Allergy and Immunology

AIMS Allergy and Immunology, 1(3): 108-123.
DOI: 10.3934/Allergy.2017.3.108
Received: 26 July 2017
Accepted: 10 October 2017
Published: 17 October 2017

http://www.aimspress.com/journal/Allergy

Research article

Platelet-activating factor and oxidized phosphatidylcholines do not suppress endotoxin-induced pro-inflammatory signaling among human myeloid and endothelial cells

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Abstract: Platelet-activating factor (PAF) and related phospholipid oxidation products termed oxidized phospholipids (OxPLs) promote inflammation. PAF is made in response to bacterial endotoxin-lipopolysaccharide (LPS) that is recognized by Toll-like receptor-4 (TLR-4) whose activation leads to translocation of transcription factor NF-KB to the nucleus—a key regulator of multiple pro-inflammatory genes including COX-2 and IL-8. Paradoxically, PAF and OxPLs are claimed to inhibit LPS-mediated signaling, questioning the very pro-inflammatory roles of PAF and OxPLs and anti-inflammatory nature of PAF-acetylhydrolase (PAF-AH), an enzyme that attenuates both PAF and OxPLs signaling. We investigated the effect of PAF and representative OxPLs: 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC) and 1-alkyl-2-butanoyl-*sn*-glycero-3-phosphocholine PAF (C_4 PAF) on LPS-induced expression of NF-KB mediated inflammation in isolated human myeloid cells: polymorphonuclear leukocyte (PMNs), monocytes and human umbilical vein endothelial cells (HUVECs). Using intracellular calcium transients, we show that POVPC and PGPC dose-

dependently activate the PAF-receptor (PAF-R) in PMNs, that can beblocked by the PAF-R antagonist WEB-2086 and rPAF-AH pre-treatment. All the three cell types express minute or no detectable COX-2 when stimulated with either PAF (0.1 μM) or OxPLs (0.1 μM) alone. While LPS (100 ng/mL) induced expression of COX-2 in all the cell types, pre-activation of PAF-R with PAF (0.1 μM) or OxPLs (0.1 μM) did not suppress LPS (100 ng/mL)-induced COX-2 expression and in fact we obresved incereased PGE_2 levels in an NS-398 sensitive manner. In addition, pre-activation of PAF-R significantly augmented LPS (100 ng/mL)-induced IL-8 production in PMNs. Thus, PAF and OXPLs do not suppress the ability of LPS to exert its pro-inflammatory effects in isolated human vascular cells.

Keywords: platelet activating factor (PAF); oxidized phospholipids (OxPLs); endotoxin; cyclooxygenase-2 (COX-2); vascular inflammation

Abbreviations

COX-2	Cyclooxygensae-2
GPCR	G-Protein Coupled Receptor
HUVEC	Human Umbilical Vein Endothelial Cells
LPS	Lipopolysaccharide
NF-KB	Nuclear Factor-Kappa B
OxPL	Oxidized Phospholipid
PAF	Platelet-Activating Factor
PAF-AH	PAF-Acetylhydrolase
PAF-R	PAF-Receptor
PAMP	Pathogen Associated Molecular Pattern
PMN	Polymorphonuclear Leukocytes
PUFA	Polyunsaturated Fatty Acid
TLR	Toll-Like Receptor

1. Introduction

A meticulously concerted network consisting of monocytes, macrophages and polymorphonuclear leukocytes (PMNs) make up the innate immune system that is highly sensitive to microbial invasion/injury and responds by synthesizing a variety of inflammatory mediators [1]. Among the various pathogen associated molecular patterns (PAMPs) recognized by Toll-like receptors (TLRs), lipopolysaccharide (LPS)—a major membrane lipoglycan of Gram-negative bacteria is of special importance and is most thoroughly studied [2,3]. The pro-inflammatory effect of LPS is conveyed via Toll-like receptor-4 (TLR-4) [4], leading to the downstream activation of NF-KB, the master regulator for the induction of a repertoire of inflammatory genes [5,6]. The NF-KB mediated inflammatory genes are known to play a vital role in the initiation and amplification of many systemic inflammatory diseases [7–10]. A key lipid mediator involved in these events is PAF, an autocoid, usually present in low levels in quiescent cells but either expressed or secreted by innate immune cells upon appropriate stimulation [11]. PAF synthesis in response to

inflammatory stimuli is rapid and vigilantly regulated owing to its multi-faceted function and unique ability to activate immune cells at sub-picomolar concentrations [11]. Considerably, dysregulated PAF signaling is evidently the underlying cause for a host of inflammatory diseases [12,13]. PAF exerts its action via platelet activating factor receptor (PAF-R)—a typical GPCR [14] that specifically recognizes the *sn*-1 ether/ester bond and a short *sn*-2 moiety of this unusual phospholipid. However, PAF homologs with ester bond at *sn*-1position are several fold less potent than their ether counter parts [11]. The functional requirement to activate PAF-R allows several structural analogs of PAF termed OxPLs to substantially activate the PAF-R with varying potencies [15–18], although a separate receptor for the selected OxPLs has also been suggested [19]. More recently, OxPLs with a slightly longer *sn*-2 residue up to 9 carbon length such as KHdiA-GL PAF (7-carbon long), KOdiA-LPAF (8-carbon long) and HAz-LPAF (9-carbon long) have been shown to bind PAF-R and invoke responses that mimic PAF [20].

Endogenously, these bioactive lipid species are generated by unregulated non-enzymatic oxidation of polyunsaturated fatty acid (PUFA) residues by reactive oxygen species (ROS) and H_2O_2 [16]. Since PUFAs are targets for oxidation and mostly esterified at *sn*-2 position of the glycerol backbone of alkyl/acyl phosphatidylcholines, their oxidation yields truncated phospholipids with short *sn*-2 residues, making them appropriate PAF-R ligands. Relevantly, OxPLs mimic many of the biological effects of PAF and contribute to inflammatory pathophysiology [16–23], hence are collectively referred as "PAF-mimetics or PAF-like lipids". Oxidation of phospholipids such as 1-palmitoyl-2-arachidonoyl-*sn*-glycerol-3-phosphocholine (PAPC) generates a series of OxPAPC, predominantly 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-*sn*-glycerol-3-phosphocholine (PGPC) [17,24]. Additionally, fragmentation of hexadecyl-arachidonoyl-phosphatidylcholine yields butanoyl/butenoyl analogs of PAF (C-4 PAF) that are only ten to hundred folds less potent than PAF [15]. Such truncated phospholipids can activate PMNs, monocytes, eosinophils as well as platelets and are validated to be the major components of oxidized low density lipoprotein (OxLDL) that promotes atherogenesis [16,25,26] in atherosclerotic plaque [27], smokers and alcoholic blood [28,29], inflamed tissues [30] and in models of cutaneous inflammation [31].

Curtailing the potency of this extended family of PAF and related OxPLs in circulation is primarily achieved by the hydrolysis of their *sn*-2 moiety by a family of related anti-inflammatory enzymes—"PAF-acetylhydrolase (PAF-AH)", of which the plasma form is thoroughly characterized [32]. The exceptionally extended substrate specificity of PAF-AH thus terminates the inappropriate signaling of OxPLs that are likely to circumvent other cellular controls [23]. Decrease in circulating PAF-AH levels together with the susceptibility of PAF-AH deficient subjects to asthma and other inflammatory diseases, nevertheless explains the very anti-inflammatory nature of this enzyme [23].

Studies have shown that OxPLs upregulate the expression of pro-inflammatory cytokines including IL-6 [22], IL-8 [33] and chemokines such as Monocyte chemoattractant protein-1 (MCP-1) [34] and inducible cyclooxygenase-2 (COX-2) [33]. Further evidence implicating the upregulation of COX-2 by OxPLs comes from the study by Pontsler et al. [35], where oxidized alkyl phospholipids derived from OxLDL enhanced COX-2 induced prostaglandin E_2 (PGE_2) in human monocytes via peroxisome proliferator activated receptor gamma (PPARγ), a receptor highly expressed in atherosclerotic plaques [36]. Presence of OxLDL along with PPARγ and COX-2 [37] in atherosclerotic plaques probably suggests their synergistic participation in atherogenesis.

Additionally, phospholipids derived from OxLDL induce intracellular calcium transients and also cause PMNs to adhere to activated endothelium [38]. Furthermore, OxPLs have also been identified to promote TNF-α-induced cell death [39].

Despite, substantial evidences affirming the pro-inflammatory roles of PAF and OxPLs, few conflicting reports claiming the inhibitory actions of OxPLs on LPS-induced effects [40–43], questions the very anti-inflammatory nature of PAF-AH and pro-inflammatory properties of PAF and OxPLs. We examined the influence of the three representative OxPLs-POVPC, PGPC and C-4 PAF on LPS-induced effects in neutrophils and monocytes, that express both functional PAF-R and TLR-4, and human umbilical vein endothelial cells (HUVECs) that lack canonical PAF-R [19], but still express functional TLR-4. We show that PAF and related OxPLs do not suppress, but rather amplify LPS-induced expression of NF-KB mediated inflammatory readouts in these isolated cell types in vitro. Oxidatively generated phospholipids are thus likely to exaggerate LPS-induced effects and thereby intensify the progression of inflammation.

2. Materials and Methods

LPS from *Escherichia coli* O111:B4 was purchased both from List Biological Laboratories, Inc. (Campbell, CA) and from Sigma Chemicals Co. (St. Louis, MO). Dextran and MCDB/12 medium were from Sigma Chemicals Co. (St. Louis, MO). PAF (C16), LysoPAF, C-4 PAF and Lyso PC were obtained from Avanti Polar Lipids (Alabaster, AL). Hanks Balanced Salt Solution (HBSS) was purchased from HiMedia (Mumbai, India) and also from Cleveland Clinic Media Core (Cleveland, OH). Human serum albumin was from Baxter Healthcare (Glendale, CA). rPAF-AH was from ICOS Corp. (Bothell, WA) and Ficoll-Paque was obtained from GE Healthcare Bio-Sciences(Uppsala, Sweden). Sterile tissue culture plates were purchased from Nest Biotech Co. (Jiangsu, China). COX-2 specific monoclonal antibody, arachidonic acid, NS-398, POVPC, PGPC and PGE_2 ELISA kit were from Cayman chemicals (AnnArbor, MI) and β-Actin antibody was from MP Biomedicals (SantaAna, CA). Secondary antibodies were from Biosource (Camarillo, CA). Protease inhibitor cocktails-Roche Complete Mini was from Roche (Basel, Switzerland), Protease Arrest was from GenoTechnology, Inc. (St. Louis, MO), and Halt Protease was from Pierce (Rockford, IL). M199 was from Mediatech, Inc. (Herndon, VA), BCA protein assay kit was from Pierce (Rockford, IL) and Immobilon-P was from Millipore Corp. (Bradford, MA). Fura 2-AM was from Santa Cruz Biotechnology (Dallas, TX). Human IL-8 Duoset ELISA kit was obtained from R&D Systems (Minneapolis, MN). Human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr. Paul DiCorleto (Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH).

2.1. Isolation of human PMNs and monocytes

Approval for the use of isolated human cells was obtained from the Institutional Human Ethical Clearance Committee (IHEC) of the University of Mysore, India (No. IHEC-UOM No. 104Ph.D/2015-16). Venous blood was drawn from healthy human volunteers (non-smokers) with informed consent as per the guidelines of IHEC, University of Mysore, Mysuru, and PMNs were isolated by dextran sedimentation and centrifugation over Ficoll as described by Zimmerman et al. [44]. The isolated PMNs were resuspended in HBSS/A (HBSS containing 0.5% human serum

albumin) or in M199. Mononuclear cells were collected at the inter phase of the Ficoll gradient, washed and resuspended in HBSS/A and used as monocyte enriched cell population.

2.2. Measurement of intracellular Ca^{2+} transients in human PMNs

Changes in intracellular Ca^{2+} flux in isolated PMNs in response to POVPC and PGPC were measured according to Marathe et al. [38]. Briefly, isolated PMNs (2.5×10^6 cells/mL) were incubated with Fura 2-AM (1 μM) at 37 ℃ for 45 min in dark. A concentration-dependent change in Ca^{2+} flux in these cells in response to indicated concentrations of POVPC and PGPC were measured at 340 and 380 nm with emission collected at 510 nm. In parallel experiments, PMNs were pre-treated with a PAF-R antagonist, WEB-2086 (10 μM) for 30 min and then tested for POVPC (1 μM) or PGPC (1 μM) stimulated calcium transients or POVPC and PGPC were pre-treated with rPAF-AH (10 ng/mL) for 30 min and then used to stimulate PMNs. Subsequently, to check if these cells were responsive, they were stimulated with 1 nM/1 μM PAF at indicated time.

2.3. Western blotting for cyclooxygenase-2

Human PMNs (5×10^6 cells/mL) were suspended in M199 medium and pre-treated with or without PAF or OxPLs (0.1 μM) for 30 min followed by LPS (100 ng/mL) for 3 hours at 37 ℃. In few experiments, we added 20 μM exogenous arachidonic acid before the cell harvest for measuring PGE_2. In other set of experiments, PMNs were treated with NS-398 (10 μM) before harvesting the cells. PMNs were treated with a combination of three protease inhibitor cocktail (Protease arrest, Roche Complete mini and Halt Protease) containing EDTA (10 mM) and DTT (5 mM) 30 min prior to cell harvest (This combination of these three protease inhibitors substantially control proteolysis in this protease loaded cell type). Cells were centrifuged at 300 × g for 5 min at 37 ℃ and media were aspirated and used to measure PGE_2 as described below. Cell pellet was lysed with lysis buffer containing protease inhibitor cocktail. The proteins were resolved on a 7.5% SDS-PAGE and transferred to an Immobilon-P membrane by Bio-Rad semidry transfer apparatus. The membrane was blocked with 5% non-fat dry milk and probed with monoclonal COX-2 specific antibody at 1:1000 dilution and β-actin antibody at 1:20,000 dilution. Appropriate horseradish peroxidase conjugated secondary antibody was used and blots were developed by chemiluminescence (Bio-Rad) according to manufacturer's instructions. In a few experiments, monocytes (5×10^6 cells/mL) were stimulated with or without PAF or PAF-like lipids (0.1 μM) for 30 min followed by LPS (100 ng/mL) for 18 hours at 37 ℃. Culture supernatants were aspirated for ELISA analysis while cellular contents were lysed in the presence of protease inhibitor cocktail and blotted for COX-2 expression as described above. When HUVECs were used for COX-2 expression, they were plated overnight in 6-well plates in MCDB/12 media supplemented with 15% foetal bovine serum. The next day, cells were washed twice with PBS (pH 7.4) containing low serum (2%) and pre-incubated with or without indicated lipids for 30 min followed by LPS for 4 hours. Culture supernatants were used for PGE_2 ELISA as described below. HUVECs were washed twice with PBS and then with ice cold cell lysis buffer containing protease inhibitors. Cellular materials were scraped and removed. The lysates were kept on ice for 30 min and then centrifuged at 4 ℃ for 5 min at 10,000 × g. Protein content of the supernatant was quantitated using BCA protein assay kit and blotted for COX-2 as described above.

2.4. Quantification of PGE_2

The culture supernatants of stimulated human PMNs, monocytes or HUVECs from their respective media containing arachidonic acid (10 μM), added just before harvest, were aspirated, followed by an immediate acidification to pH 3.0 with formic acid. The prostaglandins were selectively extracted with ethyl acetate and dried under nitrogen. The residue was then resuspended in buffer provided in the ELISA kit and was used according to manufacturer's instructions.

2.5. Quantification of IL-8

Human PMNs were suspended in M199 media containing HEPES (25 mM, pH 7.2) and pre-incubated with POVPC, PGPC or C-4 PAF (0.1 μM) for 30 min followed by LPS (100 ng/mL) for 3 hours. Culture supernatants were collected and used either directly in ELISA or concentrated using Amicon Ultra 5,000 MWCO filters (Millipore). IL-8 protein levels were quantitatively assessed by ELISA according to the manufacturer's protocol. The quiescent PMNs had undetectable IL-8 on ELISA.

3. Results

3.1. POVPC and PGPC cause intracellular calcium flux via PAF-R in PMNs

PAF-R agonists are potent inducers of rapid intracellular Ca^{2+} mobilization where, as low as 1 pM PAF causes rapid and significant increase in intracellular Ca^{2+} in PMNs [15]. Using isolated PMNs, we checked the ability of POVPC and PGPC to directly activate PAF-R and cause changes in intracellular Ca^{2+} levels. Both POVPC and PGPC efficiently provoked intracellular Ca^{2+} flux in a concentration–dependent way (0.01–1 μM) (Figure 1) that could be inhibited by pre-treating the PMNs for 30 min with PAF-R antagonist, WEB-2086 (10 μM). The response of POVPC and PGPC was also effectively abolished by pre-incubating them with selective phospholipase A_2, PAF-AH (10 ng/mL). Subsequent addition of PAF (1 nM–1 μM) to these cells showed that the cells were still responsive to PAF. This implies that OxPLs-POVPC and PGPC appear to signal via PAF-R, but are however relatively weak agonists of PAF-R when compared to PAF.

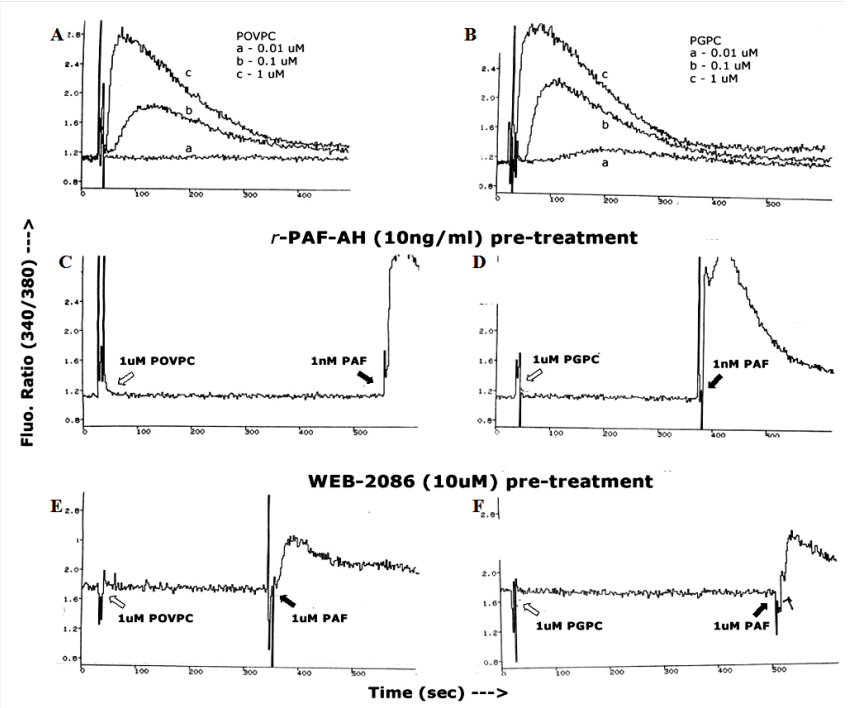

Figure 1. POVPC and PGPC signal via PAF-R in PMNs. Freshly isolated PMNs (2.5×10^6 cells/mL) were loaded with Ca^{2+} sensitive dye Fura 2-AM and stimulated with increasing concentrations (0.01 μM–1 μM) of POVPC (A) and PGPC (B). Rapid changes in intracellular Ca^{2+} were detected by dual excitation at 340 and 380 nm with emission at 510 nm. In parallel experiments, POVPC (1 μM) and PGPC (1 μM) were pre-treated with rPAF-AH (10 ng/mL) for 30 min and then added to PMNs to check for changes in intracellular Ca^{2+} (C) and (D). To show POVPC and PGPC caused intracellular Ca^{2+} via PAF-R, PMNs were treated with WEB-2086 (10 μM) before stimulating with POVPC (1 μM) (E) and PGPC (1 μM) (F). To show cell responsiveness, PAF (1 nM C and D; 1 μM E and F) was added at time indicated by second solid arrow. Data represented as 340/380 nm ratios on the Y-axis and 5 min time period on X-axis.

3.2. COX-2 as an inflammatory marker

COX-2 is an inducible enzyme expressed by a variety of cells in response to various stimuli such as endotoxins, interleukins, growth factors and many more [45,46]. Human leukocytes such as PMNs and monocytes are known to express both PAF-R [38] and TLR-4 and possess inducible COX-2 [47]. Hence, we examined the expression of COX-2 in response to PAF and PAF-like lipids in human PMNs, monocytes and also in cells that do not express canonical PAF-R, yet possess functional TLR-4 such as umbilical vein endothelial cells [19]. Stimulation of PMNs (Figure 2A) and HUVECs (Figure 2C) with PAF or OxPLs (0.1 μM) for 30 min expressed minute or no COX-2, while monocytes expressed considerable amount of COX-2 (Figure 2E). Alternatively, we assessed the effect of pre-activation of PAF-R on LPS-induced COX-2 expression in these cell types. Pre-incubation of PMNs, monocytes and HUVECs with PAF or PAF-like lipids (0.1 μM), for 30 min followed by LPS (100 ng/mL) exposure for indicated time points did not suppress expression of

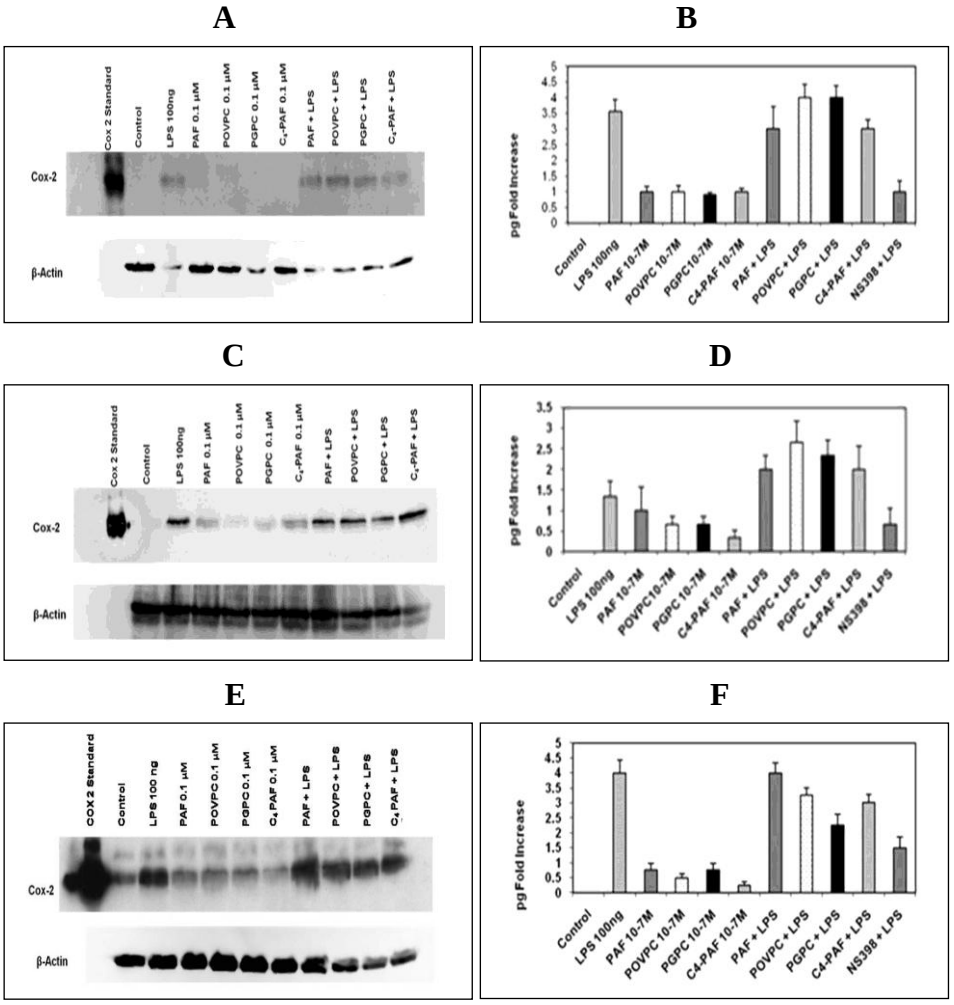

Figure 2. COX-2 expression in stimulated (A) PMNs (B) Monocytes (C) HUVECs and their corresponding PGE_2 levels (B, D and F). Human PMNs, monocytes and HUVECs in the irrespective medium were pre-treated with or without PAF, POVPC, PGPC and C_4 PAF at 0.1 μM for 30 min followed by LPS (100 ng/mL) and serum at 37 ℃ for indicated time points as mentioned in "Materials and Methods". In a few experiments, 20 μM arachidonic acid was added before the cell harvest and in a few experiments the cells were treated with NS-398 (10 μM) before stimulating with agonists. The cells were treated with a combination of three protease inhibitor cocktail containing EDTA (10 mM) and DTT (5 mM) 30 min prior to the cell harvest and centrifuged at 300 × g for 5 min at 37 ℃. Cell suspension was aspirated and used to measure PGE_2 after selectively extracting prostaglandins (see Materials and Methods) by ELISA. Cell pellets were lysed with lysis buffer containing protease inhibitor cocktail. The proteins were resolved on a 7.5% SDS-PAGE and transferred to an Immobilon-P membrane. The membrane was blocked with 5% non-fat dry milk and probed with monoclonal COX-2 specific antibody (1:1000 dilution) and β-actin antibody (1:20,000 dilution). Appropriate horseradish peroxidase conjugated secondary antibody was used and blots were developed by chemiluminescence. Each experiment was repeated more than 3 times with similar results.

LPS-induced COX-2 (Figure 2A, C and E). Stimulation of all the three cell types to LPS (100 ng/mL) alone for indicated time points showed appreciable amount of COX-2 expression.

3.3. Expression of COX-2 correlates with PGE_2 levels

COX-2 expression results in the synthesis of several prostaglandins from arachidonic acid that is released from membrane phospholipids during an inflammatory insult [48]. Hence, we measured one of the major products, PGE_2 in the supernatants of stimulated PMNs, monocytes and HUVECs by ELISA. All the three cell types secreted PGE_2 upon stimulation with LPS (100 ng/mL), while prior exposure of the cells to specific COX-2 inhibitor NS-398 (10 μM) resulted in reduction of LPS-induced PGE_2 release (Figure 2B, D and F) suggesting, that the induced COX-2 was responsible for the PGE_2 being produced. Subsequently, pre-activation of PAF-R in these cell types by PAF or OxPLs (0.1 μM) augmented LPS (100 ng/mL)-induced production of PGE_2 levels (Figure 2B, D and F). Although, corresponding increase in COX-2 expression is difficult to see in these myeloid cells loaded with proteases. However, production of PGE_2 was increased in all the cell types. As the substrate arachidonic acid is limiting, it is necessary to add exogenous arachidonic acid to see measurable amount of eicosanoids.

3.4. PAF and PAF-like lipids prime LPS-induced IL-8 secretions in PMNs

LPS is a potent inducer of IL-8 synthesis in a multitude of cell types [49]. Apparently, PAF-R activation in PMNs stimulates the synthesis of various pro-inflammatory cytokines, most important among them being IL-8 [50,51]. This occurs via mTOR pathway as demonstrated previously by Yost et al. [51]. We examined the effect of PAF and OxPLs on LPS-induced IL-8 secretion in PMNs. Stimulating PMNs with LPS (100 ng/mL) for 30 min resulted in significant increase in IL-8 levels that was efficiently blocked by IL-10, while 0.1 μM of PAF, PGPC, POVPC and C-4 PAF induced minute or no IL-8. However, pre-activation of PAF-R in PMNs with PAF (0.1 μM) or OxPLs (0.1 μM) for 30 min followed by stimulation with LPS (100 ng/mL) for 3 hours, resulted in enhancement of LPS-induced IL-8 secretion. As expected, anti-inflammatory cytokine IL-10 inhibited LPS-induced IL-8 production.

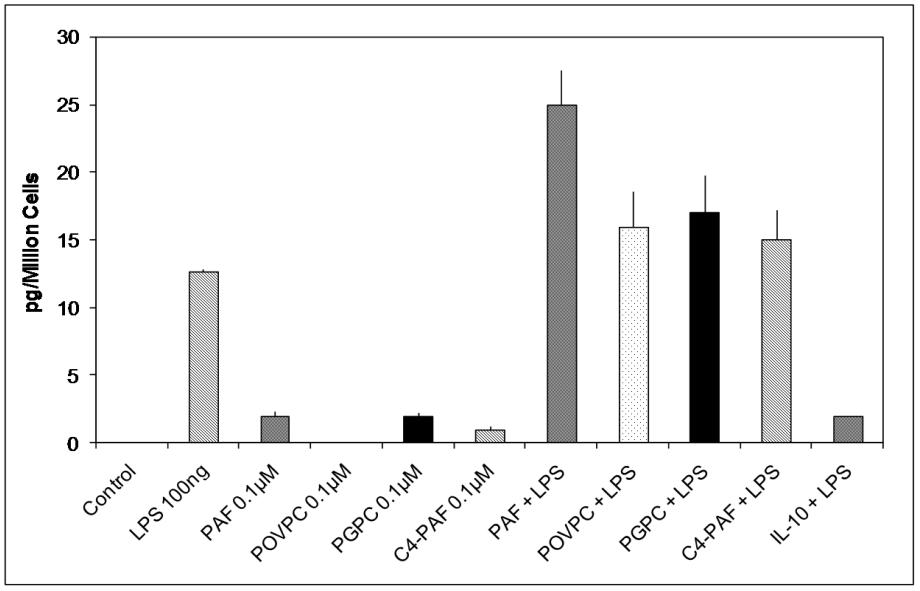

Figure 3. PAF and PAF-like lipids enhance LPS-induced IL-8 secretion in PMNs. Human PMNs in M199 buffer containing HEPES were incubated with PAF (0.1 μM), POVPC (0.1 μM), PGPC (0.1 μM) and C_4 PAF (0.1 μM) for 30 min. In a few experiments PMNs were pre-incubated with PAF (0.1 μM), POVPC (0.1 μM), PGPC (0.1 μM) and C_4 PAF (0.1 μM) for 30 min followed by stimulation with LPS (100 ng/mL) for 3 hours at 37 °C. Few PMN aliquots were pre-treated with IL-10 (10 μg/mL) for 30 min prior to LPS (100 ng/mL) stimulation. The PMNs were pelleted (200 × g for 5 min) and the supernatants were assayed for IL-8 using an IL-8 duoset ELISA kit. Pre-incubation of PMNs with PAF (0.1 μM) for 30 min followed by LPS (100 ng/mL) stimulation resulted in 2-fold increase of IL-8 production compared to LPS (100 ng/mL) alone. In addition, pre-incubation with PAF-like lipids (0.1 μM) also enhanced LPS-induced production of IL-8. Inclusion of IL-10 successfully inhibited LPS-induced IL-8 production. Each experiment was repeated more than 3 times with similar results. The data presented are mean ± SD.

4. Discussion

PAF and related OxPLs are predominantly formed during inflammatory conditions. Unlike, biosynthetic PAF, the formation of oxidized alkyl/acyl phosphatidylcholines is unregulated and accumulates during oxidative insult [16]. The signaling events initiated by such modified lipids profoundly modulate the immune cells to a pro-inflammatory phenotype. Oxidative modification of lipids is the primary and deadly event in atherosclerosis, where, high levels of OxLDL bearing OxPLs constitutes the fatty streaks found in atherosclerotic lesions in humans and experimental animals [33]. Previously, OxPLs were believed to be mediators of chronic inflammation. Of late, a remarkable role for OxPLs has also been suggested in an array of infectious diseases [52] that is consistent with the fact that ROS generated as a host defence mechanism during infection contributes to the formation of OxPLs. Indeed, increased serum PAF levels have been observed in septic patients and animal models of endotoxemia [53]. Consequently, exogenous PAF-AH administration to

hydrolyze PAF and related lipids and blocking PAF-R using specific PAF-R antagonists have been shown to protect animals from endotoxemia [54,55]. A sense of ambiguity opens up with studies claiming the inhibitory actions of OxPLs on LPS-induced effects, both in vivo [40,41] and in vitro [42,43]. Correspondingly, significant efforts have been directed to inhibit the hydrolysis of PAF using darapladib (a specific PAF-AH inhibitor) to retain PAF in circulation and thereby reduce inflammation underlying atherosclerosis [56], but in vain [57]. Besides, use of oxidized phospholipids to treat sepsis [58] is unlikely to show a positive outcome since, OxPLs themselves are shown to impair phagocytosis in Wiskott-Aldrich syndrome protein (WASP) family verprolin-homologous protein1 (WAVE1) dependent manner and diminish outcome in sepsis [59]. Additionally, our recent study indicates that intraperitoneal (i.p.) administration of a minute amount of PAF (5 μg/mouse) is lethal to Swiss albino mice [60].

In the present study, we examined the influence of POVPC, PGPC and C-4 PAF on LPS-induced effects on isolated human immune cells *viz.* monocytes and neutrophils, and also non immune cell-HUVECs. We show that POVPC and PGPC dose-dependently invoke intracellular calcium transients in human PMNs due to PAF-R activation (Figure 1). This is consistent with our previous data where we show C-4 PAF to signal via PAF-R [15]. PMNs respond quickly to agonists like PAF that cause rapid change in intracellular calcium levels that in turn causes their adhesion to activated endothelium [38]. Analogously, OxPLs derived from minimally modified OxLDL have been previously shown to increase monocyte-endothelial interactions, although there appears to be a difference in the action of PGPC and POVPC on leukocyte and endothelium via different receptors [61]. Moreover, bacterial endotoxins such as LPS and lipoproteins stimulate PMNs to synthesize PAF and PAF-like oxidized phospholipids [62], suggesting their role in amplifying endotoxin responses. Thus, LPS-induced PAF/OxPLs may further synergistically upregulate inflammatory genes including IL-8 and COX-2. We next questioned, if pre-activation of PAF-R influenced LPS-induced NF-KB signaling in isolated cells. Interestingly, as opposed to Eligini and her co-workers [42], where they find OxPOPC to inhibit LPS-induced COX-2 expression in human macrophages, OxPLs in our study did not suppress LPS-induced IL-8 as well as COX-2 (Figure 2A, C and E) but rather augmented these readout as seen by increase in PGE_2 levels (Figure 2 B, D and F). However, macrophages secrete PAF-AH [63] and this may have contributed to the hydrolysis of OxPOPC thereby nullifying the effects of the lipid in their study. Also, PAF and OxPLs themselves were unable to significantly induce IL-8 and COX-2 in our study.

The reason behind OxPLs exerting anti-inflammatory effects against LPS is believed to be due to the inhibition of interaction of LPS with LPS binding protein (LBP) and CD14 [40], vital components of the LPS-signaling complex. The authors of this study employed sterile LPS that limit the understanding the role of OxPLs in complex clinical settings like sepsis, where multiple mediators are often involved. Interestingly, the priming effect of PAF and OxPLs on LPS-induced signaling in vascular cells observed in our studies indicates the detrimental effects exerted by them. This is in accordance with, Knapp and co-workers [22] where they observed OxPAPC to rather render $CD14^{-/-}$ mice susceptible to *E.coli*-induced sepsis. In fact, OxPLs were found to increase mortality of mice intraperitoneally injected with viable *E. coli* cells and additionally promote bacterial growth. Besides, OxPLs also upregulated pro-inflammatory cytokine IL-6 and TNF-α and strongly attenuated the phagocytosing capacity of PMNs and macrophages [22]. Further, OxPLs are recognized to contribute to air way injury and inflammation in asthma and related pulmonary disorders [64] where, scavenger receptors on alveolar macrophages such as macrophage receptor

with collagenous structure (MARCO) and scavenger receptor AI/II (SRA-I/II) are primarily responsible to clear oxidatively modified lipids and thereby limit lung inflammation [65]. CD36 [66] and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) [67] are other scavenging receptors identified to play a role in clearing OxPLs in circulation and their enhanced expression is observed in pro-atherogenic conditions.

Considering the ill effects of PAF and OxPLs in inflammatory conditions, the credibility of PAF-AH appears to have a positive impact. Decreased levels of PAF-AH has been observed in a number of diseases such as asthma, coronary heart disease, stroke, systemic lupus erythematosus and Crohn's disease and is suggested to be a potential risk marker rather than risk factor [23]. Exogenous administration of rPAF-AH promotes bacterial clearance in septic mice [68]. The beneficial role of PAF-AH has also been demonstrated in vitro, where endothelial cells were protected from undergoing apoptosis when exposed to OxLDL pre-treated with PAF-AH [69]. In the present study, we show PAF and OxPLs do not impair LPS-induced pro-inflammatory responses but rather amplify (increased PGE_2 levels) LPS-mediated signaling in PMNs, monocytes and HUVECs, implying the pro-inflammatory nature of this class of bioactive lipids.

5. Conclusion

PAF and related PAF-like lipids do not oppose LPS signaling but indeed participate in aggravating inflammation, thus contributing to progression of inflammatory diseases. With our findings and other substantial reports suggesting the involvement of PAF and OxPLs in inflammatory disorders, using them as treatment option against sepsis is questionable. This invites a better understanding of the role of PAF and OxPLs in complex inflammatory disorders like sepsis to be advantageous for therapeutic interventions. Moreover, inhibiting PAF-AH in inflammatory scenario including CVD needs to be revisited [23].

Acknowledgements

The authors gratefully acknowledge the technical assistance of Jessica M Cemete, Stacy Macecevic, Manisha Sharma, Vidyanath Thirumala of Cleveland Clinic Foundation, USA. Dr. Paul Di Corleto (Cleveland Clinic Foundation, Lerner Research Institute, Cleveland, OH) is greatly acknowledged for providing HUVECs.

This work was supported by University Grants Commission Non-NET Fellowship (UGC-NonNETFS)—[F. 87-1-2012(SU-1)2] to SPJ; Basic Science Research fellowship [F.7-366/2012] to LCL; UGC-Major Research Project [F.No: 41-128/2012-13] to GMK, Vision Group of Science and Technology (VGST) [VGST/K-FIST(2010-11)/GRD-36/2013-14], Government of Karnataka, UGC-Special Assistance Program (UGC-SAP) [F3-14/2012(SAPII)].

Conflict of Interest

The authors have no financial conflict of interest.

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