N-linked glycosylation of macrophage-derived PAF-AH is a major determinant of enzyme association with plasma HDL

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Abstract Human plasma PAF-AH (platelet-activating factor-acetylhydrolase) is a Ca^{2+}-independent phospholipase A_2 of hematopoietic origin associated with LDL and HDL; it degrades PAF and oxidizes phospholipids. We show that human macrophages synthesize PAF-AH as a premedial Golgi precursor containing high mannose N-linked glycans. Secreted PAF-AH possesses a molecular mass of ~55 kDa and contains mature N-linked glycans. Secreted PAF-AH activity (90 ± 4% of the total) bound to a wheat germ lectin column and could be eluted with *N*-acetylglucosamine, whereas digestion with *N*-acetylneuraminidase II completely abolished enzyme absorption. Tunicamycin significantly reduced cell-associated PAF-AH activity and inhibited enzyme secretion; but it did not alter the ratio of secreted to cell-associated enzyme (1.8 at 6 h and 3.1 at 24 h), suggesting that glycosylation is not essential for PAF-AH secretion. Digestion of cell-associated PAF-AH or secreted PAF-AH with peptide *N*-glycosidase F affected neither catalytic activity nor its resistance to proteolysis with trypsin or proteinase K; in addition, it did not affect PAF-AH association with LDL, but significantly increased its association with HDL. We suggest that macrophage-derived PAF-AH contains heterogeneous asparagine-conjugated sugar chain(s) involving sialic acid, which hinders its association with HDL but does not influence the secretion, catalytic activity, or resistance of PAF-AH to proteases.—Tselepis, A. D., S-A. P. Karabina, D. Stengel, R. Piédagnel, M. J. Chapman, and E. Ninio. **N-linked glycosylation of macrophage-derived PAF-AH is a major determinant of enzyme association with plasma HDL. *J. Lipid Res.* 2001.** 42: **1645–1654.**

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Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent lipid mediator involved in allergic and inflammatory diseases (1) that may play an equally crucial role in atherogenesis (2). PAF is rapidly hydrolyzed and converted to lyso-PAF by PAF-acetylhydrolase (PAF-AH) (EC 3.1.1.47). PAF-AH represents a family of Ca^{2+}-independent phospholipase A_2-like enzymes that have a marked preference for phospholipids with short-chain moieties at the *sn*-2 position of glycerol (3). There are intracellular and extracellular (secreted) forms of this enzyme. Among the secreted forms, plasma PAF-AH, in contrast to other plasma phospholipases, circulates in active form complexed with lipoproteins, particularly with LDL and HDL (4, 5). With the exception of PAF, plasma PAF-AH can also effectively hydrolyze oxidized phospholipids produced by peroxidation of phosphatidylcholines containing an *sn*-2 polyunsaturated fatty acyl residue (6). Such oxidized phospholipids are formed during the oxidative modification of LDL and play key roles in various aspects of atherogenesis (7). Consequently, PAF-AH may be implicated in atherogenesis and on a biochemical and pathophysiological basis, this enzyme would appear to have a dual role. First, it degrades PAF and oxidized phospholipids, and thus it can act as a potent antiinflammatory and antiatherogenic enzyme as shown by adenoviral overexpression of human PAF-AH in apolipoprotein E-deficient ($apoE^{-/-}$) mice (8). On the other hand, PAF-AH may play a proinflammatory and proatherogenic role because, during the hydrolysis of oxidized phospholipids, it generates lysophosphatidylcholine leading to the enrichment of oxidized LDL in this phospholipid (9). Lysophosphatidylcholine contributes to several biological effects of oxidized LDL (10). Also contradictory are the results from clinical studies, because some authors suggest that the

Abbreviations: BFA, brefeldin A; NANase II, *N*-acetylneuraminidase II; PAF, platelet-activating factor; PAF-AH, PAF-acetylhydrolase; PNGase F, peptide *N*-glycosidase F.

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G^{994}→T mutation in the PAF-AH gene, which results in a V^{279}→F substitution and in a loss of the enzyme catalytic activity, could be one of the genetic determinants for atherosclerotic disease in the Japanese population (11), whereas others suggest that the mass of plasma PAF-AH could be a potential risk factor for coronary artery disease (12).

The cellular origins of plasma PAF-AH remain to be defined, although several studies have suggested that the principal sources could be monocyte-derived macrophages (13), platelets (14), and liver cells (15, 16). More recently it was shown that PAF-AH activity in plasma originates mostly from cells of the hematopoietic lineage (17). The cDNA of human plasma PAF-AH encodes a 45-kDa protein having a typical signal sequence and a serine esterase consensus motif, GXSXG (18). Plasma PAF-AH is N-glycosylated (19, 20); however, the role of such glycosylation remains to be established.

In the present study, we investigated the nature and potential role of glycosylation in the cellular secretion and catalytic activity of PAF-AH and the impact of such glycosylation on its association with plasma lipoproteins. As a cell model, we chose human peripheral blood monocyte-derived macrophages, because these cells are a major source of plasma PAF-AH. Our studies reveal that PAF-AH secreted from macrophages contains asparagine-conjugated carbohydrate chains of the complex type, containing sialic acid residues. Such glycosylation hinders association of the enzyme with HDL, but does not appear to play a significant role in the cellular secretion or resistance of the enzyme to proteases.

MATERIALS AND METHODS

Materials

Restriction enzymes, plasmids, and molecular markers for DNA size (Promega, Charbonnières, France) were used according to the manufacturer specifications. RNA Plus, phenol, and oligonucleotides were from Bioprobe Systems (Montreuil sous Bois, France). Thermostable DNA polymerase was obtained from Appligene (Appligene Oncor, Illkirch, France). Superscript Moloney murine leukemia virus (Mo-MuLV) reverse transcriptase was obtained from GIBCO-BRL (Bethesda, MD). PAF (hexadecyl), obtained as a powder from Sigma (St. Louis, MO), was dissolved at a final concentration of 20 mM in ethanol (80%, v/v). This solution was mixed with 1-*O*-hexadecyl-2-[^{3}H-acetyl]-*sn*-glycero-3-phosphocholine solution in ethanol (10 Ci/mmol; New England Nuclear, Boston, MA), dried under a stream of nitrogen, and redissolved in a solution containing fatty acid-free BSA/saline (0.25%), to obtain [^{3}H-acetyl]PAF solution at a concentration of 1 mM. Human recombinant PAF-AH and rabbit polyclonal antisera raised against the recombinant acetylhydrolase were from Cayman (Ann Arbor, MI). Rainbow protein molecular mass markers (14.3 to 220 kDa) were obtained from Amersham (Arlington Heights, IL), whereas Pefabloc SC [4-(2-aminoethyl)-benzenesulfonyl fluoride; Pefabloc] and bicinchoninic acid (BCA) assay reagent were from Pierce (Rockford, IL). Fatty acid-free BSA, cycloheximide, actinomycin D, tunicamycin, and brefeldin A (BFA) were purchased from Sigma. Kifunensine was from Toronto Research Chemicals (Toronto, Canada), endoglycosidase H (endo H) was from Boehringer Mannheim (Indianapolis, IN), HiTrap lectin columns (Amersham Pharmacia Biotech, Piscataway, NJ), DEAE-Sepharose CL-6B, and hydroxylapatite were from Pharmacia LKB Biotechnology (Uppsala, Sweden), BioGel A-1.5m was from Bio-Rad (Hercules, CA), whereas liquid scintillation fluid (OptiPhase HiSafe 3) was supplied by Wallac (Turku, Finland). RPMI 1640 was obtained from BioWhittaker (Walkersville, MD), human pooled serum was from ATGC, 75-cm^2 culture flasks were obtained from Costar (Cambridge, MA), and plastic tissue culture dishes from Primaria Falcon (Becton Dickinson Labware, Franklin Lakes, NJ).

Isolation and culture of human blood monocytes

Monocytes were isolated from the blood of healthy, normolipidemic volunteers (buffy coats) as previously described (21). Cells were cultured and grown in 24-well plastic tissue culture dishes (1×10^6/well) (for PAF-AH activity) or in 6-well plastic tissue culture dishes (3×10^6/well) (for mRNA purification) with RPMI medium, containing L-glutamine, gentamicin (40 μg/ml), and 10% human serum. After 3 days, the medium was replaced and at 6 days of culture it was removed, and the cells were washed twice with PBS. Treatments of these cells with BFA or glycosylation inhibitors were performed for several time intervals up to 24 h in RPMI medium containing 10% human serum in which the PAF-AH was completely and irreversibly inactivated by incubation with 1 mM Pefabloc as described (22). After treatment, supernatants were recovered and the cell layers were washed twice with PBS and then detached and lysed by the addition of 0.2 ml of a lysis solution containing 1% EDTA and 0.1% Triton X-100. Both supernatants and cell lysates were centrifuged (500 × g for 10 min at 4°C), stored at 4°C, and analyzed within 2 days of collection, including protein content determination with BCA. In some experiments, the cells were preincubated for 3 h with BFA or glycosylation inhibitors, and then washed with PBS and reincubated for 6 h with fresh control- or inhibitor-containing medium. Finally, in some experiments, monocytes (3×10^6/ml) were cultured and grown in 75-cm^2 culture flasks with 12 ml of RPMI medium as described above. After 6 days of culture the medium was removed, and the cells were washed twice with PBS and cultured for two more days in the presence of RPMI medium in which serum was replaced by 1% Nutridoma (Boehringer Mannheim). All supernatants as well as cell lysates were collected, centrifuged as described above and used for PAF-AH semipurification. Viability under all culture conditions was determined by trypan blue dye exclusion and the absence of lactate dehydrogenase release; viability was >95%.

PAF-AH semipurification

PAF-AH in pooled supernatants or in pooled cell lysates was semipurified by using batch and column DEAE-Sepharose CL-6B steps, a gel-filtration step on a BioGel A-1.5m column, and a hydroxylapatite column step, essentially as previously described (23). In all purification steps, 0.1% Triton X-100 was used as a detergent.

Characterization of PAF-AH carbohydrate

Semipurified secreted or cell-associated PAF-AH was subjected to digestion with peptide *N*-glycosidase F (PNGase F), *O*-glycosidase DS, or *N*-acetylneuraminidase II (NANase II), using an enzymatic deglycosylation kit (Bio-Rad) according to the denaturing protocol described in the instruction manual. In addition, PAF-AH was subjected to digestion with endo H (40 mU/ml) in 100 mM sodium acetate buffer containing 0.1% 2-mercaptoethanol and 0.1% SDS, pH 5.6, for 12 h at 37°C. The efficiency of the deglycosylation reaction was checked by running the samples (before and after deglycosylation) on SDS-polyacrylamide gels, followed by Western blot analysis as described below. Further characterization of the carbohydrates of secreted PAF-AH was performed by examining its binding affinity to immobilized lec-

tin columns (HiTrap lectin test kit), according to the manufacturer instructions. The binding affinity of PAF-AH was studied before and after digestion with PNGase F or NANase II. In these experiments, enzymatic treatments were performed according to the nondenaturing protocol described in the instruction manual of the enzymatic deglycosylation kit (Bio-Rad). Twelve fractions of 1 ml were collected from each column and analyzed for PAF-AH activity.

Gel electrophoresis and Western blot analysis

Semipurified secreted or cell-associated PAF-AH before and after digestion with endoglycosidases, as well as the secreted or cell-associated acetylhydrolase from macrophages treated with the inhibitors of glycosylation, were analyzed by gradient SDS-PAGE on 5–19% gels followed by Western blot analysis with PAF-AH antibody (diluted 1:1,000) and detection with a chemiluminescence kit (Renaissance; New England Nuclear).

Effect of glycosylation on the association of PAF-AH with lipoproteins

Plasma was first incubated for 30 min at 37°C in the presence of 1 mM Pefabloc, and extensively dialyzed for 24 h against PBS containing 2 mM EDTA on completion of incubation. The activity of PAF-AH was abolished completely by this treatment (data not shown). Samples of treated plasma were then mixed at a ratio of 100:1 (v/v) with aliquots of semipurified secreted acetylhydrolase, untreated or pretreated with PNGase F (nondenaturing protocol), containing PAF-AH activity equivalent to 4 ± 1 nmol of [^{3}H-acetyl]PAF degraded per minute, and incubated for 60 min at 37°C. In some experiments, recombinant PAF-AH was used. Lipoproteins from the mixture of plasma with PAF-AH were fractionated by isopycnic density gradient ultracentrifugation, using a Beckman (Fullerton, CA) SW 41 Ti rotor at 40,000 rpm for 44 h in a Beckman L7-65 ultracentrifuge at 15°C, by a slight modification of the method of Chapman et al. (24). After ultracentrifugation, gradients were collected by successive aspiration of 30 fractions, 0.4 ml each, from the meniscus downward. All fractions were analyzed for their protein content, using the BCA method. Subsequently, equal volumes of certain gradient fractions were pooled to constitute the following lipoprotein subfractions: fractions 1 and 2 (VLDL + IDL; d < 1.019 g/ml); 3 and 4 (LDL-1; d = 1.019–1.023 g/ml); 5 and 6 (LDL-2; d = 1.023–1.029 g/ml); 7 and 8 (LDL-3; d = 1.029–1.039 g/ml); 9 and 10 (LDL-4; d = 1.039–1.050 g/ml); 11 and 12 (LDL-5; d = 1.050–1.063 g/ml); 13 to 15 (HDL_{2b}; d = 1.063–1.091 g/ml); 16 and 17 (HDL_{2a}; d = 1.091–1.100 g/ml); 18 and 19 (HDL_{3a}; d = 1.100–1.133 g/ml); 20 and 21 (HDL_{3b}; d = 1.133–1.156 g/ml); 22 and 23 (HDL_{3c}; d = 1.156–1.179 g/ml); 24 to 30 (proteins; d > 1.179 g/ml). Subfractions were extensively dialyzed in 10 mM PBS containing 2 mM EDTA, pH 7.4, at 4°C, and then filter sterilized and analyzed for their protein content and PAF-AH activity.

PAF-AH assay

PAF-AH activity was measured by the trichloroacetic acid precipitation procedure as previously described (5), with the exception that the pH of the HEPES buffer was adjusted to 7.4; the [^{3}H-acetyl]PAF concentration was 100 μM (specific activity, 81,000 ± 2,000 dpm/nmol) and routine assays were performed for 1 h. Fifty microliters from each sample was used as the source of the enzyme. In some experiments, the PAF-AH assay was performed in the presence of the various glycosylation inhibitors or in the presence of endoglycosidases. The effect of proteases on acetylhydrolase activity before and after treatment with PNGase F was determined for the semipurified secreted acetylhydrolase pretreated with PNGase F by using the nondenaturing protocol and then incubated with trypsin (5 mg/ml) for 60 min at 37°C or with proteinase K (0.25 mg/ml) for 30 min at 37°C. As a positive control for these treatments we used erythrocyte lysate, prepared as previously described (25), that contains a PAF-AH isoform sensitive to both proteases (25).

RNA isolation, first-strand cDNA synthesis, and RT-PCR

Total RNA was isolated from adherent macrophages with RNA Plus and its concentration was determined by spectrophotometry at 260 nm. First-strand cDNA synthesis was performed with 5 μg of total RNA in the presence of oligo(dT) (2 μg) and 500 μU of SuperScript Mo-MuLV reverse transcriptase (26). Detection and quantification of PAF-AH mRNA was performed by RT-PCR in the presence of two specific oligonucleotides, AHPAF2 (TCT TGGAACACACTGGCTTATGGGC) and AHPAF6R (GGACTGA ACCCCTGATTGTAA) and compared with the amplification of the PAF receptor or actin as described (26, 27). Incubations were performed as described (26).

Statistical analysis

Results are expressed as means ± SD. Mean values were compared by the Student's *t*-test, with significance defined at a value of $P < 0.05$.

RESULTS

Secretion of PAF-AH from macrophages in culture

We first studied cell-associated and secreted PAF-AH activity as well as enzyme mRNA levels during the differentiation of adherent monocytes into macrophages. The conditioned medium obtained from freshly adherent cells (30 min) did not contain detectable amounts of PAF-AH activity, whereas enzyme activity in the cell lysate was as low as 10 ± 4 nmol/mg of cell protein per hour. After 24 h of culture, cell-associated and secreted PAF-AH activities significantly increased, attaining 52 ± 16 and 86 ± 27 nmol/mg cell protein per hour, respectively (n = 5). After 48 h of culture, cell-associated enzyme activity attained a plateau of 68 ± 13 nmol/mg cell protein per hour, whereas there was a linear increase in secreted PAF-AH activity (182 ± 41 nmol/mg cell protein per hour), which reached a plateau of 221 ± 53 nmol/mg cell protein per hour at 72 h (n = 5). We ruled out the possibility that secretion of the enzyme into the medium could be due to cell damage rather than to secretion from viable cells: first, increased PAF-AH secretion was associated with elevated cell-associated enzyme activity; second, adherent cells excluded trypan blue and did not release significant amounts of lactate dehydrogenase into the medium during culture (data not shown). Furthermore, increase in both cell-associated and secreted acetylhydrolase activities was inhibited by 0.1 μM actinomycin D or cycloheximide (data not shown).

In parallel, we evaluated the time course and level of expression of PAF-AH mRNA by RT-PCR in cultured mononuclear cells (**Fig. 1**). Specific mRNA for PAF-AH was undetectable after ethidium bromide staining in nonadherent cells (mainly lymphocytes) and in 30 min-adherent cells (mainly monocytes), but a weak, reproducible signal was detected by PCR performed in the presence of [^{32}P]dCTP, showing that the message is present at extremely low levels

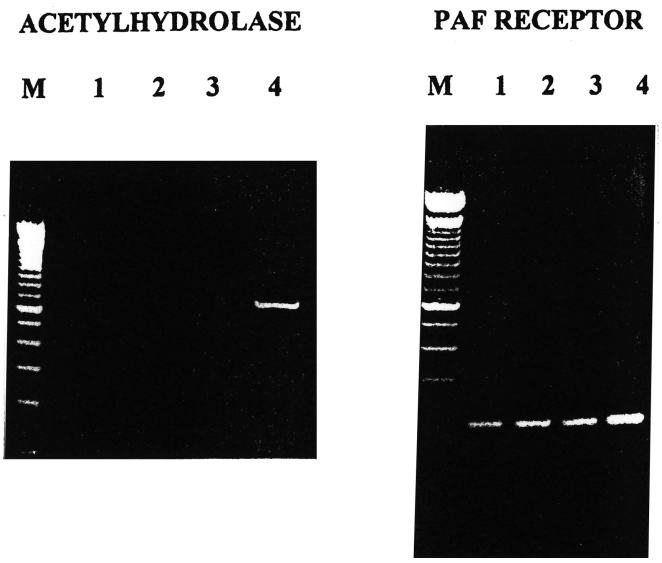

Fig. 1. PCR analysis of the tissue specificity of expression of human PAF-AH mRNA. Amplification products were obtained from (lane 1) mononuclear cells, (lane 2) lymphocytes, (lane 3) adherent monocytes (30 min), and (lane 4) 48-h cultured macrophages, using primer pairs specific for acetylhydrolase and the PAF receptor. Primer pairs for PAF-AH were AHPAF-2/AHPAF-6R (724 bp); primer pairs for PAF receptor were L1/C1 (191 bp). PCR was performed as described in Materials and Methods and products were examined by electrophoresis on 2% agarose gels. After separation, DNA transcripts were detected by ethidium bromide staining. Lane M, Molecular weight markers.

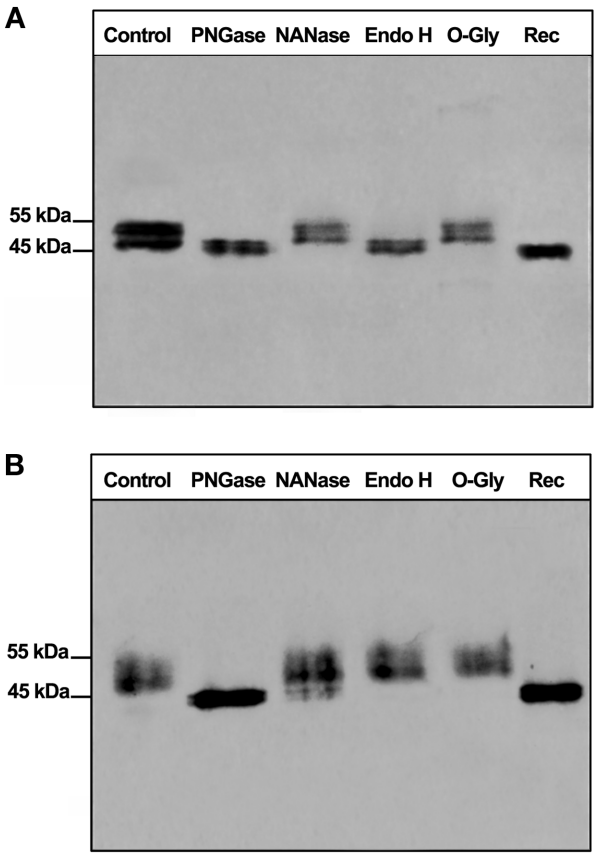

Fig. 2. Endoglycosidase digests of the precursor and mature forms of PAF-AH from human macrophages. A: Cell-associated PAF-AH. B: Secreted PAF-AH. Both forms of PAF-AH were first semipurified and then digested with PNGase F, NANase II, endoglycosidase H (Endo H), or *O*-glycosidase (*O*-Gly). Untreated (Control) or endoglycosidase-digested enzyme as well as human plasma recombinant PAF-AH (Rec) were subjected to gradient 5–19% SDS-PAGE followed by Western blotting analysis with a PAF-AH-specific antibody [diluted 1:1,000 (v/v)].

in both cell types (data not shown). In contrast, mRNA was clearly measurable after 48 h of culture (Fig. 1), and maximal expression of PAF-AH occurred at 6 days of culture. In conclusion, the amount of PAF-AH produced by adhering monocytes is controlled at the transcriptional level, confirming earlier findings (27).

Analysis of the carbohydrate content of PAF-AH

On the basis of the observation that plasma PAF-AH is glycosylated and that macrophages represent a major source of the plasma enzyme, we next examined the possibility that macrophage PAF-AH is glycosylated. In preliminary experiments using SDS-PAGE followed by Western blotting analysis of the cell supernatant containing the secreted enzyme, we observed that PAF-AH migrated as a broad band with a molecular mass ranging from 47 to 55 kDa (data not shown). To investigate the possibility that this heterogeneity was due to glycosylation, and to confirm the structural relationship between the precursor and secreted forms of PAF-AH, the cell-associated as well as the secreted enzymes were first semipurified 150-fold, using four different column chromatography steps. Subsequently, the two forms of PAF-AH were digested with various endoglycosidase enzymes. Both the cell-associated and secreted enzymes were sensitive to digestion with PNGase F, which removes all N-linked glycans from proteins, and insensitive to digestion with *O*-glycosidase which is specific for O-linked glycans (**Fig. 2A** and **B**). Furthermore, cell-associated PAF-AH was sensitive to digestion with endo H, which specifically cleaves high mannose N-linked sugars (Fig. 2A). Because resistance to endo H is acquired in the medial Golgi apparatus (28), these results suggest that the PAF-AH precursor represents a premedial Golgi form of the enzyme. Consistent with this finding, the PAF-AH precursor was resistant to digestion with NANase II, which removes sialic acid residues from glycoproteins (Fig. 2A). In contrast to cell-associated PAF-AH, the secreted enzyme was resistant to endo H digestion, whereas a small shift in the molecular mass of the enzyme was observed after treatment with NANase II (Fig. 2B), consistent with the presence of sialic acid residues on the mature N-linked glycans. Deglycosylated secreted PAF-AH (digested with PNGase F) migrates on SDS-PAGE as a much tighter band (with a molecular mass of 45 kDa, similar to that of recombinant PAF-AH), compared with the undigested enzyme, which exhibits a heterogeneous molecular mass ranging from 47 to 55 kDa (Fig. 2B).

The carbohydrate content of the semipurified secreted PAF-AH was further analyzed by affinity chromatography, using HiTrap lectin columns. In these experiments, PAF-AH

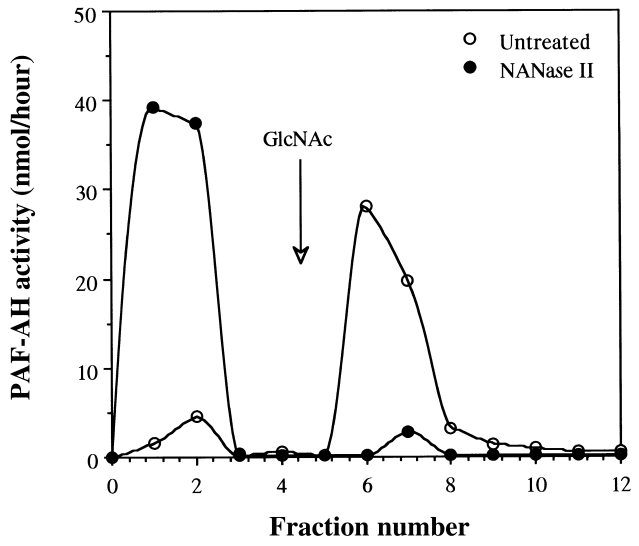

Fig. 3. Elution profile of secreted PAF-AH from human macrophages on a wheat germ lectin column. Semipurified secreted PAF-AH, untreated or digested with NANase II by the nondenaturing protocol, was loaded onto a wheat germ lectin column pre-equilibrated with binding buffer: 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4. The column was washed with 5 ml of the binding buffer and then PAF-AH was eluted with 7 ml of elution buffer containing 0.5 M *N*-acetylglucosamine (GlcNAc). Twelve fractions of 1 ml were collected and analyzed for PAF-AH activity.

was used untreated or after digestion with PNGase F or NANase II, which was performed according to the nondenaturing protocol. Most of the untreated PAF-AH (87 ± 6%) was not absorbed to a lentil lectin column [specific for branched mannoses with fucose-linked α (1,6) to the *N*-acetylglucosamine] or to a peanut lectin column (specific for oligosaccharides with terminal β-galactose). Furthermore, it was not absorbed to a concanavalin column (specific for carbohydrates with terminal mannose or glucose). By contrast, as shown in **Fig. 3**, 90 ± 4% of the activity of the untreated enzyme was bound to a wheat germ lectin column and eluted with a 0.5 M *N*-acetylglucosamine (GlcNAc) solution in 20 mM Tris-HCl, 0.5 M NaCl buffer, pH 7.4. When PAF-AH was first digested with NANase II, and then loaded onto a wheat germ lectin column, 95 ± 5% of enzyme activity was not retained (Fig. 3). Similar results were observed when PAF-AH was digested with PNGase F instead of NANase II (data not shown). Thus, the majority of the asparagine-conjugated sugar chains in the macrophage-secreted enzyme are of the complex type involving sialic acid.

Role of glycosylation in PAF-AH activity and in resistance to proteolysis

Semipurified cell-associated PAF-AH was digested with endo H or PNGase F and the semipurified secreted PAF-AH was digested with PNGase F or NANase II, using the nondenaturing protocol. After these treatments, the enzyme activity of both forms of PAF-AH was not significantly changed, showing that the carbohydrate content of acetylhydrolase is not essential for enzyme activity. Next, we asked whether the glycosylation of PAF-AH plays any role in the resistance of this enzyme to proteolysis. The semipurified secreted PAF-AH was incubated with trypsin or proteinase K before or after digestion with PNGase F. The erythrocyte lysate, containing PAF-AH activity equivalent to 8.3 nmol/ml per min, was also treated with the above-described proteinases and used as a control. The PAF-AH activity before and after digestion with PNGase F was 4.9 ± 0.9 and 5.2 ± 1.3 nmol/ml per min, respectively. Neither trypsin nor proteinase K treatment affected PAF-AH activity even after digestion with PNGase F (5.1 ± 1.0 nmol/ml per min for control treated with trypsin and 5.0 ± 1.4 nmol/ml per min for the deglycosylated PAF-AH treated with trypsin, n = 3). By contrast, treatment of the erythrocyte PAF-AH with either trypsin or proteinase K resulted in a significant decrease in PAF-AH activity by 87 ± 8%, a finding similar to that previously published (25). These results indicate that removal of the N-linked sugar chain(s) does not affect the resistance of the macrophage-derived PAF-AH to proteases.

Role of glycosylation in the association of PAF-AH with plasma lipoproteins

We next asked whether glycosylation of PAF-AH secreted from macrophages could affect the association of the enzyme with plasma lipoproteins. Semipurified secreted PAF-AH (activity, 4 ± 1 nmol/min) untreated or digested with PNGase F was incubated with plasma in which endogenous PAF-AH activity had been completely abolished by treatment with Pefabloc. Each incubation mixture was then fractionated by gradient ultracentrifugation and individual fractions were assayed for PAF-AH activity. As shown in **Fig. 4A**, untreated PAF-AH was distributed primarily in LDL and principally in LDL gradient fractions 11 and 12 (i.e., LDL-5). Furthermore, only a small portion of total enzyme activity was associated with HDL subfractions (HDL_{2b}, 5.3 ± 1.1%; HDL_{2a}, 2.4 ± 0.4%; HDL_{3a}, 4.0 ± 0.8%; HDL_{3b}, 7.2 ± 1.6%; HDL_{3c}, 12.4 ± 3.6%) whereas a significant amount of activity (33.9 ± 5.2% of total) did not associate with lipoproteins and was isolated in fractions 24–30 containing plasma proteins (Fig. 4A and B). Similar results for the LDL fractions (3 to 13) were obtained when PAF-AH was digested with PNGase F before incubation with plasma (Fig. 4A). By contrast, a significant increase of 22 ± 5% in the portion of the total enzyme activity associated with all HDL subfractions was observed (Fig. 4B). Furthermore, a significant decrease from 33.9 ± 5.2% to 11.2 ± 3.1% of total enzyme activity was observed in the portion of the PAF-AH activity isolated in fractions 24–30 (Fig. 4B). Similar results were obtained when a mixture of plasma and recombinant PAF-AH was subjected to ultracentrifugation (Fig. 4B).

Role of glycosylation in the secretion of PAF-AH by macrophages

Nascent N-linked glycan chains ($Glc_3Man_9GlcNAc_2$; where NAc is *N*-acetylglucosamine, Man is mannose, and Glc is glucose) are added en bloc to secretory proteins as they are cotranslationally translocated across the endo-

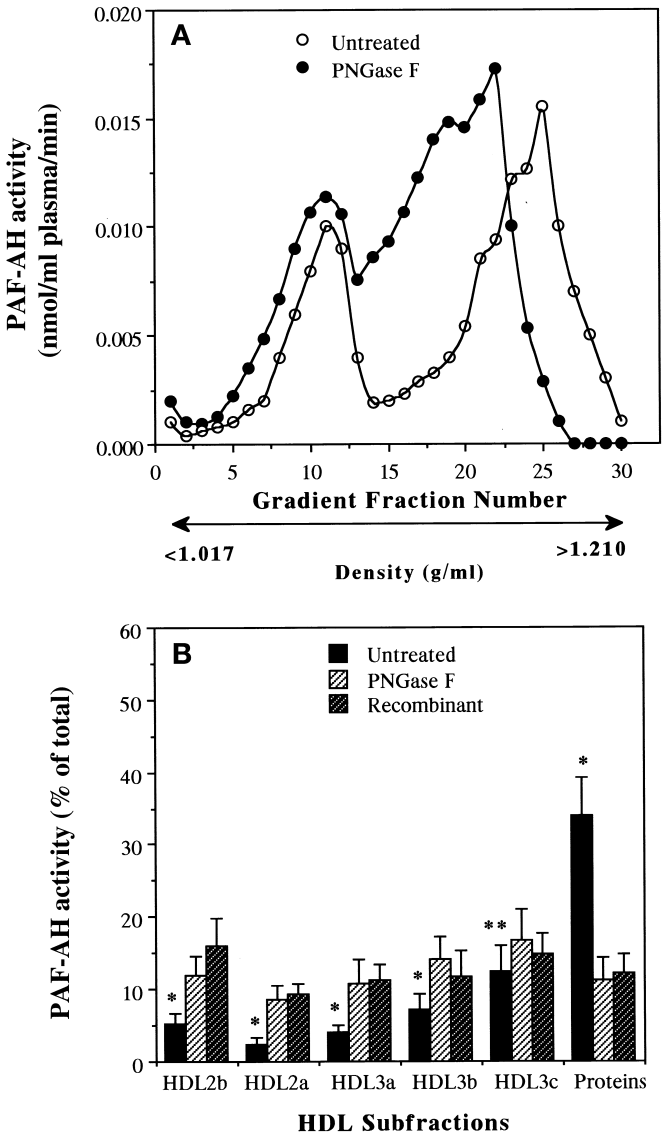

Fig. 4. Effect of glycosylation on the association of PAF-AH secreted from macrophages with human plasma lipoprotein subfractions. Semipurified secreted PAF-AH, untreated or digested with PNGase F, as well as human plasma recombinant PAF-AH, were incubated with plasma in which endogenous PAF-AH activity had been completely abolished by treatment with Pefabloc. Each incubation mixture was then subjected to fractionation by isopycnic gradient ultracentrifugation and individual fractions were assayed for PAF-AH activity as described in Materials and Methods. A: Representative profile of PAF-AH activity as a function of density. B: Distribution of PAF-AH activity among HDL subfractions constituted from individual gradient fractions as described in Materials and Methods. Values represent the means ± SD from four different experiments. * $P < 0.01$ and ** $P < 0.03$ compared with either PNGase F-digested PAF-AH or recombinant PAF-AH.

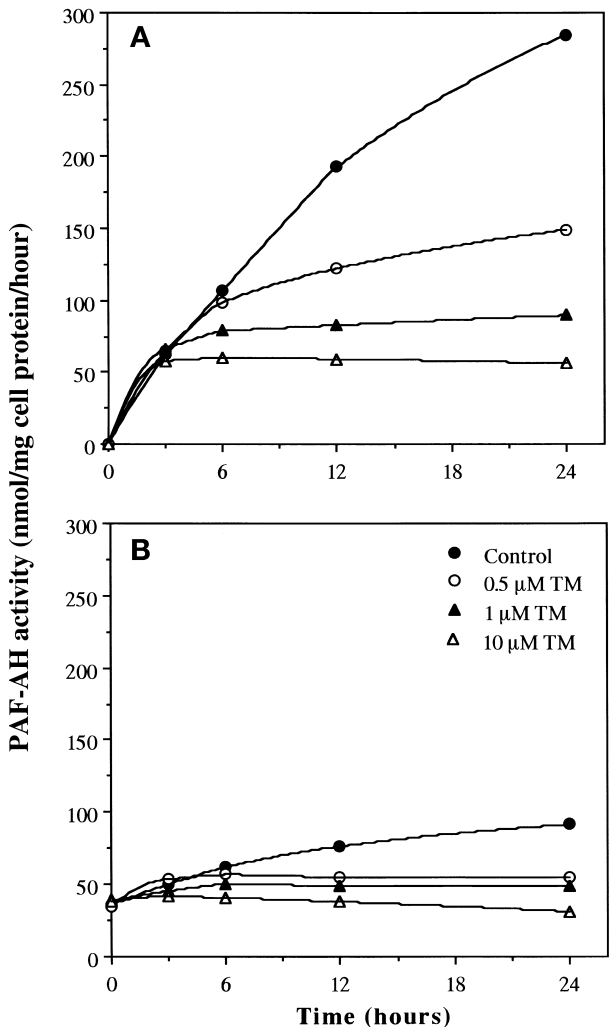

Fig. 5. Effect of tunicamycin on PAF-AH secretion from human macrophages in culture. Cells were incubated in the absence (Control) or in the presence of tunicamycin (TM) for several time intervals up to 24 h. Activity secreted into the medium (A) and cell-associated PAF-AH activity (B) were determined and the results are representative of six separate experiments.

plasmic reticulum (ER) membrane (28). To determine the role of N-linked glycans in PAF-AH secretion, we studied the cell-associated and secreted enzyme activity in macrophages treated with tunicamycin, a drug that prevents protein glycosylation (29). As shown in **Fig. 5**, tunicamycin significantly inhibits PAF-AH secretion in a time- and dose-dependent manner. Thus, after 24 h of incubation with 0.5 μM tunicamycin, secretion of PAF-AH was reduced by 47 ± 9% ($P < 0.01$) and cell-associated enzyme activity was reduced by 40 ± 8% ($P < 0.01$, n = 6) as compared with the corresponding levels in untreated cells (Fig. 5). Overall, total enzyme activity (secreted plus cell associated) was reduced by 45.8% in the presence of 0.5 μM tunicamycin (204 ± 23 vs. 377 ± 41 nmol/mg cell protein per hour in untreated cells, $P < 0.01$) and by 76.8% in the presence of 1 μM tunicamycin (87 ± 17 vs. 377 ± 41 nmol/mg cell protein per hour in untreated cells, $P < 0.003$). Interestingly, the ratio of secreted to cell-associated enzyme activity (1.8 at 6 h and 3.1 at 24 h in untreated cells) was not altered in the presence of tunicamycin, thereby providing evidence that despite the significant decrease in both cell-associated and secreted enzyme activity, tunicamycin does not affect PAF-AH secretion. In some experiments, cells were incubated for 3 h in the presence 1 μM tunicamycin (to deplete preexisting PAF-AH) and

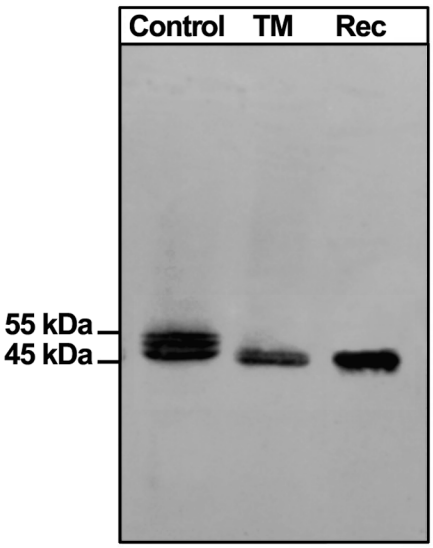

Fig. 6. Effect of tunicamycin on the molecular mass of the cell-associated PAF-AH. Macrophages were incubated at 37°C in the presence (TM) or in the absence (Control) of 1 μM tunicamycin for 3 h. The medium was replaced by fresh medium without tunicamycin or in the presence of 1 μM tunicamycin, followed by a further 6-h incubation. The cell lysates as well as human plasma recombinant PAF-AH (Rec) were subjected to gradient 5–19% SDS-PAGE followed by Western blotting analysis with a PAF-AH-specific antibody [diluted 1:1,000 (v/v)].

then for a further 6 h with fresh drug-containing medium. As a result of this treatment, the molecular mass of cell-associated PAF-AH in tunicamycin-treated cells was lower than that in untreated cells, being similar to that of recombinant enzyme, because of the absence of N-linked glycans (**Fig. 6**). Furthermore, as shown in Fig. 6, tunicamycin significantly decreased the amount of cell-associated PAF-AH and such reduction was calculated to be more than 60%. It must be noted that in enzyme assays performed in the presence of 0.5 to 10 μM tunicamycin, no effect on PAF-AH activity was observed. In addition, in all experiments tunicamycin did not affect the expression of PAF-AH mRNA and did not significantly influence total cell protein as compared with untreated cells at the same time of incubation (data not shown).

We next studied the effect of BFA on PAF-AH secretion. BFA blocks protein transport to the Golgi apparatus by causing the resorption of most of the Golgi structure into the ER (30, 31). Macrophages incubated for 6 h in the presence of BFA (5 μg/ml) showed an intracellular accumulation of PAF-AH activity associated with a significant decrease in secreted enzyme activity (**Table 1**). Despite differences in the distribution of PAF-AH activity between untreated and BFA-treated cells, total activity (secreted plus cell associated) was similar (112 ± 18 nmol/mg cell protein per hour for untreated cells vs. 103.3 ± 12 nmol/mg cell protein per hour for BFA-treated cells). Hence, PAF-AH retained within the ER by BFA treatment was fully active. The Golgi components reabsorbed into the ER by the action of BFA included enzymes involved in oligosaccharide processing. These enzymes might modify the carbohydrate structure of PAF-AH retained within the ER of BFA-treated cells. To test this possibility, the extent of glycan modification of acetylhydrolase from BFA-treated cells was evaluated (**Fig. 7**). In untreated cells (controls), most of the PAF-AH contained high mannose oligosaccharides (endo H sensitive), implying that the majority of the enzyme was still present within the ER/*cis*-Golgi boundaries. In contrast, PAF-AH within BFA-treated cells exhibited only complex oligosaccharides (endo H resistant) and was indicative of processing by the Golgi enzymes re-

TABLE 1. Effect of brefeldin A and kifunensine on the cell-associated and secreted PAF-AH activity from human macrophages in culture

Inhibitor	PAF-AH Activity	
	Secreted	Cell Associated
	nmol/mg protein/h	
None	69.0 ± 18.1	43.8 ± 9.6
BFA, 5 μg/ml	39.3 ± 8.5[a]	64.0 ± 12.7[b]
Kifunensin, 0.1 mM	74.4 ± 27.9	48.4 ± 11.3

Macrophages were incubated for 6 h in the presence or absence of either BFA (5 μg/ml) or 0.1 mM kifunensine. The media were recovered and the cells were lysed; PAF-AH activity was measured and expressed as mean values ± SD from five separate experiments.

[a] $P < 0.01$ compared with untreated cells.

[b] $P < 0.03$ compared with untreated cells.

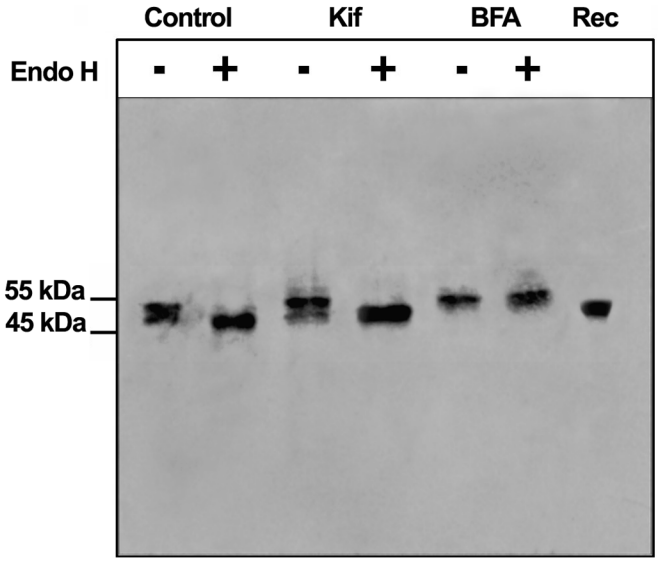

Fig. 7. Effect of kifunensine and brefeldin A on the PAF-AH oligosaccharide-processing pathway in human macrophages. Macrophages were incubated for 3 h in the presence of 0.1 mM kifunensine (Kif) or brefeldin A (BFA, 5 μg/ml) or without any inhibitor (Control). The medium was replaced by fresh medium without inhibitor or in the presence of the above-described inhibitors, followed by a further 6-h incubation. The cell lysates were collected and incubated with (+) or without (−) endoglycosidase H (Endo H, 40 mU/ml) in 100 mM sodium acetate buffer containing 0.1% β-mercaptoethanol and 0.1% SDS, pH 5.6, for 12 h at 37°C. All samples as well as human plasma recombinant PAF-AH (Rec) were then subjected to gradient 5–19% SDS-PAGE followed by Western blotting analysis with a PAF-AH-specific antibody [diluted 1:1,000 (v/v)].

distributed to the ER. PAF-AH activity in the in vitro assay was not affected by BFA. In addition, BFA affected neither the PAF-AH mRNA level nor total cell protein content.

Earlier stages in the oligosaccharide-processing pathway were further investigated with kifunensine which inhibits the activity of the ER and *cis*-Golgi mannosidase I, resulting in glycoproteins bearing high mannose oligosaccharide chains ($Man_9GlcNAc_2$) (32, 33). Incubation of macrophages with 0.1 mM kifunensine had no detectable effects on cell-associated or secreted PAF-AH activity (Table 1), indicating that PAF-AH has achieved full hydrolytic potential even at this early stage of oligosaccharide processing. The degree of oligosaccharide processing of the intracellular enzyme was assessed in control cells and in kifunensine-treated cells. While a portion of intracellular PAF-AH in control cells was processed to the endo H-resistant stage, cells incubated in the presence of kifunensine contained only unprocessed, endo H-sensitive PAF-AH (Fig. 7). PAF-AH activity in in vitro assays was not affected by kifunensine.

DISCUSSION

In the present study, we show for the first time that human macrophage-derived PAF-AH contains N-linked carbohydrate chains of the complex type containing sialic acid residues. Surprisingly, such glycosylation influenced neither enzyme activity nor its resistance to proteases; in addition, glycosylation was not a prerequisite for PAF-AH secretion. By contrast, glycosylation significantly diminished the association of PAF-AH with all HDL subspecies.

According to the predicted amino acid sequence (18), two N-linked glycosylation sites (N^{423}-T-T) and (N^{433}-S-S), close to the carboxyl terminus, are conserved in human macrophage PAF-AH. In the present study we show that the carbohydrates of macrophage-derived PAF-AH have a total mass of 10 kDa, and that they are of the complex type containing sialic acids. Previous studies have shown that purified human LDL-associated PAF-AH, which represents more than 85% of the total plasma enzyme activity, is N-glycosylated (19, 20) and that it contains about 9-kDa heterogeneous asparagine-conjugated sugar chains(s) involving sialic acid (20). Thus our results combined with others (19, 20) are consistent with the hypothesis that macrophage-derived PAF-AH represents one of the major sources of the enzyme pool present in plasma (13).

As we had previously shown, incubation of monocyte/macrophage-derived PAF-AH with plasma leads to exclusive association of the enzyme with LDL and preferentially with small dense LDL particles (5). The molecular basis for the association of PAF-AH with lipoproteins remains still unknown, and only more recently it was reported that two domains within the primary sequence of PAF-AH, Tyr-205 and residues 115 and 116, are important for its binding to LDL (34). In addition, in the latter study it was shown that the 21 amino acids at the carboxyl terminus of the enzyme do not play any role in PAF-AH binding to LDL. In the present study we show that N-linked glycosylation of PAF-AH, which could occur at two asparagine residues (N^{423} and N^{433}), that is, within the 21 amino acids at the carboxyl terminus, does not influence PAF-AH association with LDL. In contrast, we provide evidence for the first time that glycosylation significantly reduces association of the enzyme with HDL subfractions, especially with HDL_{2b}, HDL_{2a}, HDL_{3a}, and HDL_{3b}. This is further supported by the observation that recombinant PAF-AH, which is not glycosylated, binds to these HDL subfractions. Furthermore, a low level of PAF-AH activity associated with HDL_2 and HDL_3 after gradient ultracentrifugation of plasma lipoproteins, found in our previous study (5), might be related to heterogeneity in the degree of glycosylation of PAF-AH.

The factors that influence the PAF-AH distribution between proatherogenic LDL and antiatherogenic HDL, as well as the pathophysiological relevance of such distribution for the atherosclerotic diseases, remain to be established. Although the role of LDL-associated PAF-AH is currently under some debate, there is an accumulation of data supporting the contributory role of HDL-associated PAF-AH activity in the HDL protective effect against LDL oxidation as well as in the HDL-induced inhibition of the biological activities of oxidized LDL [(8); reviewed also in ref. (35)]. Consequently, the factors that promote the preferential distribution of PAF-AH on HDL versus LDL could enhance the HDL antioxidant and antiinflammatory potencies. On the basis of the results of the present study, one of these factors could be the lack of glycosylation. Because glycosylation does not affect secretion of the enzyme, it is possible that factors that influence the glycosylation in vivo could increase the enzyme pool on HDL. Furthermore, other nonglycosylated forms of PAF-AH could represent the major enzyme isoforms associated with HDL in human plasma, a hypothesis that needs further investigation.

Several studies have shown that glycosylation is essential for the catalytic activity of several enzymes such as hepatic lipase and lipoprotein lipase (36, 37). In addition, it is well established that glycosylation can protect some proteins from degradation by proteases (38). In the present study we show that glycosylation plays no role in the expression of macrophage PAF-AH catalytic activity or in its resistance to proteases, a finding that is consistent with previously published results on plasma enzyme (20).

Another important role of protein glycosylation within cells is to facilitate secretion. For this purpose, N-linked glycans ($Glc_3Man_9GlcNAc_2$) are added to secretory proteins cotranslationally in the ER lumen (28). Addition of N-linked carbohydrate is often necessary to maintain protein solubility in the ER (39). To examine the requirement for N-linked glycosylation in PAF-AH secretion from macrophages, we utilized tunicamycin, which inhibits the formation of GlcNAc-containing lipid intermediates involved in the generation of N-linked oligosaccharide chains (29). We showed that tunicamycin decreased the amount of cell-associated PAF-AH and reduced the enzyme activity. Despite this reduction, PAF-AH was secreted with relatively normal efficiency. It is unlikely that the de-

crease in cell-associated enzyme activity by tunicamycin was due to the production of inactive unglycosylated PAF-AH or to an inhibition of enzyme production at the transcriptional or translational level, because glycosylation of PAF-AH does not affect its enzymatic activity and tunicamycin did not block PAF-AH transcription. It has been shown that blocking N-linked glycosylation with tunicamycin induces misfolding of unglycosylated proteins in the ER (39). The pathway for degradation of misfolded ER-associated proteins involves translocation across the ER membrane into the cytosol and subsequent degradation by the proteasome (40). However, it is unlikely that the decrease in cell-associated PAF-AH activity induced by tunicamycin was due to an enhancement in cotranslational or posttranslational enzyme degradation, because the catalytic activity of the unglycosylated PAF-AH was not affected by treatment with proteases. It has been suggested that tunicamycin can induce a mild to moderate inhibition of protein synthesis that may be related to a drug effect on the glycosylation of protein factors involved in protein translation (41). Although tunicamycin treatment did not significantly affect total cell protein, we cannot exclude the possibility that it might impair PAF-AH synthesis. In contrast to tunicamycin, BFA inhibited PAF-AH secretion, inducing an accumulation of PAF-AH activity within the cell (increase in cell-associated enzyme activity). However, in the presence of BFA, the enzyme was retained within the ER. It is known that many proteins that fail to be transported into the Golgi system are degraded by a prelysosomal process referred to as "ER degradation" (42, 43). The extent of PAF-AH turnover within the ER could be assessed because BFA does not interfere with ER degradation but prevents access of retained proteins to the lysosomes (44). However, the accumulation of PAF-AH activity in BFA-treated cells indicates that the enzyme was not degraded.

The importance of early processing events occurring in the ER and involving trimming of the high mannose chain was next examined with kifunensine, the potent inhibitor of the glycoprotein-processing enzyme mannosidase I (32, 33). Our experiments showed that inhibition of the mannose-trimming reactions by kifunensine had no effect on the ability of the enzyme to be secreted. Consequently, PAF-AH secreted in the absence of mannose trimming was fully active, showing that catalytically active PAF-AH can be secreted from human macrophages even in the absence of processing by ER mannosidase enzymes.

In conclusion, the results of the present study show that PAF-AH secreted from peripheral blood monocyte-derived macrophages contains heterogeneous asparagine-conjugated sugar chain(s) involving sialic acid. Using inhibitors of glycosylation and glycoprotein processing, we found that human macrophages synthesize PAF-AH as a low molecular weight, premedial Golgi-associated precursor containing high mannose N-linked glycans that is processed to a higher molecular mass form containing mature N-linked glycans before secretion. Such glycosylation acts to reduce the association of PAF-AH with HDL but it does not seem to influence enzyme secretion or to play any role in the expression of the catalytic activity or the resistance of the enzyme to proteases.

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