Analysis of oxidized and chlorinated lipids by mass spectrometry and relevance to signalling

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Abstract

Oxidized and chlorinated phospholipids are generated under inflammatory conditions and are increasingly understood to play important roles in diseases involving oxidative stress. MS is a sensitive and informative technique for monitoring phospholipid oxidation that can provide structural information and simultaneously detect a wide variety of oxidation products, including chain-shortened and -chlorinated phospholipids. MS^n technologies involve fragmentation of the compounds to yield diagnostic fragment ions and thus assist in identification. Advanced methods such as neutral loss and precursor ion scanning can facilitate the analysis of specific oxidation products in complex biological samples. This is essential for determining the contributions of different phospholipid oxidation products in disease. While many pro-inflammatory signalling effects of oxPLs (oxidized phospholipids) have been reported, it has more recently become clear that they can also have anti-inflammatory effects in conditions such as infection and endotoxaemia. In contrast with free radical-generated oxPLs, the signalling effects of chlorinated lipids are much less well understood, but they appear to demonstrate mainly pro-inflammatory effects. Specific analysis of oxidized and chlorinated lipids and the determination of their molecular effects are crucial to understanding their role in disease pathology.

Background

The interest in lipids as mediators and signalling molecules goes back many years, and the effects of enzymatically oxidized fatty acid derivatives such as PGs (prostaglandins) and leukotrienes in the cardiovascular system are well known. In parallel, studies of hyperlipidaemia and elevated LDL (low-density lipoprotein) in CVD (cardiovascular disease) led to the understanding that oxidative modification of the LDL contributed to the formation of foam cells and cytotoxic effects, and stimulated studies of the underlying mechanisms [1]. In the late 1990s, it was discovered that oxPAPC [oxidized PAPC (palmitoylarachidonoyl-glycerophosphocholine)] had inflammatory biological activity mimicking that of oxLDL (oxidized LDL) [2], and this marked the start of a new focus on the identification of specific oxPLs (oxidized phospholipids) derived from LDL that could contribute to atherogenesis. These and similar products may also be generated from cell membrane phospholipids during situations where increased oxidant levels occur, such

as inflammation and apoptosis, and are thought to contribute to physiological and pathological processes [3].

The phospholipids containing polyunsaturated fatty acyl chains, such as PAPC, are most vulnerable to oxidative attack, although hypohalites (e.g. HOCl) derived from the phagocyte enzyme myeloperoxidase also react with MUFAs (mono-unsaturated fatty acids), the vinyl ether bond of plasmalogens and the head group ethanolamine. There is an enormous variety of possible products, including full-length oxidation products, chain-shortened phospholipids and the corresponding non-esterified fragments of the oxidized fatty acyl chains, of which the aldehydes malondialdehyde and HNE (4-hydroxy-trans-2-nonenal) are well-known examples [4,5]. oxPL products, both esterified and non-esterified, that contain reactive moieties such as aldehydes or alkenals can also generate adducts with proteins by Schiff base or Michael addition reactions. A good understanding of the role of phospholipid oxidation products in disease requires methods to detect specific oxidation products in biological and clinical samples. Not surprisingly, there are many available methods for detecting a variety of oxidized lipid products, as described previously [4]. As a general rule, the simplicity of the method is inversely proportional either to the specificity, sensitivity or quantity of information available. Some of the most convenient and routinely used methods involve spectrophotometric or fluorimetric assays; these often detect several similar oxidation products [e.g. DNPH (2,4-dinitrophenylhydrazine) reacts with most carbonyl-containing compounds] and are susceptible to interference from compounds that are similar in structure but not products of oxidative lipid damage. Antibody-based methods such as the ELISAs for protein carbonyl

groups (DNPH-dependent) and lipid hydroperoxides also detect a range of different compounds. HPLC-based methods with detection by using fluorescence, absorbance or chemiluminescence are able to separate and detect a variety of products in one assay, but may not discriminate closely related forms (e.g. isoprostanes). More expensive and complex methods involving MS [GC–MS, MALDI–MS (matrix-assisted laser-desorption ionization–MS) and ESI–MS (electrospray ionization–MS)] are likely to give the most specific analysis, and are also becoming increasingly sensitive as instrument technology improves [4].

MS measures the mass-to-charge ratio (m/z), and this can be used to discriminate oxidized products from native lipids or phospholipids. The soft ionization techniques ESI and MALDI are preferable for the analysis of phospholipids, which are non-volatile but readily charged; this allows intact molecular ions to be detected without the need for fragmentation. The formation of hydroperoxides corresponds to the addition of one or more molecular oxygens (O_2) with a corresponding m/z increase of multiples of 32 Da [6]. Formation of chlorohydrins by electrophilic addition of HOCl results in an m/z increase of 52 Da, and the ^{37}Cl isotope peak at +54 is a useful diagnostic indicator of the presence of chlorine [7,8]. Other oxidation and fragmentation products also have m/z values different from those of the unmodified lipids, and following oxidation of a polyunsaturated phospholipid, such as PAPC, many products both larger and smaller than the native lipid can be detected simultaneously in a spectrum [2]. Phosphatidylcholines have a constitutive positive charge and give strong signals in positive-ion mode, and phosphatidyl-ethanolamines readily acquire a positive charge. In contrast, cardiolipin (diphosphatidylglycerol), phosphatidylglycerol, phosphatidylserine and phosphatidylinositol are usually analysed in negative-ion mode [9].

As some compounds may be isobaric, such as a larger native phospholipid and a smaller oxidized one (stearoylarachidonoylglycerophosphocholine at m/z 810 compared with a chlorohydrin of palmitoyl-linoleoyl-glycerophosphocholine at m/z 758 + 52), it is also useful to couple HPLC separation with detection by MS. Normal-phase chromatography is effective for separating the different classes of lipid according to head group, while reverse-phase chromatography on C_8 or C_{18} columns works well for separating oxidized and more polar lipids, which are eluted earlier than the less polar unmodified species [2,10–12]. Further information and confirmation of the identity of oxPLs can be obtained using fragmentation techniques (MS^2 or MS^n), which requires an instrument with two mass analysers and a collision chamber. Phospholipids tend to fragment most readily at the ester bonds to release the head group or the fatty acids; for phosphatidylcholines, this gives a strong signal at m/z 184 as phosphocholine retains charge on fragmentation or signals in the region of m/z 500 corresponding to the lysolipids (m/z 496 for lysopalmitoylglycerophosphocholine and m/z 524 for lysostearoylglycerophosphocholine). The sn-2 chain is most

readily lost, especially after oxidation, and so the lysolipid commonly contains a saturated fatty acyl chain. The combination of LC with MS and fragmentation techniques has been used to detect oxPLs in a variety of biological samples, including atherosclerotic plaque samples, plasma and tissue samples, and cells stressed *in vitro*, as reviewed recently [4].

In addition to a product-ion scan, in which a particular molecular ion is selected, fragmented and the resulting fragment ions analysed, tandem instruments can carry out other scan routines that allow a targeted approach to identifying species of interest. Precursor ion scanning detects molecular ions that give rise to a specific product ion of interest. This involves scanning through a mass range in the first mass analyser, fragmentation in the collision chamber, and setting the second mass analyser to the desired m/z so that the molecular ions (precursors) that fragment to yield the product ion of interest can be identified. For example, a precursor ion scan for m/z 184 (phosphocholine) can be used to identify the phosphatidylcholines in a mixture of phospholipids; this approach will select all phosphocholine species whether oxidized or unmodified, and the precursor ion m/z will facilitate identification of the structure [13]. Alternatively, fragmentation of the oxPL at the sn-2 position to release an oxidized fatty acid can be used. Precursor ion scanning in negative ion for HETE (hydroxyeicosatetraenoic acid) at m/z 319 identified precursor ions at m/z 738, 764, 766 and 782, which were found to correspond to plasmalogen phosphatidylethanolamines containing 15-HETE [14].

Another good approach to target phospholipids containing oxidations is to use neutral loss scanning; this scan routine scans both the first and second mass analysers, but with a fixed mass offset between them, so that molecular ions that fragment by losing a specific moiety can be identified. For example, the neutral loss of 36 Da (H^{35}Cl) is diagnostic for chlorinated ions such as chlorohydrins of phosphatidylcholines, and the presence of chlorine can additionally be checked by neutral loss of 38 Da for the H^{37}Cl form; this profile should match that of neutral loss of 36 Da [15,16]. Similarly, phospholipid hydroperoxides can fragment by loss of 18 Da (H_2O) to form an epoxide or loss of 34 Da (H_2O_2) [17]. Hydroxides also fragment by loss of water, introducing an extra double bond into the structure. A good diagnostic fragmentation for a phospholipid hydroperoxide is neutral loss scanning for − 34 Da.

The advantage of precursor ion and neutral scanning routines is that they enable specific phospholipid types and oxidative modifications (hydroperoxide and chlorination) to be identified without knowing the exact structure of the phospholipid, so that the phospholipids containing such modifications can be identified in mixtures of lipids. However, if particular oxPLs or fatty acids are of interest, these can also be selected more precisely by MRM (multiple reaction monitoring). In this scan routine, both the first mass analyser is fixed for the m/z of the molecular ion of interest, and the second mass analyser is fixed for the m/z of a fragmentation product diagnostic for this

oxPL; the combination of the two related scans is called a transition. The transitions selective for each oxidized lipid are identified by product ion scanning, and then multiple transitions can be monitored in a single MS experiment to detect a number of different oxidized lipids. MRM is highly focused and therefore has higher sensitivity than the other MS scan experiments, and it is more suitable for quantification, but it does require prior knowledge of the oxPLs likely to be present in the sample. It has been used to study the formation or occurrence of oxPL products in a variety of different conditions [18,19]. For example, a study investigating the role of oxPLs in regulating macrophage function in leprosy used multiple reaction monitoring to identify them in human macrophages infected with BCG (Bacille Calmette–Guérin), with the following transitions: PEIPC [1-palmitoyl-2-(5,6)-epoxyisoprostane E2-sn-glycero-3-phosphocholine; m/z 828.6→184], LysoPC (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine; m/z 496.3→184) and PAPC (m/z 782.5→184), where m/z 184 corresponds to the phosphocholine product ion [20]. The same approach (transitions corresponding to the molecular ion m/z→184) has been used to show that levels of hydroxy and hydroperoxy phosphatidylcholines were increased in plasma from patients suffering from chronic alcohol exposure and alcoholic liver disease [21].

Biological properties and signalling by oxPLs

The broad biological importance and effects of oxPLs and the rapidly increasing interest in this field are excellently illustrated by the recent extensive review by Bochkov et al. [5]. Since the discovery in the late 1990s that oxPAPC had biological effects that mimicked oxidized LDL by inducing leucocyte–endothelial adhesion, researchers have been investigating the molecular mechanisms underlying this effect and attempting to elucidate the receptors and signalling pathways involved. Early work focused on the PAF (platelet-activating factor) receptor, owing to the structural similarities between chain shortened oxPLs and PAF, and indeed it has been shown that oxPAPC products can stimulate the PAF receptor, albeit with lower affinity than PAF [22,23]. This can result in the production of inflammatory cytokines and platelet aggregation, but is clearly not the sole mechanism of action of oxPLs, as they also show a variety of actions that do not match PAF activity. In the last 10–15 years, interactions with a variety of other receptors and signalling via several pathways have been investigated, and oxPLs have been found to cause a multitude of end effects as well as up-regulation of adhesion molecule expression [ICAM (intercellular adhesion molecule) and E-selectin], such as toxicity, apoptosis, ROS (reaction oxygen species) generation, cytokine production [IL-6 (interleukin-6), IL-8, MCP-1 (monocyte chemoattractant protein-1), MIP-1 and -2 (macrophage inflammatory proteins 1 and 2) and RANTES (regulated upon activation, normal T-cell expressed

and secreted)] and other inflammatory and stress-related effects [5].

In view of the pro-inflammatory effects that have been observed for oxPAPC and some of its individual components, and the similarity in structure between oxPLs and LPS (lipopolysaccharide), it was hypothesized that oxPLs might exert inflammatory effects by activating TLRs (Toll-like receptors). These are innate immune receptors responsible for sensing PAMPs (pathogen-associated molecular patterns), and also endogenous tissue damage [24]. TLR4 is responsible for detecting a Gram-negative LPS (e.g. from *Escherichia coli*) as well as other PAMPs such as zymosan, while TLR2 together with TLR 1 or TLR6 is able to detect cell wall lipopeptides from Gram-positive bacteria. TLRs have been implicated in atherosclerosis, as genetic deletion of the TLR-signalling adaptor MyD88 (myeloid differentiation factor 88) or deficiency of TLR2 or TLR4 results in decreased plaque burden in mice [24]. Early studies suggested that TLR4 was involved in macrophage responses to mmLDL (minimally modified LDL) [25] or HeLa cell production of IL-8 in response to oxPAPC [26], although in both cases NF-κB (nuclear factor κB) was found not to be involved in the signalling mechanism. This is in agreement with the finding that although oxPAPC can induce the production of IL-8 and MCP-1/MIP-2 and expression of E-selectin in myeloid and endothelial cells, it does not induce production of TNFα (tumour necrosis factor α), which is a typical response to LPS-mediated via NF-κB [27]. However, Erridge et al. [27] also found that transfection of a TLR-deficient cell line, HEK (human embryonic kidney)-293 cells, with TLR4 or TLR1, TLR2 and TLR6 did not increase responsiveness to oxPAPC as measured by expression of the IL-8 promoter, in contrast with the previous findings. Thus the effects of oxPLs appear to be cell-specific, and one possible explanation for the observed differences could be the differential responses of the cell types to the cholesterol depletion and lipid raft disruption induced by oxPLs: HeLa cells respond by activation of NF-κB, whereas in many other cell lines this prevents TLR dimerization and signalling [28]. Although oxPLs may not interact directly with TLRs to induce pro-inflammatory responses, it appears that, in some cellular and physiological models, TLR signalling does play a part in the response, and it has been suggested that this may depend either on indirect effects by inducing other TLR agonists or co-stimulation responses [5]. For example, a recent study supporting a role for TLRs in oxPL-induced inflammation reported that OxPAPC can cause lung injury and IL-6 production by mouse lung macrophages in a mechanism involving the TLR4-TRIF [TIR (Toll/interleukin-1 receptor)]-TRAF6 (tumour-necrosis-factor-receptor-associated factor 6) pathway [29].

There is also clear evidence that oxPLs can interfere with LPS signalling via TLRs. It has been known for some years that oxPAPC and other phospholipids modified at the sn-2 position prevent LPS-induced neutrophil–endothelial adhesion and E-selectin up-regulation [30], and the pathological relevance was demonstrated by the fact

that oxPLs improve the outcome in a murine model of endotoxaemia [31]. It has been suggested that this effect is mainly due to competitive binding of oxPLs to three accessory proteins involved in delivering LPS to TLR2 and TLR4: LBP (LPS-binding protein), CD14 and MD-2 [28,32]. Additionally, in dendritic cells, it has been found that oxPAPC can inhibit the production of TNFα and IL-12p40 induced by the TLR3 ligand poly(I:C), and TLR-independent inhibitory effects have also been reported when cells were stimulated by CD40 ligand [5].

Clearly, interactions and signalling via TLRs only account for some of the reported effects of oxPLs, and a number of other receptors and pathways have also been investigated. Scavenger receptors are a candidate group, and there is good evidence that several oxPLs bind to CD36 on macrophages, resulting in uptake and formation of foam cells. A family of chain-shortened oxPLs containing hydroxyalkenal or oxo-alkenal moieties have been found to bind with high affinity [33], although other oxPLs including POVPC (palmitoyloxovaleroylglycerophosphocholine), oxidized phosphatidylserine and phosphatidic acid have also been shown to act as ligands [34]. oxPLs also interact with other scavenger receptors such as SR-BI (scavenger receptor class B type I) and compete with ligands such as HDL (high-density lipoprotein) and cholesterol esters via a complex mechanism [35]. In addition to cell-surface receptors, oxPLs have been reported to interact with nuclear receptors, of which the best understood examples are PPARs (peroxisome-proliferator-activated receptors). Most studies point to activation of PPARα and PPARγ by a variety of full-length and chain-shortened oxPLs, leading to effects such as up-regulation of CD36 and COX-2 [36]. Ligand binding to PPARs results in binding to retinoid X receptors to form an active transcription factor that translocates to the nucleus and binds to PPREs (PPAR-responsive elements) on target genes. PPARα is known to have anti-inflammatory effects, as it can interact directly with p65 to form an inactive complex and also induces expression of IκBα (inhibitor of NF-κBα), thus interfering with NF-κB signalling [37] and resulting in decreased levels of IL-1, IL-6, TNFα and iNOS [inducible NOS (nitric oxide synthase)]. Thus PPARs could contribute to some of the anti-inflammatory effects of oxPLs, although currently it is not clear whether this may involve hydrolysis of the oxidized moiety at the *sn*-2 position to release the modified fatty acid as the active component.

Oxidized phospholipids such as oxPAPC have also been suggested to have effects through non-receptor-dependent pathways. Phospholipids oxidized in the *sn*-2 fatty acyl chain have increased polarity, and so if they are present within or inserted into the cell membrane, they are likely to disrupt the packing of membrane lipid bilayers. The lipid whisker model [38] suggests that oxidized chains are excluded from the membrane region and stick out from the cell surface, allowing oxidatively damaged cells to be recognized. Oxidized phospholipids have been found to cause cholesterol depletion from cell membranes [26], which disrupts lipid rafts and caveolae and interferes

with a variety of signalling pathways that are caveolae-dependent. This has been proposed as a mechanism for oxPAPC-induced expression of IL-8 and LDL receptor in endothelial cells, via the SREBP (sterol-regulatory-element-binding protein), which is activated by cholesterol depletion [39]. However, further studies have indicated that the mechanism is much more complex than originally anticipated, and may in fact involve interactions with other receptors and signalling pathways, including roles for endothelial nitric oxide synthase, PI3K (phosphoinositide 3-kinase)–Akt (protein kinase B), VEGFR2 (vascular endothelial growth factor receptor 2), ERK1/2 (extracellular-signal-regulated kinase 1/2) and NOX-4 [40]. Other non-membrane receptor-mediated pathways by which oxPLs can exert regulatory effects have also been proposed. The electrophilic stress response is relevant for electrophilic oxPLs containing α,β-unsaturated aldehydes or ketones that are capable of reacting by Michael addition with thiol groups on proteins. Interaction with specific thiol groups on KEAP-1 (Kelch-like enoyl-CoA hydratase-associated protein 1) results in the transcription factor Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2) being released from it, allowing it to translocate to the nucleus and bind to AREs [antioxidant-response elements; also called EpREs (electrophile-response elements)], resulting in transcription of genes such as HO-1 (haem oxygenase-1) and glutamate-cysteine ligase. OxPAPC has been shown to induce such effects [41]. The UPR (unfolded protein response) is also a stress response, centred on the ER (endoplasmic reticulum) and incorrect folding of the proteins synthesized there; it leads to up-regulation of inflammatory cytokines including MCP-1 and IL-1, and in macrophages can induce apoptosis. It is triggered by the binding of unfolded proteins to three transmembrane ER signalling proteins: PERK [PKR (double-stranded-RNA-dependent protein kinase)-like ER kinase], IRE1α (inositol requiring 1α) and ATF6 (activating transcription factor-6). Oxidized phospholipids, as well as the non-esterified electrophilic compounds HNE and 15-deoxy-$\Delta^{12,14}$-PGJ$_2$, have been found to up-regulate several proteins involved in this response [42]; it is not yet clear how they induce activation of this signalling, although it is known that electrophilic lipids can form adducts with proteins, which could result in unfolding as well as aggregation.

Thus pathways responsible for the effects of oxPLs are emerging following considerable work in this area, although the signalling is extremely complex, as illustrated by the observation that more than 1000 genes have been reported to be activated by oxPAPC in endothelial cells [42].

Effects of chlorinated lipids

Myeloperoxidase is present in atherosclerotic plaques at all stages, and is thought to contribute to the pathology [43]. As mentioned previously, it is able to generate hypohalites, especially HOCl and HOBr (hypobromous acid). There is good evidence for the formation of HOCl at inflammatory loci, based on the detection of chlorotyrosine (a marker of

attack on proteins) and also of 2-chloro fatty aldehydes and lysophosphatidylcholines generated from HOCl attack on plasmalogens. While there is extensive information available on the biological effects of oxPLs, comparatively little is known about chlorinated lipids, reflecting the perception that they are of less relevance in physiology and pathology. Nevertheless, some interesting effects have been reported. Phospholipid and fatty acid chlorohydrins have been found to cause cell lysis, toxicity and depletion of ATP levels in various cell types [44,45], and, in U937 cells, activation of caspase 3 suggested that apoptosis was involved [46], although other studies reported that necrosis was the most likely mechanism of cell death [47]. Some pro-inflammatory effects have been reported, in particular, the induction of leucocyte endothelial adhesion via a mechanism involving P-selectin and to a lesser extent ICAM-1; increased generation of ROS from leucocytes was also observed [48]. There has been some work on the effects of α-chlorohexadecanal [49], which has been found to inhibit release of nitric oxide in endothelial cells by causing delocalization of eNOS (endothelial NOS) [50]. Unlike oxPLs, the chlorinated lipids do not appear to interfere with LPS signalling via TLRs: preliminary evidence suggests that SOPC (stearoyloleoyl-glycerophosphocholine) chlorohydrin does not reduce LPS-induced production of IL-8, whereas oxPAPC at the same concentration causes a significant reduction in cytokine production by myeloid cells (Figure 1). Overall, the emerging picture is that chlorinated phospholipids have more limited biological effects than oxPLs, and do not demonstrate anti-inflammatory or protective effects. This further supports the conclusion that many of the effects observed with oxPLs are specific, as opposed to generic effects resulting from increasing the polarity of the lipid and destabilizing the membrane bilayer.

Summary

Advances in soft-ionization MS and fragmentation routines have been fundamental in identifying oxPLs that are relevant to inflammatory pathologies. A wide variety of oxPLs have already been found *in vivo*, and additional novel structures continue to be elucidated. Further development of targeted MS approaches will facilitate the identification and quantification of these products in biological and clinical samples. There is extensive knowledge on the biological effects of oxPLs as a group: many interactions with receptors and signalling pathways are now well established and thought to be biologically relevant. oxPLs have a multitude of pro-inflammatory and pro-atherogenic effects, but also interfere with LPS signalling via TLRs and have protective effects such as increasing HO-1 expression. The effects of the oxPL mixture derived from autoxidation of PAPC have been particularly extensively studied, but there are also reports on the effects of individual oxidized species such as POVPC, PEIPC and hydroxyalkenals. Comparatively little is known about chlorinated phospholipids: they have been reported to

Figure 1 | The effect of chlorinated and oxPLs on LPS-induced IL-8 production

U937 cells were treated with native SOPC (**A**), monochlorohydrin (ClOH) of SOPC (**B**) or autoxidized PAPC (**A** and **B**) for 18 h in the presence of 10 ng/ml *E. coli* LPS, and the concentration of IL-8 in the cell supernatant was measured by ELISA. Chlorohydrin and autoxidized PAPC were prepared and analysed as described previously [45,46]. The results are normalized to the positive control (10 ng/ml LPS) as 100%
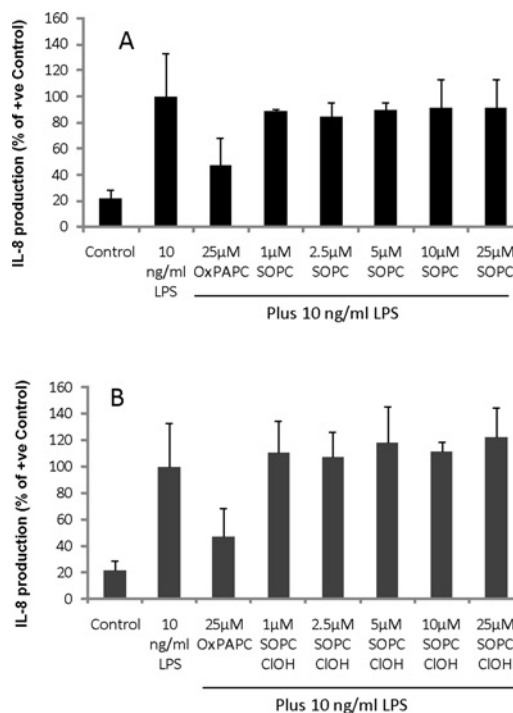
show some pro-inflammatory effects, but do not appear to induce the protective effects observed with oxPLs.

Funding

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