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Fatty Acid Epoxyisoprostane E2 Stimulates an Oxidative Stress Response in Endothelial Cells

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Abstract

Atherosclerosis is the main underlying cause of major cardiovascular diseases such as stroke and heart attack. Oxidized phospholipids such as oxidized 1-palmitoyl-2-arachidonoyl-*sn*-Glycero-3-phosphorylcholine (OxPAPC) accumulate in lesions of and promote atherosclerosis. OxPAPC activates endothelial cells, a critical early event of atherogenesis. Epoxyisoprostane E2 (EI) is an oxidized fatty acid contained at the *sn*-2 position of 1-palmitoyl-2-epoxyisoprostane E2-*sn*-glycero-3-phosphorylcholine (PEIPC), the most active component of OxPAPC in regulating inflammation. OxPAPC and its components including PEIPC activate endothelial cells to express an array of genes in different categories including oxidative stress response genes such as tumor suppressor gene OKL38 and Hemeoxygenase-1 (HO-1). EI can be released by lipase from PEIPC. In this study, we examined the ability of EI to stimulate oxidative stress response in endothelial cells. EI released from OxPAPC and synthetic EI stimulated the expression of oxidative stress response gene OKL38 and antioxidant gene HO-1. Treatment of endothelial cells with EI increased the production of superoxide. NADPH oxidase inhibitor Apocynin and superoxide scavenger N-acetyl-cysteine (NAC) significantly attenuated EI-stimulated expression of OKL38 and HO-1. We further demonstrated that EI activated oxidative stress-sensitive transcription factor Nrf2. Silencing of Nrf2 with siRNA significantly reduced EI stimulated expression of OKL38 and HO-1. Thus, we demonstrated that EI induced oxidative stress in endothelial cells leading to

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Conflict of interest

The authors report no conflict of interest.

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increased expression of oxidative stress response gene OKL38 and HO-1 via Nrf2 signaling pathway relevant to atherosclerosis.

Keywords

Epoxyisoprostane E2 (EI); oxidized phospholipids; OxPAPC; OKL38; HO-1 oxidative stress; Nrf2

Introduction

Documented *in vivo* and *in vitro* studies support that oxidized phospholipids (OxPLs) play an important role in atherosclerosis [1,2]. Specific OxPLs such as Oxidized 1-palmitoyl-2-arachidonoyl-*sn*-Glycero-3-phosphorylcholine (OxPAPC) have been demonstrated to accumulate in atherosclerotic lesions and other sites of chronic inflammation and been identified as major regulators of vascular cells[3,4]. Multiple bioactive oxidized phospholipids are formed from the oxidation of polyunsaturated fatty acids (PUFAs) at the *sn*-2 position [1,2]. These oxidized products at *sn*-2 position could be released by Phospholipase A2 (PLA2), enzymes that are closely associated with atherosclerosis[5], and regulate the function of vascular cells. For example, increased levels of hydroxyeicosatetraenoic (HETEs) and hydroxyoctadecadienoic (HODEs) acids, oxidation products of arachidonic and linoleic acids, are associated with reduced high density lipoprotein (HDL) antioxidant capacity [6,7] and intimately involved in atherogenesis.[6,8]. A large number of oxidation products can arise from the oxidation of each PUFA and effects of many of these products remains to be examined.

Three major active inflammatory lipids in OxPAPC were identified: 1-palmitoyl-2-epoxyisoprostane E2-*sn*-glycero-3-phosphorylcholine (PEIPC), 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphorylcholine (POVPC) and 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphorylcholine(PGPC) with PEIPC as the most active lipids in activating endothelial cell inflammatory function [9,10,11]. These three lipids differ only at the *sn*-2 position. We have previously shown that the *sn*-2 position is required for the activity of OxPAPC in stimulating monocyte binding and gene expression in endothelial cells[9,10]. Our most recent studies demonstrate that EI, the PLA2 hydrolysis product of PEIPC, though poorly regulating inflammatory function, is able to regulate 40% of the genes regulated by PEIPC[12]. This study examines the ability of EI to regulate oxidative stress.

We previously identified the tumor suppressor gene OKL38 as an oxidative stress response gene stimulated by OxPAPC and its component lipid PEIPC via Nrf2 signaling pathway[13]. In this study, we examined if Epoxyisoprostane E2 (EI), could activate endothelial cells and induce oxidative stress. We demonstrated that EI stimulated oxidative stress and the expression of oxidative stress response gene OKL38 and HO-1 via Nrf2 signaling pathway in endothelial cells.

Materials and Methods

Materials

Cell culture media and reagents were obtained from Invitrogen Inc. FBS was obtained from Hyclone Inc. OxPAPC and PEIPC were prepared and analyzed as previously described [11]. EI was synthesized as previously reported[14,15,16]. Apocynin, and N-acetylcysteine were purchased from Calbiochem. Protease inhibitor (PI) cocktail and superoxide dismutase (SOD) was purchased from Sigma Inc. Antibody against Nrf2 was obtained from Santa Cruz Biotech. HRP-conjugated secondary antibodies were obtained from Cell Signaling Inc.

Scrambled control siRNA was obtained from Invitrogen. SiRNA of Nrf2 (Hs_NFE2L2_4 HP) and HiPerFect® were obtained from Qiagen Inc.

Phospholipase A2 Hydrolysis of OxPAPC and fractionation of oxidized fatty acids

OxPAPC were dried under argon and resuspended in phosphate-buffered saline containing 5 mM CaCl₂. To this solution were added 5 units of phospholipase A2 (#P8913, Sigma). The solution was mixed and incubated at 37 °C for 45 min. After incubation, the lipids were extracted with chloroform. Oxidized free fatty acids from the extraction were separated by Reverse phase high performance liquid chromatography (RP-HPLC) using a C18 column (Betasil, C18, 250 x 10-mm, 5 mm, Keystone Scientific, Inc.). A mobile phase of 60% methanol containing 1 mM ammonium acetate changed linearly over 60 min to 100% methanol containing 1 mM ammonium acetate was used. Fatty acid fractions were collected every minute. Fatty acids in the fractions were analyzed by direct infusion ESI-MS using a Thermo LCQ Advantage Max equipped with an ESI source.

Cell culture and treatment

Human aortic endothelial cells (HAEC) were prepared and cultured as previously described [17]. In most case, HAEC were treated with EI or OxPAPC for 4 hours in M199/0.2% FBS. In studies with inhibitors, HAEC were pretreated with the indicated concentration of inhibitors for one hour before co-treatment with EI and inhibitors.

Quantitative RT-PCR(qRT-PCR)

Total RNA was isolated with RNeasy® mini kit from Qiagen following the manufacturer's instructions. Potential genomic DNA contamination was removed with on-column DNase I digestion. 0.5–1 µg of total RNA was reverse transcribed with Bio-Rad's iScript cDNA synthesis kit. The expression of OKL38 and HO-1 was measured at the mRNA level using semi-quantitative real-time PCR essentially as described previously [18]. The same experiment was repeated three or more times. Primers used to measure OKL38, HO-1 and Nrf2 expression were as following: OKL38: forward: TCCTCTACGCCCCGCGACTACAACATCC, reverse: GGTCTGGAACACGGCCTGGCAGTCTTC. HO-1: forward: GGCAGAGAATGCTGAGTTCATGAGGA, reverse: ATAGATGTGGTACAGGGAGGCCATCA. Nrf2: forward: AGCATGCCCTCACCTGCTACTTTA. reverse: ACTGAGTGTCTGGTGATGCCACA. The expression of target genes was calculated as fold increase relative to controls and normalized to GAPDH.

Cell lysates, nuclear extract and western blot

Nuclear extract was prepared according to Osborn *et al* with modification [19]: Cells washed with cold PBS were suspended in Buffer A (10mM Hepes, pH7.9, 1.5mM MgCl₂, 10mM KCl, 1mM DTT, 0.1% NP40, plus freshly prepared PI cocktail and 1mM PMSF). After 10 min incubation in ice, the suspension was centrifuged at 10,000g for 5 min at 4°C. The supernatant was collected as cytosolic extract. The pellet was resuspended in proper volume of buffer B (20mM Hepes, pH7.9, 1.5mM MgCl₂, 25% glycerol, 0.42M NaCl, 0.5mM EDTA, 1mM DTT, with PI cocktail and 1mM PMSF added immediately before use) and put on ice for 10 min. After centrifugation at 12,000g for 10 min at 4°C, the supernatant was collected as nuclear extract. Protein concentration was determined with a Bio-Rad DC protein assay kit. SDS-PAGE and western blot were essentially done as described [20]. Lysates and extracts were run on a 4–12% gradient SDS-PAGE gel. The proteins were then transferred to PVDF membrane and blotted with indicated primary and secondary

antibodies. Signal was developed with ECL™-Plus (GE Healthcare) and recorded with VersaDoc™ (Bio-Rad Inc).

siRNA transfection in HAEC

HAEC were plated at 12,500 cells/cm² in 6-well plate one day before transfection. The next day, cells were washed with and cultured in M199/10% FBS before transfection. siRNA was transfected into HAEC with HiPerFect® using 20 nM of scrambled control RNA or Nrf2 siRNA and 10uL of HiPerFect® lipid for each well of 6-well plate. The next day, the media was replaced with regular culture media. 48 hours after transfection, the cells were used for analysis of gene expression or for treatment.

Measurement of reactive oxygen species (ROS)

Extracellular superoxide was determined with cytochrome C reduction assay[21]. Briefly, HAEC were plated in 96-well plate and grow to confluence. The cells were washed with serum-free M199 media and then treated with cytochrome C solution with or without EI in the presence or absence of superoxide dismutase (SOD). Absorbance at 550nm was continuously measured. Superoxide was calculated from the slope difference of absorbance with or without SOD.

Intracellular ROS was assessed by 2',7'-dichlorofluorescein (DCF) assay as previously described[22]. Briefly, cells were grown in 4 well-glass chamber slides to confluence. Cells were pre-incubated with 5mM CM-H2DCFDA (Molecular Probes) for 30 min in assay media. After removing DCF solution, cells were treated with or without EI in assay media. The green fluorescence (excitation: 488 nm, detection: 505 nm) was visualized using an inverted confocal microscope (Olympus).

Statistical analysis

Data were expressed as mean $\pm$ SD. Comparisons of multiple values were made by one-way analysis of variance (ANOVA), and statistical significance for pairwise comparison was determined by using the Turkey test. P-values of < 0.05 were considered statistically significant.

Results

Epoxyisoprostane E2 (EI) stimulated the expression of oxidative stress response gene OKL38 and HO-1

Specific structures of *sn*-2 fatty acid of OxPAPC were shown to be required for the activity of OxPAPC in activating endothelial cells[9,10]. To see if the fatty acid moiety alone at the *sn*-2 position of OxPAPC is active in stimulating endothelial cells, We released the *sn*-2 fatty acids of OxPAPC with PLA2. The fatty acids were separated with HPLC and collected into 60 fractions. We analyzed the ability of these fractions to stimulate the expression of OKL38 and HO-1. The fraction 43 (F43) seemed to have the highest activity among the fractions(Fig. 1A). Mass spectrometry identified this fraction contained a relatively pure chemical with *m.z.* 349 (Fig. 1B), the expected molecular weight of EI from PEIPC. Furthermore synthetic EI dose dependently increased the expression of OKL38 and HO-1(Fig. 1C). These data demonstrate that EI alone stimulates the expression of oxidative stress response gene OKL38 and HO-1 in endothelial cells.

EI increases oxidative stress leading to the regulation of OKL38 and HO-1 expression

We have demonstrated that OxPAPC and PEIPC can activate multiple pathways leading to the modulation of gene expressions including the oxidative stress response pathway. We

therefore examined if EI could also stimulate oxidative stress in endothelial cells. Cytochrome C reduction assay demonstrated that EI dose dependently increased superoxide production (Fig. 2A). EI-stimulated ROS production was further confirmed by DCF fluorescence (Fig. 2B).

To see if EI-stimulated oxidative stress mediated EI-induced expression of OKL38 and HO-1, HAEC were treated with EI in the presence or absence of NADPH oxidase inhibitor Apocynin or antioxidant N-acetylcysteine(NAC). Both Apocynin and NAC significantly blocked EI stimulated expression of OKL38 (Fig. 3A) and HO-1 (Fig. 3B). Thus oxidative stress was implicated in EI-stimulated OKL38 and HO-1 expression.

EI stimulated the expression of OKL38 and HO-1 via the Nrf2 signaling pathway

We previously showed that OxPAPC and PEIPC activated Nrf2 signaling pathway[13]. To see if EI mimics this activity, we measured nuclear levels of Nrf2 after EI treatment. Like OxPAPC treatment, EI treatment resulted in Nrf2 translocation into nucleus (Fig. 4A). To examine if Nrf2 activation mediated EI-stimulated expression of OKL38 and HO-1, we knocked down Nrf2 expression with siRNA(Fig. 4B). Knockdown of Nrf2 significantly attenuated EI-stimulated expression of OKL38 and HO-1(Fig. 4B). Thus, our data indicated that EI stimulated OKL38 and HO-1 expression via Nrf2-dependent pathway.

Discussion

Endothelial cell dysfunction plays a critical role in the initiation and progression of atherosclerosis. The pro-atherogenic oxidized phospholipids OxPAPC and components such as PEIPC have been shown to activate endothelial cells to express a number of genes including oxidative stress response gene OKL38 and HO-1 [13,23,24]. In this study, we demonstrated that epoxyisoprostane E2 (EI), the fatty acid component at the *sn*-2 position of PEIPC could be released by phospholipase A2 and stimulated the expression of OKL38 and HO-1 in endothelial cells via oxidative stress response and the activation of Nrf2 signaling pathway.

OxPAPC contains a pool of lipid oxidation products that possess different biological activities in endothelial cells. Several receptors and signaling pathways associated with OxPAPC action have been identified, and were shown to be up-regulated in human lesions[1]. OxPAPC regulates the expression of more than 1000 genes in human aortic endothelial cells(HAEC) [23,24]. Some of these genes are pro-atherogenic such as IL-8 and others anti-atherogenic such as HO-1. In this study, we showed that fatty acid fraction of OxPAPC, specifically epoxyisoprostane E2, possess the same activity of OxPAPC/PEIPC in stimulating superoxide production and the expression of OKL38 and HO-1 in endothelial cells. We most recently showed that EI also modulated the expression of other genes regulated by OxPAPC[12]. The net effect of EI on atherosclerosis will be the interest of future study.

PLA2 is a group of enzymes that hydrolyze the *sn*-2 position of glycerophospholipids to yield fatty acids and lysophospholipids. Different PLA2 has been shown to be associated with the development of atherosclerosis. We previously showed that the removal of the structure at *sn*-2 position of oxidized lipid by PLA2 abolished several inflammatory effects of OxPAPC component lipids[9,10]. In this study, we showed that EI could be released from PEIPC by PLA2 and stimulate oxidative stress and stress related gene expression in endothelial cells.

The phospholipid oxidation products such as EI could either act as ligands or might cause local membrane disruption to exert their biological functions. Previously we have

demonstrated that OxPAPC/PEIPC stimulate monocyte binding through prostaglandin receptor EP2 and gene expression via VEGF receptor 2 (VEGFR2) dependent signaling[18,25]. There is also evidence that OxPAPC is able to form protein adducts [26,27,28], which may affect the function of the target proteins. It is not clear how EI signals to endothelial cells. 15d-PGJ₂, another arachidonic acid metabolite with some structure similarity to EI, exerts its anti-inflammatory activity through activation of peroxisome proliferator-activated receptor γ (PPAR γ) [29,30] or through inhibition of nuclear factor kappa B (NF- κ B) activation by direct covalent binding to the I κ B kinase[31]. 15d-PGJ₂ also modify cysteine residue of Keap1 and thus activate the Nrf2 signaling pathway[32]. EI, possessing an active unstable epoxide structure, may enter the cells and activate signaling pathways via covalent binding like 15d-PGJ₂[27].

The Keap1-Nrf2-ARE is a major cell signaling pathway that sense oxidative stress and protect cells from oxidative stress via stimulating the expression of antioxidant genes. In this study, we showed that EI activated Nrf2 and EI-induced expression of OKL38 and HO-1 expression is inhibited by silencing Nrf2. Similar to OxPAPC/PEIPC, EI stimulated superoxide production in endothelial cells, and the inhibition of superoxide production by Apocynin or scavenging of superoxide by N-acetylcysteine (NAC) abolished EI stimulated expression of OKL38 and HO-1. Thus EI could activate Nrf2 via the stimulation of superoxide production that depends on NADPH oxidase. In addition, EI may also enter the cells like 15d-PGJ₂, covalently bind to cysteine of Keap1 and thus activate Nrf2[26,27].

In the current study, silencing of Nrf2 did not completely inhibit EI-stimulated expression of OKL38 and HO-1, suggesting presence of Nrf2-independent pathway for these genes. We previously reported PMET (plasma membrane electron transport) involvement in HO-1 regulation in endothelial cells by Ox-PAPC [33]. The depletion of intracellular NAD(P)H levels, induced by Ox-PAPC activation of PMET system, is associated with oxidative stress and HO-1 regulation. PEIPC showed the biggest activation of PMET activity in the endothelial cells[33]. It was also reported that PPAR γ and CREB are involved in regulating HO-1 expression induced by Ox-PAPC in some types of endothelial cells [34,35]. The role of PMET, PPAR γ and CREB in EI-induced oxidative stress response and the detailed mechanism(s) of EI-stimulated expression of oxidative stress response genes await further investigation.

In summary, we demonstrated in this study that epoxyisoprostane E2 can induce oxidative stress and stimulate the expression of oxidative stress response gene OKL38 and HO-1 via Nrf2 signaling pathway in endothelial cells.

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Abbreviations and Terminology

EI	epoxyisoprostane E2
OxPL	Oxidized phospholipids
OxPAPC	Oxidized 1-palmitoyl-2-arachidonoyl- <i>sn</i> -Glycero-3-phosphorylcholine
PEIPC	1-palmitoyl-2-epoxyisoprostane E2- <i>sn</i> -glycero-3-phosphorylcholine
POVPC	1-palmitoyl-2-oxovaleroyl- <i>sn</i> -glycero-3-phosphorylcholine

PGPC	1-palmitoyl-2-glutaroyl- <i>sn</i> -glycero-3-phosphorylcholine
PUFA	polyunsaturated fatty acids
PLA2	Phospholipase A2
HETE	hydroxyeicosatetraenoic acid
HODE	hydroxyoctadecadienoic acids
HDL	high density lipoprotein
HAEC	human aortic endothelial cells
ROS	reactive oxygen species
Nox	NADPH Oxidase
Nrf2	Nuclear factor E2-related factor
ARE	Antioxidant response element
HO-1	Heme Oxygenase-1
NAC	N-acetyl cysteine

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Highlights

1. Epoxyisoprostane E2(EI) induced oxidative stress in endothelial cells
2. EI stimulated the expression of oxidative stress response gene OKL38 and HO-1
3. EI stimulated oxidative stress response genes via Nrf2 signaling

Fig. 1. Epoxyisoprostane E2 (EI) stimulated the expression of OKL38 and HO-1

(A). HAEC were treated for 4 hours with selected fatty acid fractions of OxPAPC released by phospholipase A2. The expression of OKL38 and HO-1 were measured by qRT-PCR. (B). ESI-MS spectrum of OxPAPC/phospholipase A2 fraction 43(F43), indicating that the main content in F43 was EI. (C). HAEC were treated with different dose of synthetic EI for 4 hours. The expression of OKL38 and HO-1 were measured by qRT-PCR.

Fig. 2. EI stimulated oxidative stress in HAEC

(A). HAEC were treated without or with different dose of EI and the extracellular superoxide was measured by cytochrome C reduction assay. (B). HAEC pre-incubated with CM-H2DCFDA were treated with or without 4 $\mu\text{g/mL}$ of EI. The production of intracellular ROS was visualized via green fluorescence. Shown were pictures taken at 10 minutes after treatment. C=Control.

Fig. 3. EI-stimulated expression of OKL38 and HO-1 was a response to oxidative stress HAEC were treated with or without 1 μ g/mL of EI in the presence or absence of 1mM Apocynin(Apo) or 5mM of N-Acetyl cysteine (NAC). The mRNA expression of OKL38 (A) and HO-1(B) were measured by qRT-PCR.

Fig. 4. Nrf2 mediated EI-stimulated expression of OKL38 and HO-1

(A). HAEC were treated with or without 1 μ g/mL of EI or 50 μ g/mL of OxPAPC for 2 h. Nuclear extracts were prepared and Nrf2 protein translocated into the nucleus was assessed by Western blot. (B) HAEC were transfected with 20nM of scrambled RNA (Scr) or Nrf2 siRNA (si-Nrf2) for 48 hours and then treated with or without 1 μ g/mL of EI for 4 hours. The expression of Nrf2, OKL38 and HO-1 were measured by qRT-PCR.