Epoxisoprostane and Epoxycyclopentenone Phospholipids Regulate Monocyte Chemotactic Protein-1 and Interleukin-8 Synthesis

FORMATION OF THESE OXIDIZED PHOSPHOLIPIDS IN RESPONSE TO INTERLEUKIN-18

Monocyte recruitment to the vessel wall, mediated by monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8), plays an important role in atherogenesis. We have shown previously that minimally oxidized low density lipoprotein, oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (Ox-PAPC), activates endothelial cells to produce MCP-1 and IL-8. By using liquid chromatography/mass spectrometry methods coupled with bioassay, we report a family of epoxisoprostane (PEIPC) and epoxycyclopentenone (PECPC) phospholipids that are the components of Ox-PAPC responsible for the majority of this activity. Ox-PAPC contains five chromatographically distinguishable active PEIPC components (m/z 825.5) and four PECPC components (m/z 810.5). All nine components induced endothelial cell synthesis of IL-8 and MCP-1 in a dose-dependent fashion between 0.1 and 5 μM concentrations. The five PEIPC components had identical functional groups and all underwent dehydration to produce m/z 810.5. We present evidence that these phospholipids are regioisomers with epoxide groups at the 5,6-, 8,9-, 11,12-, or 14,15-positions of the sn-2 fatty acid and their epoxide groups are important for biological activity. We have shown previously that peroxisome proliferator-activated receptor α is involved in MCP-1 synthesis in response to Ox-PAPC. We now show that PEIPC and PECPC isomers are potent activators of peroxisome proliferator-activated receptor α. PEIPC and PECPC isomers are strongly recognized by specific circulating murine natural autoantibodies (EO6) and accumulate in cells treated with IL-1β. These studies demonstrate that PEIPC and PECPC isomers are potent activators of endothelial cells increasing synthesis of IL-8 and MCP-1. Their accumulation in cells exposed to cytokines and in atherosclerotic lesions suggests that these lipids may play a role in a number of chronic disease processes.

Monocytes/macrophages are the major inflammatory cells in the atherosclerotic lesion and have been shown to be important mediators of lesion initiation and progression (1–3). It has been shown that monocytes enter the vessel wall by a multistep process involving molecules mediating monocyte rolling, activation, and binding (4). All of these steps are necessary to cause monocyte transmigration. Our group has demonstrated previously, using in vitro studies, that three phospholipid oxidation products of 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (Ox-PAPC) present in minimally modified/oxidized LDL (MM-LDL), namely 1-palmitoyl-2-oxovaleroyl-sn-glycero-3-phosphorylcholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC), and 1-palmitoyl-2-(5,6-epoxysopropane E3)sn-glycero-3-phosphorylcholine (PEIPC, m/z 828.5), were responsible for the induction of monocyte-binding molecules on endothelial cells (5–8). There is also considerable evidence that these lipids may play a role in atherogenesis in vivo. These lipids were increased in atherosclerotic lesions; apoE null mice produced antibodies to at least one of these lipids (6, 7); and fat-fed LDL receptor null mice given WEB 2086 (an inhibitor of the action of platelet-activating factor and POVPC) in their drinking water developed less atherosclerosis (9).

A major goal of our present study was to identify the components of Ox-PAPC that are responsible for the production of monocyte activators, since chemokines are critically necessary for transmigration. Monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8) were shown to be important regulators of atherogenesis (10). Both chemokines are increased in human atherosclerotic lesions (11, 12). Furthermore, mice lacking receptors for homologous chemokines or the chemokines them-
nels are more resistant toward atherosclerosis and have fewer monocytes in their lesions (2, 3, 13). Our group has previously shown (14) that MM-LDL and Ox-PAPC increased synthesis of MCP-1 and IL-8 by HAEC. Two components of MM-LDL and Ox-PAPC, POVP and PGPC, increased IL-8 and MCP-1 but were much less active than Ox-PAPC suggesting there were additional active molecules present in Ox-PAPC. These studies identify epoxysprostane and epoxycyclopentenone phospholipids as the major components of Ox-PAPC inducing MCP-1 and IL-8. We have shown previously (14) that the Ox-PAPC induction of MCP-1 was dependent on PPARα. The present studies demonstrate that epoxysprostane and epoxycyclopentenone phospholipids are potent activators of PPARα.

Another goal of the current studies was to determine whether bioactive phospholipids regulating chemokine synthesis could be produced in cell membranes in response to agents known to increase free radical production. There is evidence that oxidized phospholipids may be present in chronic diseases other than atherosclerosis, and we hypothesized that these might arise from cell membrane oxidation. To test this hypothesis, the ability of interleukin-1β (IL-1β), an important regulator of many processes of chronic inflammation, to induce the formation of bioactive phospholipids in endothelial cells was examined.

**EXPERIMENTAL PROCEDURES**

**Materials—**Acetonitrile, chloroform, methanol, ethyl acetate, water (all Optima or HPLC grade), and PrepSep-C18 columns were obtained from Fisher. Tissue culture media, sera, and supplements were obtained from Irvine Scientific and HyClone Laboratories, Inc. Tissue culture grade endothin-free gelatin, phenylmethysulfonyl fluoride, and butylated hydroxytoluene (BHT) were obtained from Sigma. PAPC and dimyristoyl-sn-glyero-3-phosphorylcholine (DMPC) were obtained from Avanti Polar Lipids (Alabaster, AL). Ox-PAPC (6) and copper-oxidized LDL (15) were prepared as described previously. The concentration of bacterial endotoxin in each treatment solution was <20 pg/ml (determined by chromogenic assay), which is ~100-fold less than that required for the significant levels of chemokine production in endothelial cells (data not shown).

**Isolation, Derivatization, and Analysis of Phospholipids—**Normal and reverse phase high performance liquid chromatography coupled mass spectrometer (NP- and RP-LC/MS, respectively) analysis and quantitative electrospray ionization mass spectrometric (ESIMS) flow injection analysis of oxidized phospholipids with an API III triple quadrupole biomolecular mass analyzer (Sciex, Toronto, Ontario, Canada) were performed as described previously (5–7). Sodium borohydride reduction and derivatization of oxidized phospholipids with methoxyamine hydrochloride and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were performed as described previously (5, 7, 16). Selective epoxide hydrolysis of oxidized phospholipids was performed as described previously using PCA (1.66 mM) in HEPES buffer (17). The sn-2 fatty acids of oxidized phospholipids were released by PLα hydrolysis as described previously (5, 7). PLα-hydrolyzed fatty acids were analyzed by negative ion mode tandem mass spectrometry as described previously (5). LC/MS/MS analyses for hydrolyzed fatty acids were performed with a C18 column (Keystone Scientific t-butylsil 250 × 10 mm, 5 µm) equilibrated in 60% MeOH containing 1 mM ammonium acetate. The column was eluted at 0.4 ml/min with a linear gradient to 100% MeOH containing 1 mM ammonium acetate over 45 min. This HPLC method was also used for the isolation of fatty acids from PLα-hydrolyzed oxidized phospholipids.

**Cell Culture and Chemokine Assays—**HAEC and HeLa cells were cultured as described previously (14, 18). In all experiments HAEC were used at passage levels from 4 to 7. HAEC were treated (with indicated amounts of oxidized phospholipids or phospholipid fractions from HPLC separation) for 4 h at 37 °C in M199 containing 1% fetal bovine serum (FBS), 5% lipoprotein-deficient serum (LPDS, 0.8 mg/ml). The supernatants were collected and assayed for IL-8 and MCP-1 protein levels by employing Quantikine kits (R & D Systems) according to the manufacturer’s direction. Each treatment was tested in triplicate and repeated at least 2 times.

**PFAα Activation by Oxidized Phospholipids—**HeLa cells were plated in 24-well plates in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. Expression plasmids that produce fusion proteins with yeast GAL4 DNA binding domain and PPAR γ ligand binding domain and reporter plasmid with GAL4-binding element upstream of a luciferase gene were prepared as described previously (19). After 2 h of transfection, the cells were washed with PBS; medium was changed, and FBS was added to a final concentration of 0.8%. The cells were allowed to recover for 4 h. The cells were transfected with the indicated amounts of lipids in Dulbecco’s modified Eagle’s medium supplemented with 1% FBS. Cells were harvested after an additional 15 h of growth, and luciferase activity was determined and normalized to the β-galactosidase activity.

**Chemiluminescent Competition Immunoassay for Binding to Monoclonal Antibody EO6—**OxLDL at 5 μg/ml was plated overnight in 24-well plates that were treated previously (20). Prior to use in assay, the unbound OxLDL was removed by repeated washing with an automated plate washer. EO6 at a final concentration of 1 μg/ml was incubated in the absence or presence of the indicated antigens at varying concentrations in siliconized microcentrifuge tubes. After an overnight incubation at 4 °C, all incubations were centrifuged in a microcentrifuge for 30 min. Aliquots of the supernatant (~200 µl) were then dried, and the bound EO6 was detected using a goat anti-mouse IgM alkaline phosphatase antibody, followed by incubation with LumiPhos 530 (Lumigen, Inc.) 1:1 with distilled water (20). The data are expressed as a ratio of the counts bound (B) in the presence of competitor divided by the counts in the absence of competitor (B/A).

**Analysis of Oxidized Phospholipid Levels in IL-1β-treated HAEC—**HAEC were cultured in 100-mm dishes in M199 supplemented with 10% FBS. At ~80% confluence, the cells were treated with medium alone or IL-1β (20 ng/ml) and incubated for 48 h at 37 °C. Attached cells were removed by scraping, pelleted by centrifugation (500 × g, 6–8 min), washed with PBS, and re-centrifuged. The cell pellets were resuspended in 1 ml of water containing phenylmethysulfonyl fluoride (4 mM), and chloroform (4 ml) containing 0.1% BHT was added. Samples were homogenized and then centrifuged (2,000 × g, 10 min). The chloroform phase was transferred to a clean glass tube, and the remaining upper (aqueous) phase was re-extracted with chloroform/methanol (4 ml, 2:1, v/v) containing 0.1% BHT. The organic layers were pooled; aqueous formic acid (1 ml, 0.7 M) was added, and the samples were centrifuged. The lower aqueous phase was placed in a clean glass tube under a stream of argon. The dried extracts were redissolved in MeOH/H2O (200 µl, 98:2) and loaded onto PrepSep-C18 extraction column previously activated with 3 ml of MeOH/H2O (98:2). The columns were eluted with MeOH/H2O (98:2, 2 ml) and then 10 ml of MeOH. The pooled eluate was analyzed by quantitative LC/MRM analysis using DMPC as an internal standard. Samples in 50 µl of MeOH containing DMPC (10 ng) were injected onto a C8 column (Keystone Scientific t-butylsil 250 × 2 mm, 5 µm) equilibrated in 1% MeOH containing 1 mM ammonium acetate. The column was eluted at 0.4 ml/min with a linear gradient to 100% MeOH containing 1 mM ammonium acetate over 45 min. The column effluent was split, and about 20% of the sample was directed to the LonsprayTM mass spectrometer source, and the rest was collected for additional analyses including inorganic phosphate measurement. Argon containing 10% nitrogen was used as collision gas (instrumental CQT settings of 110) with an orifice voltage of 100. The ion currents for the transition of each pre-selected parent ion (m/z 712.4, 549.6 (lyso-PC), 593.5 (POVP), 611.3 (POPC), 679.5 (DMPC), 782.5 (PAPC), 810.5 (dehydrated PEIPC), and 828.5 (PEIPC)) to the corresponding most abundant MS/MS fragment ion (in all cases this was the ion for phosphocholine (OP(OH)2CH2CN(CH2)3OH) at m/z 141.3 (21) were recorded. Software provided with the instrument was used for the extraction of MS/MS traces from each data set. Retention times for each phospholipid were determined using synthetic phospholipid (lyso-PC, POVP, POPC, DMPC, and PAPC) or bioactive oxidized phospholipids isolated from Ox-PAPC (PEIPC and PEPC). The individual extracted MRM profiles were smoothed, and the peak areas relative to that for DMPC were determined after exporting the data to the IGOR Pro™ computer program (version 5, WaveMetrics, Inc., Lake Oswego, OR). For the most precise treatment of the results, we export the data to IGOR program to measure MRM peak areas. The amount of each LC-resolved phospholipid was calculated using external calibration curves that were constructed with known amounts of authentic or isolated phospholipids and DMPC. Total inorganic phosphate in samples was measured by phosphorous assay as described previously (22).

**Statistical Analysis—**Data were analyzed using one-way ANOVA. The levels of significance were calculated using StatView (Abacus Concepts, Inc., Berkeley, CA).
FIG. 1. LC/MS analysis of the active fractions of Ox-PAPC. A, Ox-PAPC (10 mg) was fractionated by NP-LC/MS, and active fractions 10–22 were collected, pooled, and dried to provide NP-LC/MS-enriched Ox-PAPC fraction. This fraction was further fractionated using RP-LC/MS as described under “Experimental Procedures,” and 70 1-min fractions were collected. HAECs were treated with 1/10th of RP-LC/MS fractions, Ox-PAPC (25 µg/ml), NP-LC/MS-enriched active fractions 10–22 of Ox-PAPC (equivalent to 25 µg/ml Ox-PAPC), or medium alone (control) for 4 h at 37 °C. After incubation IL-8 enzyme-linked immunosorbent assay was performed as described under “Experimental Procedures.” For each condition n = 3. Data were analyzed using one-way ANOVA and are presented as mean ± S.D. above control (control = 1.464 ± 0.173 ng/ml of IL-8 protein). Analysis of RP-LC/MS data showed that the fractions 31–38 and 42–45 were active (upper panel), which were enriched with ions with m/z 828.5 (peaks 1–5) and 810.5 (peaks 1–4) (lower panel). The experiment is representative of two such analyses. B, structures of 5,6-PEIPC (m/z 828.5(2)) and 5,6-PECPC (m/z 810.5(3)) are shown.
FIG. 2. Dose response for isolated bioactive oxidized phospholipids. HAEC were treated with medium alone (control) or the isolated molecules with m/z 828.5 (peaks 1–5) and 810.5 (peaks 1–4) at the indicated concentrations and were tested for their ability to produce IL-8 (A and B) and MCP-1 (C and D) as described under "Experimental Procedures." Data were analyzed using one-way ANOVA and are presented as mean ± S.D. The experiment is representative of two such analyses.
M199 alone, and M199 + LPDS upon 1 h of incubation (data not shown). There was a small decrease (10–15%) in the levels of oxidized phospholipids for incubations with M199 + 1% FBS compared with PBS. These studies suggest that the effect of Ox-PAPC is rapid, and these oxidized lipids are relatively stable under the assay conditions.

Structural Characterization of Bioactive Oxidized Phospholipids—All m/z 828.5 peaks possessed nearly identical UV maxima at 252 nm (mean ε = 19,800), and all m/z 810.5 peaks possessed an identical UV maxima at ~257.5 nm (mean ε = 18,300) (5). Based on the chromatographic characteristics, ESI-MS data, and previous work (5), we hypothesized that the different m/z 828.5 peaks are stereo- or regio-isomers of PEIPC and that the m/z 810.5 peaks are the corresponding dehydration products. MS/MS, in which molecules of a selected mass undergo collision-induced fragmentation yielding characteristic fragment ions, was used to confirm this hypothesis. Positive ion ESI-MS/MS of m/z 828.5 and 810.5 parent ions from each of the nine RP peaks produced in all cases a single major m/z 184.1 fragment ion corresponding to phosphocholine (OP(OH)2OCH2CH2N(CH3)3, data not shown). This behavior is characteristic of all PC derivatives (23). Negative ion ESI-MS fragmentation of PC derivatives is slightly more complicated. At high orifice voltages PC derivatives lose a terminal methyl group. With the resulting loss of the constitutive positive charge on the nitrogen atom, they can then be induced to adopt a single negative charge on the phosphate moiety if the pH of the infusing solution is above the pKₐ value of the phosphate group. Under these conditions MS/MS decomposition of these (M – CH3)⁻ anions typically generate three characteristic fragment anions as follows: those corresponding to the sn-1 and sn-2 fatty acid carboxylate anions and an anion corresponding to lyso-PC (23). Under these conditions, the five m/z 828.5 peaks all produced an (M – CH3)⁻ anion at m/z 812.5 (derived from 828.5 by the loss of 16 Da), which in turn produced characteristic MS/MS fragments at m/z 331.1 (epoxycyclopentenone), 255.1 (palmitate), and 480.2 (lyso-PC) (Fig. 3A). The four m/z 810.5 peaks all produced an (M – CH3)⁻ anion at m/z 794.5 (derived from 810.5 by the loss of 16 Da), which in turn produced characteristic MS/MS fragments at m/z 331.1 (epoxycyclopentenone), 255.1 (palmitate), and 480.2 (lyso-PC) (Fig. 3B). These studies confirmed that at the sn-2 positions all five m/z 828.5 peaks contain a 349.1-Da moiety, and all four m/z 810.5 peaks contain a 331.1-Da moiety. Henceforth, the five m/z 828.5 peaks and the four m/z 810.5 peaks will be referred to as isomers, respectively.

We have described previously that at least two of the m/z 828.5 isomers underwent dehydration to produce m/z 810.5 as the major decomposition product (5). In the present study all five m/z 828.5 isomers were isolated separately and reanalyzed after partial dehydration during storage for 48 h in chloroform at room temperature. RP-LC/MS employing the same chromatographic conditions that were used for isolation showed that all m/z 828.5 isomers underwent dehydration to produce an ion at m/z 810.5 as the major product. Treatment with BSTFA demonstrated the presence of one derivatizable hydroxyl group in each m/z 828.5 isomer that was lost in the corresponding m/z 810.5 products (data not shown). The conversion of each 828.5 isomer to specific 810.5 isomers was deduced from the RP-HPLC retention times: dehydration of 828.5(1) produced 810.5(1); 828.5(2) produced 810.5(3); 828.5(3) produced 810.5(2); 828.5(4) produced 810.5(3) as major and 810.5(2) as a minor product; 828.5(5) produced 810.5(4) as major and 810.5(2) as a minor product (data not shown). These studies demonstrate that all molecules with m/z 828.5 undergo dehydration to produce m/z 810.5, which is consistent with formation of α,β-unsaturated enones from β-hydroxyketones as described previously (5).

We next examined the functional groups on these molecules by employing chemical derivatization methods. Each m/z 828.5 isomer reacted with two molecules of methoxylamine hydrochloride (measured mass increase of 58 Da) as one of the products, as shown previously for PEIPC (828.5(2)) (5), suggesting the presence of two carbonyl and/or epoxide groups.

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**Fig. 3. Tandem mass spectrometric analysis of m/z 828.5 and 810.5.** Tandem mass spectrometric analysis of 5,6-PEIPC (828.5 peak 2) (A) and 5,6-PEPC (810.5 peak 3) (B). Oxidized phospholipids were analyzed in the negative ion mode, and the collision-induced decomposition spectra for the pseudo parent ion (M – 16)⁻ are shown. The proposed structures of each molecule are shown in the insets.
Sodium borohydride treatment of each m/z 828.5 isomer produced a major product ion at m/z 832.5, suggesting the presence of two reducible oxygen-containing functional groups such as carbonyl and/or epoxide groups. Sodium borohydride treatment of each m/z 810.5 isomer produced m/z 816.5 as the major product ion (data not shown). Considering that these molecules possessed only two reducible oxygen groups, a reasonable explanation for this result is the presence of a reactive double bond in m/z 810.5 molecules such as an enone, which was also reduced (5). It is known that treatment of enones with sodium borohydride results in the reduction of both carbonyl groups as well as adjacent double bonds (24). Selective hydrolysis conditions (PCA in HEPES buffer) that convert epoxyeicosatrienoic acid to dihydroxyeicosatrienoic acid (17) were used to test for the presence of an epoxide moiety in the m/z 828.5 and 810.5 isomers. Under these conditions, all m/z 810.5 isomers produced an ion at m/z 828.5 as a major product, and all m/z 828.5 isomers produced an ion at m/z 846.5 as a major product (Fig. 4, A-D). The extent of hydrolysis, as judged by the relative intensity of the remaining substrate, varied between 60 and 70% for the different isomers. Together, these studies confirmed that all nine active isomers had both an epoxide group and a carbonyl group.

The negative ion MS/MS spectra of the fatty acids derived from the PLA₂ hydrolysis of m/z 828.5 isomers (parent ion [M – H]⁻ at m/z 349.1) all revealed prominent fragment anions (assignments in brackets) at m/z 331.1 [M – H – H₂O]⁻, 305.3 [M – H – CO₂]⁻, 287.3 [M – H – H₂O – CO₂]⁻, and 269.3 [M – H – 2H₂O – CO₂]⁻ (Fig. 5A). Similarly, negative ion MS/MS spectra of the fatty acids derived from the PLA₂ hydrolysis of m/z 810.5 isomers (parent ion [M – H]⁻ at m/z 331.1) had prominent fragment anions at m/z 313.1 [M – H – H₂O]⁻, 287.1 [M – H – CO₂]⁻, and 269.1 [M – H – H₂O – CO₂]⁻. These fragment ions are also evident in the negative ion MS/MS spectra of the parent phospholipids (Fig. 3, A and B).

The similarity of these fatty acid MS/MS spectra suggests that the parent m/z 828.5 and 810.5 isomers are structurally closely related.

The fatty acid MS/MS spectra were screened for smaller MS/MS fragmentation products that might distinguish between the different isomers. For these studies both free fatty acids and PCA-treated free fatty acids were used. Our previous studies have shown that the fatty acids derived from m/z 828.5(2) and 810.5(3) both produced a fragment anion at m/z 115 that is indicative of an oxygen at the 5-position or an epoxide at the 5,6-position (structure shown in Fig. 1B) (5, 25). In the present study, we performed LC/MS/MS analyses of the hydrolyzed fatty acids of m/z 828.5 or 810.5 peaks and monitored the formation of characteristic fragments for 5,6-PEIPC (m/z 115), 8,9-PEIPC (m/z 155), 11,12-PEIPC (m/z 207 or 225), and 14,15-PEIPC (m/z 247 or 265) resulting from the cleavage of epoxide group. LC/MS/MS analysis of 810.5(3) fatty acid (as shown previously) gave only one characteristic fragment at m/z 115 in the absence of characteristic fragments for other regioisomers. The MS/MS spectra of the fatty acid derived from 828.5(3) revealed a major fragment at m/z 207.2 (Fig. 5A). Similarly, MS/MS spectra of the fatty acid derived from 810.5(2) (dehydration product of 828.5(3)) revealed a major fragment at m/z 207.2 (data not shown). This fragment is consistent with the presence of an 11-carbon aldehyde generated upon fragmentation of 11,12-epoxide group followed by dehydration. In addition to the m/z 207.2, less abundant characteristic fragment ions corresponding to 5,6-PEIPC (m/z 115) and 8,9-PEIPC (m/z 155) are also present in 828.5(3) (Fig. 5A).

LC/MS/MS analysis of fatty acids derived from m/z 828.5(5) and its dehydration product, m/z 810.4(4), produced an anion at
m/z 155, which is characteristic for the presence of an oxygen at the 8- or 8,9-position (25). However, this represented a minor product (data not shown). Epoxide to diol conversion has been shown to facilitate fragmentation under mass spectrometric conditions (26). Therefore, 828.5(5) was treated with PCA and then hydrolyzed with PLA₂, and MS/MS was performed on the free fatty acid (parent (M – H)⁻ ion at m/z 367, see Fig. 5B). Major fragment ions are present at m/z 155 and m/z 127.1,

**Fig. 5. Tandem mass spectrometric analysis of the hydrolyzed fatty acids m/z 828.5.** The molecules with m/z 828.5 were isolated by sequential NP and RP-LC/MS and were hydrolyzed with PLA₂ or treated with PCA and then hydrolyzed with PLA₂, as described under “Experimental Procedures.” The released fatty acids (m/z 349 or 367) were analyzed by negative ion MS/MS. The spectra of 828.5(3) fatty acid (A) and PCA-hydrolyzed 828.5(5) fatty acid (B) are shown. The specific fragments formed are indicated in each spectrum with the assigned structures of 11,12-PEIPC for 828.5(3) and 8,9-PEIPC for 828.5(5).
both of which are characteristic for molecules with a hydroxyl group at the 8-carbon or 8,9-epoxides (25, 27). These studies confirmed the presence of an 8,9-epoxide in the 828.5(5) and 810.5(4) isomers. Analysis of hydrolyzed fatty acids from m/z 828.5(1), 828.5(4), and 810.5(1) did not provide conclusive results. We hypothesized that this might be due to the presence of a number of isomers in these peaks. To test this, LC/MS/MS analyses were performed on 828.5(1) and 828.5(4) fatty acids. LC/MS/MS analysis of 828.5(1) fatty acid produced m/z 115, 155, 207, 225, and 247 ions. LC/MS/MS analysis of 828.5(4) fatty acid produced m/z 115, 155, 207 and 247. These results showed that 828.5(1) and 828.5(4) contain all four regioisomers. Thus overall, we conclude that some HPLC peaks obtained from molecules of m/z 828.5 or 810.5 represent mainly one regioisomer, whereas others are much more complex. These studies and our previous studies (5) thus demonstrated that 828.5(2), 828.5(3), and 828.5(5) and their dehydration products contained predominantly one isomer: epoxide at the 5,6-position for 828.5(2), at the 11,12-position for 828.5(3), and at the 8,9-position for 828.5(5). Taken together these studies confirm the structures of four regioisomers of PEIPC (m/z 828.5) and PEIPC (m/z 810.5) containing epoxides at the 5,6-, 8,9-, 11,12-, or 14,15-positions. These molecules are henceforth referred to as 5,6-, 8,9-, 11,12-, or 14,15-PEIPC and 5,6-, 8,9-, or 11,12-, or 14,15-PECP.

Effects of Reduction and Hydrolysis on the Bioactivity of Oxidized Phospholipids—We have tested the effects of PLA₂ hydrolysis, epoxide hydrolysis, and reduction on the bioactivity of oxidized phospholipids. Treatment of Ox-PAPC (50 μg/ml), 5,6-PEIPC (5 μM), and 5,6-PECPC (5 μM) with PLA₂ resulted in 75–90% loss of ability of these oxidized phospholipids to cause IL-8 production (Fig. 6). In addition, isolated fatty acids did not cause significant increases in IL-8 production (data not shown). Treatment of these lipids with sodium borohydride resulted in 60–80% loss of activity. In addition, treatment with PCA abolished ~75% of 5,6-PEIPC and 5,6-PECPC activity and reduced Ox-PAPC activity by about 50%. Similar results were obtained with hydrolyzed and reduced oxidized phospholipids for MCP-1 induction in HAEC (data not shown). Taken together, these studies demonstrate the importance of the epoxide group and an intact phospholipid structure for the biological activity of PEIPC and PECPC and suggest that the epoxide derivatives are major biologically active components of Ox-PAPC.

Activation of PPARα by Oxidized Phospholipids—Previous studies (14) have shown that MM-LDL, Ox-PAPC, POVP, and PGF activate PPARα in transfected CV-1 cells. In the present study we examined the effects of Ox-PAPC, NP-LC/MS-enriched mixed isomers of m/z 828.5 and 810.5, individual isomers of m/z 828.5 and 810.5 on PPARα activation in transfected HeLa cells. Ox-PAPC (40 μg/ml), mixed, and individual isomers of m/z 828.5 and 810.5 isomers (1 or 2 μM) activated PPARα by 2–17-fold (Fig. 7). These studies show that these oxidized phospholipids are potent activators of PPARα.

Recognition of Oxidized Phospholipids by Monoclonal Antibody EO6—Previous studies (6, 20) have demonstrated that a series of autoantibodies cloned from apoE-deficient mice, termed EO antibodies, bound to OxLDL and specifically to oxidized phospholipids. In particular, as represented by EO6, they bound to MM-LDL, OxLDL, Ox-PAPC, POVP, and protein bound-POVP adducts (6, 20). EO6 specifically recognizes the isolated phosphorylcholine (PC) head group but does not recognize lyso-PC. In the present study we have tested whether monoclonal EO6 could recognize m/z 828.5 and 810.5 isomers. All the isomers of m/z 828.5 and 810.5 effectively bound by EO6 with IC₅₀ values of 1–10 μM (Fig. 8). These data show that the PEIPC and PECPC isomers are epitopes for monoclonal EO6, which recognizes these molecules as free phospholipids. EO6 recognizes other oxidized PC-containing phospholipids in the free form or when covalently attached to protein, though our studies do not yet address this latter point with respect to PEIPC and PECPC. In addition, EO6 does not discriminate the various regioisomers of PEIPC and PECPC, suggesting that the conformational changes induced by oxidation made the PC head group available for antibody binding.

Formation of Bioactive Oxidized Phospholipids in Response to IL-1β—We have hypothesized that the treatment with pro-inflammatory cytokines might produce biologically active oxidized phospholipids in endothelial cells. To test this hypothesis, we measured the levels of oxidized phospholipids in the lipid extracts of control (medium alone) and IL-1β (20 ng/ml)-treated HAEC. The levels of bioactive oxidized phospholipids (POVP, PGF, PEIPC, and PEIC) in the lipid extracts of HAEC were quantitated by LC/MRM analysis and normalized with total phospholipids (measured by phosphorous assay). The levels of four bioactive oxidized phospholipids were increased by 40–70% in the lipid extracts of endothelial cells that were treated with IL-1β compared with control cells (Fig. 9). These studies demonstrate the accumulation of bioactive oxidized phospholipids in response to pro-inflammatory cytokines in endothelial cells.
FIG. 7. Activation of PPARα by oxidized phospholipids. The transcriptional activation of PPARα by oxidized phospholipids was compared in transient transfection experiments as described under "Experimental Procedures." The fold increase in the normalized luciferase activity above control is presented as mean ± S.D. The experiment is representative of two such analyses.

FIG. 8. Recognition of oxidized phospholipids by monoclonal autoantibody EO6. Competition immunoassay for binding of EO6 to OxLDL by various competitors including Ox-PAPC, lyso-PC, PC, m/z 828.5 (1–5) and 810.5 (1–4) was performed as described under "Experimental Procedures." The data are expressed as (B/B₀) × 100. Each point is the mean of triplicate determinations, and the experiment is representative of two such analyses.

DISCUSSION

These studies were designed to identify the most active oxidized phospholipids in Ox-PAPC that induce synthesis of IL-8 and MCP-1 in HAEC. We have now identified a family of PEIPC and PECPC derivatives that were more potent inducers of chemokine production than the two previously identified biologically active phospholipids present in MM-LDL (POVPC and PGPC). Significant bioactivity of PEIPC and PECPC was obtained at 100 nM (Fig. 2, A–D) and isomers of m/z 828.5 (λ_max = 252 nm) and m/z 810.5 (λ_max = 257.5 nm) possessed similar UV characteristics, which is attributable to the presence of a γ,δ-epoxy, α,β-unsaturated enone in m/z 828.5 isomers and γ,δ-epoxy, α,β-unsaturated dienone m/z 810.5 isomers (5). These values were comparable with the structurally related Δ(12)-prostaglandin J_2 (λ_max = 248 nm, ε = 17,000), which contains a δ-hydroxy, α,β-unsaturated enone (29). Tandem mass spectrometric analysis of the fatty acid isolated from m/z 828.5 and 810.5 demonstrated the presence of epoxide at the 5,6-, 8,9-, 11,12-, or 14,15-positions (Fig. 5). Taken together these studies confirm the structures of four regioisomers of PEIPC (m/z 828.5) and the corresponding dehydration products, PECPC (m/z 810.5) with epoxide groups at 5,6-, 8,9-, 11,12-, and 14,15-positions (Fig. 5).

The proposed mechanism for the formation of regioisomeric PEIPC and PECPC during autodigestion of PAPC is shown in Fig. 10. Oxidation of arachidonoyl phospholipids were shown to generate four regioisomers of F_2 or E_2/D_2 isoprostanes via the prostaglandin endoperoxide phospholipid intermediates (G_2–IsoP-PC and H_2–IsoP-PC) (30, 31). It is known that allylic hydroperoxides can undergo dehydration to generate allylic epoxyeicosatrienoic acids (32, 33). Decomposition of endoperoxide nucleus and rearrangement of allylic hydroperoxide of G_2–IsoP-PC is expected to form an allylic epoxide containing 5,6-, 8,9-, 11,12-, and 14,15-epoxyisoprostane E_3 phospholipids (PEIPC isomers, m/z 828.5). Dehydration of PEIPC isomers can generate the corresponding epoxycyclopentenone phospholipids (PECPC isomers, m/z 810.5). One of the unique characteristics of epoxyisoprostane phospholipids and their dehydration products is that the UV absorption maxima of 252–258 nm, which is consistent with the presence of γ,δ-epoxy, α,β-unsaturated enone. This moiety is present in only in the E-type epoxyisoprostanes of 5,6-, 8,9-regioisomers and in the D-type epoxyisoprostanes of 11,12-, 14,15-regioisomers. Even though alternative type epoxyisoprostanes are possible, we do not have any presence of one hydroxyl group in the m/z 828.5 isomers, which is absent in the m/z 810.5 isomers. Reduction of the m/z 828.5 isomers with sodium borohydride added 4 daltons equivalent to four hydrogen atoms, and treatment of the m/z 828.5 isomers with methoxylamine hydrochloride added 58 daltons equivalent to two methoxylamine groups which suggested the presence of carbonyl and/or epoxide groups (5). The presence of one epoxide group in these molecules was confirmed by specific hydrolysis of epoxide to diol (Fig. 4). All isomers of m/z 828.5 (λ_max = 252 nm) and m/z 810.5 (λ_max = 257.5 nm) were isolated from Ox-PAPC, lyso-PC, PC, m/z 828.5 (1–5) and 810.5 (1–4) was performed as described under "Experimental Procedures." The data are expressed as (B/B₀) × 100. Each point is the mean of triplicate determinations, and the experiment is representative of two such analyses.

We demonstrate that the active molecules are regioisomers of PEIPC and the corresponding dehydration products (PECPC) with epoxide groups at four different locations on the 20-carbon chain. The structural characterization of these molecules was obtained by detailed mass spectrometric analysis of the active m/z 828.5 isomers, their m/z 810.5 dehydration products, and the fatty acids liberated from these phospholipids (Figs. 3–5). Derivatization with BSTFA demonstrated the presence of one hydroxyl group in the m/z 828.5 isomers, which is absent in the m/z 810.5 isomers. Reduction of the m/z 828.5 isomers with sodium borohydride added 4 daltons equivalent to four hydrogen atoms, and treatment of the m/z 828.5 isomers with methoxylamine hydrochloride added 58 daltons equivalent to two methoxylamine groups which suggested the presence of carbonyl and/or epoxide groups (5). The presence of one epoxide group in these molecules was confirmed by specific hydrolysis of epoxide to diol (Fig. 4). All isomers of m/z 828.5 (λ_max = 252 nm) and m/z 810.5 (λ_max = 257.5 nm) possessed similar UV characteristics, which is attributable to the presence of a γ,δ-epoxy, α,β-unsaturated enone in m/z 828.5 isomers and γ,δ-epoxy, α,β-unsaturated dienone m/z 810.5 isomers (5). These values were comparable with the structurally related Δ(12)-prostaglandin J_2 (λ_max = 248 nm, ε = 17,000), which contains a δ-hydroxy, α,β-unsaturated enone (29). Tandem mass spectrometric analysis of the fatty acid isolated from m/z 828.5 and 810.5 demonstrated the presence of epoxide at the 5,6-, 8,9-, 11,12-, or 14,15-positions (Fig. 5). Taken together these studies confirm the structures of four regioisomers of PEIPC (m/z 828.5) and the corresponding dehydration products, PECPC (m/z 810.5) with epoxide groups at 5,6-, 8,9-, 11,12-, and 14,15-positions (Fig. 5).

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Evidence to support alternative E/D-type structures. There are four possible regioisomers for epoxyisoprostanes with characteristic $\gamma,\beta$-epoxy, $\alpha,\beta$-unsaturated enone. Each regioisomer will have 16 possible diastereoisomers, and the total number of possible isomers is 64. There will be 32 possible PECPC isomers, which are the dehydration products of 64 PEIPC isomers.

We demonstrate that the peaks enriched in at least three regioisomers of PEIPC and PECPC are quite effective in causing MCP-1 and IL-8 synthesis in HAEC (Fig. 2, A–D). These molecules are active as the intact phospholipid rather than as a free fatty acid and require epoxide for their biological activity (Fig. 6). The common structural motif in all these molecules is the presence of $\gamma,\beta$-epoxy, $\alpha,\beta$-unsaturated enone moiety. In previous studies only one of the isomers of $m/z$ 828.5, 5,6-PEIPC was active in causing endothelial cells to bind monocytes (5), and all other fractions of $m/z$ 828.5 and 810.5 were inactive. This could be due in part to the differences in the pathways involved in monocyte binding versus chemokine production. Oxidized phospholipid-mediated monocyte binding involves a cAMP pathway (8), whereas a PPAR$\alpha$ pathway is involved in oxidized phospholipid-mediated chemokine production (14).

Recent studies (14) have shown that oxidized phospholipids are potent activators of PPAR$\alpha$ pathway and that PPAR$\alpha$ plays an important role in mediating the effects of these lipids. Our previous studies (14) demonstrate that PPAR$\alpha^{\gamma,-/-}$ mouse aortic endothelial cells do not produce MCP-1/JE in response to oxidized lipids. In the present study, we demonstrate that PEIPC and PECPC isomers were potent activators of PPAR$\alpha$ (Fig. 7). These results are consistent with recent studies that PPAR$\alpha$ deficiency reduced atherosclerosis and lowered MCP-1 levels in the atherosclerotic lesions of ApoE-deficient mice (34).

To determine the in vivo importance of PEIPC and PECPC isomers, we examined the recognition of these lipids by monoclonal autoantibody EO6 as well as the ability of cells stimulated by cytokines to produce PEIPC and PECPC isomers. In addition to atherosclerosis, circulating natural antiphospholipid antibodies and/or their epitopes are increased in several disease states including antiphospholipid antibody syndrome, diabetes, and rheumatoid arthritis (35–38). EO6 strongly recognizes PEIPC and PECPC isomers (Fig. 8). The cross-reactivity of monoclonal antibody EO6 with several oxidized phospholipids has been reported previously (6, 20). The PC head group is an absolute requirement for binding of EO6, and phosphatidylcholine (PC), as a salt, can totally abolish the binding of EO6 to oxidized LDL (39). As shown previously (20), it recognizes a variety of oxidized PC-containing phospholipids, such as POVPC, but does not recognize non-oxidized PAPC. We have
prepared an extensive number of phospholipid analogues and determined the requirements for EO6 binding (40). In brief these data document the absolute requirement for the intact PC head group, but this is not sufficient in and of itself for antibody binding. Lyso-PC does not bind, nor do oxidized PS or PE phospholipids. Phospholipids with very short chain fatty acids in the sn-1 and sn-2 positions, such as diC3PC do bind, although with greatly reduced affinity, compared with PC or to POVPC. EO6 does not recognize PGPC. In addition, when oxidized phospholipids undergo Schiff base formation through the reactive aldehyde generated by oxidation, the resultant compound is an excellent antigen, and in addition such oxidized phospholipids undergo aldehyde self-condensation reactions that also render the oxidized lipid highly antigenic. Thus, the key seems to be the mode of presentation of the PC head group. When oxidized the PC head group becomes available for EO6 recognition.

Oxidative stress has been implicated to play an important role in several disease states, and several agents including peroxide, high glucose, angiotensin II, oysterolks, and cytokines were shown to cause oxidative stress in cells (41–45). Because oxidative stress has been shown to generate free radicals in cells, we reasoned that bioactive oxidized phospholipids might be produced in response to this stress. In the present study we demonstrate that the levels of bioactive oxidized phospholipids were increased in cells that were treated with IL-1β (Fig. 9). The presence of oxidized phospholipids in unstimulated endothelial cells may be due to cell culture conditions that do not provide the same antioxidant environment as obtained in vivo. These results suggest that in addition to the presence in MM-LDL, biologically active oxidized phospholipids are produced in response to cytokines in cells and could promote the synthesis of inflammatory chemokines at the sites of inflammation.

In summary, we have characterized a family of oxidized phospholipids with potent biological activity. Because these molecules are formed by free radical oxidation, they may be important in the regulation of pro-inflammatory processes involving oxidative stress such as atherosclerosis.

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Epoxyisoprostane and Epoxycyclopentenone Phospholipids Regulate Monocyte Chemotactic Protein-1 and Interleukin-8 Synthesis: FORMATION OF THESE OXIDIZED PHOSPHOLIPIDS IN RESPONSE TO INTERLEUKIN-1β

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