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Oxidized phospholipids on alkyl-amide scaffold demonstrate anti-endotoxin and endothelial barrier-protective properties

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A B S T R A C T

Oxidized phospholipids (OxPLs) containing enzymatically or non-enzymatically oxidized fatty acids (oxylipins) are increasingly recognized as lipid mediators involved in pathogenesis of diseases. Further understanding of structure-activity relationship and molecular mechanisms activated by OxPLs is hampered by the complexity of synthesis of individual molecular species. Although dozens of individual free oxylipins are commercially available, their attachment to the phospholipid scaffold requires relatively harsh conditions during activation of carboxy-group, which may lead to decomposition of unstable oxylipins. Furthermore, additional protection-deprotection steps are required for oxylipins containing hydroxy-groups. In this work we describe synthesis of OxPLs containing oxylipins bound at the sn-2-position via an amide-bond that is characteristic of sphingophospholipids. Activation of oxylipins and attachment to the phospholipid scaffold are performed under mild conditions and characterized by high yield. Hydroxy-groups of oxylipins do not interfere with reactions and therefore no protection/deprotection steps are needed. In order to prevent oxylipin migration, a fatty acid residue at the sn-1 was bound through an alkyl bond, which is a common bond present in a large proportion of naturally occurring phospholipids. An additional advantage of combining alkyl and amide bonds in a single phospholipid molecule is that both types of bonds are phospholipase A2-resistant, which may be expected to improve biological stability of OxPLs and thus simplify analysis of their effects. As proof of principle, several alkyl-amide oxidized phosphatidylcholines (OxPCs) containing either linear or prostane ring oxylipins have been synthesized. Importantly, we show here that alkyl-amide-OxPCs demonstrated biological activities similar to those of di-acyl-OxPCs. Alkyl-amide-OxPCs inhibited pro-inflammatory action of LPS and increased endothelial cellular barrier in vitro and in mouse models. The effects of alkyl-amide and di-acyl-OxPCs developed in a similar range of concentrations. We hypothesize that alkyl-amide-OxPLs may become a useful tool for deeper analysis of the structure-activity relationship of OxPLs.
1. Introduction

Oxidation of lipids is a common mechanism generating biologically active molecules. Some lipid oxidation products are genuine mediators that are recognized by cellular receptors (e.g., prostaglandins and isoprostanes), while other act less specifically by non-selective modification of biomolecules leading to activation of signaling stress pathways. The majority of oxidation-prone PUFAs in the cell are bound to phospholipids (PLs), which can be oxidized through enzymatic or non-enzymatic mechanisms leading to the formation of oxidized PLs (OxPLs) that contain oxygenated fatty acids. Accumulation of OxPLs has been described in a number of pathological conditions [1–4]. Recent advances in analysis of OxPLs as disease biomarkers in combination with animal disease model data support the notion that OxPLs play causative pathogenic role in a number of disease states [5–9]. More detailed understanding of the biological role of OxPLs and mechanisms of their action is hampered by the lack of synthetic individual molecular species. Just a few OxPLs are commercially available. Although syntheses of OxPLs are established [10–16], they are laborious and performed in a limited number of laboratories. Here we describe a relatively simple and fast method for synthesis of alkyl-amide OxPLs.

Fig. 1. Scheme for the synthesis of alkyl-amide-OxPLs. A) Synthesis of a common precursor 1-O-hexadecyl-2-amino-2-deoxy-sn-glycero-3-phosphocholine (6). Reagents: a) Boc₂O, TEA; b) [(isoPr)₂N]POCH₂CN, (isoPr)₂NH salt of 1H-tetrazole, CH₂Cl₂; c) choline tosylate, 1H-tetrazole; d) tert-BuOOH; e) TEA; f) TFA in CH₂Cl₂. B) General scheme for synthesis of alkyl-amide-PLs. Reagents: g) oxylipin containing one carboxylic group, EDC, CH₂Cl₂; h) lyso-amino-phospholipid (6), THF, TEA.
backbone via this synthetic pathway under mild conditions and with high yield. Here we show that alkyl-amide-OxPLs demonstrate biological activities previously described for di-acyl-OxPLs, such as antagonism of bacterial lipopolysaccharide (LPS) and protection of endothelial barrier.

2. Methods

Synthetic procedures, products, and methods of biological testing are described in Supplementary materials.

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Fig. 2. Anti-LPS activity of synthetic alkyl-amide-OxPLs. A) HUVECtert cells were pre-incubated with synthetic alkyl-amide-PLs (A) for 20 min in medium with 2% serum before the addition of LPS (30 ng/ml). After 6 hrs, IL-8 in the cell culture medium was determined by ELISA. Mean values of 3–6 replicates are indicated. Representatives of one to four independent experiments are shown. B) Primary HUVECs were pre-incubated with 8-isoPGE₂-NH-PC, 8-isoPGA₂-NH-PC or OxPAPC (10 µM final concentrations) for 20 min in medium with 2% serum, followed by addition of TNFα, IL-1β or LPS to the end concentrations shown in the Figure. After 4 hrs, the medium was collected and secreted IL-8 was quantified by ELISA. C) HUVECtert cells were pre-treated with PGF₂α-NH-PC (7.5 µM) in 2% serum for 30 min before the addition of LPS (30 ng/ml). After 6 h, cells were harvested in RNAzol and total RNA was isolated. After reverse transcription, levels of specific mRNAs were measured using SYBR Green-based rt-qPCR. The data are normalized to β2-microglobulin and expressed as % of LPS stimulation. D) and E) Primary HUVECs were incubated with 8-isoPGE₂-NH-PC, 8-isoPGA₂-NH-PC or OxPAPC in the presence of 2% serum for 20 min, followed by addition of LPS (30 ng/ml). Final concentrations of PLs and LPS are indicated in the Figures. After 4 hrs, IL-8 in the cell culture medium was measured by ELISA (D). The cells were fixed with 0.05% glutaraldehyde, and E-Selectin expressed on the cell surface was determined by cell-based ELISA (E). The results are expressed as % of LPS stimulation. (B–E) Means ± SD values are indicated. Representative results of one to three independent experiments are shown.
3. Results

Synthesis of alkyl-amide-OxPCs was performed in 2 stages. First, lyso- 
amino-PC (6) was synthesized. This stage consisted of 6 individual re- actions, three of which were performed in one pot (Fig. 1A). During the second stage (Fig. 1B), oxylipins were attached to the lyso-amino-PC scaffold using 2 reactions. First, an oxylipin was treated with N-hydroxy- 
succinimide leading to formation of respective activated ester (8). This reaction was characterized by complete conversion of oxylipins (Sup- 
plementary Fig. 1, Step 1). The resulting N-hydroxysuccinimide ester of an oxylipin (8) was purified by liquid-liquid extraction. In the second reaction, isolated activated ester (8) reacted with lyso-amino-PC (6), which was preliminary deprotonated using triethylamine. The method was successfully applied to several prostanooids and linear oxylipins containing ω-terminal hydroxyl groups, altogether 9 compounds (Fig. 1 and Supplementary Fig. 2).

We further tested the influence of different solvents on the last (coupling) reaction using PGE2 as a sample oxylipin. It was found that the coupling of oxylipins to the phospholipid scaffold was characterized by comparable yield in different solvents (tetrahydrofuran/dichloromethane (1:1 or 2:1 by vol.), tetrahydrofuran-methanol (10:1 by vol.), tetrahydrofuran-acetonitrile methanol (3:1:0.5 by vol.), and in the presence of water (up to vol. 10% in THF) or tertiary amines (up to 10% TEA). In all cases of the yield of the final (coupling) reaction in the presence of equimolar amounts of oxylipins and lyso-amino-PC was around 50%. Addition of a > 2-fold molar excess of lyso-amino-PC was essentially in essential quantitative incorporation of oxylipins (Supplementary Fig. 1, Step 2).

In order to check if alkyl-amide-OxPCs demonstrate biological ac- tivities described for their di-acyl homologs, we first tested if alkyl- 
amide-OxPCs can inhibit pro-inflammatory effects of LPS [17–19]. Indeed, alkyl-amide-OxPCs containing prostanooid derivatives of arach- idonic (PGE2, PGF2α, PAG2, 8-isoPG2A, 8-isoPE2α, 8-epiPG5α) and docosatetraenoic (1α,1β-dihomoPGF5α) acids, as well as linear ω-termi- nal hydroxy fatty acids (8-hydroxy-octanoic and 12-hydroxy-dodeca- noic acids) inhibited secretion of IL-8 in LPS-treated HUVEC tert cells (Fig. 2A and Supplementary Fig. 3) and primary HUVECs (data not shown). In contrast, free prostanooids did not inhibit LPS-induced IL-8 secretion in HUVEC tert cells or primary HUVECs (data not shown). The inactivity of free prostanooids suggests that alkyl-amide-OxPCs inhibit effects of LPS through a mechanism that is independent of classical prostanooid receptors. This hypothesis is in a good agreement with data showing direct binding of OxPLs to the TLR4 accessory proteins which prevented their interaction with LPS [17,19].

An important question was whether the inhibitory effects of alkyl- 
amide-OxPCs may be explained by their toxic action on cells. This is unlikely for two reasons. First, direct analysis of toxicity has shown that none of the tested alkyl-amide-OxPCs was toxic at concentrations used in our experiments (Supplementary Figs. 4A–4D). In addition, we found that the inhibitory action of alkyl-amide-OxPCs was observed in cells stimulated by LPS, but not in those treated with TNFα or IL-1β (Fig. 2B).

In addition to suppression of IL-8 protein production, PGF2α-NH-PC (Fig. 2C) and PGE2-NH-PC (data not shown) inhibited induction by LPS of mRNA encoding for IL-8 and other pro-inflammatory chemokines and leukocyte adhesion molecules. The data are in a good agreement with proposed action of OxPLs as antagonists of TLR4 [17,19] and rule out non-specific inhibition resulting from compound toxicity.

We further compared concentration dependence of the anti-LPS ef- fects of alkyl-amide-OxPCs with that of well-characterized di-acyl 
OxPC, OxPAPC [19]. Alkyl-amide-OxPCs inhibited LPS-induced secretion of IL-8 protein and expression of E-selectin on the surface of endothelial cells at similar concentrations as OxPAPC (Fig. 2D and E). The data suggest that modification of sn-1 and sn-2 bonds does not significantly change structural features of OxPLs that are important for their anti-LPS activity.

To ensure that the anti-LPS activity is not limited to PLs containing a cyclic prostate ring, linear fatty acids with a ω-terminal hydroxide have been attached to the alkyl-amide-OxPC scaffold. The data presented in Supplementary Figs. 3A–3D demonstrate that these 12-hydroxy-C2- 
or 8-hydroxy-C4-containing alkyl-amide-PCs inhibited induction of IL-8 protein and mRNA in LPS-treated HUVEC tert, and that this effect was not due to toxicity (Supplementary Figs. 3E and 3F).

Ability to protect endothelial barrier in vitro and in vivo is a char- acteristic property of di-acyl-OxPLs [20–25]. We found that also alkyl-amide-OxPCs demonstrated barrier-protective activity. The barrier-enhancing effect of PGE2-alkyl-amide-PC was sustained and lasted for at least 20 h (Fig. 3A). The action of PGE2-alkyl-amide-PC was accompanied by enhancement of cell junctions and peripheral cytoskeleton associated with barrier enhancement (Fig. 3B) as well as activation of intracellular signaling that stimulates barrier function of endothelial cells (Fig. 3C), which are similar to the action of di-acyl-OxPLs [21,25]. PGE2-alkyl-amide-PC also inhibited disturbance in endothelial barrier caused by LPS (Fig. 3D), IL-6 (Fig. 3E), or thrombin (Fig. 3F). Moreover, PAG2-NH-PC reversed damage of the endothelial monolayer induced by heat-killed Gramm-positibe bacte-
rria (Supplementary Fig. 5).

Di-acyl-OxPCs are known to inhibit inflammation in several in vivo models including LPS-induced peritonitis [18,19]. In agreement with these data, alkyl-amide-OxPCs inhibited systemic upregulation of infla-
munatory cytokine KC induced by intraperitoneal LPS injection in mice (Fig. 4A). Furthermore, similarly to di-acyl-OxPCs [25], alkyl-amide-OxPCs reduced severity of lung edema induced by LPS instillation in mice (Fig. 4B and C). The data demonstrate protective activity of alkyl-amide-OxPCs in vivo that is similar to that di-acyl-OxPCs.

4. Discussion

OxPLs are known to be present in vivo and are increasingly recog- nized for their involvement in disease pathogenesis [26,27]. Here we describe a novel procedure for synthesis of OxPLs based on alkyl-amide 

scaffold. These compounds mimic various biological effects described previously for OxPLs. An important advantage of our method is a minimal number and mild conditions of synthetic steps involving oxylipin precursors. These are i) activation of a carboxylic group of oxylipin and ii) coupling to the PL backbone. Both reactions are performed under significantly milder conditions as compared to methods involving protection/deprotection steps [10,16]. Another advantage of the procedure is the application of a simple liquid-liquid extraction instead of chro-
matography for purification of activated oxylipin intermediate. Alto-
gether these experimental improvements aim at reducing the loss of oxylipin, which is especially important considering the complexity of synthesis and high commercial price of certain oxylipins.

Another advantage of this method is flexibility, i.e., applicability to a wider variety of oxylipins as compared to existing methods. A standard method of coupling oxylipins to the glycerol backbone using DCC/DMAP [10,16,28] is limited to molecules without free OH groups. In case of OH-containing oxylipins, which are very common, hydroxyl groups should be first protected, e.g., by silyl groups, followed by deprotection at the end of synthesis. In contrast, our method is more universal and allows coupling of aldehyde-, keto-, epoxy-, as well as hydroxyl-containing oxylipins (e.g., PGF5α) without additional protection-deprotection cycles.

A further advantage of this method is that it is faster as compared to existing procedures. Most synthetic steps are needed for the synthesis of a single universal precursor lypo-amino-PC (6). This precursor can be directly coupled to all possible oxylipins having one carboxylic group. Lyso-amino-PC (6) is stable and can be stored for at least 1 year. The use of a single precursor and robustness of coupling reaction simplify the parallel synthesis of multiple individual alkyl-amide-OxPCs, for example for analysis of structure-activity relationship.

What are the limitations of the method? First, the method can be directly applied only for synthesis of PC, PA, PG, and PL. Generation of PE- and PS-OxPLs is possible, but requires additional protection.
Fig. 3. Effect of PGE$_2$-NH-PC on endothelial barrier and on enhancement of cell junctions and intracellular signaling. A) Primary HPAECs cultured on electrodes were incubated with different concentrations of PGE$_2$-NH-PC (A) in EBM-2 medium containing 2% serum. The graph shows electrical resistance of the endothelial monolayer. B) HPAECs grown on gelatin-coated glass coverslips were exposed to 5 μM of PGE$_2$-NH-PC in EBM-2 and 2% FCS for indicated time points and subjected for immunostaining with VE-cadherin antibody (Cayman, 1:500) to visualize adherens junctions. F-actin and nuclei were stained with Texas Red phalloidin and DAPI, respectively. EC barrier in cell monolayers is marked by arrows. C) left: HPAECs were stimulated with 3 μM of PGE$_2$-NH-PC for 30 min. Normalized cell homogenates were immunoprecipitated against VE-cadherin, and samples were analyzed by Western blotting using antibodies for ZO-1 (Cell Signaling Technology; 1:1000 dilution), p120-catenin (BD Transduction Laboratories; 1:1000 dilution), β-catenin (Santa Cruz, 1:1000 dilution), and VE-cadherin (Santa Cruz, 1:1000 dilution). C) right: Primary HPAECs were incubated with 3 μM PGE$_2$-NH-PC in EBM-2 with 2% FCS for 15 min or 60 min. Cell homogenates were harvested and analyzed by Western blotting using antibodies against pPAK1/2 (Cell Signaling Technology; 1:1000 dilution), VASP (Cell Signaling Technology; 1:1000 dilution), and α-tubulin (Proteintech; dilution 1:5000). D-F) HPAECs grown on ECIS electrode arrays were incubated with 5 μM of PGE$_2$-NH-PC for 30 min followed by addition of agonists: 100 ng/ml of LPS (D), combination of 25 ng/ml of IL-6 and 50 ng/ml of IL-6 soluble receptor (E), and 0.2 U/ml of thrombin (F). EC monolayer permeability was determined by monitoring TER over indicated time periods in ECIS system. Normalized resistance = 1 is shown as a dashed line. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Fig. 4. Alkyl-amide oxidized phospholipids inhibit LPS-induced inflammation and protect lung endothelial barrier in vivo. A) C57BL/6J wild type mice were injected i.p. with 200 μl of LPS in PBS (20 μg, 1 mg/kg mouse) in the presence or absence of PGE₂-NH-PC (200 μg, 10 mg/kg mouse). After 2 and 4 hrs, blood was collected retro-orbitally, and the KC cytokine in blood plasma was determined by ELISA. n = 5–6 mice. B) C57BL/6J wild type mice were challenged with LPS (0.7 mg/kg, i.t.) in the presence or absence of PGE₂-NH-PC (20 μg/kg, i.v.) and after 20 hrs Evans blue dye (30 μl/kg) was injected i.v. Photographs depict the accumulation of Evans blue dye in the lung tissue reflecting increased vascular permeability. (C) C57BL/6J wild type mice were treated i.t. with LPS (0.7 mg/kg body weight, n = 11) or without LPS (n = 6) in the absence or presence of 8-isoPGA₂-NH-PC, n = 5; PGA₂-NH-PC, n = 7; or PGE₂-NH-PC, n = 3). After 24 hrs, bronchoalveolar lavage (BAL) fluids were collected and total cell count and protein content were determined. Mean values ± SD are indicated; *p ≤ 0.05, ***p ≤ 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
deprotection steps. The latter, however, is also required in all currently known chemical methods of OxPLs synthesis [16]. The same applies to synthesis of OxPLs containing α-terminal carboxylic group: both in published methods and in our procedure one of COOH groups in di-carboxylic oxylipins has to be protected.

Another potential limitation of our method is the type of sn-1 and sn-2 bonds, namely ether- and amide bonds replacing two ester bonds that are present in the majority of natural PLs. However, ether- and amide bonds in PLs are absolutely physiological: up to 20 % of total phospholipids in human cells contain sn-1,2-alkyl residues [29]. Furthermore, binding of fatty acid residues through the amide bond is characteristic of the common phospholipid sphingomyelin, which is comparable in abundance with PC in cells [30] and blood plasma [31]. Additionally, the non-cleavable ether bond prevents acyl migration, which is a known characteristic of acyl-PLs [32]. This stability may be advantageous for analysis of structure-activity relationships of OxPLs.

In support of the notion that properties of alkyl-amide-OxPLs are generally similar to those of di-acyl species, we have found that alkyl-amide-OxPCs demonstrated biological activities similar to those of di-acyl-OxPCs. In particular, we tested effects of alkyl-amide-OxPCs on pro-inflammatory action of LPS and on the endothelial barrier. In both assays, alkyl-amide-OxPLs induced in vitro and in vivo similar biological effects and acted at similar concentrations as di-acyl-OxPLs. The mechanisms of barrier-protective and anti-LPS action of OxPLs have been reviewed [26,33]. Di-acyl-OxPLs have been shown by several research groups to antagonize LPS action in different cell types and animal models [17,18,22]. The inhibitory mechanism at least partially is based on the release of electrophilic oxylipins from the phospholipid scaffold, followed by activation of NRF2 [34] that is increasingly abundantly with PC in cells [30] and blood plasma [31]. Additionally, the common phospholipid sphingomyelin, which is comparable in characteristic of acyl-PLs [32]. This stability may be advantageous for analysis of structure-activity relationships of OxPLs.

Another potential limitation of our method is the type of

References


Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2021.07.041.

Declarations of competing interest

OVO, AB, KB and VB are inventors on a patent related to work on OxPLs.

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