Evidence That Phospholipid Oxidation Products and/or Platelet-Activating Factor Play an Important Role in Early Atherogenesis

In Vitro and In Vivo Inhibition by WEB 2086

Ganesamoorthy Subbanagounder,* Norbert Leitinger,* Peggy T. Shih, Kym F. Faull, Judith A. Berliner

Abstract—The goal of the present studies was to determine whether phospholipid oxidation products and/or platelet-activating factor (PAF) are mediators of early atherogenesis in vivo. Monocyte–endothelial cell interactions have been shown to play an important role in early atherogenesis. We and others have demonstrated that PAF and phospholipid oxidation products, present in atherosclerotic lesions, including 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC), and 1-palmitoyl-2-epoxyisoprostane E2-sn-glycero-3-phosphocholine (PEIPC), mediate the activation of monocytes and/or endothelial cells in vitro. Previous studies have shown that the action of PAF and PAF-like ether-containing phospholipids was inhibited by WEB 2086. We now demonstrate that pretreatment of human aortic endothelial cells with WEB 2086 (10 μmol/L) and several other PAF antagonists before treatment with POVPC and PEIPC but not PGPC prevented the activation of the endothelial cells to bind monocytes. We present evidence to suggest that this inhibition is not mediated by the PAF receptor. The role of bioactive oxidized phospholipids in fatty streak formation was tested using C57BL/6J LDL R−/− mice fed a chow or Western diet for 5 weeks with or without WEB 2086 mixed with drinking water. Quantitative electrospray ionization mass spectrometry showed similar concentrations of WEB 2086 in the plasma of mice on both diets (≈4 to 5 μmol/L); this concentration was inhibitory in vitro. Administration of WEB 2086 did not affect the lipid composition of mouse plasma. However, fatty streak formation was reduced by 62% in animals fed a Western diet, whereas no change was observed in the small lesions of mice on a chow diet. These studies provide evidence that PAF and/or PAF-like phospholipid oxidation products are important mediators of atherosclerotic lesion development in vivo and that specific receptor antagonists for these molecules may represent a novel therapeutic modality. (Circ Res. 1999;85:311-318.)

Key Words: atherosclerosis ■ oxidized phospholipid ■ platelet-activating factor ■ WEB 2086 ■ platelet-activating factor–like lipid

The goal of the present studies was to examine the role of platelet-activating factor (PAF) and phospholipid oxidation products in the initiation of atherosclerosis. Although cholesterol accumulation has been the focus of many previous studies, phospholipids1 and their oxidation products2,3 are also increased in early lesions. Our group has shown that three biologically active phospholipid oxidation products, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC), and 1-palmitoyl-2-epoxyisoprostane E2-sn-glycero-3-phosphocholine (PEIPC), are present in minimally modified LDL (MM-LDL) and increased in atherosclerotic lesions.4,5 PEIPC is one of five isomers with a molecular mass of 828.5 Da. Lyso-phosphatidylcholine, another phospholipid oxidation product, has also been shown to be increased in atherosclerotic lesions.1 PAF is a phospholipid with proinflammatory and thrombogenic properties. PAF levels were found to be higher in the coronary arteries from some patients with severe atherosclerosis.6 However, the levels of PAF in early atherosclerotic lesions are not known. There are several reports demonstrating the generation of ether- and ester-containing PAF-like lipids during oxidation of LDL7–9 and polyunsaturated fatty acid–containing phospholipids.10–13 Smoking, a risk factor for atherosclerosis, has been shown to cause an increase in the levels of PAF and PAF-like lipids in plasma lipoproteins.14

Received January 25, 1999; accepted June 11, 1999.

*Both authors contributed equally to this study.

From the Departments of Medicine/Cardiology (G.S., N.L., P.T.S., J.A.B.), Pathology (J.A.B.), and Psychiatry and Biobehavioral Sciences and the Neuropsychiatric Institute (K.F.F.), University of California, Los Angeles, Calif.

Correspondence to Ganesamoorthy Subbanagounder, PhD, Department of Medicine/Cardiology, Center for the Health Sciences, Room 47-123, UCLA Medical Center, Los Angeles, CA 90095-1679. E-mail gsubbana@pathology.medsch.ucla.edu

© 1999 American Heart Association, Inc.

Circulation Research is available at http://www.circresaha.org

311
tions. PAF and PAF-like ether lipids can activate neutrophils and monocytes to bind to endothelial cells, and POVPVC, PGPC, and PEIPC at concentrations between 10^{-6} and 10^{-7} mol/L activate endothelial cells to bind monocytes and stimulate synthesis of monocyte chemoattractant protein-1 (MCP-1). Lyso-phosphatidylcholine has also been shown to activate monocyte–endothelial cell interactions by increasing expression of the vascular cell adhesion molecule-1 (VCAM-1). PAF and, in some assays, PAF-like oxidized phospholipids have previously been shown to activate monocytes via the PAF receptor. These effects were blocked by WEB 2086, a previously identified PAF receptor antagonist, WEB 2170, a structurally related PAF receptor antagonist to WEB 2086, was shown to strongly inhibit leukocytes to the endothelium of venules and arterioles in hamsters. In previous studies, we reported that WEB 2086 inhibited the ability of oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (Ox-PAPC) and partially purified POVPVC and 828.5 (bioactive oxidized phospholipid with a molecular mass of 828.5 Da) mixed isomers to activate endothelial cells to bind monocytes. However, because levels of the partially purified oxidized phospholipids were not determined, WEB 2086 concentrations might not have been appropriate to obtain specific inhibition. We now demonstrate strong inhibition by WEB 2086 at appropriate concentrations. The in vitro studies cited above taken together demonstrate that PAF and PAF-like lipids including POVPVC and PEIPC activate monocytes and/or endothelial cells to increase monocyte–endothelial cell interactions. In the present study, we document the importance of this activation in vivo by showing that WEB 2086 inhibits fatty streak development in the LDL receptor null mouse fed a Western diet.

Materials and Methods

Materials

PAF, PAF receptor antagonists, FR 49175, trans-2,5-bis-(3,4,5-trimethoxyphenyl)-1,3-dioxolane, PCA 4248, octylonium bromide, 1-O-hexadecyl-2-acetyl-N,N-trimethoxyphenyl)-1,3-dioxolane, PCA 4248, octylonium bromide, and a suspension of human monocytes (2 to 3 × 10^5 /well) from Biomol Research Laboratories, Inc.

Cell Culture and Monocyte Adhesion Assay

Human aortic endothelial cells (HAECs) were cultured as previously described. In all experiments, HAECs were used at passage levels from 4 through 7. Blood monocytes were obtained by modification of the Recalde procedure, and binding assays of human monocytes to endothelial cells were performed as previously reported. In most studies, HAECs were treated with WEB 2086 at the concentrations indicated for 1 minute before the addition of active lipids. Cells were treated with Ox-PAPC (125 μg/mL), POVPVC (8.4 μmol/L), PGPC (8.2 μmol/L), or PEIPC (3.6 μmol/L) for 4 hours at 37°C with and without WEB 2086 or other inhibitor pretreatment. Lipopolysaccharide (LPS; 2 ng/mL) was used as a positive control. After the treatment media were removed, the cells were rinsed twice with medium, and a suspension of human monocytes (2 to 3 × 10^5 /well) was added for 12 minutes. Unbound monocytes were removed by washing, and the number of bound monocytes was determined by counting under a microscope. In one set of experiments, WEB 2086 at 10 nmol/L and 10 μmol/L was added to HAECs during monocyte binding; thus, both endothelial cells and monocytes were exposed to WEB 2086.

Animals, Diets, and WEB 2086 Treatment

All animal procedures were conducted in accordance with regulations of the University of California Animal Research Committee. Ten-week-old female LDL R−/− mice (Jackson Laboratories, Bar Harbor, Maine) of a C57BL/6J background were individually housed in a controlled temperature room with a 12-hour light/dark cycle. All mice were kept on a standard chow diet containing 4% fat (Purina No. 5001) until the start of the study. Mice were then separated into the following four groups: (1) chow diet with regular water, (2) chow diet with WEB 2086 in the water, (3) Western diet (Food-Tek, Inc) with regular water, and (4) Western diet with WEB 2086 in the water. For groups 2 and 4, WEB 2086 (4.3 mg in 100 mL) was given 48 hours before initiation of group 4 on the Western diet. WEB 2086 (4.3 mg in 100 mL) was continued in the drinking water for 35 days. The water was changed weekly, and the bottles were covered with aluminum foil. After 5 weeks on the diet, animals were fasted for 18 hours before they were killed. Plasma samples were collected by orbital bleeding, and the hearts were isolated, embedded in OCT, and stored at −78°C until sectioned. WEB 2086 was kindly provided by Boehringer Ingelheim Pharmaceuticals, Inc (Ridgefield, Conn).

Plasma Lipid Levels

A fasting blood draw was collected from the mice on the day they were killed by previously established methods. Total cholesterol, unesterified cholesterol, HDL cholesterol, free fatty acid, and tri-glyceride levels were determined by previously described enzymatic methods.

Electrospray Ionization Mass Spectrometry (ESI-MS)

A triple-quadrupole biomolecular mass analyzer (API III, Perkin-Elmer Sciei Instruments) was used for mass analysis and quantification of WEB 2086 in plasma samples. Mouse plasma samples (50 μL) were extracted with chloroform/methanol (2:1, vol/vol) containing 0.01% BHT at room temperature. The lower organic layer was isolated, the solvent was evaporated under a stream of nitrogen, and the samples were stored at −78°C until analysis. For flow injection analysis, plasma extracts (from 50 μL of plasma) were dissolved in 50 μL of acetonitrile/water/trifluoroacetic acid (50:50:0.1, vol/vol/vol), after addition of triazolam (0.5 μg/10 μL of solvent) as an internal standard, and 20 μL was injected into the electrospray ion source at a flow rate of 20 μL/min (solvent: acetonitrile/water/trifluoroacetic acid 50:50:0.1, vol/vol/vol). Ratio of signal intensities for ions at m/z (mass/charge ratio) 456.1 and 458.1 (WEB 2086) to triazolam (m/z 343.1, 345.1, and 347.1) was computed from the reconstructed ion profiles for each sample, and the concentration of WEB 2086 was calculated with respect to triazolam. To confirm the ions detected at m/z 456.1 and 458.1 from mouse plasma were WEB 2086, we performed tandem mass spectrometry (MS/MS) analysis. For MS/MS analysis, total lipid extracts from 50 μL of plasma or WEB 2086 (1 μg) were analyzed in 50 μL acetonitrile/water/trifluoroacetic acid (50:50:0.1, vol/vol/vol).

Measurement of Lipid Accumulation in Frozen Sections of C57BL/6J LDL R−/− Mice

Animals were killed and hearts were perfused and removed as described above. After perfusion and removal from the body cavity, the hearts were not fixed but quickly embedded into OCT freezing medium and placed at −78°C. The hearts were sectioned at 10 μm in thickness starting at the level of the mitral valves and ending at the descending aorta. All of the sections, including those that spanned the aortic sinus (40 sections), were stained with Oil Red O for lipids. The area covered by lipids was assessed using the NIH Image program. Sections corresponding to the region of the aortic sinus were captured by an Olympus BH-2 video camera and measured using the Optimas 6.1 program.

Measurement of Lipid Accumulation in Frozen Sections of C57BL/6J LDL R−/− Mice

Animals were killed and hearts were perfused and removed as described above. After perfusion and removal from the body cavity, the hearts were not fixed but quickly embedded into OCT freezing medium and placed at −78°C. The hearts were sectioned at 10 μm in thickness starting at the level of the mitral valves and ending at the descending aorta. All of the sections, including those that spanned the aortic sinus (40 sections), were stained with Oil Red O for lipids. The area covered by lipids was assessed using the NIH Image program. Sections corresponding to the region of the aortic sinus were captured by an Olympus BH-2 video camera and measured using the Optimas 6.1 program.
Phospholipid Preparations
MM-LDL was prepared according to previously reported methods. 

PAPC was obtained from Avanti Polar Lipids, Inc, or Sigma. Ox-PAPC, POVPC, and PGPC were synthesized as described previously. 

The molecule of mass 828.5 was isolated using normal-phase, high-performance liquid chromatography/mass spectrometry (HPLC/MS) and reverse-phase HPLC/MS. Normal-phase HPLC/MS was performed using a preparative column (Adsorbosphere, 250 mm×10 mm, 5 μm; Alltech Associates, Inc) at a flow rate of 5.0 mL/min. Phospholipids were applied in chloroform and eluted isocratically with acetonitrile/methanol/water (77:8.15, vol/vol/vol). 

Fractions enriched in m/z 828.5 were collected from the column, dried under a stream of nitrogen, and the biologically active isomer of m/z 828.5 (PEIPC), which is one of the five isomers of m/z 828.5, was further purified by reverse-phase HPLC/MS. 

PEIPC was quantitated by ESI-MS using dimyristoyl-sn-glycero-3-phosphocholine as an internal standard.

Statistical Analysis
Data were analyzed using the Statview 4.5 program. All probability values were calculated using ANOVA and Fisher’s PLSD significance test.

Results
Effect of PAF Receptor Antagonists on Monocyte Binding to HAECs
The goal of the present studies was to examine the effect of WEB 2086 and other PAF receptor antagonists on POVPC, PGPC, and PEIPC activation of endothelial cells to bind monocytes. For these studies, we have used POVPC and PGPC that was obtained by chemical synthesis and PEIPC that was isolated from Ox-PAPC by normal-phase HPLC/MS and reverse-phase HPLC/MS. 

To determine the effects of WEB 2086 on the actions of POVPC, PGPC, and PEIPC, HAECs were preincubated with the indicated concentrations of WEB 2086 for 1 minute and then incubated for 4 hours in the presence of WEB 2086 and LPS or active lipids. The concentrations of active lipids were selected based on the dose-response curves obtained for their ability to induce endothelial cells to bind monocytes (data not shown). WEB 2086 had no effect on binding to untreated cells (data not shown) or to LPS-treated cells (Figure 1). Treatment of HAECs with WEB 2086 (10 μmol/L) inhibited the effect of POVPC (8.4 μmol/L) and PEIPC (3.6 μmol/L) to induce monocyte binding by 96% and 57%, respectively (Figure 1). The effect of PGPC (8.2 μmol/L) was not inhibited by WEB 2086 (Figure 1). No additional inhibition was seen at higher concentrations of WEB 2086 (data not shown). The inhibitory effect of WEB 2086 was dose dependent, inhibiting 65% of POVPC action at 100 nmol/L. The effects of additional PAF receptor antagonists were also tested using POVPC as an activator. PAF receptor antagonists (10 μmol/L), trans-2,5-bis-(3,4,5-trimethoxyphenyl)-1,3-dioxolane, octylonium bromide, 1-O-hexadecyl-2-acetyl-sn-glycero-3-phospho-(N,N,N trimethyl)hexamethyamine, CV 6209, and WEB 2086 inhibited 25%, 33%, 46%, 73%, and 96% of the action of POVPC (8.4 μmol/L), respectively. Two PAF receptor antagonists, FR 49175 and PCA 4248, caused no significant inhibition of the ability of POVPC to induce endothelial cells to bind monocytes.

Role of the PAF Receptor in WEB 2086 Inhibition
We examined in more detail the question of whether the endothelial PAF receptor was responsible for the observed effects of bioactive oxidized phospholipids on monocyte binding. With our standard stimulation medium (M199 +10% FBS), 10 μmol/L PAF did not stimulate monocyte binding (Figure 2). However, it was possible that platelet-activating factor-acetylhydrolase (PAF-AH) present in the FBS of our medium could have partially degraded the PAF. Therefore, we treated HAECs with 5 μmol/L PAF in medium with or without the addition of WEB 2086 (concentrations as indicated). LPS was used as a positive control. WEB 2086 was added to HAECs 1 minute before addition of oxidized phospholipids or LPS. Monocyte adhesion was performed as described in Materials and Methods. These data are representative of 4 experiments that gave similar results. For each condition, n=12. Data are expressed as a percentage of control (mean±SD), where control represents cells treated with medium only. *P<0.001 as calculated by one-way ANOVA.
without serum and again found PAF to be ineffective under both conditions (control: 20±1; PAF with serum: 21±3; PAF without serum: 23±2 [monocytes per field±SE]). Furthermore, PAF receptor expression was not detected in Northern blots that had 15 μg/fane total HAEC RNA, whereas the monocyte PAF receptor was readily detectable (data not shown). Although PAF could not activate monocyte binding, it was possible that it could compete with the bioactive lipids for binding to the relevant receptor and thus reduce the activity. To test this possibility, HAECs were treated with a combination of PAF and Ox-PAPC, POVPC, or PGPC; the effect on monocyte binding was then determined (Figure 2). No significant reduction in binding in seen in the presence of PAF.

We also examined the possibility that the monocyte PAF receptor might be involved in WEB 2086 inhibition of monocyte binding to endothelial cells treated with the phospholipid oxidation products. The hypothesis being tested was that treatment of endothelial cells with POVPC (used as a test agonist) increased endothelial surface expression of PAF, which induced monocyte binding. In endothelial cells preincubated with WEB 2086, some of that antagonist might remain attached to endothelial cells and inhibit monocyte binding. Alternatively, monocyte- monocyte interaction by the PAF receptor might contribute to monocyte binding. We tested this in 2 ways. In one study, endothelial cells incubated for 4 hours with 10 μmol/L WEB 2086 and POVPC were washed 3 times (as opposed to the single rinse used in previous studies) to further reduce levels of residual antagonist; then a monocyte binding study was performed. This additional washing did not reduce the inhibitory effect of WEB 2086 (96% inhibition). In a separate study, endothelial cells were untreated, treated with POVPC, or treated with LPS for 4 hours in the absence of WEB 2086. Monocytes were then suspended in 10 μmol/L or 10 nmol/L WEB 2086 and added to the endothelial cells. At 10 nmol/L (a concentration higher than that would be expected after washing WEB 2086–pretreated endothelial cells 3 times), there was no effect on monocyte binding. At 10 μmol/L, WEB 2086 caused an approximately 12% inhibition of both LPS and POVPC action (LPS: 103±5; LPS with 10 nmol/L WEB 2086: 100±3; LPS with 10 μmol/L WEB 2086: 91±3; POVPC: 47±2; POVPC with 10 nmol/L WEB 2086: 47±3; and POVPC with 10 μmol/L WEB 2086: 41±2 [monocytes per field±SE]). Thus, these studies suggested that neither the PAF receptor on endothelial cells nor on monocytes was involved in WEB 2086 inhibition.

In Vivo Studies on the Effect of WEB 2086

Given that WEB 2086 was shown to reduce strongly monocyte–endothelial cell interactions in vitro, we examined its effect in vivo. Ten-week-old female C57BL/6j LDL R−/− mice were placed on a chow diet or Western diet for 5 weeks; half of the mice received normal drinking water and half received drinking water containing WEB 2086. The animals were caged separately, and thus we could compare the amount of food and water intake by the animals. The general health and size were comparable, and no effect of WEB 2086 was observed. Previous in vivo studies from other groups indicated that WEB 2086 was relatively stable in plasma. WEB 2086 and triazolam were detected by positive-ion mode of ESI-MS. WEB 2086 was detected as a positive ion (MH+ [protonated molecule], m/z 456.1 and 458.1) relative to triazolam (MH+, m/z 343.1, 345.1, and 347.1) (Figure 3). Equivalent levels of WEB 2086 were detected in plasma from mice on the chow diet supplemented with WEB 2086 in the drinking water (1.98±1.30 μg/mL, 4.3 μmol/L) as well as in mice on the Western diet supplemented with WEB 2086 in the drinking water (2.38±1.06 μg/mL, 5.2 μmol/L) (Table). To further confirm that the ions detected at m/z 456.1 and 458.1 from mice plasma were WEB 2086, we performed MS/MS experiments by ESI-MS. Analysis of the m/z 456.1 parent from both WEB 2086 and mice plasma extract showed characteristic daughter ions at m/z 341.1, 327.0, and 308.1 (Figure 4A and 4B). Similarly, analysis of m/z 458.1 parent from both WEB 2086 and mice plasma extract showed characteristic daughter ions at m/z 343.0, 329.0, and 310.0 (Figure 4C and 4D).

Lipid analysis of the plasma showed higher levels of total cholesterol, unesterified cholesterol, and triglycerides in the mice on the Western diet than the mice on the chow diet. WEB 2086 did not significantly alter cholesterol levels or lipoprotein profiles. No significant differences were found in free fatty acid and HDL cholesterol levels between the various groups (Table).
The formation of fatty streak lesions in the heart valves of these animals was then examined. Animals on the chow diet with or without WEB 2086 in the drinking water had small fatty streak lesions (Figure 5A and 5B). Animals on the Western diet without WEB 2086 had much larger fatty streak lesions (Figure 5C) but no fibrous plaques. In animals on the Western diet given WEB 2086 in the drinking water, the fatty streaks were smaller (Figure 5D).

### Analysis of Plasma Lipid and WEB 2086 Levels in Mice

<table>
<thead>
<tr>
<th>Plasma Lipids/WEB 2086</th>
<th>Chow (n=6)</th>
<th>Chow+WEB (n=5)</th>
<th>Western (n=7)</th>
<th>Western+WEB (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>287±21.4</td>
<td>308.8±45.5*</td>
<td>1109.3±119.3</td>
<td>1228.6±92.8*</td>
</tr>
<tr>
<td>Unesterified cholesterol, mg/dL</td>
<td>83.8±15.45</td>
<td>72.2±12.63†</td>
<td>290.7±35.97</td>
<td>324.8±26.67†</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>80.0±18.56</td>
<td>96.2±19.51</td>
<td>96.86±13.98</td>
<td>91.20±9.12</td>
</tr>
<tr>
<td>Free fatty acid, mg/dL</td>
<td>58.20±28.17</td>
<td>77.00±11.94‡</td>
<td>89.57±15.90</td>
<td>105.20±18.21‡</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>70.00±16.00</td>
<td>61.6±19.76§</td>
<td>102.43±44.79</td>
<td>116.80±41.55§</td>
</tr>
<tr>
<td>WEB 2086, μg/mL</td>
<td>...</td>
<td>1.98±1.30</td>
<td>...</td>
<td>2.38±1.06</td>
</tr>
</tbody>
</table>

Total cholesterol, unesterified cholesterol, HDL cholesterol, triglyceride, free fatty acid, and WEB 2086 levels from various groups of mice plasma. The 4 groups of mice used for the study were (1) chow diet with regular water, (2) chow diet with WEB 2086 water (43 mg/L), (3) high-fat diet with regular water, and (4) high-fat diet with WEB 2086 water (43 mg/L). Mice plasma samples (50 μL) were extracted with chloroform/methanol (2:1, vol/vol) containing 0.01% BHT at room temperature. The solvent was evaporated under a stream of nitrogen, and the samples were analyzed for WEB 2086 levels by ESI-MS using an internal standard (triazolam) as described in Materials and Methods. Statistical comparison was made to the chow-fed animals using the Statview 4.5 program, and P values were calculated using ANOVA and Fisher’s PLSD significance test.

P values for chow vs Western: *P < 0.0001, †P < 0.0001, ‡P = 0.316, and §P = 0.021.

The formation of fatty streak lesions in the heart valves of these animals was then examined. Animals on the chow diet with or without WEB 2086 in the drinking water had small fatty streak lesions (Figure 5A and 5B). Animals on the Western diet without WEB 2086 had much larger fatty streak lesions (Figure 5C) but no fibrous plaques. In animals on the Western diet given WEB 2086 in the drinking water, the fatty streaks were smaller (Figure 5D). The size of the lesions was

---

**Figure 4.** ESI-MS/MS spectra of WEB 2086 and WEB 2086 extracted from mouse plasma. A solution of WEB 2086 (1 μg) or total lipid extract of plasma (50 μL) from mice on a Western diet supplemented with WEB 2086 in the drinking water in acetonitrile/water/trifluoroacetic acid (50:50:0.1, vol/vol/vol) was used for MS/MS experiments. Daughter ion analyses were performed for WEB 2086 (m/z 456.1) (A), m/z 456.1 from mice plasma (B), WEB 2086 (m/z 458.1) (C), and m/z 458.1 from mice plasma (D).
then quantitated using an image analyzer. The mean lesion area from mice on the Western diet with WEB 2086 showed a 62% decrease ($P=0.0012$) compared with that from mice on a Western diet alone. However, WEB 2086 had no significant effect on the small lesions in mice fed the chow diet (Figure 6). These results demonstrate that WEB 2086 strongly reduces fatty streak formation in LDL R$^{-/-}$ mice on the Western diet.

**Discussion**

In previous studies, WEB 2086 has been demonstrated to block the action of PAF and ether-containing PAF-like lipids including Ox-PAPC. In the present study, we demonstrate an inhibitory effect of WEB 2086 on the activation of endothelial cells by 2 ester-containing phospholipid oxidation products, POVPC (96%) and PEIPC (57%), which represent 2 of the active lipids in MM-LDL and Ox-PAPC (Figure 1). Inhibition was maximal at approximately equal concentrations of agonist and antagonist. Several other PAF receptor antagonists were also shown to inhibit the effect of POVPC to induce monocyte binding. In contrast, WEB 2086 did not block the action of PGPC on monocyte binding. We have previously reported several differences in the action of POVPC and PGPC, including their effects on neutrophil binding, hemoxygenase-1, and E-selectin expression. The present results suggest that the aldehyde, epoxysprostane, and carboxylic acid groups at the sn-2 position of the bioactive phospholipids play an important role in determining their receptor interactions.

WEB 2086 has been identified as a powerful competitive inhibitor of the PAF receptor; however, we have presented evidence that the in vitro effects of POVPC and PEIPC are probably not mediated by the PAF receptor. We and others have found extremely low to undetectable levels of this receptor in large-vessel endothelial cells; PAF was not active in inducing monocyte binding or competing for the effects of POVPC (Figure 2). Furthermore, the inhibitory effect of WEB 2086 does not appear to be due to blocking PAF expressed on POVPC-treated endothelial cells, because addition of WEB during monocyte binding to POVPC-treated endothelial cells was only minimally inhibitory. Despite this evidence against a role for the PAF receptor in the action of POVPC and PEIPC, a role for this receptor cannot be excluded. Other investigators have shown that WEB 2086 is specific for the inhibition of the action of PAF receptor. It is possible that POVPC and PEIPC can bind to different areas of the PAF receptor not bound by PAF. Nonetheless, the data are suggestive of a separate WEB 2086–sensitive receptor for POVPC and PEIPC.
Because the previous and present studies demonstrated the strong inhibitory effect of WEB 2086 on monocyte–endothelial cell interactions in vitro, we examined its ability to inhibit fatty streak formation in vivo. WEB 2086 in the drinking water of LDL R−/− mice on a Western diet showed a 62% reduction in the formation of fatty streak lesions in the aortic valve (Figure 6) while having no significant effect on lipoprotein levels (Table). This major reduction suggests an important role, in fatty streak formation, for molecules whose action is inhibited by WEB 2086. On the basis of in vitro studies, this may include PAF17,18 or certain phospholipid oxidation products reported in the present study. The lack of complete inhibition of lesion formation may be due to molecules other than WEB 2086–inhibitable phospholipids. We have shown that the effects of PGPC on monocyte binding are not inhibited by WEB 2086 and those of PEIPC are only partially inhibited. It is also likely that lipids other than oxidized phospholipids are increased in fat-fed animals and may independently contribute to the induction of the fatty streak formation. Alternatively, the relatively modest levels of WEB 2086 detected in the animals might not have been sufficient to inhibit the effects of all of the target lipids. In vitro studies suggest that the level was sufficient when tested with particular lipids, but the effective concentration for inhibition of monocyte–endothelial cell interactions may differ in vivo. The amount of WEB 2086 detected in mouse plasma (4 to 5 μmol/L) was much higher than the IC50 value (0.17 μmol/L) reported for WEB 2086 inhibition of PAF receptor.37 More importantly, the dose of WEB 2086 used is effective in reducing lesion size in the present study. However, it is possible that a higher dose of WEB 2086 may result in more effective reduction of lesion development in vivo. We have used a relatively small group of mice (n= 5 to 7) for the present study because of the limited supply of WEB 2086. It is likely that an in vivo study that uses a larger group of animals and different doses of WEB 2086 might provide additional understanding of the beneficial effects of WEB 2086 in controlling lesion development in vivo.

In addition to the present results, previous studies also suggest an important role for phospholipid oxidation products and PAF in atherogenesis. Both PAF-AH and paraoxonase (PON) have been shown to degrade oxidized phospholipids.38–41 It has recently been reported that atherosclerosis is increased in a Japanese population with strongly reduced PAF-AH activity.42 In addition, PON null mice have been demonstrated to display increased fatty streak formation.43 In summary, the results presented in previous studies9,22 and in the present study demonstrate the ability of WEB 2086 to block the induction of monocyte–endothelial cell interactions by PAF or POVPVC and PEIPC. The results further demonstrate that oral administration of WEB 2086 in the drinking water of LDL R−/− mice on a Western diet effectively reduces fatty streak formation, a process dependent on monocyte-endothelial cell interactions. Thus, these in vivo studies strongly implicate an important role for PAF and PAF-like bioactive oxidized phospholipids in the early stages of atherogenesis. The in vivo studies do not address whether or not the canonical PAF receptor or a separate WEB 2086–inhibitable receptor was involved in the effects of WEB 2086 on lesion formation. Although the present study has focused on the role of phospholipid oxidation products in fatty streak formation in mice, the presence of antibodies to phospholipid oxidation products in patients with atherosclerosis and several other chronic diseases underlines the potential importance of these molecules in mediating several types of chronic inflammation.34,44 The present study also suggests a potential role of receptor antagonists for bioactive oxidized phospholipids as a novel therapeutic approach in the control of lesion development in vivo.

Acknowledgments

This work was supported by United States Public Health Service grant HL 30568. We thank Dr Alan M. Fogelman for helpful suggestions. We also thank Dr Xuping Wang for exceptional help with histological studies and Dr Larry Castellani for lipid analysis.

References


Evidence That Phospholipid Oxidation Products and/or Platelet-Activating Factor Play an Important Role in Early Atherogenesis: In Vitro and In Vivo Inhibition by WEB 2086
Ganesamoorthy Subbanagounder, Norbert Leitinger, Peggy T. Shih, Kym F. Faull and Judith A. Berliner

Circ Res. 1999;85:311-318
doi: 10.1161/01.RES.85.4.311

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/85/4/311

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/