Fatty acid epoxyisoprostane E2 stimulates an oxidative stress response in endothelial cells

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

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\section{1. 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 PUFAs 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-glyce-
ro-3-phosphorylcholine (PGPC) with PEIPE as the most active
lipids in activating endothelial cell inflammatory function [9–11].
These three lipids differ only at the sn-2 position. We have previous-
ly shown that the sn-2 position is required for the activity of OxPA
C in stimulating monocyte binding and gene expression in endo-
thelial cells [9,10]. Our most recent studies demonstrate that ESI,
the PLA2 hydrolysis product of PEIPE, though poorly regulating inflam-
atory function, is able to regulate 40% of the genes regulated by
PEIPE [12]. This study examines the ability of ESI to regulate oxida-
tive stress.

We previously identified the tumor suppressor gene OKL38 as
an oxidative stress response gene stimulated by OxPA and its com-
ponent lipid PEIPE 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.

2. Materials and methods

2.1. Materials

Cell culture media and reagents were obtained from Invitrogen
Inc. FBS was obtained from Hyclone Inc. OxPA and PEIPE were
prepared and analyzed as previously described [11]. ESI was synthe-
sized as previously reported [14–16]. Apocynin, and N-acetylcyste-
ine were purchased from Calbiochem. Protease inhibitor (PI)
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in endothelial cells.

2.2. Phospholipase A2 hydrolysis of OxPA and fractionation of
oxidized fatty acids

OxPA 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 lip-
ids 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 × 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.

2.3. Cell culture and treatment

Human aortic endothelial cells (HAEC) were prepared and cul-
tured as previously described [17]. In most case, HAEC were trea-
ted with ESI or OxPA for 4 h in M199/0.2% FBS. In studies with
inhibitors, HAEC were pretreated with the indicated concentration
of inhibitors for 1 h before co-treatment with ESI and inhibitors.

2.4. 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 mea-
sured 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 follows: OKL38: forward:
TCCTTACGCCGCCACTACAACATCC, reverse: GGTCCTGAAACCGG
CCTGCAGCTTCTC. HO-1: forward: GGCAGAGAATGCTGAGTTCAT
GAGGA, reverse: ATAGATGTGGTACAGGGAGGCCATCA. Nrf2: for-
ward: AGCATGCCCTACCTGCTACTTTA. reverse: ACTGAGTGTTCTG
GTATGCCACA. The expression of target genes was calculated as
cold increase relative to controls and normalized to GAPDH.

2.5. 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 (10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM
DTT, 0.1% NP40, plus freshly prepared PI cocktail and 1 mM 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 cyto-
sollic extract. The pellet was resuspended in proper volume of buf-
fer B (20 mM Hepes, pH 7.9, 1.5 mM MgCl2, 25% glycerol, 0.42 M
NaCl, 0.5 mM EDTA, 1 mM DTT, with PI cocktail and 1 mM 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).

2.6. 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 cul-
tured in M199/10% FBS before transfection. siRNA was transfected
into HAEC with HiPerFect™ using 20 nM of scrambled control RNA
or Nrf2 siRNA and 10 μL of HiPerFect™ lipid for each well of 6-well
plate. The next day, the media was replaced with regular culture
media. 48 h after transfection, the cells were used for analysis of
gene expression or for treatment.

2.7. 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 ESI in the presence or absence of superoxide dismutase
(SOD). Absorbance at 550 nm was continuously measured. Super-
oxide 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-incu-
bated with 5 mM CM-H2DCFDA (Molecular Probes) for 30 min
in assay media. After removing DCF solution, cells were treated with
or without ESI in assay media. The green fluorescence (excitation:
488 nm, detection: 505 nm) was visualized using an inverted confocal microscope (Olympus).

2.8. Statistical analysis

Data were expressed as mean ± 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.

3. Results

3.1. 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.

3.2. 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.

3.3. 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.

4. 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 (Fig. 4B). Thus, our data indicated that EI stimulated OKL38 and HO-1 expression via Nrf2-dependent pathway.

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 [26–28], 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 (PPARγ) [29,30] or through inhibition of nuclear factor kappa B (NF-κB) activation by direct covalent binding to the IκB kinase [31]. 15d-PGJ2 also modify cysteine residue of Keap1 and thus activate the Nrf2 signaling pathway [32]. EI possessing an active
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 PPARγ 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, PPARγ 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.

Conflict of interest

The authors report no conflict of interest.

Acknowledgments

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