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Omega-3 fatty acid oxidation products prevent vascular endothelial cell activation by coplanar polychlorinated biphenyls

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Abstract

Coplanar polychlorinated biphenyls (PCBs) may facilitate development of atherosclerosis by stimulating pro-inflammatory pathways in the vascular endothelium. Nutrition, including fish oil-derived long-chain omega-3 fatty acids, such as docosahexaenoic acid (DHA, 22:6ω-3), can reduce inflammation and thus the risk of atherosclerosis. We tested the hypothesis that cyclopentenone metabolites produced by oxidation of DHA can protect against PCB-induced endothelial cell dysfunction. Oxidized DHA (oxDHA) was prepared by incubation of the fatty acid with the free radical generator 2,2-azo-bis(2-amidinopropane) dihydrochloride (AAPH). Cellular pretreatment with oxDHA prevented production of superoxide induced by PCB77, and subsequent activation of nuclear factor-κB (NF-κB). A_4/J_4-neuroprostanes (NPs) were identified and quantitated using HPLC ESI tandem mass spectrometry. Levels of these NPs were markedly increased after DHA oxidation with AAPH.. The protective actions of oxDHA were reversed by treatment with sodium borohydride ($NaBH_4$), which concurrently abrogated A_4/J_4-NP formation. Up-regulation of monocyte chemoattractant protein-1 (MCP-1)by PCB77 was markedly reduced by oxDHA, but not by un-oxidized DHA. These protective effects were proportional to the abundance of A_4/J_4NPs in the oxidized DHA sample. Treatment of cells with oxidized eicosapentaenoic acid (EPA, 20:5ω-3) also reduced MCP-1 expression, but less than oxDHA. Treatment with DHA-derived cyclopentenones also increased DNA binding of NF-E2-related factor-2 (Nrf2)and downstream expression of NAD(P)H:quinone oxidoreductase (NQO1), similarly to the Nrf-2 activator sulforaphane. Furthermore, sulforaphane prevented PCB77-induced MCP-1 expression, suggesting that activation of Nrf-2 mediates the observed protection

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Conflict of Interest Statement
The authors declare they have no actual or potential competing financial interests.

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against PCB77 toxicity. Our data implicate A_4/J_4-NPs as mediators of omega-3 fatty acid-mediated protection against the endothelial toxicity of coplanar PCBs.

Keywords

Polychlorinated biphenyls(PCBs); endothelial cells; docosahexaenoic acid (DHA); monocyte chemoattractant protein-1 (MCP-1); NF-E2-related factor-2 (Nrf2); oxidative stress

Introduction

Chronic exposure to persistent organic pollutants, such aspolychlorinated biphenyls (PCBs), contributes to the development of cardiovascular diseases in humans (Hennig *et al.,* 2007). It has been well established that inflammation is an important mechanism contributing to the pathology of atherosclerosis, an underlying cause in the majority of cardiovascular deaths (Wilson, 2008). Coplanar PCBs can exacerbate early development of atherosclerosis by increasing production of inflammatory mediators, such as monocyte chemattractant protein-1 (MCP-1), in the vascular endothelium (Hennig *et al.,* 2002; Majkova *et al.,* 2009).

Changing the composition of dietary lipids is a promising strategy to prevent negative outcomes of exposure to environmental chemicals(Wang *et al.,* 2008). There is a substantial number of epidemiological studies demonstrating that fish-derived omega-3 polyunsaturated fatty acids (PUFAs) can reduce cardiovascular morbidity and mortality(Wang *et al.*, 2006; Marik and Varon, 2009). Docosahexaenoic acid (DHA, 22:6ω-3), and eicosapentaenoic acid (EPA, 20:5ω-3)are the major components of fish oil, and their anti-inflammatory properties contribute to the cardioprotective effects of fish oil(Mori and Beilin, 2004).

Long-chain PUFAs in the body are subject to free radical-initiated oxidation, leading to the production of prostaglandin-like compounds called isoprostanes (IsoPs)(Morrow *et al.,* 1992). This reaction proceeds through the formation of an unstable endoperoxide intermediate, which can then be be reduced to generate IsoPs containing F-type prostane rings(F -IsoPs) (Roberts *et al.*, 1998). Alternatively these intermediates can undergo isomerization to form molecules with E-type and D-type prostane rings(E/D -IsoPs) (Reich *et al.,* 2000). E/D-IsoPs are subsequently dehydrated resulting in A -type and J-type compounds (A/J-IsoPs) (Fam *et al.,* 2002). Oxidation of DHA specifically leads to formation of neuroprostanes (NPs) which are IsoP-like compounds found commonly in DHA-rich tissues, in particular brain (Roberts *et al.,* 1998; Musiek *et al.*, 2008). A_4/J_4-NPsare cyclopentenone metabolites of DHA, that contain electrophilic α,β-unsaturated carbonyl moieties, which allow them to form Michael adducts with nucleophiles, including thiol groups in signaling proteins(Musiek *et al.,* 2008). As a result, they can inhibit inflammatory responses, for example by binding to IκB kinase β (IKKβ), thus inhibiting transcription factor nuclear factor -κB (NF-κB) (Musiek *et al.,* 2008).

Reactive oxygen species (ROSs)are critical mediators of PCB -induced endothelial inflammation (Slim *et al.,* 1999). Redox imbalance leads to activation of oxidative stress-sensitive kinases and transcription factors, including NF-κB, and increased production of inflammatory cytokines and adhesion molecules(Gloire and Piette, 2009). Nuclear factor erythroid 2-related factor 2 (Nrf2) plays a major role in cellular response to oxidative stress by binding to its cognate antioxidant response element (ARE) in promoters of genes encoding cytoprotective proteins, including glutathione synthesis and metabolism enzymes, or NAD(P)H:quinone oxidoreductase (NQO1) (Kensler *et al.,* 2007). Nrf2 is present in aortic endothelial cells, where its activation inhibits inflammatory signaling(Zakkar *et al.,* 2009). Several naturally occurring chemoprotective compounds can activate Nrf-2 and

stimulate antioxidant responses(Mann *et al.,* 2009). Interestingly, DHA-derived cyclopentenones increased Nrf -2 transcriptional activity by direct binding to sulfhydryl groups on Keap1, a negative regulator of Nrf2 (Gao *et al.*, 2007). In this report we test the hypothesis that cyclopentenone products of DHA oxidation prevent PCB toxicity in endothelial cells by activation of antioxidant responses and inhibition of PCB-induced oxidative stress.

Materials and Methods

Materials and chemicals

PCB77 was a generous gift from Dr. Larry W. Robertson, University of Iowa, Iowa City, IA. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DHA and EPA (>99% pure by gas-liquid chromatography) were obtained from Nu-Chek Prep (Elysian, MN). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA), and all other chemicals from Sigma-Aldrich Corporation(St. Louis, MO), unless otherwise specified.

Cell culture

Primary endothelial cells were isolated from porcine pulmonary arteries as described previously (Hennig *et al.,* 1984). The basic culture media consisted of medium 199 (M199) containing 10% fetal bovine serum (FBS). At confluency, cells were incubated overnight with treatment media, followed by an exposure to tested compounds in treatment media (M199 with 0.5% FBS for parent fatty acids, and M199 with 5% FBS for oxidized fatty acids, respectively). PCB77 was solubilized in dimethyl sulfoxide(DMSO), and subsequently diluted in cell culture media to 5 μM. Similar PCB levels were found in human serum after acute exposure to PCBs (Wassermann *et al.*, 1979; Jensen, 1987; Vaman Rao and Banerji, 1989).

Fatty acid treatments

Fatty acids were diluted in EtOH (50 mg/ml), aliquoted, and stored at −80°C. Treatment with parent fatty acids was performed as described previously (Mattos *et al.,* 2003). Briefly, the ethanol was evaporated with nitrogen gas, and the fatty acids were diluted to 1 mM in M199 cell culture medium containing 33 mg/ml of fatty acid -free bovine serum albumin (BSA) to achieve a molar fatty acid to BSA ratio of 2:1. This solution was incubated for 2 h at 37°C to allow binding of the FA to BSA, and then further diluted in treatment media to final treatment concentrations. For the experiments with oxidized fatty acids, ethanol stock solutions were diluted to 1 mM in phosphate-buffered saline (PBS), containing 2 mM of a free radical generator free radical generator 2,2-azo-bis(2-amidinopropane) dihydrochloride (AAPH; Cayman Chemical, Ann Arbor, MI), a methods that was reported to produce cyclopentenone IsoPs (Musiek *et al.,* 2008). The solutions were incubated at 37°Cfor 16 h, unless otherwise indicated, diluted in treatment media, filtered, and exposed to the cells. Sodium borohydride ($NaBH_4$) reduction of oxidized DHA (oxDHA) was performed as before (Musiek *et al.,* 2008)with minor modifications. Oneml of 18% (w/w) NaBH4 in water was added to 0.66 mg of previously oxidized DHA in 2 mM AAPH/PBS, vortexed, and incubated on ice for 30 min. Then, a molar excess of HCl was added to neutralize $NaBH_4$, and lipids were extracted into the chloroform phase by the addition of chloroform and methanol (a final ratio of 1:1:0.9 of chloroform:methanol:acidic aqueous phase), followed by vortexing and centrifugation for 5 min each. The lower phase was dried under nitrogen, and the lipid extracts were re -dissolved in EtOH/PBS/treatment media, and exposed to the cells. Both of these resulted in comparable levels of albumin in the experimental media (5% FBS or 30 μM of albumin for parent fatty acids and 20 μM albumin

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for delivery of oxidized fatty acid), which is the critical predictor of fatty acid availability (Hostmark, 1995).

Assessment of superoxide ($O_2^{-\cdot}$) levels

Cells were grown to confluence in 4-chamber culture slides (BDB iosciences, Bedford, MA). After treatments, the cells were rinsed 2x with Krebs-Ringer buffer (KRB; 118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 12 mM $MgCl_2$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose, pH = 7.4), followed by incubation with 5 μM dihydroethidium (DHE) or KRB (blank) at 37°C for 30 min. Cells were then rinsed with KRB, fixed with 10% buffered formalin, and washed with PBS. Slides were mounted with ProLong Gold Antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA) to visualize the nuclei. The slides were evaluated under an Olympus BX61W1 fluorescence microscope and the images were captured digitally using a Retiga-EXi camera and QCapture Pro 5.1.1.14 sotware (QImaging, Surrey, BC, Canada). Mean fluorescence intensity was quantified using ImageJ 1.42q (NIH, Bethesda, MD).

Electrophoretic mobility shift assay (EMSA)

After treatments, nuclear extracts were prepared as described previously (Oesterling *et al.*, 2008). DNA-binding activities of NF-κB and Nrf2 were assessed using LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL) and binding reactions were carried out as published before (Sauzeau *et al.,* 2003), with 8 μg of antibodies against p65 (NF-κB subunit) or Nrf2 used to confirm band specificity. Synthetic 5′-biotinylated complementary oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The cognate DNA sequence for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was described previously (Lim *et al.,* 2007), and antioxidant response element (ARE) from porcine NQO1 promoter (5′-TAGTCACAGTGACTCAGCGAGATTC-3′) was identified based on conserved ARE sequence (Wasserman and Fahl, 1997).

Analysis of A_4/J_4-NPs

Analysis of neuroprostanes and DHA was carried out using a Shimadzu UFLC coupled with an ABI 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. DHA and neuroprostanes were separated using a Zorbax Eclipse XDB C8 column, 5 um, 4.6 × 150 mm (Agilent). The mobile phase consisted of 63/37/0.5 v/v/v: Water/Acetonitrile/Formic acid as solvent A and 50/50 v/v: Acetonitrile/IPA as solvent B. For the analysis of DHA and neuroprostanes the separation was achieved using a gradient of 100 to 0 % solvent B in 6 min and maintaining at 0 % B for the next 9 min and equilibrated back to the initial conditions in 3 min. The flow rate was 0.5 mL/min with a column temperature of 30 C. The sample injection volume was 10 uL. The mass spectrometer was operated in the negative electrospray ionization mode with optimal ion source settings with a declustering potential of −80 V, entrance potential of −10 V, collision energy of −14 V, collision cell exit potential of −11 V, curtain gas of 20 psi, ion spray voltage of −4500 V, ion source gas1/gas2 of 40 psi and temperature of 550 C. MRM transitions monitored were as follows: Neuroprostanes - *m/z* 357/339, *m/z* 357/295, *m/z* 357/313, *m/z* 357/161, *m/z* 357/175, and for DHA – *m/z* 327/283, *m/z* 327/229, *m/z* 327/177, *m/z* 327/191, *m/z* 327/249. D4 -isoprostane was used as an internal recovery standard and a surrogate calibrator to quantitate NPs and DHA. The MRM transitions used to quantitate d4-isoprostane were *m/z* 333.5/314.6, *m/z* 333.5/296.6.

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Measurement of MCP-1 mRNA and protein levels

MCP-1 mRNA expression was assessed using real-time PCR (RT-PCR), and MCP-1 protein levels were measured in cell culture media using Quantikine ELISA kit (R&D Systems, Minneapolis, MN) as described previously (Majkova *et al.,* 2009).

Measurements of thiobarbituric acid reactive substances (TBARS)

TBARS formation in oxidized fatty acid solutions before cell treatments was assessed using the TBARS Assay Kit (Cayman Chemical) according to manufacturer's instruction.

Western blotting

NQO1 protein levels were assessed by Western Blotting as described previously (Han *et al.,* 2010). Rabbit polyclonal anti NQO1 antibody was incubated overnight at 4 °C in blocking buffer (5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20).

Statistical analysis

Values are reported as means ± SE obtained from of at least three independent experiments. Comparisons were made by one-way, two-way, or three-way analysis of variance (ANOVA), followed by post-hoc Fisher's least significant difference (LSD)test, using SigmaStat 2.0 software (Systat Software, Point Richmond, CA). Statistical probability of $p < 0.05$ was considered significant.

Results

Oxidized DHA prevents up -regulation of superoxide by PCB77 in endothelial cells

Oxidative stress is a key component of endothelial activation by coplanar PCBs (Slim *et al.,* 1999). Cyclopentenone metabolites of omega-3 PUFAs can activate antioxidant defenses in the cell (Gao *et al.*, 2007), which could provide a protection from PCB toxicity. To test this hypothesis, endothelial cells were pretreated with oxDHA, produced by free radical-initiated oxidation with AAPH, followed by exposure to vehicle or PCB77. Pre-treatments with oxDHA were performed for 4 h. This time-point was selected based on a previously published study (Gao *et al.*, 2007)that reported Nrf2 stimulation by oxDHA after 4 h exposure. Production of superoxide was assessed using dihydroethidium(D HE), a cell-permeable compound that can be oxidized by $O_2^{-\cdot}$ into a fluorescent product (Tarpey *et al.,* 2004). Antimycin A, a mitochondrial electron transport inhibitor (Piskernik *et al.*, 2008), was used as a positive control. $O_2^{-\cdot}$ levels were assessed by fluorescent microscopy (Figure 1A)and fluorescence intensity was quantified by ImageJ 1.42q software. PCB77 increased superoxide production and pre-treatment with oxDHA prevented this, demonstrating that oxDHA can protect endothelial cells form PCB-induced oxidative stress.

Activation of NF-κB by PCB77 is inhibited by oxidized DHA

ROS production is known to enhance nuclear translocation and transcriptional activity NF -κB (Gloire and Piette, 2009) a central regulator of endothelial inflammatory responses (Shin *et al.*, 2002). DNA-binding activity of NF-κB after exposure to PCB77 was measured by EMSA. Tumor necrosis factor-α (TNF-α) was used as a positive control, and supershift with antibody against NF-κB subunit p65 confirmed the band identity. In agreement with our previous studies (Lim *et al.,* 2007), PCB77 increased NF-κB activity after 6 h. Pre -treatment with oxDHA completely abolished NF-κB activation(Figure 1B), a likely result of a decreased inflammatory ROS production in the vascular endothelium to PCB77 after exposure to oxDHA.

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Oxidation of DHA in vitro leads to the production of A_4/J_4-NPs

Cyclopentenone metabolites of DHA, i.e. A_4/J_4-NPs, are uniquely active due to their ability to interact with sulfhydryl groups of signaling proteins (Gao *et al.*, 2007; Musiek *et al.*, 2008). We examined our oxDHA preparations, generated by treatment with AAPH, for the presence of A_4/J_4-NPs by infusion mode tandem mass spectrometry. We identified an ion with the predicted *m/z* ratio for the M -H-ion of J $_4$/A_4NPs (*m/z* 357) in these preparations. A product ion spectrum obtained after collisional dissociation of this *m/z* 357 species correlated well with that reported previously for J_4/A_4-NP (Fam *et al.,* 2002)(Figure 2A). Although the unavailability of synthetic standards for these molecules makes unambiguous assignment of the product ion spectrum challenging as observed by (Fam *et al.*, 2002), some of the abundant product ions derived from the *m/z* 357 species, for example *m/z* 339 $([M - H] - H2O)^-$, *m/z* 313 $([M - H] - CO2)^-$, and *m/z* 295 $([M - H] - H2O\ CO2)^-$, represent commonly observed transitions for this category of oxidized fatty acids. We used several of these precursor product ion pairs to establish selective reaction monitoring mode HPLC ESI MS/MS methods for quantitation of A_4/J_4-NPs in our oxidized DHA samples. We determined that, as also observed previously, untreated DHA contains low levels of A_4/J_4-NPs (presumably the result of auto oxidation) but levels of these compounds were increased markedly by AAPH-initiated oxidation (Figure 2B), and abrogated after reduction with $NaBH_4$(Figure 2B). These data demonstrate that oxDHA contains substantially elevated levels of A_4/J_4-NPs, which can be reduced using $NaBH_4$.

Oxidation of omega-3 PUFAs is required for the protection from PCB toxicity

The capacity of parent DHA and EPA to prevent PCB -induced inflammation was tested by measuring the expression levels of MCP-1, a cytokine mediator of monocyte recruitment into endothelium in early stages of atherosclerosis (Shin *et al.,* 2002). DHA and EPA were delivered using fatty acid-free BSA as a vehicle. After pre-treatment with control or fatty acids, cells were treated with PCB77, and mRNA levels of MCP-1 were assessed using RT-PCR. Neither DHA, nor EPA, had any effect on MCP-1 up-regulation by PCB77 (data not shown). By contrast, oxDHA decreased up-regulation of MCP-1 by PCB77 in a dose-dependent manner, with 40 μM being the most effective concentration (Figure 3A). In order to further demonstrate that only the oxidized metabolites are protective, the same concentrations of parent DHA were oxidized for increasing periods of time before treatments. The levels of oxidation are expressed as malondialdehyde (MDA) equivalents using TBARS assay and increased gradually over 24 hours (dashed line in Figure 3B). There was a direct correlation between the level of DHA oxidation measured using this assay and the effectiveness of preventing MCP-1 up-regulation by PCB77 (Figure 3B), supporting the hypothesis that DHA oxidation can result in production of anti -inflammatory compounds.

Cyclopenenone metabolites are the anti-inflammatory component of oxDHA

To test the hypothesis that A_4/J_4-NPs are the active compounds that prevent PCB toxicity, oxDHA was subjected to chemical reduction with $NaBH_4$ in order to reduce the carbonyl moiety on the cyclopentenone ring to a non-reactive alcohol (Mohanazadeh *et al.,* 2005; Musiek *et al.,* 2008). Residual $NaBH_4$ was removed by using chloroform -methanol extraction. This procedure decreased the concentration of A_4/J_4-NPs to the baseline (Figure 2B). As presented in Figure 3C, oxDHA prevented MCP-1 up-regulation, but after reduction with $NaBH_4$ (red DHA), it had no effect. This demonstrates that $NaBH_4$-sensitive components of our oxDHA preparations which clearly include A_4/J_4-NPsare responsible for prevention of the PCB-induced inflammatory response.

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Oxidized DHA is more protective than oxidized EPA

The relative potency of omega -3 PUFAs in cardiovascular prevention varies (Mori and Woodman, 2006; Gorjao *et al.*, 2009). Free radical-induced oxidation of both DHA and EPA yields cyclopentenone metabolites that can exert anti -inflammatory responses (Chaudhary *et al.*, 2004; Musiek *et al.*, 2008). Since DHA is more susceptible to oxidation due to higher number of double bonds (Visioli *et al.,* 1998), we tested the hypothesis that oxDHA is more protective than oxEPA. DHA and EPA were oxidized with 2 mM AAPH for 16 h. Under these conditions, DHA was oxidized to a greater extent than EPA measured using the TBARS assay (1.73±0.02 μM MDA per 40 μM of DHA) than EPA (1.22±0.02 μM MDA per 40 μM of EPA), which is consistent with previous reports(Visioli *et al.,* 1998). Correspondingly, oxEPA significantly inhibited PCB -mediated up-regulation of MCP-1 mRNA (Figure 4A) and protein (Figure 4B), but oxDHA had a more pronounced inhibitory effect (Figures 4A and 4B). This suggests that oxDHA is more protective than oxEPA against PCB toxicity which likely relates to its greater susceptibility to free radical -initiated oxidation and IsoP formation.

Nrf2 activation is involved in protection against PCB77 toxicity

Nrf2 is a key regulator of antioxidant defenses in cells (Kensler *et al.*, 2007), and an important anti-inflammatory mediator in vascular endothelium (Zakkar *et al.*, 2009). Previous studies suggested that A_4/J_4-NPs can activate Nrf2 by binding to sulfhydryl groups in Keap1 (Gao *et al.,* 2007). Since coplanar PCBs cause endothelial dysfunction by increasing oxidative stress, an increase inNrf2 transcriptional could prevent PCB toxicity. Nrf2 activity was assessed by EMSA using DNA sequence corresponding to Nrf2 -binding site in the promoter of NQO1, aNrf2 -responsive antioxidant enzyme (Nioi and Hayes, 2004). Nrf2 activity was significantly induced by cell exposure to oxDHA (Figure 5A). OxEPA tended to increase Nrf2 activity, but this increase was not significant. The Nrf2 data corresponded to a lesser degree of oxidation of EPA compared with DHA (1.18±0.03 μM MDA per 40 μM of EPA versus 1.93±0.07 μM MDA for DHA, respectively). Nrf2 stimulation by oxDHA was comparable to that observed in cells treated with suforaphane (SR) (Figure 5A), a dietary isothiocyanate known to stimulate Nrf2 transcriptional activity (Ahn *et al.,* 2010). The ability of oxDHA to increase protein levels of NQO1 was tested by Western Blot (Figure 5B). NQO1 was up-regulated markedly by oxDHA, and similar but less pronounced effect s were obtained by exposure to SR (Figure 5B). In order to confirm that up-regulation of Nrf2 activity can lead to protection against PCB toxicity, the ability of SR to prevent MCP-1 up-regulation by PCB77 was tested. Cells were pretreated with SR, followed by exposure to PCB77. MCP-1 mRNA expression induced by PCB77 was prevented by SR treatment, suggesting that induction of Nrf2 can protect against PCB-mediated endothelial cell activation. Additional experiments were conducted to demonstrate the importance of Nrf2 in cellular protection against PCB toxicity. Figure 6 illustrates that inhibiting Nrf2 with all-trans retinoic acid (ATRA) (Wang *et al.*, 2007) can further increase PCB-induced MCP -1 expression. Most importantly, inhibiting Nrf2 totally negated any protective properties of oxDHA, suggesting that functional Nrf2 is required for oxDHA to protect against PCB-induced induction of MCP-1.

Discussion

Humans are constantly exposed to complex mixtures of environmental chemicals with potentially deleterious effects. Diet modifications are viable means for preventing adverse outcomes of these exposures. This current work demonstrates that oxidized metabolites of long chain omega-3 polyunsaturated fatty acids, and in particular cyclopentenone NPs formed by free radical-initiated oxidation of DHA, can prevent endothelial dysfunction induced by coplanar PCBs. PCBs are ubiquitous environmental pollutants, and significant

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levels are present in human tissues (Zamir *et al.,* 2009). Furthermore, increased exposure to PCBs can contribute to cardiovascular mortality (Gustavsson and Hogstedt, 1997; Rylander *et al.*, 2009), and coplanar PCBs facilitate atherosclerotic lesion formation *in vivo* (Arsenescu *et al.*, 2008). Increased endothelial expression of adhesion molecules and cytokines, such as MCP-1, augments monocyte recruitment in early stages of atherosclerosis, and contributes to plaque formation (Aiello *et al.,* 1999). We have previously demonstrated that coplanar PCBs can induce MCP-1 expression in endothelial cells and that this effect is mediated via the aryl hydrocarbon receptor (AhR) (Majkova *et al.*, 2009).

Oxidative stress is a central mediator of PCB-induced endothelial dysfunction. Coplanar PCBs increase ROS production by up -regulation and uncoupling of cytochrome P450 monoxygenases (Schlezinger *et al.,* 2006)and/or activation of endothelial nitric oxide synthase (Lim *et al,* 2007), while non-coplanar PCBs can affect endothelial NADPH oxidase (Eum *et al.,* 2009). PCB-induced ROS can activate the transcription factor NF -κB (Hennig *et al.,* 2002; Lee *et al.*, 2003), an integral mediator of inflammatory responses in the vascular endothelium (Ding *et al.*, 2009). In particular, ROS stimulate IKKβ-independent phosphorylation of IκB and nuclear translocation NF -κB(Gloire and Piette, 2009), resulting in an increased production of inflammatory mediators, including MCP-1 (Shin *et al.,* 2002). Coplanar PCBs and other dioxin-like chemicals up-regulate MCP-1 release by endothelial cells, thus contributing to monocyte recruitment and plaque formation (Majkova *et al.,* 2009).

Diets rich in fish oil-derived omega-3 PUFAs are associated with lower rates of cardiovascular mortality (Marik and Varon, 2009), partially due to the anti-inflammatory properties of DHA and EPA (Mori and Beilin, 2004). Dietary intervention with omega-3 PUFAs also leads to reduced oxidative stress *in vivo* (Yin *et al.*, 2009). Recent evidence suggests that metabolites of omega-3 PUFAs contribute to the inhibition of an inflammatory response (Musiek *et al.*, 2008; Tian *et al.,* 2009). Most importantly, free radical-mediated oxidation of DHA and EPA in tissues has been reported to result in production of biologically active IsoPs (Musiek *et al.*, 2008; Yin *et al.,* 2009). To test the hypothesis that omega-3 PUFAs-derived IsoPs can alleviate PCB toxicity in endothelial cells, oxidized metabolites were produced by incubation of fatty acids with the free radical generator AAPH. Since increased ROS production is required for endothelial toxicity of coplanar PCBs (Slim *et al.,* 1999), the capacity of oxDHA to prevent PCB77-induced rise in $O_2^{-\cdot}$ was assessed using DHE fluorescence. Pre -treatment with oxDHA abolished PCB77-induced ROS production. The observed protection could be caused by activation of Nrf2, a transcription factor that regulates a variety of genes involved in antioxidant defenses (Kensler *et al.,* 2007). Indeed, IsoPs produced by oxidation of omega -3 PUFAs can specifically activate Nrf2 by covalently binding its regulator protein Keap1 (Gao *et al.,* 2007), and Nrf2 activation has recently been implicated in prevention of PCB toxicity (Park *et al.*, 2010).

Subsequent experiments from this study showed that oxDHA increases Nrf2 DNA-binding activity, and also protein levels of NQO1, a Nrf2-regulated enzyme involved in the detoxification of reactive quinones and replenishing antioxidants (Nioi and Hayes, 2004). Because PCB metabolism results in the formation of toxic quinones (Song *et al.,* 2008), Nrf2 activation and NQO1 induction could explain the decrease in PCB77-induced superoxide formation observed after oxDHA pre-treatment. As a result of the decreased ROS formation, oxDHA prevented PCB77 -induced activation of NF-κB. It has been shown that DHA-derived A_4/J_4-NPs can induce NF-κB directly by binding to IκB kinase β (IKKβ) (Musiek *et al.,* 2008). This mechanism, however, is not likely to counteract PCB toxicity, because coplanar PCBs induce NF-κB through oxidative stress signaling, i.e. downstream

from IKKβ (Gloire and Piette, 2009). Rather, Nrf2-mediated induction of antioxidant enzymes probably resulted in rapid detoxification of PCB77, and prevented the ROS build-up and NF-κB activation. Another possibility would be NF-κB inhibition by Nrf2 cross-talk (Kensler *et al.*, 2010). In our study, a well established Nrf2 inducer, dietary isothiocyanate sulforaphane (Ahn *et al.*, 2010), also activated Nrf2 and NQO1 expression, and subsequently prevented MCP-1 up-regulation by PCB77. This suggests that Nrf2 activation by dietary compounds can prevent environmental insult caused by coplanar PCBs in the vascular endothelium. It is quite possible that other Nrf2 targets than NQO1 would reduce PCB toxicity in endothelial cells. Nrf2 also mediates induction of various enzymes involved in cellular anti-oxidant defense, e.g., those involved in the synthesis of glutathione (Osburn and Kensler, 2008). Other genes that are regulated by Nrf2 include multiple UDP-glucuronosyl transferases (Osburn and Kensler, 2008), which can play a role in phase II metabolism and removal of PCBs from a cell (Daidoji *et al.,* 2005).

Oxidized DHA prevented MCP-1 up-regulation by PCB77 in a dose-dependent manner. Both oxDHA and oxEPA decreased MCP -1 up-regulation, but oxDHA was more potent. This was associated with a more pronounced oxidation of DHA (assessed using the TBARS assay), potentially resulting in a higher concentration of the active metabolites. Oxidation of DHA for increasing periods of time led to larger TBARS levels, and more effective inhibition of MCP-1 up-regulation. Because EPA is less susceptible to oxidation than DHA, higher levels of the parent fatty acid may be needed to produce desired levels of active metabolites. Interestingly, the parent long-chain fatty acids (DHA and EPA) had no effect on MCP -1 up-regulation by PCB77. These data support the notion that oxidized omega -3 fatty acid metabolites are uniquely protective in endothelial cells, findings consistent with a previous report where only oxidized omega-3 PUFAs prevented cytokine production (Mishra *et al.,* 2004).

Oxidation of long-chain PUFAs leads to formation of a complex mixture of metabolites. Prostaglandin-like products of free radical-mediated oxidation include F -type IsoPs with hydroxylated cyclopentane ring, or carbonyl-containing D/E-IsoPs that subsequently get dehydrated to form cyclopentenone-containing compounds (A/J -IsoPs)(Roberts and Milne, 2009). Certain factors, including oxygen tension and glutathione concentrations, affect the relative levels of different IsoPs (Morrow *et al.,* 1998). Prostaglandins and IsoPs containing cyclopentenone rings are particularly effective in reducing an inflammatory response (Musiek *et al.*, 2008). For example, arachidonic acid-derived 15-deoxy$\Delta^{12,14}$-PGJ_2(15d -PGJ_2) inhibited KF -κB activation (Rossi *et al.,* 2000)and MCP -1 production(Rovin *et al.,* 2001), while prostaglandins lacking a cyclopentenone group were ineffective in these studies. Also, DHA-and EPA-derived cyclopentenones specifically activated Nrf2 (Gao *et al.*, 2007). In the current study, we found that inhibition of Nrf2 with the antagonist ATRA further increased MCP-1 as induced by PCB77. Furthermore, inhibiting Nrf2 totally negated any protective properties of oxDHA, suggesting that functional Nrf2 is required for oxDHA to protect against PCB-induced induction of MCP-1. The limitations associated with non-specific inhibition of Nrf2 by ATRA should also be considered. It has been shown that ATRA decreases inducible Nrf2 mediated ARE activity *in vitro*, probably through direct interaction of RARα with Nrf2 (Wang *et al.,* 2007). Thus, the pleiotropic effects of ATRA treatment to inhibit Nrf2 invites the possibility of other mechanisms by which ATRA may prevent oxDHA dependent protection against PCB induced MCP-1 expression.

Omega-3 PUFAs-derived IsoPs (NPs) are present in various tissues (Fam *et al.,* 2002); and their concentrations increase after dietary supplementation with fish oil (Yin *et al.,* 2009). In order to find out whether cyclepentenone NPs are the protective metabolites of oxDHA, A_4/J_4-NPs in oxDHA were identified using tandem mass spectrometry approaches. A species of *m/z* 357, corresponding to the predicted *m/z* of the M -H- ion of A_4/J_4NPs was detected and

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its product ion spectrum revealed the presence of daughter ions consistent with the behavior of A_4/J_4-NPs reported previously (Fam *et al.,* 2002). Relative levels of A_4/J_4-NPs increased markedly after oxidation by AAPH, consistent with the observation that parent omega-3 PUFAs were ineffective in preventing MCP -1 up-regulation. Subsequently, cyclopentonone groups were reduced using $NaBH_4$, which resulted in loss of protection against PCB-induced MCP -1 up-regulation. Taken together, our data show for the first time that only oxidized DHA can counteract PCB toxicity and that cyclopentenone NPs are likely the major active DHA oxidation metabolites.

Our study shows that components of oxidized DHA, most likely A_4/J_4-NPs, can alleviate endothelial dysfunction caused by coplanar PCB77. This is likely mediated by activation of Nrf2 and and cellular antioxidant defenses, resulting in reduced ROS formation and decreased production of inflammatory chemokine MCP-1 (Figure 7). These data imply that dietary supplementation with omega -3 PUFAs, and in particular DHA, might prevent toxicity resulting from environmental exposure to PCBs.

Acknowledgments

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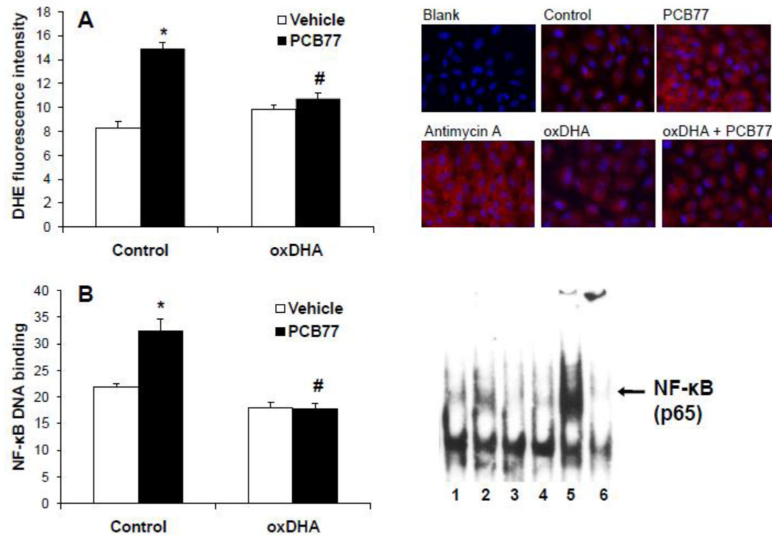

Figure 1.

Oxidized DHA prevents PCB77-induced superoxide production and activation of NF-κB. (A) Oxidized DHA (oxDHA) was generated via oxidation for 16 h in 2 mM AAPH. Cells were pre-treated with control or oxDHA (40 μM) for 4 h; followed by exposure to vehicle control (DMSO) or PCB77 (5 μM) for 8 h, or antimycin A (50 μM) for 6 h. Cells were then stained with DHE, and red fluorescence was assessed using microscope and quantified by ImageJ 1.42q. (B) Cells were pre-treated with control or oxDHA (40 μM) for 4 h, followed by exposure to control or PCB77 (5 μM) for 6 h. TNF-α (5 ng/mL) treatment for 2 h was used as a positive control. NF-κB DNA-binding was assessed by EMSA. 1, control; 2, PCB77; 3, oxDHA; 4, oxDHA + PCB77; 5, TNF-α; 6, p65 supershift. Data represent mean ± SEM of 3–4 independent experiments. Two-way ANOVA revealed a statistically significant interaction between oxDHA and PCB77. *Significantly different compared to vehicle control ($p<0.05$). [#]Significantly different compared to PCB77-treated control without oxDHA ($p<0.05$).

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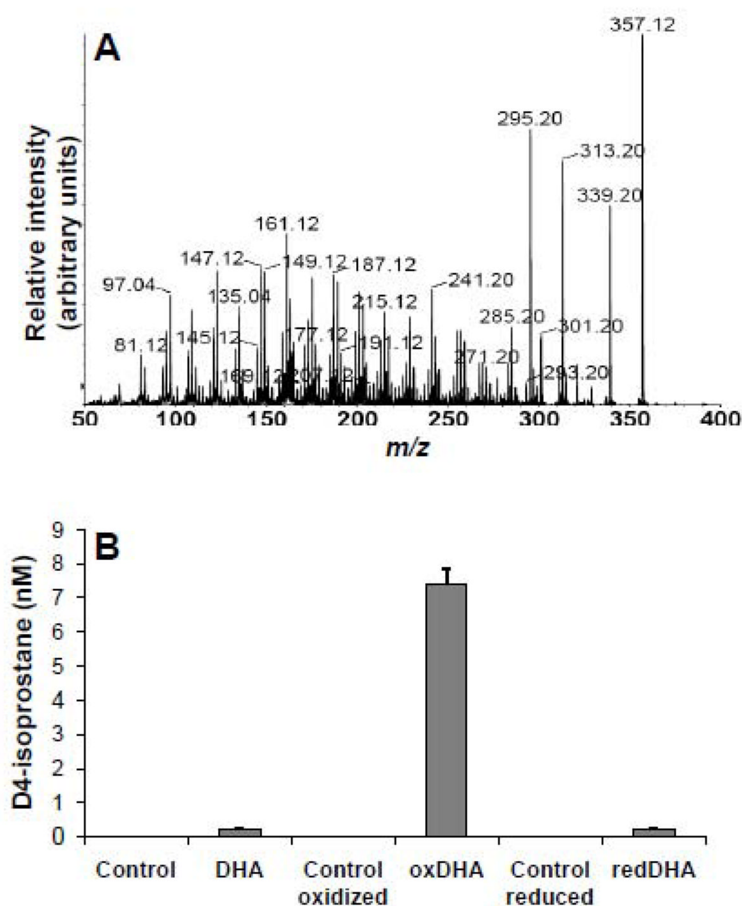

Figure 2.

Analysis of A_4/J_4-NPs by tandem mass spectrometry.(A) Oxidized DHA sample was infused into the ion source of our instrument, a species of *m/z* 357 generated in negative mode ESI was subjected to CID, and daughter ions identified from *m/z* 50 to 400. (B) A $_4$/ J_4-NP levels were quantitated in samples containing 40 μM of controls, unoxidized DHA, AAPH-oxidized DHA (oxDHA), or sodium borohydride ($NaBH_4$)-reduced DHA (redDHA). Qunatification was performed by HPLC ESI MS/MS using d4 -isoprostane as a surrogate calibration standard.

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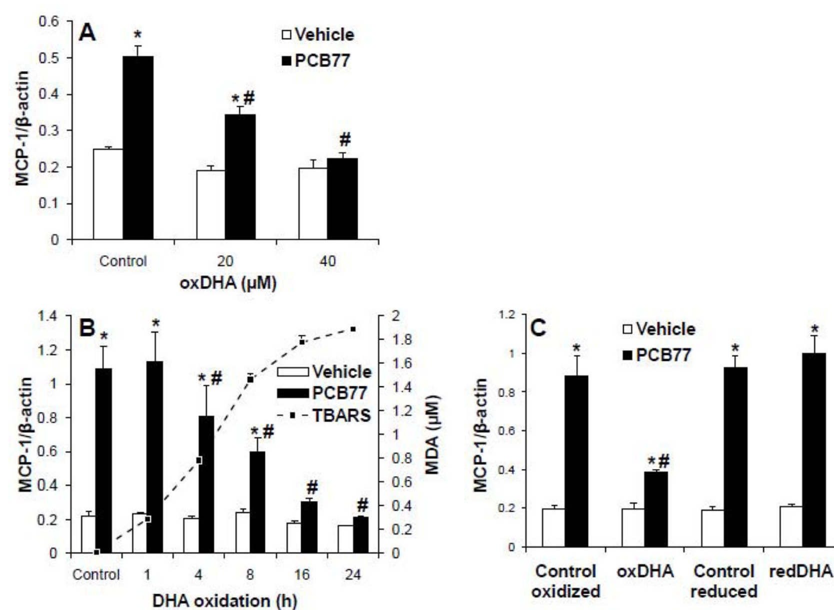

Figure 3.

Oxidized DHA prevents MCP-1 mRNA up-regulation by PCB77. (A) Cells were pre-treated with control or oxDHA (0–40 μM) for 4 h. After exposure to control or PCB77 (5 μM) for 24 hours, MCP-1 mRNA expression was assessed using RT-PCR. (B) DHA was oxidized for increasing periods of time, and levels of malondialdehyde (MDA) were assessed using TBARS assay (dashed line). Cells were pre-treated with control or oxDHA (40 μM) for 4 h, followed by PCB77 treatment and RT-PCR as described in (A). (C) DHA was oxidized by AAPH and/or reduced using $NaBH_4$ (redDHA). Cells were pre -treated with respective controls, oxDHA, or redDHA for 4 h, followed by PCB77 treatment and RT-PCR as described in (A). Data represent the mean ± SEM of 4-5 independent experiments. Two-way ANOVA revealed a statistically significant interaction between oxDHA and PCB77. *Significantly different compared to vehicle control ($p<0.05$). #Significantly different compared to PCB77-treated control without oxDHA ($p<0.05$).

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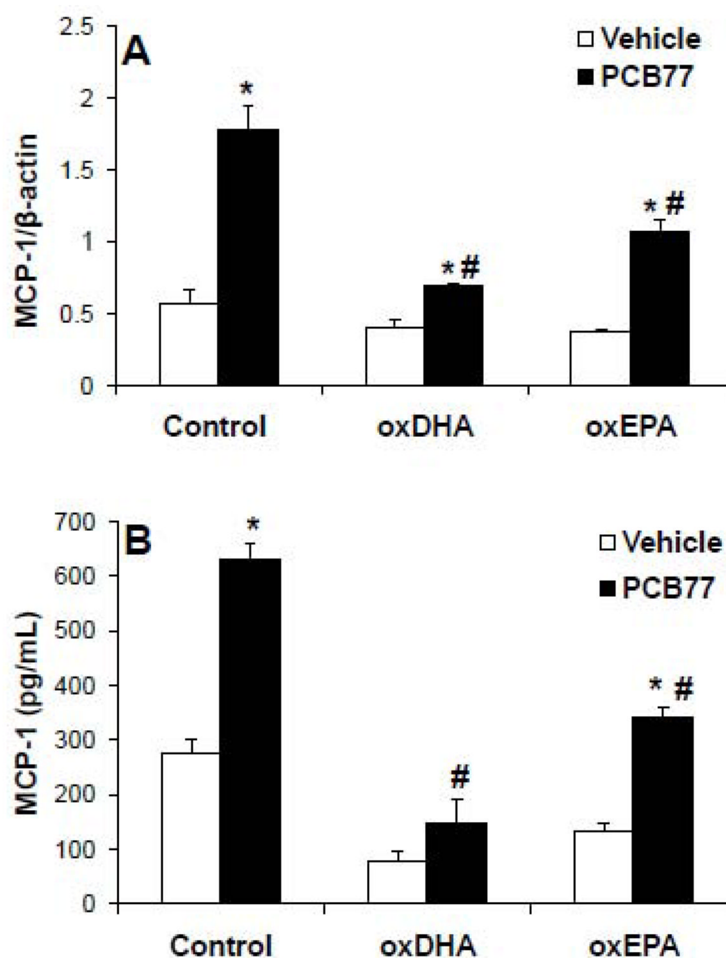

Figure 4.

Inhibition of PCB77-induced MCP-1 up-regulation by oxDHA and oxEPA. (A) Cells were pre-exposed to control, oxDHA (40 μM), or oxEPA (40 μM) for 4 h, followed by exposure to control or PCB77 (5 μM) for 24 hours. MCP-1 mRNA expression was assessed using RT-PCR. (B) Cells were treated as in (A) and MCP-1 protein levels in cell culture media were assessed using ELISA. Data represent mean ± SEM of 4 independent experiments. Two-way ANOVA revealed a significant interaction between oxidized fatty acids and PCB77. *Significantly different compared to vehicle control ($p<0.05$). #Significantly different compared to PCB77-treated control without oxidized fatty acid ($p<0.05$).

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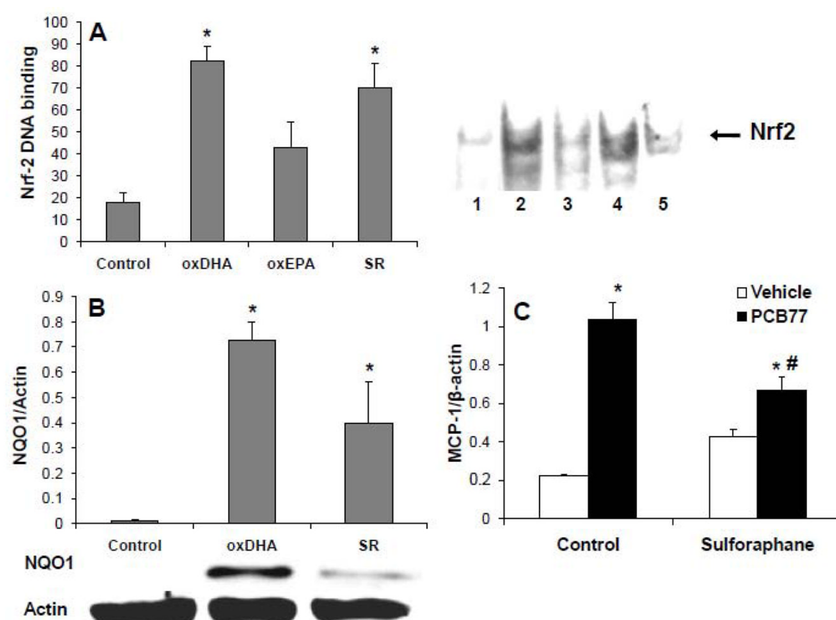

Figure 5.
Nrf2 activation in endothelial cells. (A) Cells were treated with control, oxDHA (40 μM), oxEPA (40 μM), or sulforaphane (SR, 10 μM), for 4 h; followed by an assessment of Nrf2 DNA-binding activity using EMSA. 1, control; 2, oxDHA; 3. oxEPA; 4, SR; 5, Nrf2 supershift. (B) Cells were treated for 24 h with control, oxDHA (40 μM), or SR (10 μM); and protein levels of NQO1 were measured by Western Blot. (C) Cells were pre-treated with SR (5 μM) for 4 h, followed by exposure to control or PCB77 (5 μM) for 24 hours. MCP-1 mRNA expression was assessed using RT-PCR. Data represent mean ± SEM of 3–5 independent experiments. One-way ANOVA revealed a statistically significant treatment effect (A and B), and two-way ANOVA revealed an interaction between SR and PCB77 (C). *Significantly different compared to vehicle control ($p<0.05$). #Significantly different compared to PCB77-treated control without SR ($p<0.05$).

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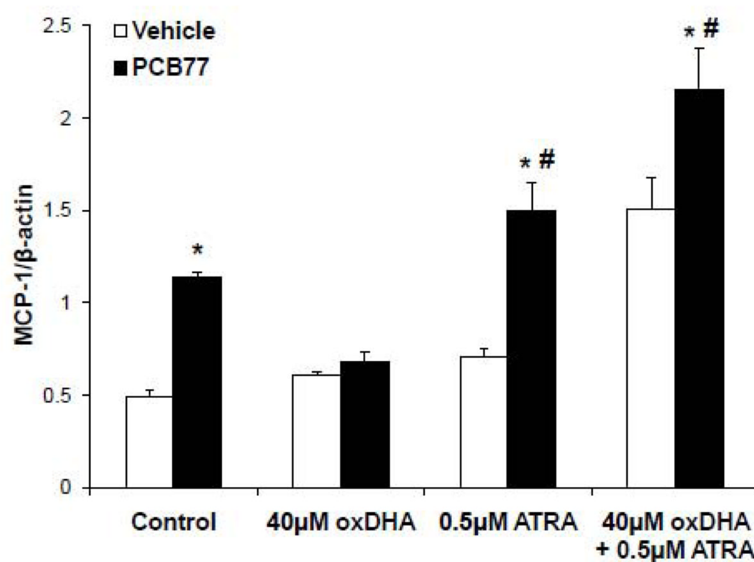

Figure 6.

Inhibiting Nrf2 negates protection by oxDHA against PCB77 -mediated induction of MCP-1. Cells were pre-treated with the Nrf2 inhibitor all-trans retinoic acid (ATRA, 0.5 μM) (Wang *et al.,* 2007)prior to exposure to oxDHA (40 μM) and PCB77 (5 μM). Data represent mean ± SEM of 3 independent experiments.*Significantly different compared to respective vehicle controls($p<0.05$). #Significantly different compared to PCB77 -treated control ($p<0.05$).

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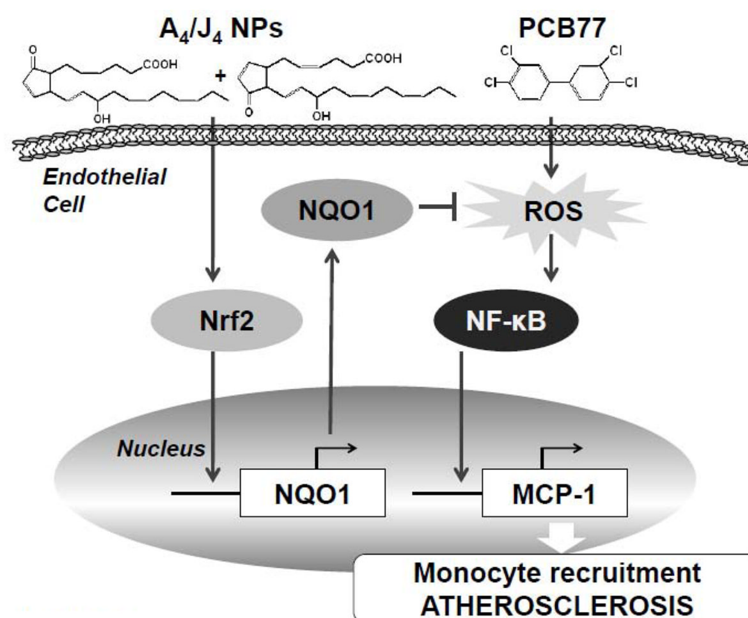

Figure 7.

Scheme illustrating the inhibition of PCB77-induced endothelial inflammatory response by A_4/J_4-NPs. A_4/J_4-NPs bind Keap1 in the cytoplasm thus increasing Nrf2 nuclear translocation and DNA-binding activity. This leads to induction of antioxidant defense genes, including NQO1. Antioxidant enzymes (NQO1) inhibit ROS production by PCB77, leading to inhibition ofPCB77 -induced NF-κB activation and expression of pro-inflammatory genes, such as MCP-1.