Local Expression of Platelet-Activating Factor–Acetylhydrolase Reduces Accumulation of Oxidized Lipoproteins and Inhibits Inflammation, Shear Stress–Induced Thrombosis, and Neointima Formation in Balloon-Injured Carotid Arteries in Nonhyperlipidemic Rabbits

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Background—Platelet-activating factor (PAF) and PAF-like phospholipids are inactivated by PAF-acetylhydrolase (PAF-AH). Using nonhyperlipidemic animals, we tested whether local expression of PAF-AH into injured arteries might induce antithrombotic and antiinflammatory effects.

Method and Results—Balloon-injured rabbit carotid arteries were infected at the time of injury with an adenovirus expressing either human plasma PAF-AH (AdPAF-AH) or bacterial β-galactosidase (AdLacZ) or infused with saline. Seven days later, shear stress–induced thrombosis was observed in all AdLacZ-infected and saline-infused arteries (controls) but eliminated in AdPAF-AH–treated contralateral arteries, even in the presence of epinephrine or an inhibitor of NO production. Injury-induced expression of tissue factor was also significantly suppressed. In AdPAF-AH–treated arteries compared with controls, the expressions of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 and macrophage infiltration were decreased by 66%, 66%, and 71%, respectively (P<0.01), and intimal area and intima/media ratio were decreased on day 21 by 43% and 52%, respectively (P<0.05). Within 1 week after injury, oxidized lipoproteins (OxLDL) had readily accumulated in the arterial wall. However, this was markedly reduced in the AdPAF-AH–treated arteries. No differences in the titers of autoantibodies to OxLDL or total cholesterol in blood were found between controls and AdPAF-AH–treated rabbits.

Conclusions—Our results show for the first time that OxLDL accumulates in arteries in nonhyperlipidemic animals within 1 week after injury and that local expression of PAF-AH reduces this accumulation and exerts antiinflammatory, antithrombotic, and antiproliferative effects without changing the plasma levels of PAF-AH activity or titers of autoantibodies to OxLDL. (Circulation. 2005;111:3302-3309.)

Key Words: gene therapy | inflammation | lipoproteins | thrombosis | platelet activating factor

Oxidation of lipoproteins including LDL, IDL, chylomicrons, and postprandial remnants and of polyunsaturated fatty acids generates platelet-activating factor (PAF) and PAF-like phospholipids (PAFs) in a number of cells, including endothelial cells, neutrophils, monocytes/macrophages, and platelets.1 PAFs in turn activate arterial wall cells. Through activation of nuclear factor-κB (NF-κB), PAFs induce expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), chemotaxis stimulants such as monocyte chemoattractant protein-1, and colony-stimulating factor,2 which may contribute to the recruitment of monocytes/macrophages and leukocytes into arterial walls.2 These are considered key events in atherosclerogenesis.3 Current information strongly suggests that PAFs should be involved in pathological changes in injured arteries and atherosclerogenesis.

PAF-acetylhydrolase (PAF-AH) catalyzes the hydrolysis of PAFs to biologically inactive molecules, lyso-PAFs.3 Two kinds of PAF-AH have been identified thus far: plasma PAF-AH and intracellular PAF-AH.3 Plasma PAF-AH is mainly produced and secreted from macrophages and Kupffer cells.
cells in liver. Interestingly, PAF-AH is contained within lipoproteins and appears to decrease bioactivity, because PAF-AH–depleted lipoproteins and lipoproteins from PAF-AH–deficient persons show increased inflammatory properties. In view of the possible actions of PAFs and the aforementioned published information, it seems reasonable to assume that the introduction of PAF-AH may have therapeutic effects. In fact, adenovirus-mediated gene transfer of PAF-AH into the liver in apolipoprotein E (ApoE)–deficient mice increased PAF-AH activity in the serum (by 1.5- to 3.5-fold), reduced the level of oxidized LDL (OxLDL) in the blood (by 82%), suppressed macrophage homing into arteries, and suppressed guidewire injury–induced neointima formation (when gene transfer was performed 7 days before injury) (77% reduction).

The authors of the aforementioned articles thought that the reduction in OxLDL achieved by systemically increased PAF-AH activity would contribute to the reduced recruitment of leukocytes and monocytes/macrophages into vessel walls, with consequent antiatherogenic and antiproliferative effects. If the therapeutic effects of PAF-AH depended solely on a reduction in OxLDL in the blood, then PAF-AH might be expected to display its beneficial effects only in OxLDL-rich conditions. One potential problem is that systemically increased PAF-AH activity could have side effects in humans. For that reason, in the present study we explored the possibilities that local expression of PAF-AH into injured arteries might suppress inflammation, thrombosis, and cell proliferation without increasing systemic PAF-AH activity in the serum and that these effects might occur irrespective of changes in indices of plasma OxLDL levels. We found for the first time that OxLDL accumulates in arteries in nonhyperlipidemic animals within 1 week after injury and that local expression of PAF-AH reduces this accumulation and exerts multiple therapeutic effects. Our results suggest that the local application of PAF-AH might result in therapeutic benefits in a wide range of subjects, including nonhyperlipidemic subjects.

Methods

Preparation of Recombinant Adenoviruses

Replication-defective E1- and E3- adenoviral vectors that express either human plasma PAF-AH (AdPAF-AH) or bacterial β-galactosidase (AdLacZ) under a CAG promoter (comprising a cytomegalovirus enhancer and chicken β-actin promoter) were prepared as previously reported.

In Vivo Gene Transfer Into Injured Arteries

A total of 67 rabbits and 36 rats (for reverse transcription–polymerase chain reaction [RT-PCR] assay only) were used in this study. All animals and experiments were in accordance with the Guidelines for Animal Experiments of the institution and the Law (No. 105) and Notification (No. 6) of the Japanese government. Japanese White rabbits (male; weight, 3.0 to 3.5 kg) or Wister rats (male; weight, 400 to 450 g) were balloon injured within their carotid arteries with the use of Fogarty catheters (3F for rabbits, 2F for rats) and then either infused with saline, then PAF-AH might be expected to display its beneficial effects only in OxLDL-rich conditions. One potential problem is that systemically increased PAF-AH activity could have side effects in humans. For that reason, in the present study we explored the possibilities that local expression of PAF-AH into injured arteries might suppress inflammation, thrombosis, and cell proliferation without increasing systemic PAF-AH activity in the serum and that these effects might occur irrespective of changes in indices of plasma OxLDL levels. We found for the first time that OxLDL accumulates in arteries in nonhyperlipidemic animals within 1 week after injury and that local expression of PAF-AH reduces this accumulation and exerts multiple therapeutic effects. Our results suggest that the local application of PAF-AH might result in therapeutic benefits in a wide range of subjects, including nonhyperlipidemic subjects.

Recurrent Thrombosis Model

Seven days after balloon injury, the shear stress–induced recurrent formation and dislodgment of thrombi were monitored by measuring carotid blood flow (6 vessels per group), as described previously. Three rabbits per group were treated with one of the following agents: N-nitro-l-arginine methyl ester (L-NAME), an NO synthase inhibitor (bolus, 3 to 100 mg/kg body wt IV), and epinephrine (continuously infused, 0.2 to 1.0 μg/kg body wt per minute). Another 3 vessels per group were resected after shear stress and subjected to a scanning electron microscopic analysis, as previously described.

PAF-AH Activity

Segments of rabbit injured carotid arteries (2 cm in length; n=6) were harvested 7 days after local gene transfer, then placed in 1 mL of serum-free medium for 24 hours at 37°C. The PAF-AH activity in the medium was measured with the use of a commercially available assay kit (Cayman Chemical). In some rabbits, we injected AdPAF-AH (5.0×10⁸ pfu per rabbit) into the femoral vein to express PAF-AH, primarily in the liver (systemic gene transfer). Plasma samples were collected before or 3, 7, and 14 days after either local or systemic gene transfer, and the activity in each sample was assayed. The rabbit PAF-AH activity, which was not significantly changed before and after injury plus...
AdLacZ infection, was subtracted from each value to show only human PAF-AH activity.

Reverse Transcription–Polymerase Chain Reaction
Balloon-injured and gene-transferred rat carotid arteries were harvested (5 to 6 vessels per group), and RNA was extracted. The sequences of tissue factor (TF) (367 bp) and GAPDH (555 bp) were amplified by RT-PCR, as described previously.8 The data were densitometrically analyzed.

Histological Analyses
Seven days after balloon injury and gene transfer, rabbit arteries (n=6 to 8 per group) were subjected to immunohistostaining with the use of antibodies raised against human PAF-AH (Santa Cruz Biotechnology), rabbit VCAM-1 or ICAM-1 (generous gifts from Dr M. Cybulsky, University of Toronto), rabbit macrophages (RAM 11; Dako), apolipoprotein B-100 (ApoB-100) (MB47),9 and malondialdehyde-modified LDL (MDA-LDL) (MDA2).10 Semi-quantitative analyses of the staining were performed morphometrically, as reported previously.8 Infiltration of macrophages was assessed by counting the RAM 11–positive cells.

Chemiluminescence Immunoassay for Autoantibodies to OxLDL
The plasma autoantibodies against copper-oxidized LDL (CuOx-LDL) and MDA-LDL were measured with chemiluminescent immunoassay, as described previously.11 The light emissions were measured as a relative light units per 100 ms with a Dynex Luminometer.11

Statistical Analysis
Values were analyzed statistically with the use of ANOVA. A value of P<0.05 was considered significant.

Results
PAF-AH Activity Was Increased Locally in AdPAF-AH–Treated Arteries but Was Minimal Systemically in Serum
Seven days after injury and gene transfer, carotid arteries were resected and cultured ex vivo. A considerable level of PAF-AH activity was detected in the medium of AdPAF-AH–treated arteries. No activity was detectable from either AdLacZ-infected or saline-infused arteries (Figure 1A). Plasma samples were collected before or 3, 7, and 14 days after either local or systemic gene transfer (intravenous injection of AdPAF-AH). On the third day after local infection, significant but minimal increases in human PAF-AH activity, which were less than the basal rabbit PAF-AH activity (0.38±0.06 nmol/mg plasma protein per minute; mean±SD; n=6), were detectable in plasma samples (Figure 1B). The activities on days 7 and 14 were not significantly increased. In contrast, when AdPAF-AH was intravenously injected (PAF-AH would be produced in livers), quite high levels of PAF-AH activity were detected. The amount of

Blood Flow and Flow Variation Frequency in Injured Carotid Arteries

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<tr>
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<th>CFV Frequency, Cycles/h</th>
<th>Carotid Blood Flow, mL/min</th>
<th>% Reduction of Carotid Blood Flow</th>
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<tr>
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<td>Before Ligation</td>
<td>After Ligation</td>
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<tr>
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<td>AdPAF-AH</td>
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<td>38.8±5.8</td>
<td>18.1±4.1</td>
</tr>
<tr>
<td>Saline</td>
<td>12.6±3.1</td>
<td>41.6±5.8</td>
<td>19.2±4.1</td>
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Balloon-injured rabbit carotid arteries were infected with AdPAF-AH or AdLacZ or infused with saline. Seven days later, blood flow was monitored before and immediately after the partial ligation. CFV was then monitored for 1 hour. Values are mean±SD (n=6 in each group).

*p<0.05 vs AdLacZ and saline groups.

AdLacZ infection, was subtracted from each value to show only human PAF-AH activity.

Figure 2. CFV was eliminated in AdPAF-AH–treated arteries, even in the presence of epinephrine or L-NAME. Rabbit carotid arteries were balloon injured and infected with AdLacZ (A) or AdPAF-AH (B, C). Seven days later, shear stress–induced CFV was measured. In some AdPAF-AH–infected rabbits, either epinephrine (B) or L-NAME (C) was infused or injected intravenously. Similar results were obtained in at least 3 rabbits in each group.
AdPAF-AH we used in a rabbit was the same others used in a mouse.5,6

Shear Stress–Induced Thrombosis Was Eliminated in AdPAF-AH–Treated Arteries Even in the Presence of Either Epinephrine or L-NAME

Seven days after balloon injury plus gene transfer, a time when inflammation would be submaximal,8 we measured cyclic flow variation (CFV), which is thought to reflect recurrent cycles of thrombus formation and dislodgment.12 There were no significant differences among the arteries tested in terms of the change in carotid blood flow before versus immediately after the establishment of stenosis (Table). After stenosis, CFV was observed in all AdLacZ-infected (Figure 2A, Table) and saline-infused arteries (Table). However, in AdPAF-AH–treated arteries, no CFV was detectable (Figure 2B and 2C, Table), even in the presence of either epinephrine (Figure 2B) or L-NAME, a NO synthase inhibitor (Figure 2C).

In our electron microscopic analysis, the luminal surface of AdLacZ-infected arteries was covered with a large quantity of aggregated platelets and fibrin, in which leukocytes and erythrocytes were entrapped (Figure 3, left). In contrast, in AdPAF-AH–treated arteries there was only a monolayer of spread-out platelets, with no visible evidence of platelet aggregation, leukocyte adhesion, or fibrin formation (Figure 3, right).

Injury-Induced Transcription of TF Was Reduced in AdPAF-AH–Treated Arteries

The enhanced expression of TF would contribute to arterial thrombosis and the proliferative response seen in injured arteries. In rat injured arteries, TF mRNA increased by 2-fold within 6 hours and was sustained for at least 3 days (Figure 4A). However, in AdPAF-AH–treated arteries, TF mRNA (at 24 hours after injury) was significantly reduced (by ~50%) compared with the signals obtained after other treatments (Figure 4B).

Injury-Induced Inflammation Was Markedly Reduced in AdPAF-AH–Treated Arteries

We examined macrophage infiltration and the expression levels of inflammatory cytokine-induced adhesion molecules ICAM-1 and VCAM-1 in the arterial walls. Representative immunostained sections are shown in Figure 5A. In saline-infused (data not shown) and AdLacZ-infected arteries, macrophages, ICAM-1, and VCAM-1 were readily detectable. In the AdPAF-AH–treated arteries, each was markedly reduced in terms of both staining area and intensity. When L-NAME was given continuously to some rabbits in each group via their drinking water (0.5 mg/mL) for 8 days (starting 1 day before injury) to suppress NO production, as previously described,8 the expression levels of ICAM-1 and VCAM-1 and the infiltration of macrophages were unaffected, indicating that the antiinflammatory effect of PAF-AH...
is NO independent. The semiquantitative values of ICAM-1, VCAM-1, and macrophages in the AdPAF-AH–treated arteries were decreased by 66%, 66%, and 71%, respectively (P<0.01) compared with those in the AdLacZ-infected vessels (Figure 5B to 5D).

PAF-AH Gene Transfer Inhibited Balloon Injury–Induced Neointima Formation
Three weeks after balloon-injury and gene transfer, the rabbit carotid arteries were analyzed by quantitative histomorphometry. Neointima formation was significantly reduced (by 43% in the intima area and 52% in the intima/media ratio; Figure 6) in the AdPAF-AH–treated arteries compared with the AdLacZ-infected ones.

Lipoproteins, Including OxLDL, Were Readily Accumulated in Arteries Within 1 Week After Injury
Immunohistostaining analysis was performed with the use of specific antibodies against ApoB-100, a major component of LDL and VLDL particles, and MDA-LDL, a chemically modified and oxidized form of LDL. No staining was detected in intact arteries (data not shown). Interestingly, within 1 week after the injury, both ApoB-100 and MDA-LDL were readily detectable in the saline-infused (Figure 7B) and AdLacZ-infected arteries (Figure 7, A and B). However, in the AdPAF-AH–treated arteries, in which expression of PAF-AH was confirmed (top row of Figure 7A), although ApoB-100 was stained to a similar extent, MDA-LDL was markedly (by 75%; P<0.001; Figure 7B) reduced versus the level in the AdLacZ-infected arteries. It should be noted that both carotid arteries in a given rabbit were balloon injured, then infected with either AdPAF-AH or AdLacZ (or saline infusion) (on the contralateral side).

Plasma OxLDL and Total Cholesterol Were Not Altered by Local PAF-AH Gene Transfer
We were unable to measure OxLDL directly in the plasma of any of the normolipidemic rabbits used in this study. We also measured the levels of autoantibodies against Cu-OxLDL and MDA-LDL, as previously described. As shown in Figure 7C, the levels of these antibodies on days 7 and 28 after local gene transfer, as well as that of total cholesterol on day 28 (inset of Figure 7C), were not significantly changed.

Discussion
The accumulated evidence indicates that PAFs are potent proinflammatory substances and would be involved in the fibroproliferative changes that follow injury and in atherosclerosis, suggesting a potential therapeutic use for PAF-AH. In the present study we found for the first time that OxLDL readily accumulates in the arterial walls, even in low-lipidemic animals, within 1 week after an injury (Figure 7). Our results also show that local expression of PAF-AH greatly reduces this OxLDL accumulation (Figure 7), eliminates shear stress–induced thrombosis (Figures 2 and 3), markedly suppresses inflammatory responses (Figures 4 and 5), and significantly reduces neointima formation (Figure 6). These desirable effects were achieved without alterations of plasma levels of cholesterol, increments in measurable levels of plasma OxLDL, autoantibody titers to OxLDL (suggesting no detectable systemic immune response to the localized OxLDL generated within the damaged arterial walls) (Figure 7C), or plasma PAF-AH activity (except within a few days) (Figure 1). The multiple beneficial actions (possibly without side effects) of locally applied PAF-AH observed in nonhyperlipidemic animals in our study demonstrate its therapeutic potential as a molecule for future clinical application into injured or atherosclerotic arteries.

Previous animal experiments have shown that (1) PAF was increased by 3- to 64-fold after arterial injury; (2) an infusion of PAF restores CFV after its abolition by thromboxane A2 or serotonin receptor antagonists; and (3) a PAF antagonist can abolish CFV in injured arteries in dogs. Furthermore, mutations in the PAF-AH gene are an independent risk factor for coronary artery disease. All of these
findings suggest that PAFs may play roles in both arterial thrombosis and acute coronary syndrome and that PAF-AH may be therapeutically useful. Indeed, we observed in the present study that shear stress–induced thrombosis was eliminated in AdPAF-AH–treated arteries. This antithrombotic effect of PAF-AH was intact under epinephrine of a submaximal dose, and it was NO independent (Figure 2), unlike the antithrombotic effect of C-type natriuretic peptide, which was NO dependent.8 We also found that TF mRNA was significantly reduced in AdPAF-AH–treated arteries (Figure 4). This finding implies that in addition to its direct inactivation of PAFs, PAF-AH should suppress the inflammation-induced expression of prothrombotic molecules (such as TF), an effect that may contribute to a complete suppression of shear stress–induced thrombosis. This suppression of TF is compatible with our finding that PAF-AH suppresses injury-induced inflammation (Figure 5) because TF is one of the NF-κB–regulated inflammation-induced genes.

It was recently reported that PAF-AH gene transfer into the liver in ApoE-deficient mice increased serum PAF-AH activity and reduced serum OxLDL and either reduced macrophage homing6 or suppressed spontaneous atherosclerosis and neointima formation after injury.5 The authors of the aforementioned studies concluded that the antiatherosclerotic and antiproliferative effects of PAF-AH were attributable to a systemic increase in PAF-AH activity and a reduction in plasma OxLDL. We observed that local application of PAF-AH exerted therapeutic effects even in the absence of measurable changes in plasma OxLDL level (Figure 7C) and increase in plasma PAF-AH activity. On the third day after local gene transfer, we detected significant increases in PAF-AH activity in serum (Figure 1). However, this minimal increase in a short period of time did not contribute to the therapeutic effect because no effect was observed in the contralateral AdLacZ-infected artery of the same rabbit. We do not know whether the PAF-AH really comes from the injured arteries because a portion of the AdPAF-AH applied into an injured artery leaks through small branches into the systemic circulation.

At first, we thought that our results supported the notion that PAF-AH may possess a direct antiinflammatory effect in addition to its inactivating action on PAFs, including OxLDL. Then, however, we unexpectedly found that OxLDL readily accumulated in the injured arterial wall and that this accumulation was greatly reduced only in the AdPAF-AH–treated contralateral artery in a given rabbit (Figure 7). An accumulation of OxLDL has been detected in arteries in athrogenic animals, such as ApoE-deficient mice18 and cholesterol-fed rabbits.19 However, to our knowledge, it has never been documented that OxLDL can accumulate in arteries in relatively low-cholesterol animals within such a short period of time (1 week) after the infliction of injury. Our finding may give rise to a new concept about the roles played by OxLDL in the pathogenesis of neointima formation and of atherosclerosis.

OxLDL is known to be atherogenic and to contain many proinflammatory oxidized molecules.20 OxLDL (1) enhances expressions of adhesion molecules and chemokines and hence enhances the infiltration of monocytes/macrophages; (2) stimulates platelet adhesion and aggregation by decreasing the productions of NO and prostacyclin and reduces fibrinolysis by decreasing the secretion of plasminogen activator and facilitating the release of plasminogen activator inhibitor-1; and (3) stimulates proliferation of vascular smooth muscle cells through its oxidized phospholipids and through an increased production of growth factors (including platelet-derived growth factor and fibroblast growth factor).20–22 To judge from the aforementioned information, the antithrombotic, antiinflammatory, and antiproliferative effects of PAF-AH observed in this study may be achieved simply by inactivation of PAFs within injured arterial walls. It is most likely that the therapeutic effects of PAF-AH are not necessarily related to a systemic modification of oxidized lipoproteins, and indeed at this time we do not know whether the therapeutic effects seen in this study were due solely to a suppression of OxLDL accumulation or whether some might have been due to as yet unidentified direct action(s) of PAF-AH. Further studies are needed. Moreover, we do not know whether the scarcity of OxLDL in the AdPAF-AH–
treated arteries was due to an inhibition of their generation and/or accumulation or to hydrolysis of oxidized phospholipids once accumulated. This clinically important issue is now under investigation in our laboratory. Our preliminary study seems to support the latter possibility.

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References


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