Atherosclerosis and Lipoproteins

Human Plasma Platelet-Activating Factor Acetylhydrolase Binds to All the Murine Lipoproteins, Conferring Protection Against Oxidative Stress

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Objective—Plasma platelet-activating factor (PAF) acetylhydrolase (AH) is an enzyme bound with lipoproteins that degrades not only PAF but also PAF-like oxidized phospholipids that are proposed to promote atherosclerosis. In this study, we investigated the distribution of PAF-AH protein among lipoprotein classes by using adenovirus-mediated gene transfer in mice, and we examined its effects on lipoprotein oxidation and foam cell formation of macrophages.

Methods and Results—Adenovirus-mediated overexpression of PAF-AH in mice resulted in a 76- to 140-fold increase in plasma PAF-AH activity. Contrary to the previous report, overexpressed human PAF-AH protein was bound to very low density lipoprotein, intermediate density lipoprotein, low density lipoprotein, and high density lipoprotein (HDL). All the lipoproteins with overexpressed human PAF-AH revealed more resistance against oxidative stress, which was associated with lower levels in autoantibody against oxidized low density lipoprotein in the plasma. In addition, HDL with human PAF-AH inhibited foam cell formation and facilitated cholesterol efflux in macrophages.

Conclusions—These results suggest that human plasma PAF-AH exerts an antiatherogenic effect by binding to all the lipoproteins and thereby protecting them from oxidation, producing less proatherogenic lipoproteins and preserving HDL functions. (Arterioscler Thromb Vasc Biol. 2003;23:829-835.)

Key Words: platelet-activating factor acetylhydrolase ■ oxidative stress ■ adenovirus ■ foam cell formation ■ cholesterol efflux

Platelet-activating factor (PAF) acetylhydrolase (AH) is a calcium-independent enzyme that degrades PAF, a bioactive phospholipid mediator for allergic and inflammatory processes, to a biologically inactive lyso-PAF. Plasma PAF-AH, 1 of the 3 PAF-AH isoforms identified so far, is produced from macrophages and exists in the plasma in the form bound with lipoproteins; the other 2 isoforms are found only in tissues. Seventy percent to 83% of the plasma PAF-AH protein exists on LDL, and 11% to 30% exists on HDL in human plasma. An interchange between the 2 lipoproteins has been reported in plasma PAF-AH. In mice, it has been recognized that PAF-AH is associated primarily with HDL and minimally with VLDL and that neither murine PAF-AH nor human PAF-AH has been proposed to bind to murine LDL.

An observational study has shown that plasma PAF-AH activity is altered in atherosclerotic diseases. Oxidation of LDL, in which PAF-like oxidized phospholipids are produced on the LDL surface, is one of the key factors in the early stages of atherosclerosis. Besides catalyzing PAF, plasma PAF-AH protein hydrolyzes PAF-like oxidized phospholipids, thereby most likely inactivating the biologically active mediator. However, the products of this reaction include oxidized fatty acids and lysophosphatidylcholine, which are potentially inflammatory mediators that could amplify atherogenesis. Therefore, it is not fully clear whether PAF-AH is antiatherogenic or proatherogenic in humans. There was one report documenting that high PAF-AH activity is associated with an increased risk of coronary artery disease in humans. However, it is not conclusive whether PAF-AH is a causative agent of coronary artery disease or just a marker. A recent animal study demonstrated that overexpression of human plasma PAF-AH protein reduced atherosclerosis in apoE-deficient mice, suggesting its role in the inhibition of atherogenesis. They also clarified that antiatherogenic mechanisms of PAF-AH include reduced macrophage adhesion and homing, reduced production of oxidized LDL, and a reduced lysophosphatidylcholine/phosphatidyl-

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829
choline ratio. However, the distribution of PAF-AH protein among lipoprotein particles and its effect on macrophage foaming have not been clarified.

In the present study, using gene transfer with second-generation adenoviruses, we examined the distribution of the overexpressed human PAF-AH among lipoprotein classes, its effect on lipid metabolism, and its properties for lipoprotein oxidation and foam cell formation. Our results demonstrate that human PAF-AH binds to all the lipoprotein in mice, conferring an antiatherogenic property to each of them.

Methods

Materials and Animals
Paraoxon (PON) was purchased from Sigma Chemical Co. Male wild-type mice (C57BL6/J) and male apoE-deficient mice were obtained from Jackson Laboratory (Bar Harbor, Me). All animal experiments were approved by the University of Tokyo Ethics Committee for Animal Experiments. Total and free cholesterol concentrations were measured by a standard enzymatic method (Wako Pure Chemical Industry, Ltd). Protein concentrations were measured by Lowry’s method. Goat polyclonal antibody against mouse apoA-I and rabbit polyclonal antibody against human plasma PAF-AH were purchased from Rockland Immunocchemicals, Inc, and Cayman Chemical Co, respectively. Secondary peroxidase-labeled goat anti-rabbit IgG and goat anti-mouse IgG antibodies were purchased from Jackson Immuno Research Laboratories, Inc. Murine macrophage RAW264.7 cells were purchased from American Type Culture Collection.

Construction of Recombinant Second-Generation Adenovirus
Human plasma PAF-AH cDNA was amplified from the human cDNA library (Clontech Inc) by polymerase chain reaction. After confirming the sequence of the cDNA, the PAF-AH cDNA was subcloned into the shuttle plasmid vector pAdCMV-link, which contains 0 to 1 mU adenovirus, the cytomegalovirus immediate-early gene enhancer and promoter, and 9 to 16 mU adenovirus. Recombinant second-generation adenovirus was constructed by the use of established methods. Briefly, the plasmid was linearized with NheI and cotransfected into 293 cells along with LacZ second-generation adenoviral DNA digested with ClaI, and the cells were overlaid with agar and incubated at 32°C for 14 days. Plaques were picked and screened by polymerase chain reaction. After the confirmation of the presence of PAF-AH cDNA and the absence of LacZ and wild-type adenovirus, the new recombinant adenovirus, designated as AdPAF-AH, was expanded in 293 cells. Cell lysates were used to infect HeLa cells for confirmation of the expression of human PAF-AH by Western blotting and activity assay of the media. The recombinant adenovirus was further expanded in 293 cells and purified by cesium chloride ultracentrifugation. AdLacZ, a second-generation adenovirus carrying the β-galactosidase cDNA, was used as a control virus.

Animal Studies
Male wild-type C57BL6/J mice and male apoE-deficient mice at 12 weeks of age were injected intravenously with 3×10^9 plaque-forming units of AdPAF-AH, AdLacZ, or PBS. Blood was drawn from the retro-orbital plexus after a 4-hour fast before virus injection and at 3, 7, 14, 21, and 28 days after injection, and it was collected in a heparin-coated tube.

Fractionation of Lipoproteins
Pooled plasma samples (120 μL) were fractionated by fast protein liquid chromatography (FPLC) gel filtration with the use of a Superose 6 column (Pharmacia LKB Biotechnology). Fractions were collected into 46 tubes in a volume of 375 μL per fraction. Each fraction was analyzed enzymatically for total cholesterol and PAF-AH activity.

PAF-AH Activity Assay
A spectrophotometric assay for plasma PAF-AH activity using 1-myristoyl-2-(p-nitrophenylsucci)nyl)phosphatidylcholine, a PAF analogue with a 4-nitrophenyl group, as a substrate was performed. Briefly, the rate of hydrolysis of the PAF analogue was determined by monitoring the liberation of p-nitrophenol succinate at 405 nm at 37°C for 2 minutes. The assay reagents included 200 mmol/L NaCl, 15 mmol/L EDTA, 9.6 mmol/L sodium 1-nanosalusulfonate, 7 mmol/L 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 100 mmol/L HEPES. The activity was calculated by using the absorbance rate per minute and the extinction coefficient (ε=12.3×10^3 L/mol per centimeter at pH 7.6) of 4-nitrophenol.

Western Blot Assay
Plasma and FPLC fractions pooled in pairs were subjected to SDS-PAGE using 10% polyacrylamide gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane and immunoblotted with antibody against mouse apoA-I or human PAF-AH, followed by incubation with the corresponding peroxidase-labeled secondary antibody. Chemiluminescence with ECL Plus Western

Figure 1. FPLC cholesterol profile before and after injection of AdPAF-AH. Pooled plasma samples were subjected to gel filtration by using a Superose 6 column, and the cholesterol level in each fraction was measured enzymatically. Aa, Wild-type mice. B, ApoE-deficient mice. Open circles indicate before injection; closed squares, day 7 after injection. For wild-type mice, Western blot assay of apoA-I was also performed to elucidate its distribution (Ab).
blotting detection reagents (Amersham Pharmacia Biotech UK Ltd) was used to detect the protein bands, and they were visualized by Image Reader LAS-1000 for Macintosh (Fuji Photo Film Co, Ltd). Densitometric analysis was performed with NIH Image software.

Isolation and Modification of Lipoproteins
Human and murine serum samples were also subjected to sequential flotation ultracentrifugation at 14°C. Each lipoprotein fraction was dialyzed against PBS (pH 7.4) at 4°C. Human LDL was oxidized by 5 μmol/L CuSO4.15

Lipid Peroxidation Assay
Each lipoprotein fraction (0.1 mg protein/mL) from apoE-deficient mice obtained through sequential flotation ultracentrifugation was exposed to CuSO4 (5 μmol/L for HDL and 1.67 μmol/L for the other lipoproteins) to initiate lipid peroxidation at 37°C. Lipid peroxidation was monitored by UV absorption spectroscopy at 234 nm as formation of conjugated dienes.

Determination of Autoantibody Titers Against Oxidized LDL
Autoantibodies against oxidized LDL were measured by ELISA as described before.16,17 Briefly, 50 μL of oxidized LDL (5 μg/mL) was plated on a microtiter plate as antigen overnight at 4°C, followed by saturation with 2% serum albumin in PBS. After incubation with 1:1000 dilutions of mouse plasma for 1 hour at room temperature, 1:500 dilutions of peroxidase-labeled secondary antibody were added to each well. One hour later, after a wash with PBS, 50 μL of freshly made substrate (0.4 mg/mL o-phenylenediamine [Sigma] and 0.045% H2O2 in 0.05 mol/L phosphate-citrate buffer, pH 5.0) was added and incubated for exactly 5 minutes at room temperature. The reaction was terminated by adding 50 μL of 2 mol/L H2SO4. The optical density (OD) was monitored at 492 nm by use of a microplate reader.

Foam Cell Formation Assay and Cholesterol Efflux Assay
RAW 264.7 cells were cultured in DMEM (Invitrogen Corp) supplemented with 10% FBS (Invitrogen Corp). For foam cell formation assay, cells were washed twice with PBS when confluent in 6-well dishes and incubated in medium with 10% lipoprotein-deficient serum (LPDS, Sigma) containing murine HDL (0, 10, 25, or 50 μg/mL) purified from wild-type mice and human oxidized LDL (50 μg/mL) for 48 hours in the absence of 8-bromoadenosine 3’:5’-cAMP (8-Br-cAMP, Sigma). For cholesterol efflux assay, cells were washed and incubated in medium with 10% LPDS containing human oxidized LDL (50 μg/mL) for 24 hours. Subsequently, the cells were washed again and incubated in medium with 10% LPDS containing murine HDL (0, 10, 25, or 50 μg/mL) with or without 0.3 mmol/L 8-Br-cAMP for 24 hours. After incubation, cells were washed, and the cholesterol was extracted from the cells with 600 μL hexane plus isopropyl alcohol (3:2).18 The solvent was evaporated, and total cholesterol and free cholesterol were measured as described above. The cells were then dissolved in 500 μL NaOH (0.3N), and protein levels were measured as described above. As a marker for foaming, intracellular cholesteryl ester was calculated by subtracting free cholesterol/protein from total cholesterol/protein.

Statistical Analysis
Measurements were analyzed by the Student t test. A value of P<0.05 was considered significant.

Results
Effect of Human Plasma PAF-AH Overexpression on Cholesterol Levels in Wild-Type Mice and ApoE-Deficient Mice
Compared with baseline levels, plasma total cholesterol levels increased significantly in wild-type mice injected with
AdPAF-AH and AdLacZ on days 7 and 14; however, no significant differences were noted between these 2 groups at the same time points (please see online Figure IA, available at http://atvb.ahajournals.org). Injection of AdPAF-AH or Ad-LacZ did not alter plasma cholesterol levels in apoE-deficient mice (please see online Figure IB). The FPLC cholesterol profile revealed an appearance of lipoprotein particles (fractions 25 to 27), which are smaller than LDL and larger than normal HDL, together with an increase in LDL cholesterol and a decreased peak cholesterol level in normal HDL fractions only on day 7 in wild-type mice injected with AdPAF-AH (Figure 1Aa). In wild-type mice injected with AdLacZ, only the increase in LDL cholesterol was observed on day 7 (data not shown). Western blot analysis of apoA-I, a specific apolipoprotein of HDL, showed that the distribution of apoA-I became wider among FPLC fractions, reaching to the lipoprotein fractions appearing between LDL and HDL, with a left shift of the peak fraction after overexpression of human PAF-AH (Figure 1Ab). In apoE-deficient mice, there was no significant change in the FPLC cholesterol profile after gene transfer with AdPAF-AH (Figure 1B) and AdLacZ (data not shown).

**Plasma PAF-AH Activity**

Overexpression of human PAF-AH resulted in a 140-fold increase in activity in wild-type mice on day 3 (1625±133 IU/L on day 0 and 23.0±4.0×10^4 IU/L on day 3) and a 76-fold increase in apoE-deficient mice on day 3 (1515±84 IU/L on day 0 and 11.6±1.8×10^4 IU/L on day 3). The significantly increased plasma activity continued up to day 28 in wild-type mice and up to day 14 in apoE-deficient mice (please see online Figure II, available at http://atvb.ahajournals.org). The results of Western blot analysis of the plasma also confirmed the expression of human PAF-AH.

**Distribution of Overexpressed Human PAF-AH on Lipoproteins**

Distribution of human PAF-AH was analyzed by using FPLC fractions. Western blot analysis revealed that human PAF-AH was found to be distributed mostly in HDL in wild-type mice; however, with the activity assay, an association of PAF-AH protein was noted not only with HDL but also with VLDL, IDL, and LDL (Figure 2A).

In apoE-deficient mice, both activity assay and Western blot assay clearly showed that overexpressed human PAF-AH was associated with all the lipoproteins (Figure 2B). Densitometric analysis of the Western blot assays demonstrated that the activity per PAF-AH protein (specific activity) in the HDL fraction was 4-fold higher than that in the other fractions in apoE-deficient mice.

**Effect of Human Plasma PAF-AH on Lipoprotein Oxidation**

To determine whether the lipoproteins with overexpressed human PAF-AH were less susceptible to oxidative stress than were those in the control group, lipoproteins were isolated from apoE-deficient mice and incubated with CuSO_4. Figure 3 demonstrates a prolonged lag time for the initiation of lipoprotein oxidation, indicating that overexpressed PAF-AH provides protection against oxidation.

To examine whether the overexpressed PAF-AH affects the oxidative state of LDL in vivo, we measured the titers of autoantibody against oxidized LDL in the plasma of apoE-deficient mice. Autoantibodies against oxidized LDL were significantly lower in the AdPAF-AH group than in the AdLacZ group on day 14 (175±15 versus 220±9 OD, respectively; P<0.03). On day 28, no significant increase in antibodies from day 14 was seen in the AdPAF-AH group, whereas a 60% increase was noted in the AdLacZ group, although the difference between the 2 groups was nonsignificant (191±26 versus 353±78 OD, respectively; P=0.11).

**Effects on Macrophage Foam Cell Formation and Cholesterol Efflux**

When RAW 264.7 cells were incubated with oxidized LDL and HDL concomitantly, HDL with overexpressed human PAF-AH reduced intracellular cholesteryl ester accumulation (which represents foam cell formation) significantly more than HDL did (Figure 4A). On the other hand, when RAW...
264.7 cells were first made lipid-loaded with oxidized LDL and sequentially incubated with HDL, the magnitude of lipid removal was greater for HDL with human PAF-AH at a higher HDL concentration in the presence of 8-Br-cAMP (Figure 4B), whereas no cholesterol efflux by either HDL was noted in the absence of 8-Br-cAMP (data not shown).

**Discussion**

Plasma PAF-AH is a lipoprotein-associated enzyme that is supposed to possess an antiatherogenic effect; it hydrolyzes hyperperoxides in LDL, thereby most likely protecting against atherosclerotic vascular diseases. No clinical studies have so far clarified the antiatherogenic role of PAF-AH in humans, and the effect of PAF-AH on atherogenesis in humans remains under debate. However, a recent investigation demonstrated the inhibitory role of human PAF-AH on atherogenesis in mice.\(^9\) This investigation clarified that antiatherogenic mechanisms of PAF-AH include reduced macrophage adhesion and homing, reduced production of oxidized LDL, and a reduced lysophosphatidylcholine/phosphatidylcholine ratio.\(^9,10\) To clarify further the roles of PAF-AH on atherogenesis, we examined the properties of human PAF-AH by using gene delivery with second-generation adenoviruses in mice.

It has been reported that the majority of the plasma PAF-AH protein exists on LDL and the rest of it on HDL in human plasma,\(^1,2\) and it is associated primarily with HDL and minimally with VLDL in mice.\(^3,4\) Stafforini et al\(^5\) reported that the carboxyl terminus of apoB plays a crucial role in the binding of human PAF-AH to LDL and human PAF-AH does not associate with murine LDL. In contrast to that report, overexpression of human PAF-AH resulted in its binding to all the murine lipoproteins in both wild-type mice and apoE-deficient mice. This association of PAF-AH on all lipoprotein classes was confirmed in apoE-deficient mice even on day 14, when the plasma activity of PAF-AH declined to approximately one sixth of that on day 3 (Figure 2), clarifying the definite preference to apoB-containing lipoproteins. The probable explanation for the association of human PAF-AH on apoB-containing lipoproteins found in the present study is that much more PAF-AH protein was introduced in the present study than in the previous study. The content of human PAF-AH used in the previous investigation was too low to be detected, although the partial contribution of murine apoB cannot be ruled out. Future studies are needed to clarify these details, including the role of apolipoproteins in the association of PAF-AH with lipoproteins.\(^1,3,5\)

Interestingly, we observed the difference in the activity of PAF-AH among the different classes of lipoproteins; that on HDL was found to function more efficiently (ie, specific activity is higher) than that on the other lipoproteins. This phenomenon was also observed in human and PAF-AH–overexpressing LDL receptor–deficient mice (authors’ unpublished data). It was possibly caused by the interaction with apoA-I and apoB, or by the different surface lipid compositions and the particle sizes; however, further research is essential to clarify this mechanism. From the viewpoint of atherogenesis, this fact may be physiologically of significance in mice because PAF-AH protein has preferential association with HDL, on which its activity is the greatest.

Rodrigo et al\(^19\) recently proposed that PON1, another antiatherogenic enzyme on HDL that also possesses a
calcium-independent PAF-AH-like activity, is the only responsible enzyme on HDL for the hydrolysis of PAF and PAF-like phospholipids. However, as was shown by Min et al., plasma PAF-AH protein possesses a broad substrate specificity, and its binding to lipoproteins including HDL does not interrupt its catalytic activity. The present study also clarified that overexpression of human plasma PAF-AH protein in mice does result in the increased hydrolyzing activity not only in LDL fractions but also in HDL fractions. Thus, we could confirm that plasma PAF-AH is absolutely functioning as a hydrolyzing enzyme on HDL for PAF and PAF-like phospholipids.

Overexpression of PAF-AH in wild-type mice resulted in an appearance of lipoprotein particles that are smaller than LDL and larger than normal HDL, a decrease in the peak cholesterol level of normal HDL, and the wider distribution of apoA-I among FPLC fractions with a left shift of the peak fraction. These observations imply that HDL particles became bigger in size with PAF-AH overexpression. Excess PAF-AH protein might have made the HDL size larger, or the abundant PAF-AH might affect the metabolism of HDL. On the other hand, elevated LDL cholesterol level, which was also observed in mice injected with AdLacZ, was probably due to a nonspecific effect of adenoviral infection; of course, it is possible that the association of PAF-AH to LDL particles modified the degradation of LDL particles. Further studies are necessary to clarify these mechanisms.

With overexpressed human PAF-AH, all the lipoproteins were found to be resistant to oxidation. Oxidation of LDL is one of the key factors in the early stages of atherosclerosis. In addition, oxidized phospholipids may be the bioactive compounds in LDL and VLDL that induce growth factor expression in smooth muscle cells, thereby resulting in smooth muscle cell migration and proliferation. Our result suggested that besides catalyzing oxidized lipids, PAF-AH exerts a novel antiatherogenic function through protecting all the lipoproteins from oxidation. We also demonstrated this protective effect of PAF-AH against oxidative stress in vivo, as evidenced by lower titers of autoantibodies against oxidized LDL. Concordant with this notion, adenosine-mediated gene transfer of human PAF-AH in mice has been reported to decrease plasma malondialdehyde-modified LDL.

In the present study, PAF-AH prevented HDL oxidation as well, which points to the possibility that PAF-AH on HDL may intensify the antiatherogenic functions of HDL, i.e., the protection of LDL from lipid peroxidation and cholesterol removal from peripheral tissues. In consequence, we further performed a macrophage foaming assay to evaluate these effects of HDL with overexpressed human PAF-AH. A previous study has shown that cholesterol efflux from RAW264.7 cells can be attained only in the presence of cAMP, which induces ABCA1 expression. In the present study, cholesterol efflux either by control HDL or HDL abundant in PAF-AH was not noted in the absence of cAMP (data not shown). HDL with overexpressed human PAF-AH exerted a more inhibitory effect on foam cell formation by oxidized LDL compared with control HDL in the absence of cAMP. It indicates that this inhibitory effect on foaming was due to the conversion of oxidized LDL to a less atherogenic lipoprotein by PAF-AH. On the other hand, HDL abundant in PAF-AH facilitated lipid removal from foam cells when incubated with cAMP. This observation, together with the finding obtained from the oxidation assay of lipoprotein particles, supports the notion that PAF-AH prevented HDL modulation by oxidative stress from activated foam cells and maintained its function. However, we can also speculate that abundant PAF-AH contributed to facilitated ABCA1-mediated cholesterol efflux. Future studies are required to clarify the importance of PAF-AH for ABCA1-mediated cholesterol efflux. These results indicate that the enrichment of PAF-AH on HDL may be crucial in preserving the function of HDL, as was observed for PON1.

In summary, we demonstrated that human PAF-AH possesses multifaceted antiatherogenic properties; it binds to all the lipoproteins and protects them from oxidation, reducing proatherogenic lipoproteins and preserving HDL functions.

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Figure I. Plasma total cholesterol levels after injection of adenoviruses. (A) Data from wild type mice (AdPAF-AH, n = 6; AdLacZ, n = 6), and (B) data from apoE deficient mice (AdPAF-AH, n = 6; AdLacZ, n = 5). (open circles) mice injected with AdPAF-AH; (closed squares) mice injected with AdLacZ. Data are mean(SEM). *: P<0.05 versus day 0. No statistical differences were observed between AdPAF-AH group and AdLacZ group on the same day.
Figure II. Changes in plasma PAF-AH activity and Western blot assay of human PAF-AH. (A) Wild type mice (AdPAF-AH, n = 6; AdLacZ, n = 6); (B) ApoE deficient mice (AdPAF-AH, n = 6; AdLacZ, n = 5). (open circles) mice injected with AdPAF-AH; (closed squares) AdLacZ group. Data are mean(SEM). *: P<0.05 versus AdLacZ group on the same day. Western blot analysis of the plasma from AdPAF-AH group is also depicted.