methods

Altered distribution of plasma PAF-AH between HDLs and other lipoproteins in hyperlipidemia and diabetes mellitus

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Abstract Platelet-activating factor acetylhydrolase (PAF-AH) is a phospholipase A_2 associated with lipoproteins that hydrolyzes platelet-activating factor (PAF) and oxidized phospholipids. We have developed an ELISA for PAF-AH that is more sensitive than previous methods, and have quantified HDL-associated and non-HDL-associated PAF-AH in healthy, hyperlipidemic, and diabetic subjects. In healthy subjects, plasma total PAF-AH concentration was positively correlated with PAF-AH activity and with plasma total cholesterol, triacylglycerol, LDL cholesterol and apolipoprotein B (apoB) concentrations (all $P < 0.01$). HDL-associated PAF-AH concentration was correlated positively with plasma apoA-I and HDL cholesterol. Subjects with hyperlipidemia (n = 73) and diabetes mellitus (n = 87) had higher HDL-associated PAF-AH concentrations than did controls ($P < 0.01$). Non-HDL-associated PAF-AH concentration was lower in diabetic subjects than in controls ($P < 0.01$). Both hyperlipidemic and diabetic subjects had lower ratios of PAF-AH to apoB ($P < 0.01$) and higher ratios of PAF-AH to apoA-I ($P < 0.01$) than did controls. Our results show that the distribution of PAF-AH mass between HDLs and LDLs is determined partly by the concentrations of the lipoproteins and partly by the mass of enzyme per lipoprotein particle, which is disturbed in hyperlipidemia and diabetes mellitus.—Kujiraoka, T., T. Iwasaki, M. Ishihara, M. Ito, M. Nagano, A. Kawaguchi, S. Takahashi, J. Ishi, M. Tsuji, T. Egashira, I. P. Stepanova, N. E. Miller, and H. Hattori. **Altered distribution of plasma PAF-AH between HDLs and other lipoproteins in hyperlipidemia and diabetes mellitus. *J. Lipid Res.* 2003. 44: 2006–2014.**

Supplementary key words platelet-activating factor acetylhydrolase • high density lipoprotein • low density lipoprotein • lipoprotein-associated phospholipase A_2 • ELISA • Invader® assay

Platelet-activating factor acetylhydrolase (PAF-AH; 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine acetylhydrolase, E.C.3.1.1.47) is a Ca^{2+}-independent phospholipase A_2 that catalyzes the conversion of platelet-activating factor (PAF) to lyso-PAF by hydrolyzing the acetyl group at the *sn*-2 position of the glycerol backbone (1–6). As PAF is a potent lipid mediator involved in inflammatory disease (7), inactivation of the bioactive phospholipid by PAF-AH has an antiinflammatory effect (8, 9). PAF-AH also hydrolyzes phospholipids containing oxidatively fragmented residues at the *sn*-2 position, suggesting that it might also have an antiatherogenic effect (10–13).

Plasma PAF-AH binds to lipoproteins with high affinity (2). Normally, most of the enzyme activity in plasma is associated with LDLs and the rest with HDLs (2). Although the physiologic role of the enzyme in lipoprotein metabolism is poorly understood, it is thought to protect LDLs from oxidative modification (8, 13). However, the plasma PAF-AH activity associated with LDLs is progressively lost during oxidative modification of the particle (14). Furthermore, oxygen radicals rapidly and irreversibly inactivate PAF-AH, providing a potential mechanism by which they might enhance the proinflammatory effects of PAF and oxidized phospholipids (15).

Approximately 4% of Japanese subjects are deficient in plasma PAF-AH activity, owing to PAF-AH gene mutations, such as V279F (3, 16) and Q281R (17). The frequency of plasma PAF-AH deficiency in children with severe bronchial asthma was 3-fold greater than normal (12%), suggesting that PAF-AH may play an important role in inflammatory and allergic responses (18). Many studies have suggested that deficiency of plasma PAF-AH is associated with inflammatory disease. It has been shown that plasma PAF-AH activity is decreased in patients with asthma (16), systemic lupus

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Abbreviations: CHO, Chinese hamster ovary; MAb, monoclonal antibody; PAF-AH, platelet-activating factor acetylhydrolase.

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erythematosus (19), and septic shock (20). These observations suggest that individuals with plasma PAF-AH deficiency are at increased risk of severe responses to allergic or inflammatory stimulation. Several groups have found that PAF-AH mutation increases susceptibility to inflammatory and allergic diseases (8, 21). More recently, an association of a PAF-AH mutation with atherosclerosis was reported in patients with abdominal aortic aneurysm (22) and in those with hypertrophic cardiomyopathy (23).

We have raised two monoclonal antibodies (MAbs) against PAF-AH purified from human plasma, and used them to develop a new sandwich ELISA that is more sensitive than previous assays and performs well on supernatants obtained after precipitation of apolipoprotein B (apoB)-containing lipoproteins from plasma. We then used the assay to compare plasma PAF-AH concentrations and the distribution of enzyme mass between HDLs and other lipoproteins in healthy, hyperlipidemic, and diabetic subjects.

METHODS

Materials

AF-Blue Toyopearl 650ML was purchased from TOSHO (Tokyo, Japan). 3-[(3-Cholamidopropyl) dimethyl-ammonio] propanesulfonic acid (CHAPS) was from Wako Pure Chemical Industries (Osaka, Japan). DEAE Sepharose, HiTrap Blue, HiTrapQ, and Protein A Sepharose FF were from Amersham Biosciences (Uppsala, Sweden).

Subjects

Blood from 100 apparently healthy volunteers (71 males, 29 females) who were taking no medications and who had fasted overnight was collected at the BML Clinical Reference Laboratory (Saitama, Japan). Blood from 73 patients with hyperlipidemia (type IIa or IIb; 45 males and 28 females) and from 87 non-insulin-dependent diabetics (52 males and 35 females) was collected in the outpatient clinic of the Hokkaido Hospital for Social Insurance (Sapporo, Japan). Fourteen of 73 hyperlipidemic subjects were taking a lipid-lowering medication, such as a statin or fenofibrate. Lipid profiles are shown in **Table 1**. Concentrations of total cholesterol, triacylglycerol, LDL cholesterol, and apoB were greater, and those of apoA-I were lower, in hyperlipidemic and diabetic subjects than in controls. HDL cholesterol was lower in diabetics than in controls.

Assay of PAF-AH activity

Plasma PAF-AH activity was determined as previously described (24). Briefly, EDTA-plasma diluted 1:10 (v/v) or purified PAF-AH was incubated with [^{3}H]PAF (hexadecyl-2-[^{3}H]acetyl-*sn*-glyceyl-3-phosphorylcholine; NEN, Boston, MA) for 30 min at 37°C. The reaction was stopped by addition of an equal volume of $CHCl_3$-CH_3OH (2:1; v/v), and after centrifugation at 1,500 *g* for 10 min at 4°C, the aqueous layer (supernatant) was removed. The supernatant was washed once with an equal volume of ice-cold $CHCl_3$. The radioactivity in the supernatant was measured in a scintillation counter (Beckman Coulter, Fullerton, CA).

PAF-AH activity was also measured in an automated analyzer (Prestage 24i; Tokyo Boeki Medical System, Tokyo, Japan) using a commercial kit (Azwell, Osaka, Japan) (25). This method uses a chromogenic substrate and, unlike the radioisotopic method just described, gives background readings of 50 IU/l or more in plasma in the absence of PAF-AH.

Purification of plasma PAF-AH

Purification of plasma PAF-AH was performed as previously described (24). Pooled LDL after apheresis was stored at −20°C. LDL was diluted in MES buffer (50 mM MES/NaOH, 0.5 M NaCl, and 10 mM CHAPS at pH 6.0). Any precipitate was removed by centrifugation. The resulting solution was applied to a preequilibrated AF-Blue Toyopearl 650ML column (2.6 × 40 cm) with MES buffer, and then the column was washed with the same buffer until absorbance of the buffer was below 0.3 at 280 nm. After replacing the buffer with MOPS buffer (50 mM MOPS/NaOH, 0.5 M NaCl, and 10 mM CHAPS at pH 7.4), the column was eluted with Tris buffer (50 mM Tris/HCl, 1.5 M NaCl, and 10 mM CHAPS at pH 8.0). The eluted fractions containing PAF-AH activity were collected, concentrated by ultrafiltration (Amicon YM 30; Millipore, Billerica, MA), and dialyzed against Tris buffer without NaCl. After dialysis, the pooled fraction was loaded onto a HiTrap Q column preequilibrated with Tris buffer without NaCl, and then eluted with a gradient of 0 to 1 M NaCl. The fractions containing PAF-AH activity were pooled and dialyzed against MES buffer. Then the pooled fraction was loaded onto a HiTrap Blue column preequilibrated with MES buffer, washed with the same buffer, and eluted with Tris buffer. The purity of the PAF-AH was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining.

Cloning of plasma PAF-AH and expression of recombinant PAF-AH

Human plasma PAF-AH cDNA encoding AA42-441 was obtained by RT-PCR from mRNA of human peripheral monocyte-derived macrophages. PCR was carried out using as the sense primer 5′-GCTGCATGCATACAAGTACTGATGGCTGCAAG-3′ and

TABLE 1. Clinical characteristics of healthy, hyperlipidemic, and diabetic subjects

	Healthy Controls	Hyperlipidemia	Diabetes
Number of subjects (M/F)	100 (71/29)	73 (45/28)	87 (52/35)
Age (y)	53.1 ± 7.8	50.3 ± 13.2	59.6 ± 11.0
Total cholesterol (mmol/l)	4.95 ± 0.86	6.79 ± 1.45[a]	5.50 ± 1.07[a]
Triacylglycerol (mmol/l)	1.35 ± 0.88	2.00 ± 1.90[a]	1.90 ± 1.38[a]
LDL cholesterol (mmol/l)	3.13 ± 0.85	4.87 ± 1.44[a]	3.74 ± 0.96[a]
HDL cholesterol (mmol/l)	1.54 ± 0.40	1.49 ± 0.39	1.38 ± 0.42[a]
apoB (g/l)	0.86 ± 0.24	1.50 ± 1.20[a]	1.12 ± 0.28[a]
apoA-I (g/l)	1.43 ± 0.25	1.33 ± 0.28[b]	1.28 ± 0.24[a]

apoB, apolipoprotein B. Results are represented as mean ± SD.
[a] $P < 0.01$, significant difference from healthy controls.
[b] $P < 0.05$, significant difference from healthy controls.

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as the antisense primer 5′-GCTGCTGACCTAATTGTATTTCTC-TATTCCTGAAG-3′. The PCR product was subcloned into the pQE30 vector (Qiagen). Isolated plasmid DNA was transformed into *Escherichia coli* M15 (pREP4), grown in 400 ml of TB medium containing 100 mg/l ampicillin and 25 mg/l kanamycin. Expression was induced with 1 mM isopropyl-β-D-galactoside, and cells were lysed with phosphate buffer (25 mM sodium phosphate, pH 8.0, and 0.5 M NaCl) containing 10 mM CHAPS, 10 mM 2-mercaptoethanol, and 10 mM imidazole. The lysate was loaded onto the Ni agarose column (Qiagen) and the recombinant protein eluted with phosphate buffer, pH 7.4, containing 10 mM CHAPS, 10 mM 2-mercaptoethanol, and 250 mM imidazole. The eluate was then loaded onto a Cibacron blue agarose column (Sigma, St. Louis, MO) equilibrated with MES buffer containing 10 mM 2-mercaptoethanol and 10 mM CHAPS, and eluted with Tris buffer containing 10 mM 2-mercaptoethanol. The eluate was applied to an Ni agarose column and eluted with 25 mM sodium phosphate buffer, pH 7.4 containing 10 mM 2-mercaptoethanol and 250 mM imidazole. Purified recombinant protein was dialyzed against 25 mM sodium phosphate buffer, pH 7.4, containing 0.5 M NaCl and analyzed by SDS-PAGE, using silver staining to visualize the proteins. The recombinant PAF-AH from *E. coli* showed one band of 45 kDa (not shown).

Chinese hamster ovary (CHO) cells transfected with plasmid pEF321 (26) inserted with plasma PAF-AH cDNA were cultured in serum-free RPMI medium, and the culture medium was collected. Recombinant PAF-AH (rhPAF-AH) was purified by Ni agarose and HiTrap Blue column chromatography as described above. The purity of rhPAF-AH was confirmed by SDS-PAGE followed by silver staining or immunoblotting. For immunoblotting, rhPAF-AH was detected with Penta- or Tetra-His antibody (Qiagen) as primary antibody and horseradish peroxidase-conjugated anti-mouse IgG (Zymed Laboratories, South San Francisco, CA) as secondary antibody. Bound antibodies were detected with an enhanced chemiluminescence kit (Perkin Elmer Life Sciences).

Preparation of MAbs against purified plasma PAF-AH

MAbs against purified plasma PAF-AH were obtained as previously described (26, 27). Balb/c mice were immunized with 25 μg purified PAF-AH, and spleen cells from the mice were fused with Sp2/0 cells (28). The supernatants of hybridoma cells were screened by ELISA using plates coated with purified PAF-AH (100 ng/well) and by immunoblotting. Positive hybridoma cells were cloned at least three times by limiting dilution and injected intraperitoneally into pristane-primed Balb/c mice. The IgG fraction was isolated from ascitic fluid using protein A-Sepharose FF according to the manufacturer's instructions, dialyzed at 4°C against PBS, and stored at −80°C. The specificities of MAb 8B1 and MAb A7G were confirmed by ELISA and immunoblotting against purified plasma PAF-AH and rhPAF-AH. MAb isotype was characterized using the IsoStrip mouse monoclonal antibody isotyping kit (Roche Diagnostics, Basel, Switzerland), and was IgG_1 and IgG_{2b} for MAb 8B1 and MAb A7G, respectively.

Measurement of plasma total PAF-AH concentration

MAb 8B1 (100 μl of 5 μg/ml solution in PBS) was coated onto a microtiter plate (Nunc Immunoplate II) by incubation at 4°C overnight. The wells were then blocked with 200 μl of PBS containing 30 g/l BSA for 2 h at room temperature. After the plate had been washed with 200 μl of PBS containing 1 ml/l Tween 20, 100 μl of the calibrator solution and diluted plasma samples (1:50) were added and incubated for 2 h at room temperature. After the plate had been washed five times, 100 μl of 1 μg/ml biotinylated MAb A7G was added to each well and the mixture was incubated for 2 h at room temperature. After the plate had been washed five times, 100 μl/l of 1 g/l horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA) was added and the mixture was incubated for 1 h at room temperature. After the plate had been washed, 100 μl of substrate solution (50 mM citrate-phosphate buffer, pH 5.0) containing 0.25 g/l *o*-phenylenediamine dihydrochloride and 0.15 ml/l H_2O_2 was added to each well. After 0.5 h, the reaction was stopped by addition of 100 μl of 4 mol/l H_2SO_4. The absorbance was measured at 492 nm by a microplate reader. Purified rhPAF-AH and pooled culture medium from CHO cells expressing rhPAF-AH served as primary and secondary calibrators, respectively.

When purified rhPAF-AH was added to samples of plasma (n = 4) in amounts sufficient to raise the total PAF-AH concentration by 0.6–3.0 μg/ml, the final concentrations given by the ELISA averaged 93.2% (82.5% to 105.9%) of those predicted. The intra- and interassay coefficients of variation of the ELISA were <4.6% and <3.8%, respectively (n = 10). No interference with the ELISA was observed with hemoglobin (5.0 g/l), bilirubin (0.3 g/l), or triacylglycerol (4.25 g/l). Storage of plasma and serum samples for 14 days did not affect the PAF-AH concentration as determined by the ELISA (data not shown).

Measurement of HDL-associated PAF-AH concentration

The ELISA was applied to the supernatant obtained when apoB-containing lipoproteins were precipitated from plasma with phosphotungstate and magnesium chloride using reagents from a commercial kit for measurement of HDL cholesterol (Daiichi Pure Chemicals, Tokyo, Japan). The supernatant was first diluted 10-fold with dilution buffer. The intra- and interassay coefficients of variation for the entire HDL-associated PAF-AH concentration assay, including the precipitation step, were 5.2% and 5.9%, respectively (n = 8). When plasma from healthy subjects was added to PAF-AH-deficient plasma (from a homozygote for the V279F mutation), the recovery of HDL-associated PAF-AH concentration by the ELISA was 95.4 ± 7.6% (mean ± SD, n = 8). Non-HDL-associated PAF-AH was then calculated by difference.

Determination of PAF-AH gene genotype by Invader® assay

The V279F and Q281R genotypes of PAF-AH were detected by the Invader® assay as previously described (29). Primary probes and Invader oligonucleotides for each mutation were designed with Invader® Creator software to have theoretic annealing temperatures of 63°C and 77°C, respectively, using a nearest-neighbor algorithm on the basis of final probe and target concentrations. The primary probes and Invader oligonucleotides used are shown in **Table 2**. Genotyping was performed by calculation, using the ratios of net counts with wild primary probe to net counts with mutant primary probe. The accuracy of each genotyping was 100%, determined by comparison with results previously obtained by PCR-restriction fragment length polymorphism analysis and direct sequencing.

Other laboratory methods

Measurements of plasma total cholesterol, triacylglycerol, and HDL cholesterol concentrations were performed in a Hitachi 7450 automated analyzer using commercial kits (Daiichi Pure Chemicals). HDL cholesterol was measured after precipitation of apoB-containing lipoproteins with dextran sulfate, phosphotungstate, and magnesium chloride. LDL cholesterol concentration was calculated according to Friedewald, Levy, and Fredrickson (30). Protein content was determined by the bicinchoninic acid (BCA) protein assay kit (Pierce) using BSA as a calibrator. SDS-

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TABLE 2. Oligonucleotide sequences of wild-type, mutant and Invader probes for detection of the PAF-AH gene mutations

Mutation	Nucleotide Change	Target	Probes	Sequences	Dye
V279F	G→T	Sense	Wild	5′-A**CGGACGCGGAG**CCGTTGCTCCACCAV-3′	RED
			Mutant	5′-**CGCGCCGAGG**ACGTTGCTCCACCAV-3′	FAM
			Invader	5′-ACTATCTTATTTTCTTACCTGAATCTCTGATCTTCACTAAGAGTCTGAATAAT-3′	
Q281R	A→G	Antisense	Wild	5′-**CGCGCCGAGG**AGACTCTTAGTGAAGATCAGAV-3′	FAM
			Mutant	5′-**ACGGACGCGGAG**GGACTCTTAGTGAAGATCAGAV-3′	RED
			Invader	5′-GCAGTAATTGGACATTCTTTTGGTGGAGCAACGGTTATTC-3′	

PAF-AH, platelet-activating factor acetylhydrolase; V, amino blocking group. The flap sequences of primary probes are represented in boldface.

PAGE was performed by the Laemmli method (31), and immunoblotting as described by Towbin, Staehelin, and Gordon. (32). Silver staining was carried out using a kit (Daiichi Pure Chemicals). The LDL fraction (d = 1.006–1.063 kg/l) was isolated from fresh human plasma by sequential preparative ultracentrifugation, as previously described (26).

Statistical analysis

Results were expressed as mean ± SD. ANOVA was used for group comparisons. Correlations were assessed by least-squares regression analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of anti-PAF-AH MAbs

The purified plasma PAF-AH and rhPAF-AH from the culture medium of CHO cells showed a major band of 50–60 kDa protein (**Fig. 1A**). These represented more than 90% of total protein after scanning of the gel. Mice were immunized with purified plasma PAF-AH, and two MAbs specific for PAF-AH were established: MAb 8B1 and MAb A7G. When rhPAF-AH and LDLs from human plasma were subjected to SDS-PAGE, both MAbs reacted with a single protein (Fig. 1B), the molecular mass of which (50–60 kDa; Fig. 1B, lanes 1 and 2) was similar to that previously reported for human plasma PAF-AH (24). By agarose electrophoresis and Western blotting, PAF-AH was detected in the portion corresponding to LDLs, but not in that corresponding to HDLs, presumably owing to the lower content of PAF-AH in the latter (data not shown). There was no evidence of recognition of other plasma proteins. Both MAbs reacted with purified PAF-AH coated onto a microtiter plate (**Fig. 2**). Neither inhibited enzyme activity, suggesting that they may react with epitopes remote from the catalytic site of the enzyme (data not shown).

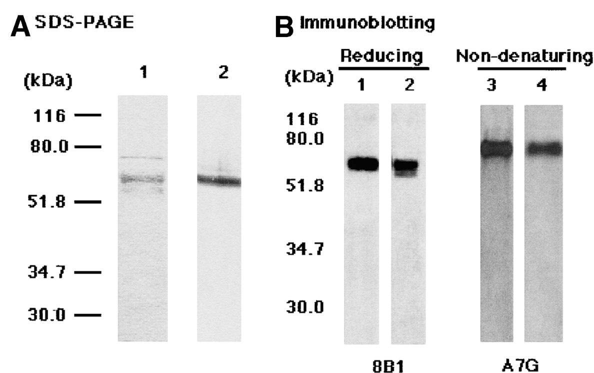

Fig. 1. Characterization of (A) purified plasma platelet-activating factor acetylhydrolase (PAF-AH) and recombinant PAF-AH (rhPAF-AH) and (B) monoclonal antibodies (MAbs). A: Purified PAF-AH (3 μg) was analyzed by SDS-PAGE and visualized by Coomassie brilliant blue (lane 1); rhPAF-AH (0.2 μg) was visualized by silver staining (lane 2). B: Purified rhPAF-AH (0.2 μg; lanes 1 and 3) and LDL (50 μg; lanes 2 and 4) from human plasma were subjected to SDS-PAGE under reducing conditions (lanes 1 and 2) or to nondenaturing PAGE (lanes 3 and 4). Immunoblotting with MAb 8B1 (lanes 1 and 2) or MAb A7G (lanes 3 and 4) was performed as described under Methods.

Standardization of ELISA for plasma PAF-AH concentration

A sandwich ELISA for plasma PAF-AH was established using MAb 8B1 for capture and biotinylated MAb A7G for detection. This system showed a dose-dependent response to purified rhPAF-AH, rhPAF-AH culture medium, and plasma (**Fig. 3**). For calibration of the ELISA, rhPAF-AH was purified from the culture medium of transfected CHO cells. When subjected to SDS-PAGE and visualized by silver staining, the purified rhPAF-AH showed a single major 50–60 kDa band (Fig. 1), which represented >90% of the total protein in the preparation (as determined by gel scanning using the Intelligent Quantifier system (Bio Image, Ann Arbor, MI). The protein concentration of this primary rhPAF-AH calibrator, assayed using a BCA protein kit with BSA as calibrator, was typically 23.1 μg/ml.

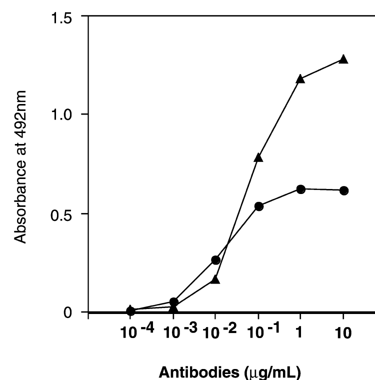

Fig. 2. The reactivity of MAbs against purified PAF-AH. Purified PAF-AH (100 ng/well) was coated onto a microtiter plate. ELISA was carried out as described under Methods. Closed circles: 8B1; closed triangles: A7G.

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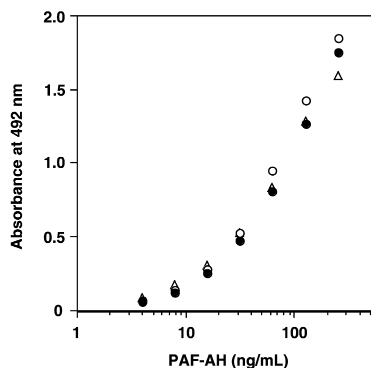

Fig. 3. Titration curves of the PAF-AH ELISA. The ELISA was performed as described under Methods. The titration curves were made using serial dilutions (1:10 to 1:640) of purified rhPAF-AH (2.0 μg/ml, closed circles), rhPAF-AH culture medium (3.1 μg/ml, open circles), or human plasma (2.7 μg/ml, open triangles). Each point represents the mean of triplicate determinations.

To obtain a calibration curve for the ELISA, dilutions of the primary calibrator were made in PBS containing 1 ml/l Tween 20 to provide 0.125–8.0 ng of rhPAF-AH protein per well (1.25–80.0 ng/ml). When the rhPAF-AH culture medium, as a secondary calibrator, was diluted in PBS containing 1 ml/l Tween 20 to cover the PAF-AH concentration range 1.25–80.0 ng /ml, the curve was identical to that obtained with the primary calibrator (**Fig. 4**). The ELISA was linear up to 80.0 ng/ml and suitable for quantifying PAF-AH concentrations as low as 0.3 ng/ml. To avoid potential nonlinearity caused by very low or high absorbance, the PAF-AH concentrations in plasma samples were measured using several dilutions (1:640 to 1:10), and the least-diluted aliquot that gave an absorbance between 0.5 and 1.2 was chosen.

The detergent Tween 20 was included in the diluent to avoid any effects of differences between samples in their lipid or apolipoprotein compositions. We examined several detergents for sample dilution, including Triton X-100, Nonidet P-40, SDS, CHAPS, deoxy-BIGCHAP, *N*-heptyl-β-D-thioglucoside, *N*-octyl-β-D-thioglucoside, *N*-dodecyl-β-D-maltoside, MEGA-8, MEGA-9, sucrose monocaprate (SM-1000), sodium cholate, and digitonin (Detergent Starter Kit II; Wako Pure Chemical Industries). Plasma samples diluted with PBS containing each detergent gave similar absorbance, and the values for PAF-AH concentration did not differ from those obtained with PBS containing 1 ml/l Tween 20 (data not shown).

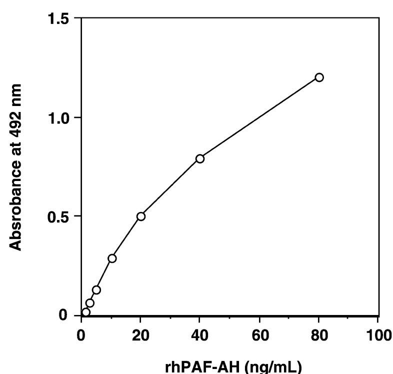

Fig. 4. Standard curve for purified rhPAF-AH concentration by ELISA. The standard curve was made using serial dilutions (1:2 to 1:64) of 80 ng/ml purified rhPAF-AH. Each point is the mean of triplicate determinations.

Correlations between PAF-AH concentration and activity

Plasma total PAF-AH concentration measured by the ELISA was strongly correlated with total PAF-AH activity. PAF-AH concentration and activity in the supernatant of plasma after precipitation of apoB-containing lipoproteins were also strongly positively associated. These relations are shown in **Fig. 5**.

Plasma total, HDL-associated, and non-HDL-associated PAF-AH concentrations in healthy subjects

Results for PAF-AH concentration in healthy men and women are presented in **Table 3**. On average, 10.7% of PAF-AH mass was associated with HDLs. The concentrations of total and non-HDL-associated PAF-AH were higher in men than in women. Plasma total PAF-AH concentration was positively correlated with plasma total cholesterol ($r = 0.48$), triacylglycerol (0.27), LDL cholesterol (0.47), and apoB (0.51) concentrations (all $P < 0.01$). These correlations reflected underlying associations with

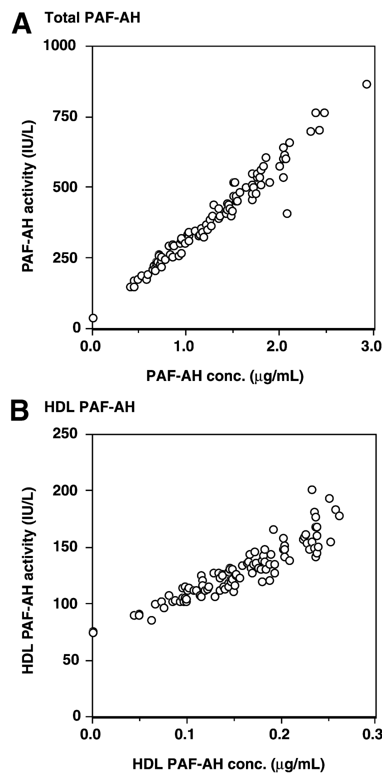

Fig. 5. Relation of the activities of total PAF-AH and HDL-associated PAF-AH to the total and HDL-associated PAF-AH concentrations in healthy subjects. Plasma total (A) and HDL-associated (B) PAF-AH activities were determined colorimetrically using a chromogenic substrate. Experimental details are provided under Methods.

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TABLE 3. Plasma PAF-AH concentrations

Subjects	n	PAF-AH Concentrations Total	HDL-Associated	Non-HDL-Associated
		μg/ml		
Controls				
Male	69	1.38 ± 0.57	0.14 ± 0.05	1.24 ± 0.53
Female	29	1.09 ± 0.45[c]	0.16 ± 0.09	0.94 ± 0.38[c]
All	98	1.30 ± 0.55	0.14 ± 0.07	1.15 ± 0.51
Hyperlipidemia				
Male	41	1.74 ± 0.70	0.66 ± 0.34[a]	1.08 ± 0.44
Female	28	1.42 ± 0.75	0.55 ± 0.29[a]	0.87 ± 0.57
All	69	1.61 ± 0.73[b]	0.61 ± 0.32[a]	1.00 ± 0.51[b]
Diabetes				
Male	50	1.52 ± 0.63	0.54 ± 0.25[a]	0.98 ± 0.50[a]
Female	33	1.11 ± 0.47[c]	0.44 ± 0.17[a]	0.68 ± 0.33[a,c]
All	88	1.36 ± 0.60	0.50 ± 0.23[a]	0.86 ± 0.46[a]

Results are represented as mean ± SD.
[a] $P < 0.01$, significant difference from healthy controls.
[b] $P < 0.05$, significant difference from healthy controls.
[c] $P < 0.01$, significant difference from males.

non-HDL PAF-AH. The correlations of non-HDL-associated PAF-AH concentration with LDL cholesterol and apoB concentrations appear in **Fig. 6**. HDL-associated PAF-AH was correlated positively with HDL cholesterol (0.54) and apoA-I (0.40) and negatively with LDL cholesterol (−0.44) and apoB (−0.60) (all $P < 0.01$) (**Fig. 7**). Non-HDL-associated and HDL-associated PAF-AH concentrations were negatively correlated with each other ($r = -0.30$, $P < 0.01$).

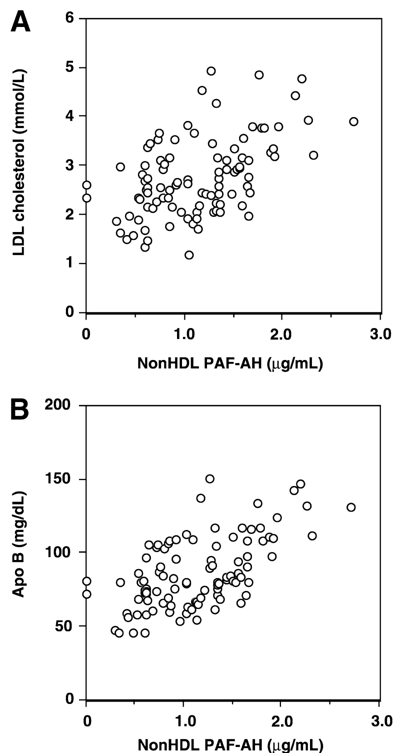

Fig. 6. Correlations of non-HDL-associated PAF-AH concentration with LDL cholesterol (A) and apolipoprotein B (apoB) (B) concentrations.

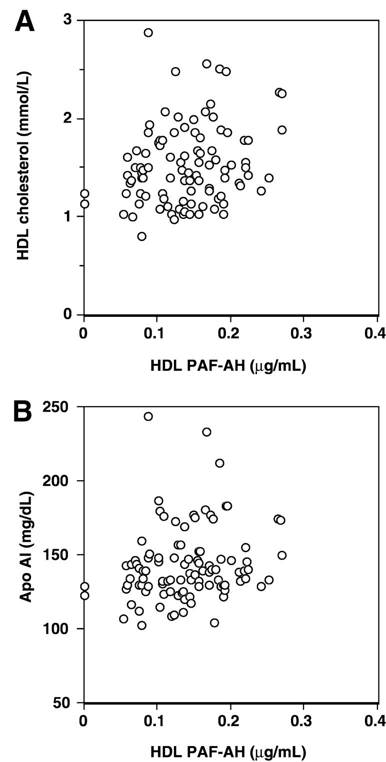

Fig. 7. Correlations of HDL-associated PAF-AH concentration with HDL cholesterol (A) and apoA-I (B) concentrations.

Plasma total, HDL-associated, and non-HDL-associated PAF-AH concentrations in hyperlipidemic subjects

In both men and women, HDL-associated PAF-AH concentration was much greater in hyperlipdemic subjects than in healthy controls, whereas there was no significant difference in non-HDL-associated PAF-AH (Table 3). This was in spite of the fact that LDL cholesterol and apoB concentrations were higher than in controls, and there was significant difference between the two groups in HDL cholesterol or apoA-I. The positive correlations of non-HDL PAF-AH concentration with total cholesterol (0.21, NS), LDL cholesterol (0.32, $P < 0.01$), and apoB (0.23, NS) were weaker in hyperlipidemic subjects than in controls, while HDL-associated PAF-AH concentration was not significantly correlated with either HDL cholesterol (−0.03) or apoA-I (0.003). The ratio of non-HDL-associated PAF-AH to apoB was significantly lower in hyperlipidemic subjects than in controls, while that of HDL-associated PAF-AH to apoA-I was significantly greater (**Table 4**). In contrast to the situation in controls, HDL-associated and non-HDL-associated PAF-AH concentrations were positively associated ($r = 0.67$, $P < 0.01$).

Plasma total, HDL-associated, and non-HDL-associated PAF-AH concentrations in non-insulin-dependent diabetes

As in the hyperlipidemic subjects, in both men and women, HDL-associated PAF-AH concentration was significantly greater, and non-HDL-associated PAF-AF concentration was significantly lower in non-insulin-dependent diabetic subjects than in healthy controls. This was despite the

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TABLE 4. Ratios of PAF-AH mass to apolipoprotein mass in healthy controls, hyperlipidemic subjects, and diabetic subjects

Subjects	V279F Genotype	Non-HDL PAF-AH/apoB	HDL-PAF AH/apoA-I
Controls	Wild-type	16.5 ± 3.1	1.3 ± 0.4
	Heterozygous	8.3 ± 1.6	0.7 ± 0.2
Hyperlipidemia	Wild-type	8.5 ± 2.5[a]	6.4 ± 2.7[a]
	Heterozygous	3.5 ± 1.5[b]	3.7 ± 1.5[b]
Diabetes	Wild-type	9.0 ± 4.2[a]	4.6 ± 2.2[a]
	Heterozygous	4.7 ± 2.0[b]	2.4 ± 0.8[b]

Results are represented as mean ± SD.
[a] $P < 0.01$, significant differences from healthy controls of the wild-type Val/Val at position 279.
[b] $P < 0.01$, from healthy controls of the Val/Phe at position 279.

fact that HDL cholesterol and apoA-I were lower and LDL cholesterol and apoB were greater than in controls. Also as in the hyperlipidemic subjects, the ratio of non-HDL-associated PAF-AH to apoB was significantly lower, and the ratio of HDL-associated PAF-AH to apoA-I was significantly greater than in the controls (Table 4), while HDL-associated and non-HDL-associated PAF-AH concentrations were positively correlated with each other ($r = 0.71$, $P < 0.01$).

Plasma PAF-AH concentration in relation to PAF-AH genotype

A missense mutation of V279F in the PAF-AH gene, resulting in complete loss of activity, is common in Japanese subjects (16, 18). In the present study, the allele frequency for Val279 was 0.80, 0.80, and 0.84 in controls, hyperlipidemic subjects, and diabetic subjects, respectively. We also identified a healthy subject and a diabetic subject who were heterozygous for the Q281R mutation. These results were consistent with those previously reported for the Japanese population (16, 18). Within each of the three clinical groups of subjects, heterozygotes for the V279F mutation had significantly lower total, non-HDL-associated, and HDL-associated PAF-AH concentrations than subjects with wild-type PAF-AH (**Table 5**). In homozygotes for the V279F mutation, PAF-AH was undetectable (data not shown).

TABLE 5. PAF-AH concentration in relation to the V279F mutation

Genotype at Codon 279	n	PAF-AH Concentrations		
		Total	HDL-Associated	Non-HDL-Associated
		μg/ml		
Controls				
Val/Val	61	1.61 ± 0.44	0.18 ± 0.06	1.44 ± 0.04
Val/Phe	37	0.79 ± 0.24[b]	0.09 ± 0.03 [b]	0.69 ± 0.22
Hyperlipidemia				
Val/Val	47	1.98 ± 0.59[a]	0.75 ± 0.30 [a]	1.23 ± 0.44[c]
Val/Phe	23	0.89 ± 0.33[b]	0.34 ± 0.12 [a,b]	0.55 ± 0.28[a,b]
Diabetes				
Val/Val	61	1.56 ± 0.56	0.58 ± 0.22 [a]	0.99 ± 0.46[a]
Val/Phe	24	0.78 ± 0.28[b]	0.29 ± 0.09 [a,b]	0.51 ± 0.24[a,b]

Results are represented as mean ± SD.
[a] $P < 0.01$, significant difference from healthy controls.
[b] $P < 0.01$, significant difference from the wild-type Val/Val at position 279.
[c] $P < 0.05$, significant difference from healthy controls.

DISCUSSION

We have developed a sandwich ELISA for plasma PAF-AH concentration, using two MAbs against PAF-AH purified from human plasma. The specificity of the antibodies was confirmed by immunoblotting. Both MAb 8B1 and MAb A7G reacted with a single protein in human plasma of 50–60 kDa molecular mass, which is the same as that previously reported for purified plasma PAF-AH (24). MAb A7G reacted with human LDLs only under nondenaturing conditions, while MAb 8B1 reacted with LDLs under reducing conditions only, even though both MAbs reacted similarly with purified rhPAF-AH and purified plasma PAF-AH coated onto a microtiter plate. Neither antibody inhibited the activities of PAF-AH in plasma or rhPAF-AH (data not shown). Our ELISA can be used to measure up to 30 μg/ml of plasma PAF-AH with linearity. A strong correlation between plasma PAF-AH concentration and its activity suggested that our assay reveals all active enzyme in plasma.

Plasma PAF-AH concentration has been measured by others with a sandwich ELISA using MAbs (33, 34). Plasma PAF-AH concentrations observed in healthy English subjects (1.03 ± 0.04 μg/ml, mean ± SEM) (33) and Scottish subjects (2.27 ± 0.57 μg/ml, mean ± SD) (34) were similar to those that we have observed in healthy Japanese subjects (1.27 ± 0.57 μg/ml, mean ± SD). Caslake et al. (33) reported that plasma PAF-AH concentration correlated positively with plasma total cholesterol, triacylglycerol, LDL cholesterol, and apoB concentrations, and negatively with HDL cholesterol in normal subjects. Similar relations were present in our data, with the exception of HDL cholesterol.

It has been reported that plasma PAF-AH activity is higher in subjects with type IIa or IIb hyperlipidemia (35) and in subjects with coronary artery disease (33, 34) than in normal subjects. We also found high concentrations of PAF-AH in hyperlipidemic subjects ($P < 0.05$). However, non-insulin-dependent diabetic subjects did not have significantly raised PAF-AH concentrations, even though they had raised plasma total cholesterol concentrations.

Four percent of the Japanese population carries a substitution of G to T at 994 nt of the PAF-AH gene, resulting in an amino acid change of Phe for Val at position 279. Homozygotes for this variation have almost complete absence of enzyme activity. In the present study, we identified seven homozygotes and 84 heterozygotes for the V279F mutation and two heterozygotes for the Q281R mutation. The allele frequency for the V279F mutation was similar to that previously described in Japan (16, 18). PAF-AH was undetectable in plasma from homozygotes by our sandwich ELISA, while concentrations in heterozygotes were about half those in the controls. Stafforini et al. (3) have shown that the V279F mutant protein is not secreted by cells. They have also shown that a Q281R mutant, expressed in *E. coli*, has about half the activity of the wild type (18). We identified four additional heterozygous subjects with the Q281R mutation, whose mean PAF-AH concentration was low (0.81 ± 0.14 μg/ml, n = 4), suggesting

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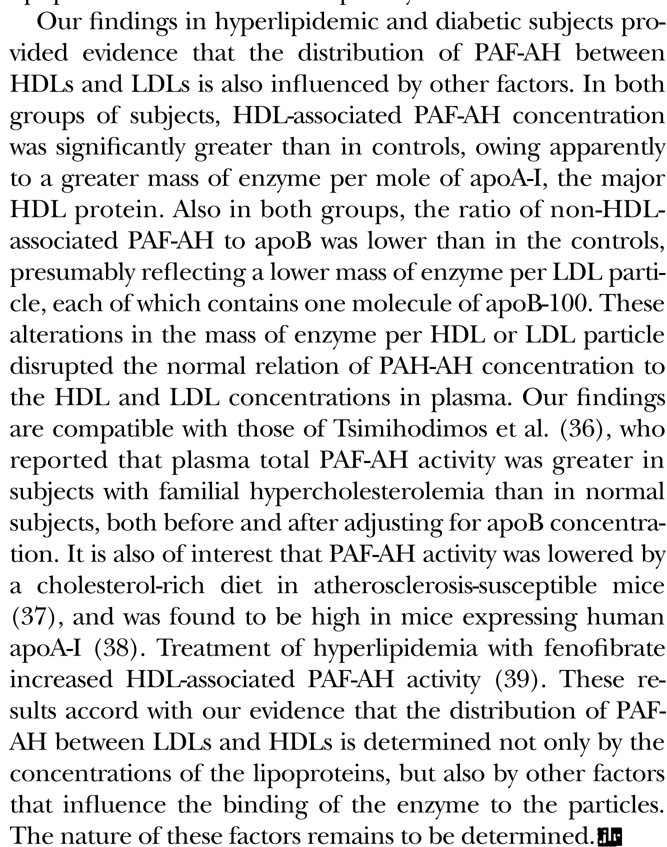

that the mutant Q281R protein may not be secreted (unpublished observation). Among subjects with wild-type PAF-AH, the concentration of PAF-AH was greater in hyperlipidemic subjects than in controls, whereas this was not the case among heterozygotes for the V279F mutation.

Plasma HDL-associated PAF-AH concentration, measured after precipitation of apoB-containing lipoproteins, was strongly positively correlated with HDL-associated PAF-AH activity. The proportion of PAF-AH activity in HDLs in healthy Japanese subjects was somewhat lower than observed in another population (2), suggesting that the distribution of the enzyme between HDLs and LDLs may be influenced by ethnicity. Our finding that in normal subjects, the mass of non-HDL-associated PAF-AH was correlated positively with LDL cholesterol and apoB concentrations, while that of HDL-associated PAF-AH was correlated positively with HDL cholesterol and apoA-I concentrations, strongly suggests that the distribution of the enzyme among lipoproteins is determined in part by their concentrations.

Our findings in hyperlipidemic and diabetic subjects provided evidence that the distribution of PAF-AH between HDLs and LDLs is also influenced by other factors. In both groups of subjects, HDL-associated PAF-AH concentration was significantly greater than in controls, owing apparently to a greater mass of enzyme per mole of apoA-I, the major HDL protein. Also in both groups, the ratio of non-HDL-associated PAF-AH to apoB was lower than in the controls, presumably reflecting a lower mass of enzyme per LDL particle, each of which contains one molecule of apoB-100. These alterations in the mass of enzyme per HDL or LDL particle disrupted the normal relation of PAH-AH concentration to the HDL and LDL concentrations in plasma. Our findings are compatible with those of Tsimihodimos et al. (36), who reported that plasma total PAF-AH activity was greater in subjects with familial hypercholesterolemia than in normal subjects, both before and after adjusting for apoB concentration. It is also of interest that PAF-AH activity was lowered by a cholesterol-rich diet in atherosclerosis-susceptible mice (37), and was found to be high in mice expressing human apoA-I (38). Treatment of hyperlipidemia with fenofibrate increased HDL-associated PAF-AH activity (39). These results accord with our evidence that the distribution of PAF-AH between LDLs and HDLs is determined not only by the concentrations of the lipoproteins, but also by other factors that influence the binding of the enzyme to the particles. The nature of these factors remains to be determined.

N. E. Miller was British Heart Foundation Professor of Cardiovascular Biochemistry.

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