammatory effects while still avoiding the unwanted effects that occur with complete NF κ B inhibition. Indeed, this study lays the groundwork defining a broader pleiotropic beneficial action of EPA supplementation much in the same way as has been demonstrated for DHA (Horrocks and Yeo 1999; Montine *et al.* 2004) and offer an excellent biological metric of efficacy given the correlation between A_{3t}-IsoP levels and anti-inflammatory activity of EPA-derived cyclopentenone IsoPs.

In this study, we report that in addition to identifying a redox sensitive site on I κ K which is modified in response to A_{3t}-IsoP treatment, we found that A_{3t}-IsoP is able to inhibit a major step in atherosclerosis – the formation of foam cells in response to treatment with oxidized lipids. Macrophages have been shown to internalize OxLDL, and this increase in lipid loading was determined to be an important first-step in the progression of atherosclerosis (Hansson *et al.* 2006). 15-A_{3t}-IsoP decreased the accumulation of cytosolic droplets, and, consequently, could delay the formation of foam cells from macrophages interacting with OxLDL *in vivo*.

We believe this work warrants further study to determine the nature and extent of the anti-atherosclerotic activity of 15-A_{3t}-IsoP. Additional studies would determine if this effect is caused by prevention of cholesterol accumulation or via other mechanisms, such as alteration of NF κ B-driven migration molecules necessary for deposition of foam cells (Brach *et al.* 1991; Bond *et al.* 1998) or the inhibition of NF κ B-controlled inflammatory mediators (Gu *et al.* 1998; Burleigh *et al.* 2002; Zhao and Funk 2004). Given that the A₃-IsoPs are not cytotoxic and do not increase the formation of other lipid-derived peroxidation products, this specific EPA product may be more useful than native EPA in eliciting anti-inflammatory and cytoprotective responses in cells.

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Abbreviations used

BCA	bicinchoninic acid
CH₂Cl₂	dichloromethane
COX-2	cyclo-oxygenase 2
DAPI	4',6-diamidino-2-phenylindole
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
GC-NICI-MS	gas chromatography-negative ion chemical ionization mass spectrometry
GST	glutathione-S-transferase
IL-1β	interleukin-1 beta
iNOS	inducible nitric oxide synthase
IsoP	isoprostane
IκBα	inhibitor of kappa b
IκK	I kappa kinase
K₃PO₄	potassium phosphate
LPS	lipopolysaccharide
LT	leukotriene
NaBH₄	sodium borohydride
NFκB	nuclear factor kappa b
OxLDL	oxidized low-density lipoprotein
PBS	phosphate-buffered saline
PG	prostaglandin
PGD₂	prostaglandin D ₂
PPAR	peroxisome proliferator activator receptor
PUFA	polyunsaturated fatty acids
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha

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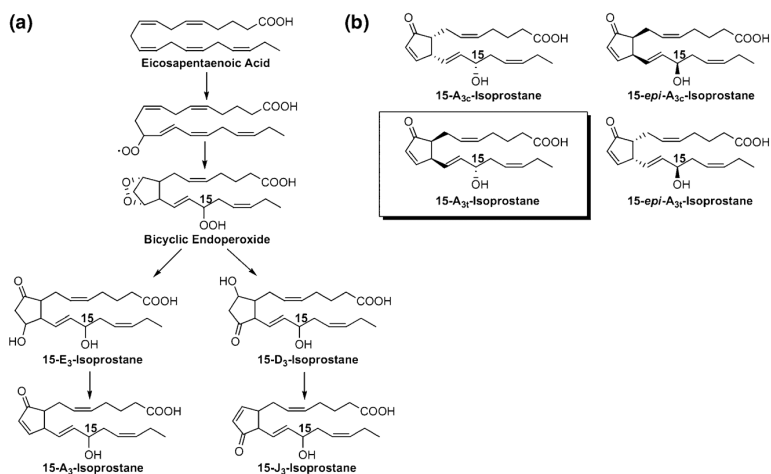
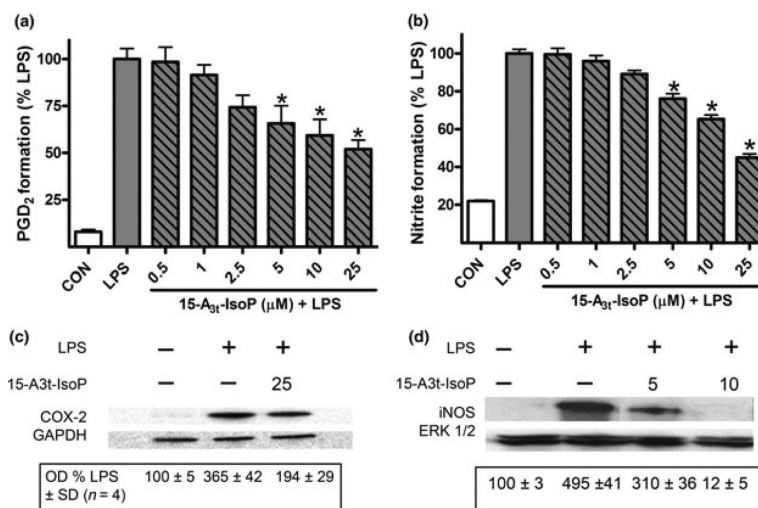
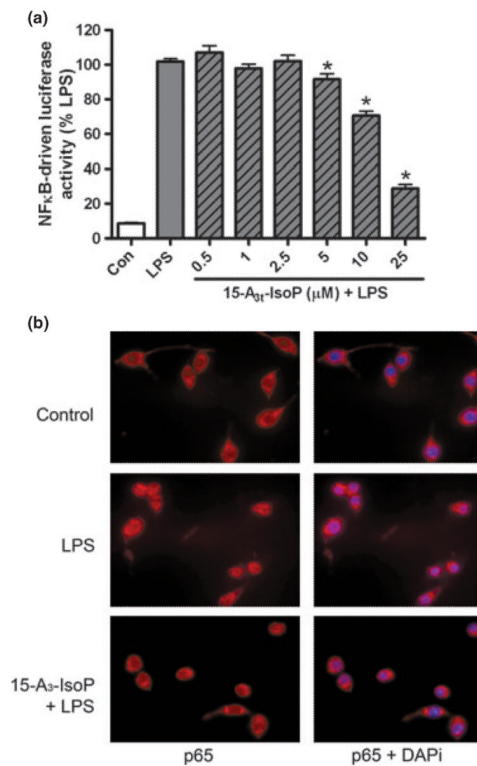


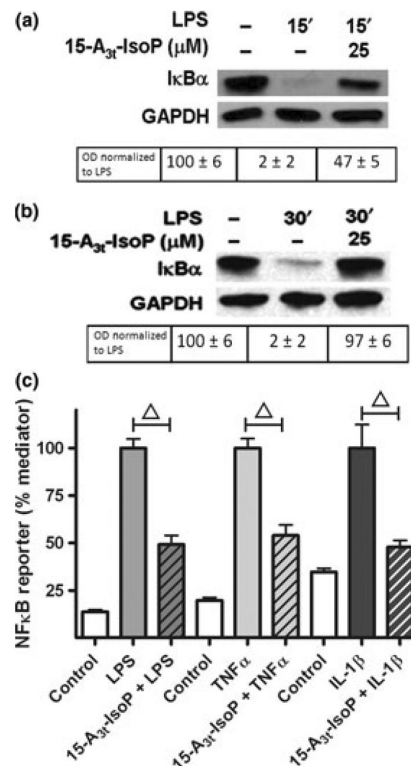
Fig. 1. Formation of cyclopentenone IsoPs through the free-radical oxidation of eicosapentaenoic acid (EPA). (a) Oxidation of EPA yields a bicyclic endoperoxide intermediate that can undergo rearrangement to form D- and E-ring IsoPs which can spontaneously dehydrate to form J- and A-ring (cyclopentenone) IsoPs. Although six regioisomers can form from oxidation of EPA, only 15-series isomers are shown for simplicity. (b) structures of the four 15-A₃-IsoP stereoisomers generated from non-enzymatic peroxidation of EPA; the boxed stereoisomer was synthesized for these studies.

**Fig. 2.**

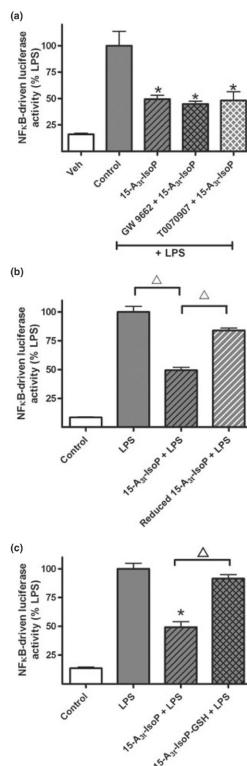
15-A_{3t}-IsoP inhibits LPS-induced production of inflammatory mediators in RAW264.7 macrophages. RAW264.7 macrophages were pre-incubated with varying concentrations of 15-A_{3t}-IsoP for 30 min and then stimulated with LPS (1 μg/mL) for 24 h in the presence of 15-A_{3t}-IsoP. **A**, 15-A_{3t}-IsoP inhibits LPS-induced PGD₂ production, the primary product of COX-2 activity in macrophages, as quantified by GC/MS. **B**, 15-A_{3t}-IsoP inhibits LPS-induced production of nitrite, a stable metabolite of iNOS activity, as quantified by Griess assay. Assays were performed 24 h after the initiation of the pre-incubation period. Statistical analysis of PGD₂ (**a**) and nitrate formation (**b**) were performed using a two-tailed between group ANOVA and demonstrated a significant treatment effect with $p < 0.01$. *Post hoc* analysis using Tukey's HSD *post hoc* testing revealed control versus LPS $p < 0.001$ for both Fig. 2A and B. Asterisks are used to denote statistically significant effects of 15-A_{3t}-IsoP + LPS compared to LPS alone. All data represent mean ± SD of four separate experiments carried out in triplicate where data were normalized to the protein concentrations of samples. (**c**, **d**) 15-A_{3t}-IsoP (5, 10, and 25 μM) inhibits LPS-induced expression of the proteins COX-2 after 6 h of LPS stimulation (**c**) and iNOS after 9 h of LPS stimulation (**d**) as analyzed by western blot. GAPDH and ERK blots are shown as loading controls. Blots in panels (**c**) and (**d**) are representative of four independent experiments. Semi-quantitative densitometric analysis of the blots was performed using Image J and data shown are the optical densities (OD) for each condition ± SD where the first lane is taken as a baseline value of 100%.

**Fig. 3.**

15-A₃₁-IsoP inhibits LPS-induced NFκB nuclear translocation and activation. (a) NFκB reporter macrophages were pre-treated with varying concentrations of 15-A₃₁-IsoP for 30 min and then stimulated with LPS (1 μg/mL) for 4 h. Luciferase assays were then performed and data represent mean ± SD of five separate experiments; data were normalized to protein concentration expressed as % LPS-induced luciferase activity increase. Statistical analysis of were performed using a one-tailed ANOVA and demonstrated a significant treatment effect with $p < 0.01$. *Post hoc* analysis revealed control versus LPS $p < 0.001$. Asterisks are used to denote statistically significant effects of various concentrations of 15-A₃₁-IsoP + LPS compared with LPS alone. (b) RAW264.7 cells were pre-treated with vehicle or 15-A₃₁-IsoP (25 μM) and then stimulated with vehicle or LPS (1 μg/mL) for 1 h. Cells were then subjected to immunofluorescent microscopy following staining of the NFκB p65 subunit (red) and nucleus (DAPI staining, blue).

**Fig. 4.**

15-A₃₁-IsoP inhibits LPS-induced inflammation in macrophages at the level of the IκK. (a, b) RAW264.7 macrophages were pre-treated with varying concentrations of 15-A₃₁-IsoP for 30 min, stimulated with LPS (1 μg/mL), and harvested at 15 (a) or 30 min (b). Protein lysates were subjected to western blot analysis with the anti-IκBα-antibody and results are representative of four independent experiments. GAPDH blots are shown as loading controls. Semi-quantitative analysis of optical densities is shown below each group with average ± SD expressed for all analyses. Data in panels (a) and (b) represent four independent experiments. (c) NFκB reporter macrophages were pre-treated with vehicle or 15-A₃₁-IsoP for 30 min and then stimulated with LPS (1 μg/mL), TNFα (10 ng/mL), or IL-1β (20 ng/mL) for 4 h. Data expressed as % inflammatory mediator-stimulated luciferase activity. Statistical analysis of was performed using a two-tailed between group ANOVA for each treatment (TNFα, LPS or IL1β) and demonstrated a significant treatment effect of each drug group with $p < 0.01$. *Post hoc* analysis using Tukey's HSD testing revealed significance of 15-A₃₁-IsoP addition compared with treatment alone with a $p < 0.01$ denoted by a bracketed Δ representing a $p < 0.01$.

**Fig. 5.**

The anti-inflammatory effects of 15-A₃₁-IsoP are independent of PPAR γ activation and are dependent on the α,β -unsaturated carbonyl moiety. (a) NF κ B reporter cells were pre-treated with vehicle or one of two PPAR γ antagonists (GW9662, 1 μ M, or T0070907, 500 nM) for 2 h, exposed to 15-A₃₁-IsoP (25 μ M) for 30 min, and then stimulated with LPS (1 μ g/mL) as in previous experiments. Data expressed as % LPS-induced luciferase activity increase. Statistical analysis was performed using a two-tailed between group ANOVA and demonstrated a significant treatment. *Post hoc* analysis revealed that LPS only control (gray bar) was significantly different from control (white bar) (a). Additional comparisons revealed 15-A₃₁-IsoP, 15-A₃₁-IsoP with GW9662 and 15-A₃₁-IsoP with T0070907 were significantly different from LPS alone but not from one another as denoted by a * representing a $p < 0.01$ compared with LPS alone. (b, c) 15-A₃₁-IsoP was subjected to reduction with NaBH₄ for 30 min (b) or incubated with GST and GSH for 2 h (c) and purified by C18 SepPak extraction before being applied to NF κ B reporter cells as in previous experiments. C18 SepPak extraction removes residual GST, GSH, and NaBH₄. LPS-stimulated NF κ B reporter activity was significantly different from control in both panels (b) and (c) and 15-A₃₁-IsoP's effect on NF κ B was significantly diminished when using the reduced form of the compound or the GSH adduct as denoted by bracketed Δ representing a $p < 0.01$ compared with 15-A₃₁-IsoP + LPS. Data are shown as \pm SD from four independent experiments carried out in triplicate.

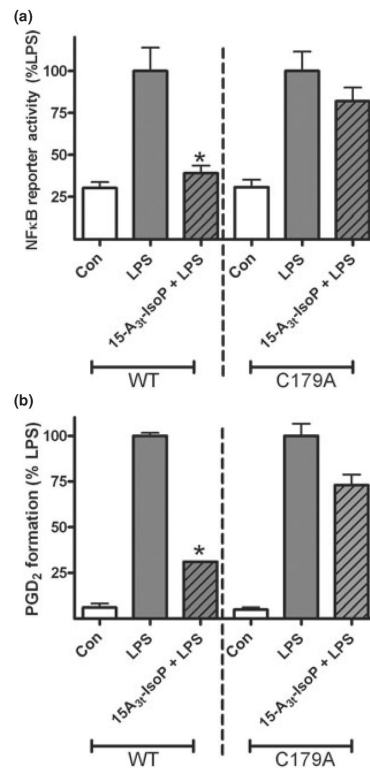


Fig. 6. Mutation of cysteine 179 on the $I\kappa K_b$ subunit inhibits 15-A₃₁-IsoP activity in inflamed macrophages. (a) RAW264.7 cells were transiently transfected with an NF κ B reporter luciferase reporter plasmid as well as either wild-type (left portion of graph) or mutant (C179A, right portion of graph) $I\kappa K_b$ expression vectors. Twenty-four hours post-transfection, cells were pre-treated with 15-A₃₁-IsoP (25 μ M) for 30 min and stimulated with LPS (1 μ g/mL) for 4 h. Cells were then assayed for luciferase activity. (b) RAW264.7 cells were transfected with either wild-type (left portion of graph) or mutant (C179A, right portion of graph) $I\kappa K_b$ expression vectors as well as a pcDNA3.1 antibiotic selection vector. Selection was carried out utilizing G418 sulfate as an antibiotic. After selection, cells were pre-treated with 15-A₃₁-IsoP (25 μ M) for 30 min and stimulated with LPS (1 μ g/mL) for 24 h. PGD₂ levels from the media were then assayed. Statistical analysis was performed using a two-tailed between groups ANOVA and Tukey's HSD *post hoc* analysis. Paired analysis revealed that LPS was significantly different from control as denoted with an * p < 0.001 but that the only difference between LPS and LPS + 15-A₃₁-IsoP was in WT cells as denoted by * representing a p < 0.01. Data represented as mean \pm SD and represents either five (a) or four (b) independent experiments.

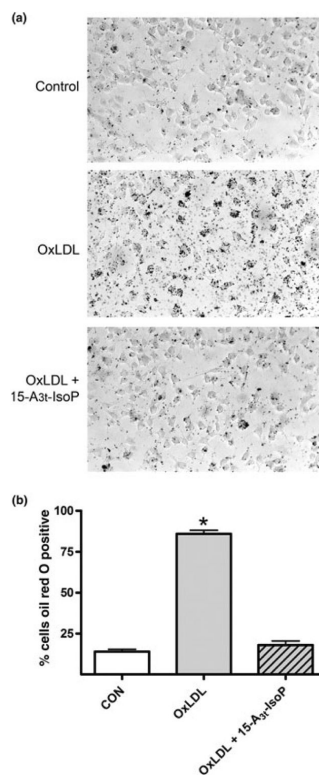


Fig. 7.

Incubation of cells with 15-A_{3I}-IsoP decreases lipid accumulation in cells treated with oxidized LDL. (a) RAW264.7 cells were pre-treated with vehicle or 15-A_{3I}-IsoP (10 μM) and then incubated with OxLDL (1 μg/mL) for 48 h. Cells were then subjected to light microscopy at 20× magnification after Oil Red O staining to measure intracellular lipid accumulation. (b) Quantification of lipid loading by macrophages demonstrating the percentage ± SD of cells positive for Oil Red O stain from three independent experiments. Statistical analysis was performed using a two-tailed between groups ANOVA and Tukey's HSD *post hoc* analysis. Paired analysis revealed that OxLDL was significantly different from control as denoted with an * $p < 0.001$. OxLDL + 15-A_{3I}-IsoP was not significantly different from control.

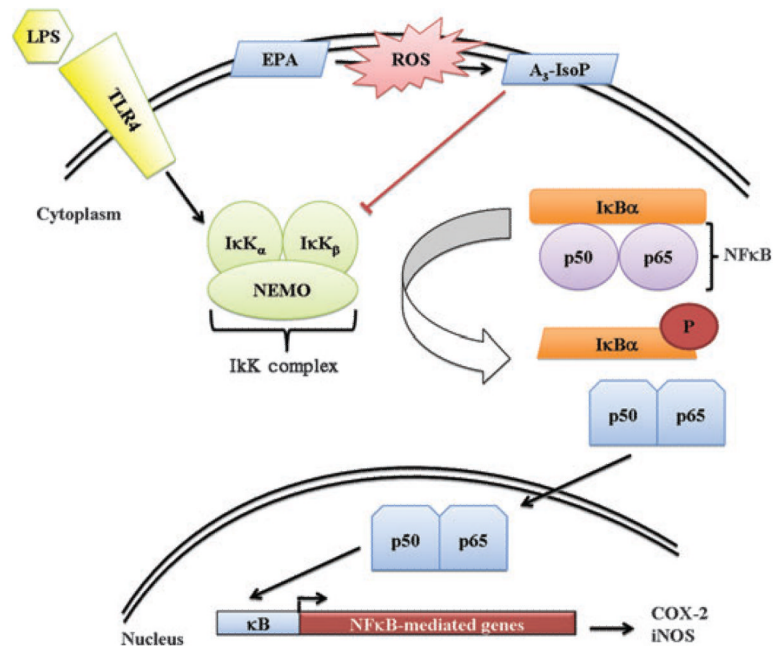


Fig. 8. Model of NFκB signaling inhibition by EPA-derived cyclopentenone IsoPs. Based on our findings, we suggest that 15-A₃₁-IsoP is formed in cellular membranes and can adduct to exposed cysteine residues on the β subunit of the IκK complex, hindering the complex's ability to activate NFκB signaling. This results in decreased iNOS and provides a beneficial effect on vascular integrity.