

# Determinants of Bioactivity of Oxidized Phospholipids

## Specific Oxidized Fatty Acyl Groups at the *sn*-2 Position

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**Abstract**—We previously described 3 bioactive oxidation products of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC) containing oxovaleroyl (POVPC), glutaroyl (PGPC), and epoxyisoprostane (PEIPC) groups at the *sn*-2 position that were increased in minimally modified/oxidized low density lipoprotein (MM-LDL) and rabbit atherosclerotic lesions. We demonstrated specific and contrasting effects of POVPC and PGPC on leukocyte-endothelial interactions and described an effect of PEIPC on monocyte binding. The major purpose of the present study was to determine the effects of structural changes on the bioactivities of these 3 lipids. We demonstrate herein that the group at the *sn*-2 position determines the specific bioactivity and that the substitution of stearoyl for palmitoyl at the *sn*-1 position or ethanolamine for choline at the *sn*-3 position of the phospholipid did not alter bioactivity. Oxidized PAPC, oxidized 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine, and oxidized 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylethanolamine stimulated monocyte binding and inhibited lipopolysaccharide-induced expression of the neutrophil-binding molecule E-selectin. Furthermore, all oxovaleroyl phospholipids but not the glutaroyl phospholipids induced monocyte binding without an increase in vascular cell adhesion molecule-1 (VCAM-1) expression and inhibited lipopolysaccharide-induced E-selectin expression. In contrast, glutaroyl phospholipids but not oxovaleroyl phospholipids stimulated E-selectin and VCAM-1 expression. We further demonstrate that all parts of the phospholipid molecules are required for these bioactivities. Hydrolysis with phospholipase (PL) A<sub>1</sub>, PLA<sub>2</sub>, and PLC strongly reduced the bioactivities of POVPC, PGPC, and mixed isomers of PEIPC. PLD had a smaller but still significant effect. The effects of POVPC and PEIPC could be abolished by sodium borohydride treatment, indicating the importance of the reducible groups (carbonyl and epoxide) in these molecules. In summary, these studies identify 6 new bioactive, oxidized phospholipids that are increased in MM-LDL and, where measured, in atherosclerotic lesions. They thus suggest that a family of phospholipid oxidation products containing oxovaleroyl, glutaroyl, and epoxyisoprostane at the *sn*-2 position play an important role in the regulation of leukocyte-endothelial interactions, bioactivity being in part controlled by several types of phospholipid hydrolases. (*Arterioscler Thromb Vasc Biol.* 2000;20:2248-2254.)

**Key Words:** atherosclerosis ■ oxidized phospholipids ■ monocyte-endothelial interactions  
■ E-selectin ■ phospholipases

Minimally oxidized LDL (MM-LDL), in contrast to native LDL, has been shown to stimulate endothelial cells to increase monocyte-endothelial interactions.<sup>1</sup> Monocytes are the major inflammatory cells in the atherosclerotic lesion and have been shown to be important mediators of lesion initiation and progression.<sup>2,3</sup> An important characteristic of atherosclerotic lesions and other chronic, inflammatory lesions is that they contain essentially no neutrophils.<sup>4</sup> Our group has shown that the major bioactive lipids in MM-LDL were derived from oxidation of arachidonoyl phospholipids.<sup>5,6</sup> We have identified 3 bioactive, oxidized phospholipids present in oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC) as 1-palmitoyl-2-oxovaleroyl-*sn*-

glycero-3-phosphorylcholine (POVPC), 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphorylcholine (PGPC), and 1-palmitoyl-2-(5,6-epoxyisoprostane E<sub>2</sub>)-*sn*-glycero-3-phosphorylcholine (PEIPC).<sup>7,8</sup> The levels of these lipids were increased in atherosclerotic lesions in rabbit aortas, and natural antibodies to these oxidized phospholipids were present in the sera of apo E-null mice.<sup>8,9</sup>

We have observed several differences in action between POVPC and PGPC. POVPC induced monocyte binding and connecting segment-1 fibronectin expression, increased the levels of cAMP, and inhibited lipopolysaccharide (LPS)-induced E-selectin expression and neutrophil binding. The action of POVPC was inhibited by the platelet-activating

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factor receptor antagonist WEB 2086.<sup>10,11</sup> In contrast, PGPC induced both monocyte and neutrophil binding and vascular cell adhesion molecule-1 (VCAM-1) and E-selectin expression, had no effect on connecting segment-1 or cAMP, and was not inhibited by WEB 2086.<sup>10,11</sup> We further demonstrated that the effects of POVPC dominated in mixtures of the active compounds.

This report examines the molecular requirements for the specific actions of POVPC, PGPC, and PEIPC. We also identify new bioactive phospholipids with oxovaleroyl, glutaroyl, and epoxyisoprostane groups, demonstrating their role in the regulation of endothelial-leukocyte interactions. We determined that the levels of these lipids are increased in MM-LDL and atherosclerotic lesions.

## Methods

### Materials

Dimyristoyl-*sn*-glycero-3-phosphorylcholine (DMPC), dimyristoyl-*sn*-glycero-3-phosphorylethanolamine (DMPE), BHT, PMSF, soybean lipoxygenase, phospholipase (PL) A<sub>2</sub> (from *Naja naja* venom), PLC (type IX, from *Bacillus cereus*), and PLD (type I, from cabbage) were obtained from Sigma Chemical Co. PLA<sub>1</sub> (lipase from *Rhizopus arrhizus*) was obtained from Boehringer Mannheim. PAPC, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (SAPC), and heart bovine PE fraction were obtained from Avanti Polar Lipids. Glutaric acid and NaBH<sub>4</sub> were obtained from Aldrich Chemical Co.

### Lipid and Lipoprotein Modification

LDL (1.019 to 1.069 g/mL) was isolated from the sera of normal blood donors by density gradient ultracentrifugation as described previously<sup>13</sup> and stored at 4°C until use within 1 to 4 weeks of isolation. To prepare MM-LDL, LDL was pretreated with PMSF (3 mmol/L) and incubated at 1 milligram protein per milliliter in PBS at 37°C with Sepharose-bound soybean lipoxygenase (20 000 U) by using a previously described method.<sup>6</sup> PMSF did not affect the levels of native and oxidized phospholipids measured in native LDL (data not shown). To isolate SAPE, bovine heart PE fraction was treated with 0.5N HCl for 15 minutes, extracted with chloroform/methanol (2:1, vol/vol), and then dried under argon.<sup>12</sup> The residue was resuspended in chloroform and subjected to normal-phase coupled high-performance liquid chromatography–mass spectrometry (HPLC/MS, described below) to provide enriched 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylethanolamine (SAPE; charge-to-mass ratio [*m/z*] 768.5). PAPC, SAPC, and SAPE were auto-oxidized as described previously.<sup>8</sup> POVPC, PGPC, SOVPC, and SGPC were prepared by ozonolysis of PAPC and SAPC by using the previously reported method.<sup>10,14</sup> PEIPC was isolated from oxidized PAPC, and epoxyisoprostane (the oxidized fatty acid present in PEIPC) was generated by PLA<sub>2</sub> hydrolysis of PEIPC as described previously.<sup>7</sup> The concentration of bacterial endotoxin in each treatment solution was <20 pg/mL.

### Analysis of Phospholipids

Normal-phase HPLC/MS analysis for oxidized PAPC and oxidized SAPC was performed as described previously,<sup>8</sup> and oxidized SAPE was fractionated with acetonitrile/methanol/water (88:4:8, vol/vol) containing 1 mmol/L ammonium acetate. PE derivatives were less stable than the corresponding PC derivatives and thus, were used for testing immediately after isolation and quantification. An API III triple-quadrupole biomolecular mass analyzer (Perkin-Elmer Sciex Instruments) was used for mass analysis and quantification of PC derivatives as described previously.<sup>8</sup> For flow-injection analysis in the negative mode, phospholipids were dissolved in 100 μL of methanol/water (50:50, vol/vol) containing 10 mmol/L ammonium acetate and analyzed in the same solvent. For quantitative analysis, DMPC at 1 μg/10 mg tissue, 10 μg/mg lipoprotein was used as an internal standard for PC derivatives, and DMPE at 1 μg/mg lipoprotein was used as an internal standard for PE derivatives.<sup>15</sup> To validate the use of DMPC as an internal standard for quantitative

analyses, we correlated the levels of various phospholipids (PAPC, oxidized PAPC, POVPC, PGPC, and mixed isomers of PEIPC) determined by electrospray ionization–mass spectrometry analysis, and their levels determined by phosphorous assay.<sup>16</sup> The values obtained by the 2 methods differed by <5% for all chemical species except PEIPC, which differed by <10%, thus demonstrating reliable quantification of these lipids with DMPC. Similarly, we used DMPE as an internal standard for the analysis of PE derivatives.

### PL Hydrolysis and NaBH<sub>4</sub> Reduction of Oxidized Phospholipids

Oxidized phospholipids were hydrolyzed by various PLs by using a previously reported method with some modifications.<sup>7,17</sup> To standardize the hydrolysis conditions, PAPC, oxidized PAPC, and PGPC were treated with different amounts of enzymes (1, 5, and 10 U/mL, with units defined by the supplier for the standard substrates) for different times (30 minutes and 1, 6, and 18 hours). On the basis of these studies, oxidized phospholipids were treated with 10 U/mL of PLs (PLA<sub>1</sub>, PLA<sub>2</sub>, PLC, or PLD) in PBS containing 5 mmol/L CaCl<sub>2</sub> at pH 5.6, 7.0, 7.3, and 5.6, respectively). The samples were incubated at 37°C for 1 hour for PLA<sub>2</sub> or overnight for PLA<sub>1</sub>, PLC, and PLD.<sup>17</sup> to achieve 70% to 100% hydrolysis. For NaBH<sub>4</sub> reduction, oxidized phospholipids were treated with NaBH<sub>4</sub> (50 mmol/L) in PBS or acetonitrile for 30 minutes<sup>7,8</sup> to achieve 90% reduction. Phospholipid hydrolysis and NaBH<sub>4</sub> reduction were monitored by the disappearance of starting phospholipids and/or the formation of hydrolysis or reduced products.

### Cell Culture, Monocyte Adhesion Assay, and E-Selectin ELISA

Monocyte binding assays were performed as described previously<sup>1</sup> with human aortic endothelial cells (HAECs) and human monocytes, except that the cells were treated overnight with 0.8 mg/mL lipoprotein-deficient serum and binding was performed in lipoprotein-deficient serum to reduce basal HAEC activation. The cells were then rinsed twice with medium, and binding of human monocytes to HAECs was performed essentially as described previously.<sup>1</sup> VCAM-1 and E-selectin expression was measured on HAECs in 96-well dishes as described previously with the use of 10% fetal bovine serum during the incubation with oxidized phospholipids.<sup>10</sup>

### Preparation of Lipids From Rabbit Aortas and Lipoproteins

The rabbits used in this study were a subset of animals used in another study reported elsewhere.<sup>18</sup> Some rabbits (n=4) were studied while they consumed a standard chow diet, whereas other rabbits (n=6) were studied after 22 weeks on a semipurified, atherogenic diet with casein, sucrose, and butter as sources of protein, carbohydrate, and fat, respectively.<sup>18</sup> At the end of treatment with the atherogenic diet, the rabbits were exsanguinated after ketamine hydrochloride anesthesia (6 mg/kg body weight). Aortas were removed, cleaned of adventitial tissue (at 4°C), weighed, frozen at –70°C under argon, and stored protected from light.<sup>18</sup> Total lipids and phospholipids were isolated from LDL, MM-LDL, and rabbit aortas as described previously.<sup>8</sup>

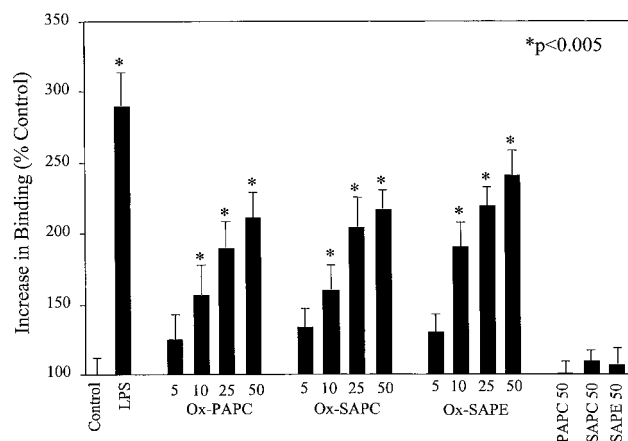
### Statistical Analysis

Data were analyzed by 1-way ANOVA, probability values were calculated by using StatView (Abacus Concepts, Inc), and *P*<0.05 was considered statistically significant.

## Results

### Comparison of Oxidation Products in Oxidized PAPC, Oxidized SAPC, and Oxidized SAPE

We first tested the hypothesis that oxidation of SAPC and SAPE would produce oxidation products similar to those in oxidized PAPC. Auto-oxidation of PAPC generated a complex mixture of products, including 3 bioactive, oxidized



**Figure 1.** Oxidized (Ox) arachidonoyl phospholipids induce monocyte-endothelial interactions. HAECs were treated for 4 hours with indicated concentrations of oxidized PAPC, oxidized SAPC, or oxidized SAPE (5 to 50  $\mu\text{g}/\text{mL}$ ); the corresponding native lipid (50  $\mu\text{g}/\text{mL}$ ); LPS (2 ng/mL) as a positive control; or medium alone (control). After incubation, monocyte binding was performed as described in Methods. Data ( $n=9$ ) were analyzed by 1-way ANOVA and are presented as percent above control mean  $\pm$  SD.

phospholipids, POVPC ( $m/z$  594.3), PGPC ( $m/z$  610.2), and mixed isomers of PEIPC ( $m/z$  828.5).<sup>7,8</sup> Here, we report that auto-oxidation of SAPC and SAPE also resulted in a similar addition of molecular oxygen or fragmentation of the arachidonate moiety at the *sn*-2 position of the phospholipid (online Figures I through III; please see [www...](#)). These results suggest that the changes at the *sn*-1 and *sn*-3 groups did not affect the oxidation patterns of arachidonoyl phospholipids. Although the  $m/z$  values of the derivatives in oxidized SAPC and oxidized SAPE suggested that they represented oxovaleroyl, glutaroyl, and epoxyisoprostane phospholipids, we confirmed this identification by using tandem mass spectrometry. Tandem mass spectrometry analyses of phospholipids generated characteristic fragment ions corresponding to the *sn*-1, *sn*-2 fatty acid groups, and an ion of lysophospholipid.<sup>8</sup> For example, the ion corresponding to 1-stearoyl-2-oxovaleroyl-*sn*-glycero-3-phosphorylcholine (SOVPC,  $\text{MH}^+$  ion with  $m/z$  622.3) in oxidized SAPC (online Figure II; please see [www...](#)) produced an  $(\text{M}-\text{CH}_3)^-$  ion at  $m/z$  606.3 in the negative mode, which gave characteristic fragments at  $m/z$  115 (oxovalerate), 283.1 (stearate), and

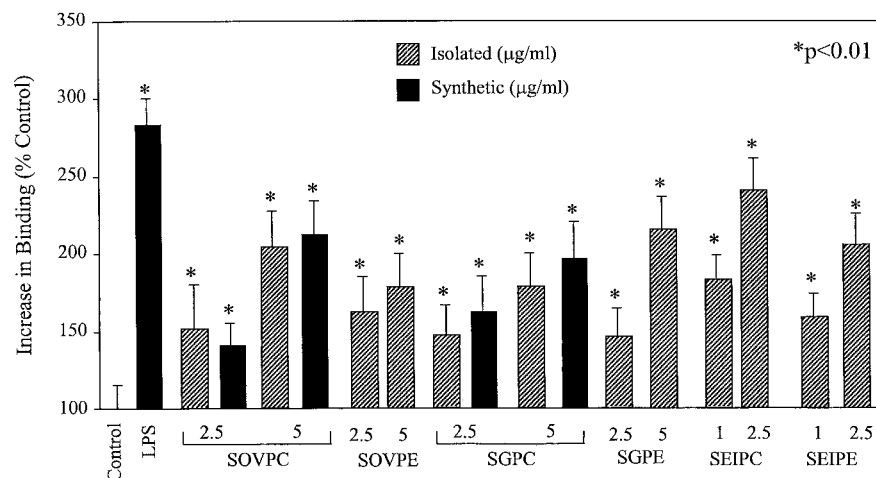
508.2  $[(\text{M}-\text{CH}_3)^-$  ion of lysophospholipid]. This result confirmed that the ion at  $m/z$  622.3 in oxidized SAPC was SOVPC. Similar analyses for ions corresponding to bioactive, oxidized phospholipids in oxidized SAPC and oxidized SAPE gave the characteristic *sn*-2 fragments at  $m/z$  115 (oxovalerate), 131 (glutarate), or 349 (epoxyisoprostane) along with ions for *sn*-1 fatty acids and lysophospholipids. These analyses confirmed the structure of molecules in oxidized SAPC as SOVPC ( $m/z$  622.3), SGPC ( $m/z$  638.2), and mixed isomers of SEIPC ( $m/z$  856.6) and in oxidized SAPE as SOVPE ( $m/z$  580.2), SGPE ( $m/z$  596.2), and mixed isomers of SEIPE ( $m/z$  814.6).

### Effect of Oxidized Phospholipids on Monocyte Binding

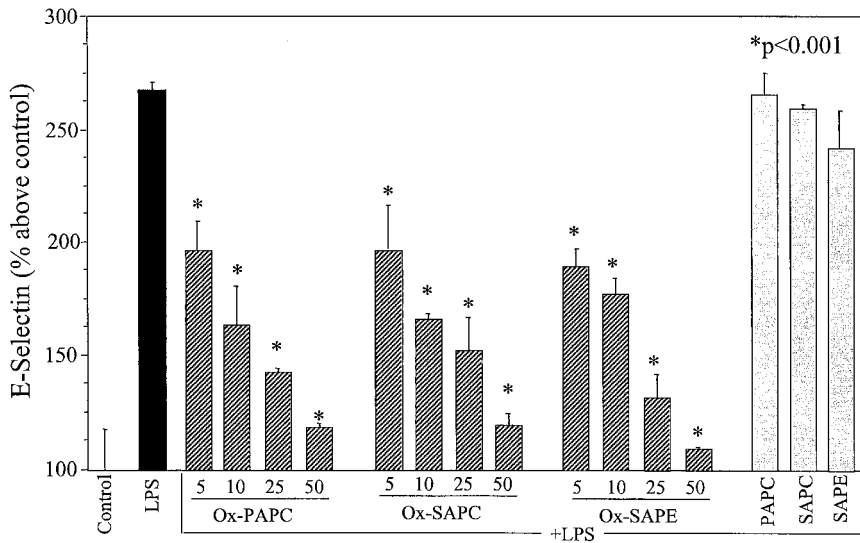
Treatment of endothelial cells with oxidized arachidonoyl phospholipids (PAPC, SAPC, and SAPE at 5 to 50  $\mu\text{g}/\text{mL}$ ) for 4 hours at 37°C induced binding of monocytes in a dose-dependent fashion. The corresponding native arachidonoyl phospholipids (50  $\mu\text{g}/\text{mL}$ ) were inactive (Figure 1). We fractionated oxidized SAPC and oxidized SAPE by normal-phase HPLC/MS and tested the fractions for their ability to stimulate endothelial cells to bind monocytes. The fractions containing the molecules with  $m/z$  values corresponding to oxovaleroyl, glutaroyl, and epoxyisoprostane derivatives (determined by reconstructed selective-ion chromatograms) stimulated endothelial cells to bind monocytes. The bioactivity of oxidized phospholipids present in oxidized SAPC and oxidized SAPE was further confirmed by dose-response studies with known amounts of isolated and synthetic molecules. SOVPC, SGPC, and mixed isomers of SEIPC ( $m/z$  856.6) were isolated from oxidized SAPC, and fractions that were enriched in SOVPE, SGPE, and mixed isomers of SEIPE ( $m/z$  814.5) were isolated from oxidized SAPE and tested for their ability to induce monocyte binding. These isolated molecules showed a dose-dependent increase in monocyte binding. Synthetic SOVPC and SGPC also showed a similar dose-dependent increase in monocyte binding similar to the corresponding isolated molecules (Figure 2).

### Effect of Oxidized Phospholipids on E-Selectin and VCAM-1 Expression

As was previously reported for oxidized PAPC, oxidized SAPC, and oxidized SAPE, all (5 to 50  $\mu\text{g}/\text{mL}$ ) effectively



**Figure 2.** Oxovaleroyl, glutaroyl, and epoxyisoprostane phospholipids induce monocyte-endothelial interactions. HAECs were treated for 4 hours with indicated amounts of isolated SOVPC, SGPC, SEIPC, SOVPE, SGPE, or SEIPE; synthetic SOVPC, synthetic SGPC, or LPS (2 ng/mL); or medium alone (control). After incubation, a monocyte binding assay was performed as described in Methods. Data ( $n=9$ ) were analyzed by 1-way ANOVA and are presented as percent above control mean  $\pm$  SD.



**Figure 3.** Oxidized (Ox) arachidonoyl phospholipids inhibit LPS-induced E-selectin expression. HAECs were treated for 4 hours with LPS (2 ng/mL); cotreated with the indicated amounts of oxidized PAPC, oxidized SAPC, oxidized SAPE (5 to 50 μg/mL), or the corresponding native phospholipids (50 μg/mL) and LPS (2 ng/mL); or treated with medium alone (control). After incubation, the medium was removed and an E-selectin ELISA was performed. Data (n=4) were analyzed by 1-way ANOVA and are presented as percent above control mean±SD.

inhibited the action of LPS on E-selectin expression in a dose-dependent fashion, whereas the corresponding native arachidonoyl phospholipids (50 μg/mL) did not have any significant effect (Figure 3). Oxovaleroyl phospholipids alone caused a similar inhibition. POVPC, SOVPC, and SOVPE (1 to 5 μg/mL) dose-dependently inhibited LPS-induced E-selectin expression (online Figure IV; please see www. . . ). In contrast, PGPC was previously shown to induce E-selectin, VCAM-1 expression, and binding of polymorphonuclear neutrophils to HAECs.<sup>10</sup> Here, we demonstrate that PGPC, SGPC, and SGPE all induced E-selectin and VCAM-1 on HAECs (Figure 4). However, oxovaleroyl phospholipids alone did not induce significant levels of VCAM-1 and E-selectin on HAECs (data not shown). These results showed that the changes at the *sn*-1 and *sn*-3 positions did not affect the ability of oxovaleroyl phospholipids to inhibit LPS action and glutaroyl phospholipids to stimulate E-selectin or VCAM-1 expression.

**Comparison of Phospholipids in LDL and MM-LDL**

POVPC, SOVPC, PGPC, SGPC, mixed isomers of PEIPC, and mixed isomers of SEIPC were increased by 2- to 11-fold in MM-LDL phospholipids compared with native LDL phospholipids (the Table , columns 1 and 2). The levels of

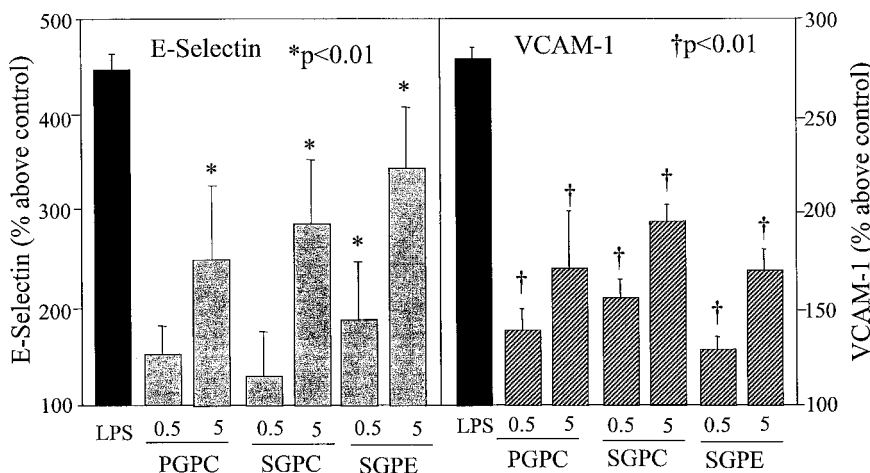
SOVPE, SGPE, and mixed isomers of SEIPE were increased by 3- to 5-fold in MM-LDL lipids compared with native LDL lipids. The levels of bioactive PC derivatives detected were ≈10- to 25-fold higher than those of the corresponding PE derivatives. The bioactive, oxidized phospholipids represent a small fraction of the arachidonoyl phospholipid oxidation products (5% to 10%). However, this amount is sufficient to account for the bioactivity of MM-LDL.

**Comparison of Phospholipid Levels in Normal Rabbit Aorta and Rabbit Atherosclerotic Lesions**

Cholesterol, PAPC, and SAPC were all increased in the rabbits fed the atherogenic diet. The levels of bioactive, oxidized phospholipids, POVPC, PGPC, mixed isomers of PEIPC, SOVPC, SGPC, and mixed isomers of SEIPC were increased by 3- to 6-fold in animals fed the atherogenic diet (the Table , columns 3 and 4). These levels correspond to ≈116, 62, and 85 μg/mL POVPC, PGPC, and mixed isomers of PEIPC, respectively (by approximate conversion of wet weight to volume). This level is roughly 10 to 20 times that required to activate the endothelium.

**Effects of PLs and NaBH<sub>4</sub> on the Bioactivity of Oxidized Phospholipids**

To further identify the structural motifs of oxidized phospholipids that are important for their bioactivity, we tested the



**Figure 4.** Glutaroyl phospholipids induce surface expression of E-selectin and VCAM-1 on HAECs. HAECs were treated for 4 hours with LPS (2 ng/mL) or the indicated amounts of PGPC, SGPC, or SGPE in medium or in medium alone (control). After incubation, the medium was removed and E-selectin or VCAM-1 ELISA was performed. Data (n=4) were analyzed by 1-way ANOVA and are presented as percent above control mean±SD.

## Levels of Bioactive, Oxidized Phospholipids in MM-LDL and in Rabbit Lesions

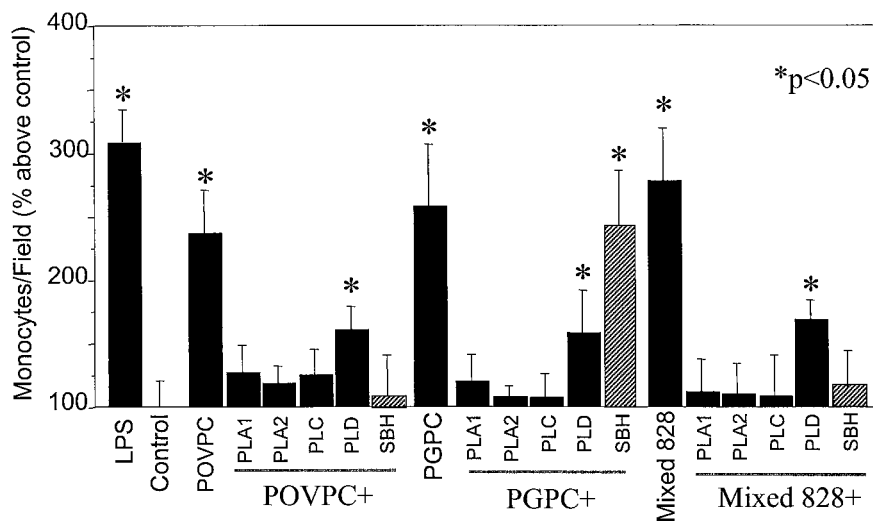
Oxidized Phospholipid, <i>m/z</i> value	Oxidized Phospholipids, ng/mg of Lipoprotein or Wet Weight			
	LDL	MM-LDL	Chow Diet-Fed Rabbit Aorta	Atherogenic Diet-Fed Rabbit Aorta
POVPC 594.3	172.4±6.6	985.6±57.6	26.8±3.4	116.2±35.5
PGPC 610.2	168.4±7.8	1966.4±132.4	20.9±2.2	61.6±16.8
PEIPC 828.6	350.9±19.9	742.1±67.9	15.6±1.2	85.3±32.6
SOVPC 622.3	83.7±9.7	650.3±77.6	15.5±3.8	70.1±19.9
SGPC 638.2	118.7±19.1	1004.3±111.8	15.1±3.5	44.6±9.9
SEIPC 856.6	197.8±13.0	363.6±28.3	14.1±2.0	58.1±13.0
SOVPE 578.2	8.9±0.6	30.0±4.8	ND	ND
SGPE 594.2	14.4±2.4	82.0±8.4	ND	ND
SEIPE 812.6	14.3±0.5	71.8±6.1	ND	ND

Columns 1 and 2 are quantitative analyses of PC (in the positive mode) and PE (in the negative mode) derivatives in LDL and MM-LDL lipids (n=3) performed by electrospray ionization-MS as described in Methods. Column 3 and 4 are quantitative analyses of PC derivatives in the phospholipid extracts of rabbit aorta performed by electrospray ionization-MS as described in Methods. Aortas from rabbits fed a chow diet (n=4) or an atherogenic diet (n=6) were used. ND indicates not determined. The results are presented as nanograms of oxidized phospholipids per milligram of lipoprotein (mean±SD) or per milligram of wet weight (mean±SEM). PEIPC, SEIPC, and SEIPE represent mixed isomers.

effects of chemical modification and enzymatic digestion. Treatment of oxidized phospholipids with NaBH<sub>4</sub> reduced the oxygenated groups such as aldehydes, ketones, and epoxides.<sup>7,8</sup> The specific PLs PLA<sub>1</sub>, PLA<sub>2</sub>, PLC, and PLD were shown to selectively hydrolyze the *sn*-1, *sn*-2, and *sn*-3 groups and the phosphate-choline bond, respectively<sup>19</sup> (online Figure V; please see www. . . ). In the present study, we tested the effects of PL and NaBH<sub>4</sub> treatments on the bioactivity of POVPC, PGPC, and mixed isomers of PEIPC by a monocyte binding assay (Figure 5). Treatment of these 3 active lipids with PLA<sub>1</sub>, PLA<sub>2</sub>, and PLC completely abolished their bioactivity, whereas PLD partially reduced their bioactivity. NaBH<sub>4</sub> treatment abolished the activity of POVPC and mixed isomers of PEIPC; however, the activity of PGPC was unaltered (Figure 5). These studies suggest that the aldehyde group in POVPC and reducible groups in PEIPC (carbonyl and epoxide) are important for their bioactivity. Taken together, these studies show that the *sn*-1, *sn*-2, and *sn*-3

groups are essential for their effects on monocyte binding; however, hydrolysis with PLD only partially reduced activity. The ability of POVPC (5 μg/mL) to inhibit LPS-induced E-selectin expression was strongly reduced by all 4 PLs and NaBH<sub>4</sub> (online Figure VI; please see www. . . ). Similarly, PGPC- (5 μg/mL) induced E-selectin expression was also significantly reduced by all 4 PLs; however, NaBH<sub>4</sub> had no significant effect (online Figure VI; please see www. . . ).

The common structural motifs for the bioactive, oxidized phospholipids are the presence of oxovaleroyl, glutaroyl, and an epoxyisoprostane at the *sn*-2 position; hence, we examined the effects of the isolated *sn*-2 fatty acids on monocyte adhesion. Oxovaleric acid (1 to 10 μmol/L, fatty acid from POVPC), glutaric acid (1 to 10 μmol/L, fatty acid from PGPC), and epoxyisoprostane (1 to 3 μmol/L, fatty acid from PEIPC) did not induce monocyte binding at the concentrations at which the corresponding intact, oxidized, phospholipids are maximally active (data not shown).



**Figure 5.** Effects of PL and NaBH<sub>4</sub> treatment on the activity of oxidized phospholipids. HAECs were treated for 4 hours with oxidized phospholipids, POVPC (5 μg/mL), PGPC (5 μg/mL), mixed isomers of PEIPC (2.5 μg/mL), with or without exposure to various PLs (PLA<sub>1</sub>, PLA<sub>2</sub>, PLC, and PLD) or NaBH<sub>4</sub> (as described in Methods). After incubation, monocyte binding was performed as described in Methods. Data (n=9) were analyzed by 1-way ANOVA and are presented as percent above control mean±SD.

## Discussion

These studies were designed to (1) examine the effects of structural changes and hydrolytic enzymes on the actions of bioactive, oxidized phospholipids and (2) to quantify the levels of these lipids in MM-LDL and in atherosclerotic lesions. In previous studies, we identified 3 oxidized phospholipids primarily responsible for the activity of oxidized PAPC.<sup>7,8</sup> Although PAPC is the major arachidonoyl phospholipid in native human LDL, SAPC and SAPE are also present. We now provide evidence for the formation of bioactive, oxidized phospholipids from the oxidation of SAPC and SAPE and for their increased levels in MM-LDL (Figure 1 and the Table).

In the present study, we have presented evidence that the *sn*-2 position of the bioactive, oxidized phospholipids is the main determinant of the type of activity shown by the lipid. The changes in saturated fatty acid at the *sn*-1 position and in the head group at the *sn*-3 position did not affect bioactivity (Figures 1 and 2). We have demonstrated that oxovaleroyl-, glutaroyl-, and epoxyisoprostane-containing phospholipids all stimulated endothelial cells to bind monocytes (Figure 2). Oxovaleroyl phospholipids were effective in inhibiting LPS induced E-selectin expression (online Figure IV; please see www. . . ). In contrast, glutaroyl phospholipids induced the surface expression of E-selectin and VCAM-1 in HAECs (Figure 4). Thus, 2 types of oxidized phospholipids, whether derived from PAPC, SAPC, or SAPE, have quite different effects, and their relative levels could control the recruitment of specific leukocytes at the sites of inflammation.

The present study demonstrates that all 3 groups, *sn*-1, *sn*-2 and *sn*-3, of bioactive, oxidized phospholipids are important for bioactivities (Figure 5). Our group has previously shown that the 2 hydrolytic enzymes, platelet-activating factor-acetyl hydrolase and paraoxonase, were able to destroy the bioactivity of oxidized phospholipids.<sup>5,6</sup> Studies with NaBH<sub>4</sub> demonstrate that the reducible functional groups (carbonyl and epoxide) are essential for bioactivity. However, certain changes at the *sn*-3 position (PC to PE) do not alter their activity, and cleavage of the choline moiety of PC by PLD partially reduced its bioactivity (Figures 4 and 5). The past and present studies show that neither lysophospholipids<sup>6</sup> nor the *sn*-2 fatty acids in the free form were active in inducing monocyte binding. Lysophosphatidic acid has been shown to exert a variety of biological responses, including stimulation of endothelial cells to bind monocytes and neutrophils and to induce E-selectin and VCAM-1 expression.<sup>20</sup> The formation of lysophosphatidic acid and/or lysophosphatidic acid analogues after treatment of oxidized phospholipids with PLD may explain the partial, and not complete, loss of bioactivity (Figure 5).

Our present and past data demonstrate that the bioactive, oxidized phospholipids responsible for stimulating monocyte binding and inhibiting neutrophil binding are increased in atherosclerotic lesions. The presence of oxidized LDL in rabbit and human atherosclerotic lesions<sup>21</sup> and increased levels of total phospholipids and lysophosphatidylcholine in rabbit lesions<sup>22</sup> have been previously demonstrated. In the present study, the levels of oxidized phospholipids in individual aortas were highly correlated with the accumulation of cholesterol and unoxidized phospholipids (data not shown).

One interpretation of this finding is that increased levels of phospholipid oxidation products in the vessel wall are due to the increase in phospholipid, which serves as the oxidative substrate. However, a number of other mechanisms may explain the increased oxidation observed. In general, our results suggest that these oxidized phospholipids in the vessel wall could serve as specific markers of oxidative injury in vivo.

In summary, these results identify 6 new bioactive, oxidized phospholipids that have specific effects on leukocyte-endothelial cell interactions. We have demonstrated that the specificity is determined by the *sn*-2 groups of oxidized phospholipids. The levels of all 6 bioactive, oxidized phospholipids are increased in MM-LDL, and the levels of at least 3 bioactive, oxidized phospholipids are increased in atherosclerotic lesions, suggesting a role for these newly identified bioactive, oxidized phospholipids in atherogenesis. Finally, these results demonstrate the importance of the integrity of the *sn*-1, *sn*-2, and *sn*-3 positions in the activation of endothelial cells.

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