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Red Blood Cells Highly Express Type I Platelet-Activating Factor-Acetylhydrolase (PAF-AH) Which Consists of the α_1/α_2 Complex

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Although red blood cells account for about 30% of total PAF-AH activity found in the blood, the physiological function of this enzyme is unknown. To understand the role and regulatory mechanism of this enzyme, we purified it from easily obtainable pig red blood cells. PAF-AH activity was mainly found in the soluble fraction of the red blood cells. Two peaks of enzyme activity appeared with increasing concentration of imidazole on column chromatography on nickel-nitroacetic acid (Ni-NTA) resin. We called these peaks of small and large enzyme activities fractions X and Y, respectively, and then further purified the enzymes by sequential chromatofocusing on Mono P and gel filtration on TSK G-3000. In the final preparation from fraction Y, two proteins bands corresponding to 26 kDa and 28 kDa were related to enzyme activity. Determination of the partial amino acid sequences of the proteins of 26 kDa and 28 kDa revealed that these proteins were identical to α_1 and α_2, respectively, both of which are catalytic subunits of Type I intracellular PAF-AH. On Western analysis, the 26 kDa and 28 kDa protein bands cross-reacted with specific monoclonal antibodies to α_1 and α_2, respectively. Since the apparent molecular weight of the natural enzyme was estimated to be about 60 kDa, the enzyme activity in fraction Y was thought to be that of a heterodimer consisting of α_1 and α_2. On the other hand, the enzyme activity in fraction X was thought to be that of a homodimer consisting of α_2. Other blood cells such as polymorphonuclear leukocytes and platelets only contained the α_2/α_2 homodimer. It has been reported that the α_1/α_2 heterodimer is poorly expressed in adult animals except for in the spermatogonium. Taken altogether, these results suggest that high expression of the α_1/α_2 heterodimer is important for the physiological function of mature red blood cells.

Key words: PAF, PAF-AH, red blood cells.

Abbreviations: PAF, platelet-activating factor; PAF-AH, platelet-activating factor-acetylhydrolase.

PAF is a phospholipid with a wide range of biological and pharmacological activities including in the aggregation and degranulation of platelets and neutrophils, bronchoconstriction, systemic hypotension, anaphylaxis, and inflammatory and allergic responses (*1*). Degradation and inactivation of PAF occur through hydrolysis of an acetyl ester at the *sn*-2 position of PAF and then conversion to an inactive metabolite, lysoPAF. The enzymes responsible for inactivation of PAF are termed PAF-AH and classified into two groups, plasma- and intracellular-types. Furthermore, two different types of intracellular PAF-AHs, namely Types I and II have been identified (*2*, *3*).

It has been reported that plasma-type and intracellular Type II PAF-AHs also degrade phospholipids with oxidatively fragmented *sn*-2 acyl chains (*4*, *5*). In addition, both types of enzymes exhibit transacetylation activity that transfers acetic acid from PAF to lysophospholipids (*6*, *7*). This similarity in substrate specificity of the two enzymes is likely to result from the homology of the amino acid sequences of the two enzymes (*8*). By contrast, Type I PAF-AH is supposed to exhibit tighter specificity for an acetyl group at the *sn*-2 position. However, this enzyme degrades PAF analogs in which the choline moiety at the *sn*-3 position is replaced with another polar head group (*9*). Type I PAF-AH, which shows less sequence homology with plasma-type and Type II intracellular PAF-AHs, does not catalyze the transacetylation that is found for both plasma-type and Type II intracellular PAF-AHs. These three kinds of PAF-AHs, which were originally identified as enzymes that degrade PAF, have consensus motif of lipase in active sites (*8*, *10–12*). This raises the possibility that these enzymes metabolize various kinds of lipid molecules due to their broad substrate specificity for besides PAF.

In human blood, about 70% of total PAF-AH activity is present in the plasma, the remaining about 30% of total PAF-AH activity being found in red blood cells (*13*). However, the subtypes of PAF-AHs in red blood cells have not been identified yet. To elucidate the physiological function and regulatory mechanism of this enzyme, it is necessary to determine the molecular structure of this enzyme. Stafforini *et al*. first attempted to purify this

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enzyme from human red blood cells (*13*). They reported that a dimer consisting of polypeptides of 25 kDa was responsible for the PAF-AH activity in human red blood cells. They also suggested that this enzyme acted as a scavenger of oxidatively fragmented phospholipids that are toxic to the cells. However, the molecular structure of this enzyme and its relationship with previously identified subtypes of other PAF-AHs remain unknown. Therefore, in this study, we used pig red blood cells as a source for enzyme purification, because a large amount of the cells was easily obtainable. As a result, we found that pig blood cells contained a heterodimer consisting of catalytic subunits of Type I PAF-AH (α_1 and α_2), which is poorly expressed in adult animals. Furthermore, we found that other blood cells such as platelets and polymorphonuclear leukocytes do not contain this heterodimer in an immunochemical study involving specific monoclonal antibodies to the individual catalytic subunits.

MATERIALS AND METHODS

Materials—1-*O*-Hexadecyl-2[^{3}H]acetyl-*sn*-glycero-3-phosphocholine was obtained from Du Pont-New England Nuclear. 1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine was obtained from Avanti. Q-Sepharose and Mono P were obtained from Amersham Biosciences. TSK G-3000 sw was obtained from Tosoh. Hydroxyapatite and Affi-Gel Blue were obtained from Bio-Rad. Nickel-nitroacetic acid (Ni-NTA) resin was obtained from Qiagen. Mouse monoclonal antibodies to human Type I PAF-AH α1 and α2 subunits were donated by Drs. Hiroyuki Arai and Junken Aoki, the University of Tokyo.

Assaying of PAF-AH Activity—Acetylhydrolase activity was measured according to the method of Stafforini *et al.* (*14*) with a slight modification. Fifty microliters of 80 μM 1-*O*-hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (2,500 dpm/nmol) dissolved in 100 mM Hepes-NaOH (pH 7.0) was combined with 10 μl of enzyme. The reaction was carried out by incubation at 37°C for 30 min, and stopped by adding 50 μl of 10 M acetic acid. After adding 1.5 ml of 0.1 M sodium acetate, the mixture was applied on a Sep-Pak C_{18} cartridge column (Waters) washed stepwise with 5 ml each of ethanol and distilled water. The column was washed with 1.5 ml of 0.1 M sodium acetate and the eluate was combined with 5 ml of ACS-II scintillation fluid (Amersham Biosciences). The radioactivity in the eluate was measured with a liquid scintillation counter (Aloka, LSC-300). The PAF-AH activity was expressed as nmol PAF hydrolysis per min per mg protein.

Preparation of Blood Cells—Fresh pig blood, which was collected using sodium citrate as an anti-coagulant (Tokyo Shibaura Zoki, Japan), was centrifuged for 10 min at 220 × *g* and 4°C. After removing the supernatant and white blood cells, the red blood cells were resuspended in two volumes of 0.13 M NaCl/5 mM KCl/10 mM Hepes-NaOH (pH 7.4). After centrifugation for 10 min at 220 × *g* and 4°C, the supernatant and white blood cells were removed. This step was repeated threetimes to remove the plasma and white blood cells completely. The washed red blood cells were dispersed in 4 volumes of 10 mM Tris-HCl (pH 7.4) containing 5 mM $MgCl_2$. After centrifugation for 10 min at 10,000 × *g* and 4°C, the resulting supernatant was used as the lysate. To obtain polymorphonuclear leukocytes, 20 ml of pig blood was combined with 6.7 ml of 6% dextran dissolved in saline, and the mixture was kept in a syringe for 30 min at room temperature. The resulting supernatant was transferred to a new tube and centrifuged for 10 min at 220 × *g* and 4°C. The precipitated cells were resuspended in PBS and then layered on Lymphoprep (Axis-shield). Polymorphonuclear leukocytes were collected by centrifugation for 30 min at 340 × *g* and 4°C, and resuspended in 1 ml of PBS. After adding 10 ml of cold water, contaminating red blood cells were subjected to hemolysis by mixing mildly for 10 s. After adding 10 ml of saline, the cell suspension was centrifuged for 10 min at 220 × *g* and 4°C. The resulting pellet was washed with PBS three times. To obtain platelets, pig blood was centrifuged for 10 min at 220 × *g* and 4°C. The supernatant was transferred to a new tube, and then diluted with wash buffer comprising 25 mM Tris-HCl (pH 7.4)/130 mM NaCl/1.5 mM EDTA. The cell suspension was centrifuged for 10 min at 1,500 × *g* and room temperature, and the supernatant was removed. The resulting pellet was washed with PBS three times.

Purification of PAF-AH—All steps were carried out at 4°C. Mono P and TSK G-3000 sw columns were connected to a Shimadzu high-performance liquid column chromatography system, LC-6A. The amount of protein was monitored by measuring the absorbance at 280 nm. The lysate was loaded onto a column of Q-Sepharose (5.0 × 20 cm), which had been equilibrated with 50 mM Tris-HCl (pH 8.0). After washing the column with the same buffer, the enzyme activity was eluted with a 0 to 0.6 M NaCl gradient in 50 mM Tris-HCl (pH 8.0) at a flow rate of 2.0 ml/min. The enzyme activity appeared as a single peak in the fractions with about 0.3 M NaCl (Fig. 1A). The active fractions were pooled, and then dialyzed against 50 mM Tris-HCl (pH 8.0) at 4°C for 16 h. The dialyzate was applied to a column of Affi-Gel Blue (2.5 × 10 cm), which had been equilibrated with 50 mM Tris-HCl (pH 8.0), and washed with the same buffer. The enzyme activity passed through the column. The pooled fractions containing enzyme activity were loaded on a column of Ni-NTA (2.5 × 20 cm), which had been equilibrated with 50 mM sodium phosphate (pH 7.5) containing 0.5 M NaCl and 10% glycerol, and the column was washed with the same buffer at a flow rate of 0.5 ml/min. Two separate peaks of enzyme activity appeared with on increase in imidazole from 0 to 50 mM and a reduction in the pH from 7.5 to 6.0. For the gradient, 50 mM sodium phosphate (pH 7.5)/0.5 M NaCl/10% glycerol and 50 mM sodium phosphate (pH 6.0)/0.5 M NaCl/10% glycerol/50 mM imidazole were used as the starting and termination buffers, respectively. Since two peaks containing enzyme activity appeared for the fractions with about 20 mM and 35 mM imidazole, the individual fractions were termed fractions X and Y, respectively (Fig. 1B). The active fractions comprising the individual peaks were pooled and dialyzed against 50 mM Tris-HCl (pH 8.0) containing 10% glycerol for 16 h. Dialyzed fraction Y was applied to a column of hydroxyapatite (2.5 × 20 cm), which had been equilibrated with 50 mM Tris-HCl (pH 8.0) containing 10% glycerol. After washing the column with the same buffer, enzyme activity was eluted with a 0 to 0.4 M potassium phosphate gradient at a flow rate of 0.5 ml/min, fractions of 4.0 ml being collected (Fig. 1C). The pooled fractions were dialyzed against 25 mM bis-Tris-HCl (pH 6.7) containing 10% glycerol for

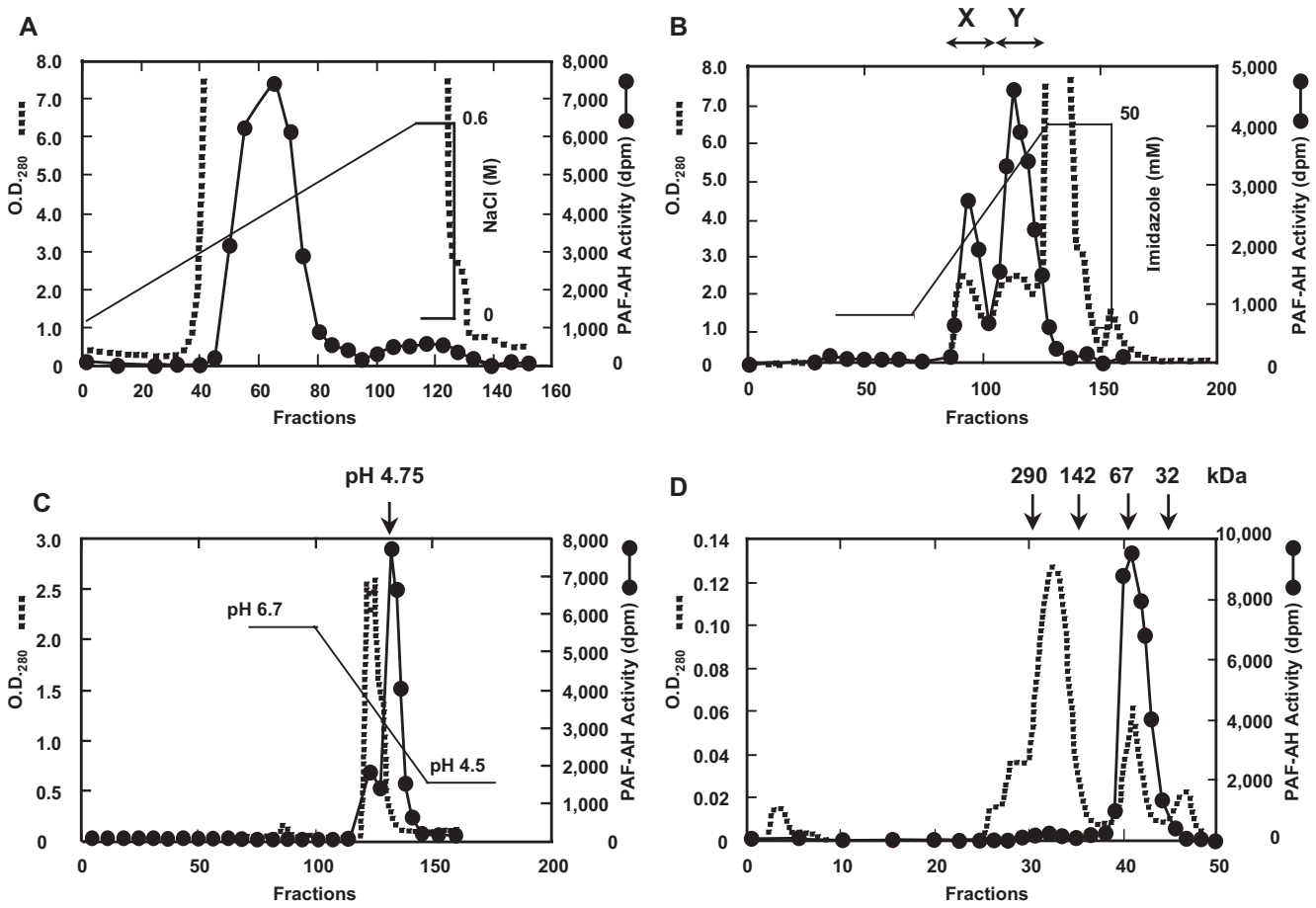

Fig. 1. **Purification of PAF-AH from pig red blood cells.** A: Q-Sepharose. The lysate obtained from pig red blood cells was loaded on a column of Q-Sepharose, as described under "MATERIALS AND METHODS." Fractions (4 ml) were collected and assayed for enzyme activity. B: Ni-NTA. After column chromatography on Affi-Gel Blue, the flow-through fraction was applied on a column of Ni-NTA. Fractions (4 ml) were collected and assayed for enzyme activity. Since two peaks with enzyme activity appeared at the fractions corresponding to about 20 and 35 mM imidazole, the individual peaks were termed fractions X and Y. C, Mono P. Fraction Y was applied on a column of hydroxyapatite and active fractions eluted from the column with a potassium phosphate gradient were pooled. The pooled fractions were loaded on a column of Mono P. Fractions (0.5 ml) were collected and assayed for enzyme activity. D, TSK G-3000 sw. The active fraction from Mono P was concentrated to 200 μl using a Vivaspin 500 and the resulting solution was loaded on a column of TSK G-3000 sw. Fractions (0.2 ml) were collected and assayed for enzyme activity. Protein concentration was monitored by measurement of the absorbance at 280 nm. PAF-AH assaying was carried out by incubating an aliquot of each fraction (10 μl) for 30 min at 37°C, as described under "MATERIALS AND METHODS."

16 h. To prevent the formation of insoluble aggregates, CHAPS was added to the dialyzed fraction [final concentration, 0.5% (w/w)]. The dialysate was applied to a column of Mono P (0.5 × 5 cm), which had been equilibrated with 25 mM bis-Tris-HCl (pH 6.7) containing 0.5% (w/w) CHAPS and 10% glycerol. The enzyme activity emerged on the addition of elution buffer (10% Polybuffer 74, 0.5% CHAPS and 10% glycerol at pH 4.5) at a flow rate of 0.5 ml/min. The active fractions were pooled and dialyzed against 50 mM Tris-HCl (pH 8.0) containing 10% glycerol for 16 h. The dialyzed solution was concentrated to 200 μl using a Vivaspin 500 (Vivascience), and the resulting solution was applied to a column of TSK G-3000 sw (7.5 mm × 60 cm), which had been equilibrated with 50 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl and 10% glycerol. The enzyme activity emerged on the addition of the same solution at a flow rate of 0.4 ml/min (Fig. 1D). The active fractions were pooled and stored at 4°C. Glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), myokinase (32 kDa), and cytochrome *c* (12.4 kDa) were used to measure the apparent molecular weight of the native enzyme.

SDS-PAGE—SDS-PAGE was carried out using a 10% polyacrylamide gel (Bio-Rad). Tris SDS 2ME sample buffer (Daiichi chemical Corp.) was added to the sample, followed by boiling at 100°C for 5 min. Precision Plus Protein standards (Bio-Rad) were used as molecular weight markers. Electrophoresis was carried out at a constant current of 20 mA. The proteins were visualized by silver staining with a Silver Stain II Kit Wako (Wako Pure Chemical Industries).

Western Blotting—After carrying out SDS-PAGE as described above, the proteins were blotted onto a PVDF membrane (Amersham Biosciences). The membrane was blocked with Tris-buffered saline (TBS) containing 5% skim milk for 16 h at 4°C. After washing the membrane

with TBS containing 0.05% Tween 20 (T-TBS), the membrane was incubated with mouse monoclonal antibodies diluted with T-TBS for 1 h at room temperature. The membrane was washed three times with T-TBS, and then incubated with a horseradish peroxidase–conjugated goat anti-mouse IgG (Amersham Biosciences) diluted 1,000-fold with T-TBS containing 5% skim milk for 1 h at room temperature. After washing the membrane six times with T-TBS, the membrane was reacted with the chemiluminescence reagent in a ECL Advance Western Blotting Detection Kit (Amersham Biosciences). The blots were visualized with an imaging analyzer (IS440CF, Kodak Digital Science).

Determination of Partial Amino Acid Sequences—Polyacrylamide gel containing the protein bands visualized on silver staining after SDS-PAGE was removed using a clean scalpel, and then placed in the tube. The protein was extracted from the gel and then subjected to digestion with trypsin or lysylendopeptidase. After separating peptide fragments by reverse-phased high-performance liquid chromatography, the amino acid sequence of each fragment was determined by LC/tandem MS.

Protein Determination—Protein was determined with a BCA protein assay kit (Pierce) using BSA as a protein standard. On the column chromatography, the protein concentration was monitored by measuring absorbance at 280 nm.

Metabolism of PAF in Human Blood—1-*O*-Hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (55,000 dpm) was added to 0.25 ml of plasma or whole blood obtained from healthy human volunteers at a final concentration of as low as 10^{-8} M. After incubation at 37°C, the remaining [^{3}H]PAF was extracted according to the method of Bligh and Dyer (*15*). The extracted lipids were dissolved in chloroform and then spotted on a TLC plate (Merck). The lipids were developed using a solvent system consisting of chloroform/methanol/acetic acid/H_2O (25:15:8:4, v/v/v/v), and the area corresponding to the PAF was removed. The removed silica was transferred to scintillation vials and then the radioactivity of the remaining [^{3}H]PAF was determined with a liquid scintillation counter.

RESULTS

We used pig as an animal to purify PAF-AH from red blood cells, because pig red blood cells showed specific enzyme activity comparable to that of human red blood cells and a large amount of red blood cells was easily obtainable. When a lysate of red blood cells was separated by centrifugation, more than 99% of the enzyme activity was found in the soluble fraction. Since the crude soluble fraction contained a large amount of hemoglobin, it was impossible to immunologically detect the PAF-AH protein using antibodies raised against previously reported PAF-AHs subtypes. Thus, we attempted to purify the PAF-AH activity from red cells to identify and compare the PAF-AH with other previously reported PAF-AHs subtypes. First, PAF-AH activity was separated from the major protein constituent, hemoglobin, by chromatography on Q-Sepharose (Fig. 1A). After removing hemoglobin by washing the column, enzyme activity appeared as a single peak at about 0.3 M NaCl. To exclude the possibility that the PAF-AH activity was a contaminant from plasma, the enzyme activity was allowed to pass through a column of Affi-gel Blue, because this column was known to tightly adsorb plasma-type PAF-AH (*16*). On column chromatography on Ni-NTA, almost all the enzyme activity was adsorbed to the column and the enzyme activity was eluted as two separate peaks with increasing imidazole concentration, from 0 to 50 mM (Fig. 1B). Thus, we named the individual fractions appearing at 20 mM and 35 mM imidazole fractions X and Y, respectively, and further purified the enzyme activity in fraction Y exhibiting the larger enzyme activity (about 79% of total enzyme activity) by column chromatography on Mono P (Fig. 1C). It was necessary to add 0.5% CHAPS to the working solution in order to prevent the formation of insoluble aggregates near the isoelectric point. Enzyme activity appeared with a good recovery as a single peak at pI 4.7. The purification of red blood cells PAF-AH from peak Y is summarized in Table 1. The specific enzyme activity of the final preparation was increased 1,740-fold, as compared with that of the red blood cells lysate. When this preparation was analyzed by SDS-PAGE/silver staining, the fraction containing enzyme activity gave two bands corresponding to 26 kDa and 28 kDa (Fig. 2B). Since the apparent molecular weight of the natural enzyme was estimated to be about 60 kDa on gel-filtration column chromatography on TSK G-3000 sw (Fig. 2A), the enzyme purified from fraction Y was thought to form a complex consisting of these two proteins. Thus, these protein bands were removed from the polyacrylamide gel and the amino acid sequences were determined. As a result, it was found that the amino acid sequences of five trypsin-treated peptide fragments obtained from the protein material of 26 kDa (PTPVQDVQGDR, LENGELEHIRRK, GQHPNPLR, RVNELVR and ALHSLLLR) were completely identical to those of the intracellular type I PAF-AH α_1 subunit that had been previously isolated from bovine brain (*11*). On the other hand, the amino acid sequences of two lysylendopeptidase-treated peptide fragments originating from the protein material of 28 kDa were EAGVQ and PLNEL. These sequences corresponded to those of another catalytic subunit, α_2, of the intracellular Type I PAF-AH

Table 1. **Purification of PAF-AH from pig red blood cells (fraction Y).**

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Purification (-fold)	Recovery (%)
RBC lysate	28,200	2,850	0.102	1.0	100.0
Q-Sepharose	585	621	1.06	10.5	21.80
Affi-Gel Blue	272	378	1.39	13.7	13.20
Ni-NTA	13.5	42.2	3.10	30.6	1.48
Hydroxyapatite	6.14	47.2	7.68	75.7	1.65
MonoP	0.401	44.4	111	1,090	1.56
TSK G3000 sw	0.041	7.14	176	1,740	0.25

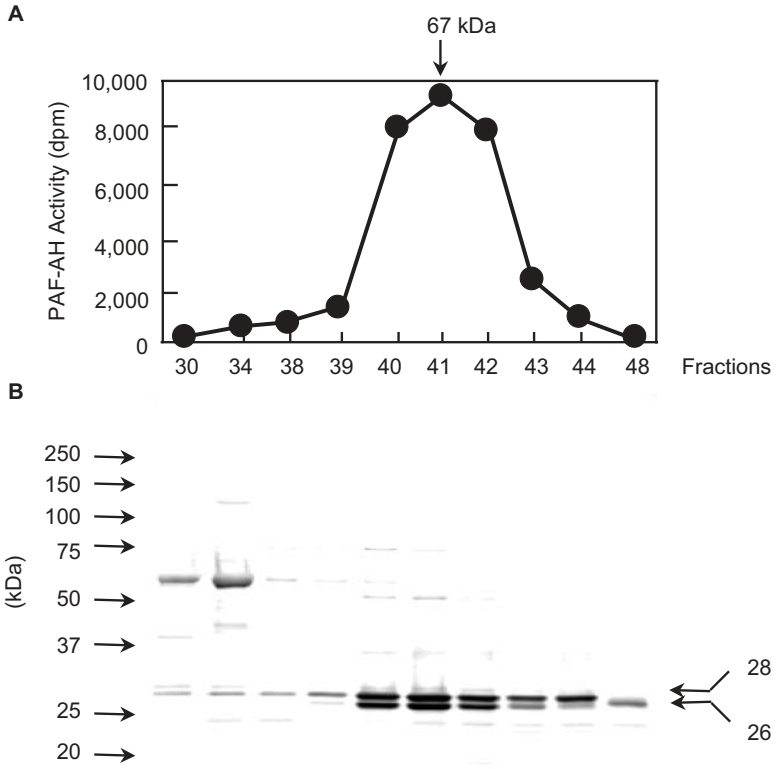

Fig. 2. **Identification of the proteins responsible for the PAF-AH activity in pig red blood cells by SDS-PAGE.** Fraction Y obtained on column chromatography on Ni-NTA was further purified and the protein materials responsible for PAF-AH activity were identified by SDS-PAGE. A shows the PAF-AH activity on gel filtration column chromatography on TSK G-3000 sw. B shows analysis of the protein materials by SDS-PAGE. An aliquot of each fraction (16 μl) was loaded on a 10% polyacrylamide gel and the proteins were visualized by silver staining.

obtained from bovine brain (*12*), although I^{121} and H^{210} were replaced by G and N, respectively, in the pig. Thus, we examined if these proteins cross-reacted with antibodies raised against the individual subunits of Type I PAF-AH. As a result, monoclonal antibodies to the human Type I PAF-AH α_1 and α_2 subunits were found to show cross-reactivity with the protein bands corresponding to 26 and 28 kDa for the final preparation from fraction Y, as shown in Fig. 3. Therefore, this protein was thought to be a heterodimer consisting of type I PAF-AH α_1 and α_2 subunits based on the partial amino acid sequences and reactivity with specific monoclonal antibodies, although the entire amino acid sequence of this protein remains undetermined. Type I PAF-AH is reported to form oligomers consisting of catalytic subunit dimers and the regulatory subunit termed the β subunit. However, the β subunit was not detected immunologically (data not shown).

Although we attempted to purify PAF-AH activity from fraction X (Fig. 1B), it was not possible to purify the enzyme to homogeneity. Specific enzyme activity was increased 306-fold, as compared with that in the red blood cells lysate, and this enzyme showed similar behavior on the individual column chromatographies performed for its purification. However, the immunochemical study showed that the final preparation from fraction X consisted of only the type I PAF-AH α_2 subunit (Fig. 3). In addition, the β subunit was not detected in this enzyme. Since the apparent molecular weight of the natural enzyme was estimated to be about 60 kDa on gel-filtration column chromatography on TSK G-3000 sw (data not shown), the PAF-AH activity in fraction X was thought to be that of the α_2/α_2 homodimer.

In addition to red blood cells, other blood cells such as platelets and polymorphonuclear leukocytes show PAF-AH

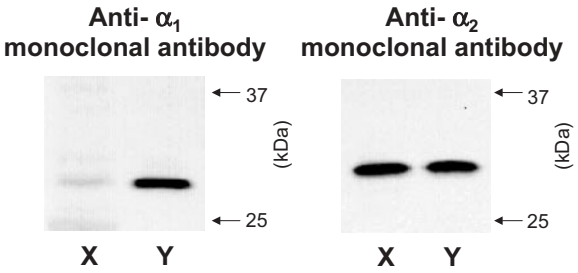

Fig. 3. **Immunochemical identification of PAF-AH from pig red blood cells.** The final preparations obtained by the further purification from fractions X and Y by Ni-NTA column chromatography (Fig. 1B) were analyzed by Western blotting using monoclonal antibodies to the human Type I PAF-AH α_1 and α_2 subunits. An aliquot (16 μl) of each preparation was subjected to Western analysis, as described under "MATERIALS AND METHODS."

activities, although the level of total enzyme activity is less than 1% of the total PAF-AH activity found in the blood in these cells. Therefore, we examined the subtypes of PAF-AHs in these blood cells by immunoblotting using specific monoclonal antibodies to the individual subunits after separation on a nickel-loaded column. As a result, both platelets and polymorphonuclear leukocytes were found to contain only the α_2 complex, while the α_1 subunit was not detected in these cells at all. Like in red blood cells, the β subunit was not detected immunologically in these cells (Fig. 4). Thus, expression of the α_1 subunit is restricted to red blood cells in pig blood. We examined if red blood cells from other animals contained the same subtypes of PAF-AHs on Western analysis using specific monoclonal antibodies to the individual proteins. As a result, human

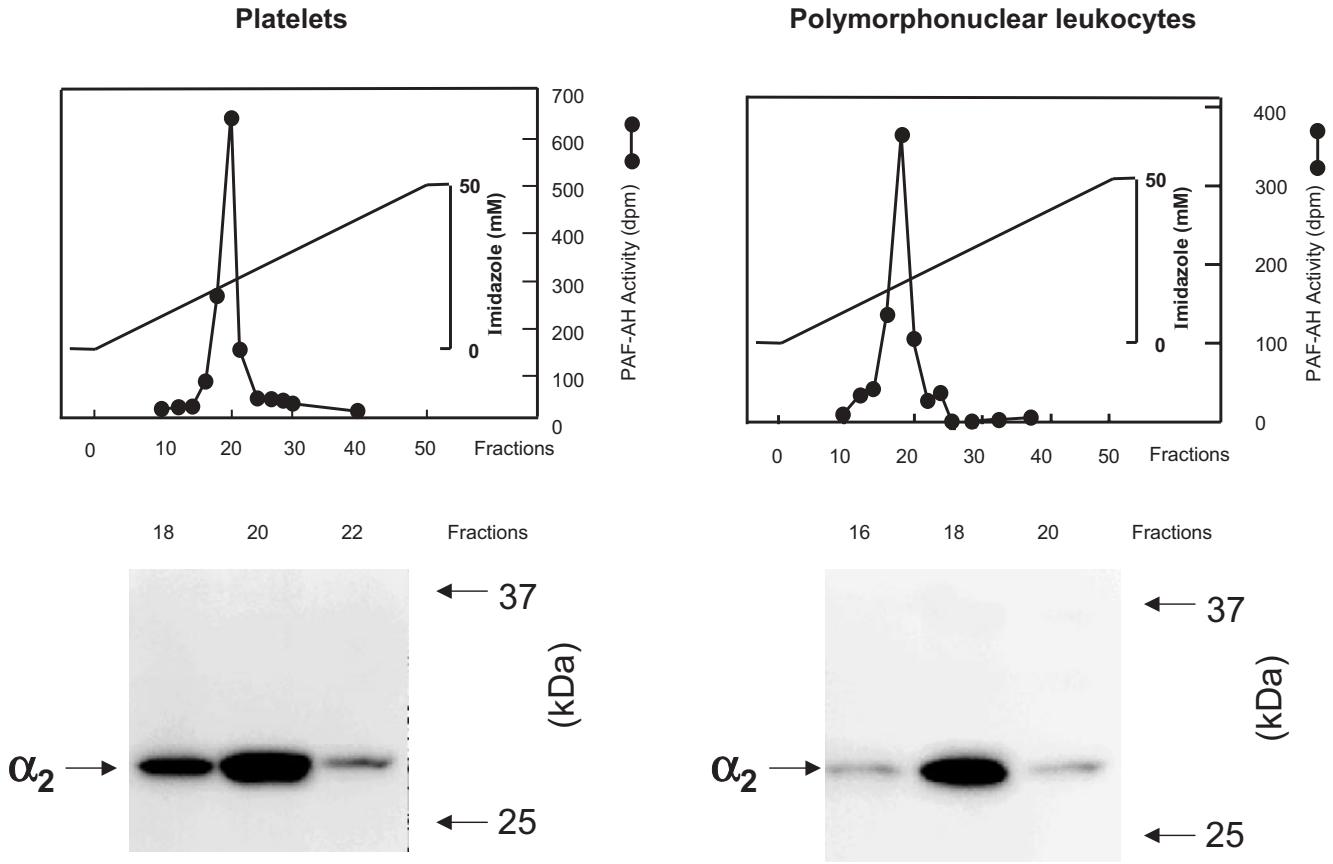

Fig. 4. **Identification of subtypes of PAF-AH in pig platelets and polymorphonuclear leukocytes by Western analysis.** Platelets were obtained from 240 ml of pig blood, as described under "MATERIALS AND METHODS." The resulting platelets were resuspended in 2.3 ml of phosphate-buffered saline and then sonicated for 1 min at 4°C. After centrifugation at 10,000 × *g* for 10 min, the supernatant was applied on a nickel-loaded column (4.6 × 100 mm), which had been equilibrated with 50 mM sodium phosphate (pH 7.5) containing 0.5 M NaCl and 10% glycerol. After washing the column with the same buffer solution, the adsorbed proteins were eluted by increasing the imidazole concentration from 0 to 50 mM and reducing the pH from 7.5 to 6.0 at a flow rate of 0.4 ml/min. Fractions (0.2 ml) were collected and assayed for enzyme activity. Then, an aliquot (16 μl) of each of the indicated fractions with enzyme activity was analyzed by Western blotting using monoclonal antibodies to the human Type I PAF-AH α_1 or α_2 subunit. Likewise, polymorphonuclear leukocytes obtained from 640 ml of pig blood were sonicated and the supernatant was separated by the same column. An aliquot (16 μl) of each of the indicated fractions with enzyme activity was analyzed by Western blotting using monoclonal antibodies to the human Type I PAF-AH α_1 or α_2 subunit.

and rabbit red blood cells were found to mainly contain Type I PAF-AH consisting of the α_1/α_2 complex (data not shown).

Finally, we examined if PAF-AH activities in red blood cells were involved in the hydrolysis of PAF accumulated in the blood. When [^{3}H]PAF was exogenously added to whole blood, about 80% of [^{3}H]PAF was degraded within 10 min. On the contrary, no degradation of [^{3}H]PAF was observed until at least 90 min in whole blood obtained from subjects with a genetic deficiency of plasma-type PAF-AH (*17*), as shown in Fig. 5. This finding indicates that PAF-AH activity in red blood cells is not involved in the degradation of PAF accumulated in the blood. Therefore, PAF-AH found in red blood cells degrades other phospholipids and related molecules produced by oxidative stress.

DISCUSSION

Although red blood cells account for 30% of the PAF-AH activity in blood, the molecular structure and the physiological function of this enzyme have remained unknown. The first trial to purify this enzyme was performed by Stafforini *et al.* (*13*). They started purification from 800 ml of human blood and identified a 25 kDa protein band which behaved as a dimer during gel filtration. In this study, we attempted purification of this enzyme from easily obtainable pig red blood cells, and identified two kinds of protein complexes which consisted of the α_1/α_2 heterodimer or α_2/α_2 homodimer. The PAF-AH activity identified in both studies should be the same, because the molecular weights of the purified proteins and the behavior of the enzyme activities on gel filtration column chromatography are very similar. Moreover, the inhibitory effect of 5,5′-dithiobis (2-nitrobenzoic acid) and diisopropyl fluorophosphates, reported in the previous study, was observed in our study involving the enzyme purified from pig blood cells. Since there was the possibility that the difference was due to the animal species, we preliminary examined if human red blood cells contained these two complexes by immunoblotting with specific monoclonal antibodies to the human α_1

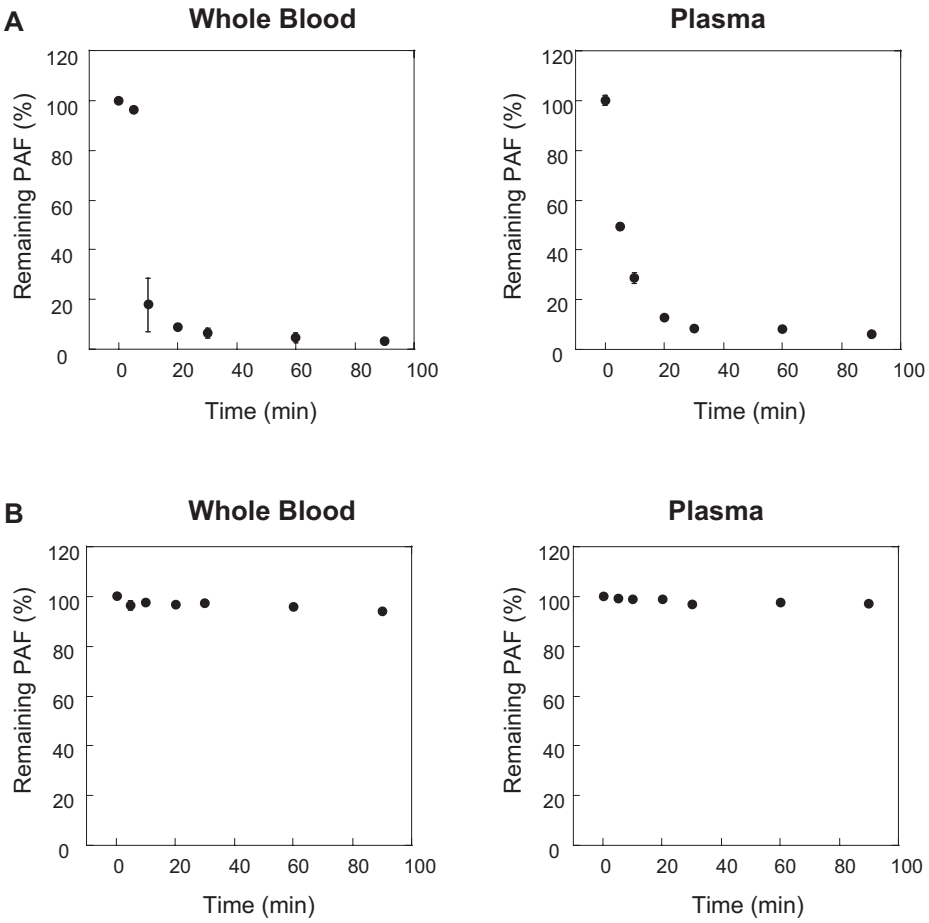

Fig. 5. **Metabolism of exogenously added PAF in human blood.** 1-*O*-Hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (55,000 dpm) was added to 0.25 ml of plasma or whole blood obtained from healthy human volunteers with normal (A) or genetically deficient plasma-type PAF-AH (B) at a final concentration as low as 10^{-8} M. After incubation at 37°C for the indicated times, [^{3}H]PAF was extracted according to the method described under "MATERIALS AND METHODS." Extracted lipids were dissolved in chloroform and then spotted on a TLC plate (Merck). The lipids were developed with a solvent system consisting of chloroform/methanol/acetic acid/H_2O (25:15:8:4, v/v/v/v), and the area corresponding to the PAF was removed. The removed silica was transferred to scintillation vials and then the radioactivity of the remaining [^{3}H]PAF was quantitated with a liquid scintillation counter.

and α_2 subunits. As a result, it was found that human red blood cells contain both complexes (data not shown), like pig red blood cells. Therefore, the PAF-AH identified in this study is thought to be the same as the previously reported one. We found that the α_1/α_2 heterodimer or α_2/α_2 homodimer was separated on column chromatography on Ni-NTA resin. Therefore, it is likely that these complexes interact with heavy metals. Although we examined copper and zinc ions as chelating metals besides nickel, enzyme activity was not desorbed from these metals with imidazole because of the high affinity to the protein complexes. Nickel not only interacted with these complexes but also influenced their enzyme activities. In addition, consistent with the affinity of the individual complexes to nickel (data not shown), the inhibitory effect of nickel on the enzyme activity of the α_1/α_2 heterodimer enzyme was greater than on that of the α_2/α_2 homodimer one. It has been reported that the PAF-AH activity in red blood cells is sensitive to oxidation, and heavy metals such as lead, cadmium and copper (*18*). Therefore, the low recovery of enzyme activity in the purification step involving chromatography on Ni-NTA resin (Table 1) might have resulted from direct inhibition and/or heavy metal-induced oxidation of this enzyme.

Type I PAF-AH was originally purified from bovine brain, and shown to form a complex consisting of two catalytic subunits and a regulatory subunit, β (*19*). However, the β subunit was not associated with the complexes consisting of the α_1/α_2 heterodimer or α_2/α_2 homodimer in pig red blood cells, although the β subunit was immunologically detected in the red blood cells. The interaction of the β subunit with catalytic complexes and the effect of the β subunit on the enzyme activity have been analyzed in detail using recombinant proteins (*20*). When the β subunit is incubated with the α_1/α_2 heterodimer or α_2/α_2 homodimer, complexes consisting of the β subunit and catalytic subunits are partially formed. Therefore, it is possible that the β subunit is associated with catalytic subunits in red blood cells under natural conditions and that this subunit is readily dissociated from the catalytic subunits during Ni–NTA column chromatography, which is conducted with a high salt concentration to prevent non-specific binding. Although the β subunit is not essential for the enzyme activity, it has been demonstrated to influence the enzyme activity of the individual catalytic complexes, especially that of the α_2/α_2 complex (*9*). Therefore, it is possible that the β subunit modulates the enzyme activity of catalytic complexes in red blood cells under natural conditions.

In this study, we found that the α_1/α_2 heterodimer was highly expressed in red blood cells. By contrast, other blood cells such as platelets and polymorphonuclear leukocytes contained the α_2/α_2 homodimer instead of the α_1/α_2 heterodimer. According to studies related to the distribution of Type I PAF-AH subunits in the rat (*20*, *21*), the α_1 subunit is specifically expressed in migrating neurons in the embryonic and postnatal stages, whereas α_2 expression

is almost constant from the fetal stages through adulthood. The resulting switching of catalytic subunits from the α_1/α_2 heterodimer to the α_2/α_2 homodimer is likely to be involved in brain development (*20*). The same type of alteration in the catalytic dimers occurs during differentiation from spermatogonia to spermatocytes (*21*). It has been shown that the α_2 subunit is observed in meiotically dividing spermatocytes and elongating spermatids but that the expression of the α_1 subunit is restricted to spermatogonia (*22*). These observations suggest that a change in the combination of two kinds of catalytic subunits is involved in the maturation of some kinds of cells. The finding that mature red blood cells also highly express the α_1/α_2 heterodimer leads us to speculate that the α_1/α_2 heterodimer plays other roles, including in proliferation and differentiation of erythroid progenitor cells.

It has been reported that PAF is metabolized after incorporation into cells through a receptor-mediated (*23*) or receptor-independent (*24*) mechanism. In addition, very recently, the expression of a specific PAF-receptor was demonstrated on human red blood cells (*25*). Therefore, we examined the possibility that the PAF-AH activity of red blood cells was involved in the degradation of PAF accumulated in the blood on incorporation of PAF into the red blood cells. As a result, it was found that exogenously added PAF remained unchanged in blood obtained from human subjects. This result indicates that PAF-AH activity in red blood cells does not contribute the degradation of PAF accumulated in the blood. Recently, it was reported that some oxidized phospholipids exhibit biological activities eliciting immune responses and promote inflammation. Most biologically active phospholipids are products with ω-aldehydic, ω-hydroxy, or ω-carboxy group at the *sn*-2 position, and these phospholipids have been detected in oxidized low density lipoproteins, atherosclerotic plaques, aged red blood cells, apoptotic cells, and the plasma of smokers (*26–28*). Previous investigations revealed that oxidized and fragmented phospholipids are hydrolyzed by plasma-type PAF-AH and intracellular Type II PAF-AH (*29*, *30*). Since red blood cells contain a large amount of oxyhemoglobin, membrane phospholipids are exposed to oxidant stress. Indeed, it has been shown that cytotoxic products such as 2-azelaoylphosphatidylcholine are produced in liposomes treated with oxyhemoglobin (*31–32*). It has been hypothesized that the PAF-AH activity in red blood cells scavenges the toxic or biologically active oxidized phospholipids generated with oxidant stress during their life span, since PAF-AH in red blood cells hydrolyzes phospholipids that contains short *sn*-2 acyl residues generated through oxidative fragmentation of polyunsaturated residues (*13*). Plasma-type PAF-AH and intracellular Type II PAF-AH preferentially hydrolyze phospholipids with ω-carboxylic acyl residues at the *sn*-2 position (*i.e.*, 1-*O*-alkyl-2-succinoyl-*sn*-glycero-3-phosphocholin and 1-*O*-alkyl-2-glutaroyl-*sn*-glycero-3-phosphocholine), while Type I PAF-AH catalytic dimers show little or no hydrolytic activity towards these oxidized phospholipids. Therefore, there is the possibility that Type I PAF-AH degrades different species of oxidized phospholipids from plasma-type and Type II PAF-AHs. Our interest is currently directed toward physiological substrates of this enzyme, which shows a different substrate specificity from other PAF-AH subtypes.

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