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Intracellular Erythrocyte Platelet-activating Factor Acetylhydrolase I Inactivates Aspirin in Blood*[S]

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Background: Aspirin circulates transiently in blood, but the identity of the enzyme(s) that hydrolyzes its acetyl residue remains unknown.
Results: Purification, mass spectrometry, and overexpression identified erythrocyte type I PAF acetylhydrolase as aspirin hydrolase.
Conclusion: Aspirin is primarily hydrolyzed within erythrocytes by PAF acetylhydrolase.
Significance: PAF acetylhydrolase and aspirin hydrolysis varies among individuals to modulate the effectiveness of aspirin.

Aspirin (acetylsalicylic acid) prophylaxis suppresses major adverse cardiovascular events, but its rapid turnover limits inhibition of platelet cyclooxygenase activity and thrombosis. Despite its importance, the identity of the enzyme(s) that hydrolyzes the acetyl residue of circulating aspirin, which must be an existing enzyme, remains unknown. We find that circulating aspirin was extensively hydrolyzed within erythrocytes, and chromatography indicated these cells contained a single hydrolytic activity. Purification by over 1400-fold and sequencing identified the PAFAH1B2 and PAFAH1B3 subunits of type I platelet-activating factor (PAF) acetylhydrolase, a phospholipase A_2 with selectivity for acetyl residues of PAF, as a candidate for aspirin acetylhydrolase. Western blotting showed that catalytic PAFAH1B2 and PAFAH1B3 subunits of the type I enzyme co-migrated with purified erythrocyte aspirin hydrolytic activity. Recombinant PAFAH1B2, but not its family member plasma PAF acetylhydrolase, hydrolyzed aspirin, and PAF competitively inhibited aspirin hydrolysis by purified or recombinant erythrocyte enzymes. Aspirin was hydrolyzed by HEK cells transfected with PAFAH1B2 or PAFAH1B3, and the competitive type I PAF acetylhydrolase inhibitor NaF reduced erythrocyte hydrolysis of aspirin. Exposing aspirin to erythrocytes blocked its ability to inhibit thromboxane A_2 synthesis and platelet aggregation. Not all individuals or populations are equally protected by aspirin prophylaxis, the phenomenon of aspirin resistance, and erythrocyte hydrolysis of aspirin varied 3-fold among individuals, which correlated with PAFAH1B2 and not PAFAH1B3. We conclude that intracellular type I PAF acetylhydrolase is the major aspirin hydrolase of human blood.

Aspirin (acetylsalicylic acid) is the classic nonsteroidal anti-inflammatory agent with an estimated 100 million tons made and consumed annually (1). The reactive acetyl group of aspirin irreversibly acetylates serine 530 residue of type I cyclooxygenase, thereby irreversibly inhibiting the first of the two reactions this enzyme catalyzes (2, 3). The cyclooxygenase product prostaglandin H_2 made in platelets is converted by their thromboxane synthase to thromboxane A_2. This lipid mediator is critical to platelet function because platelets express the TXA receptor that strongly promotes activation by incomplete agonists (3, 4). Platelets are particularly prone to the effects of aspirin because, unlike all other cells, these anucleate cells are unable to synthesize new enzyme to replace inactivate enzyme. Once a platelet is exposed to aspirin, its adhesion to activated endothelial cells, growing platelet plugs, thrombosis, and degranulation are irreversibly suppressed (5, 6).

Ingested aspirin is hydrolyzed in the gut by first pass metabolism, with about 50–70% reaching systemic circulation (7, 8). The circulating pool of aspirin is hydrolyzed with a half-life of ~15 to 20 min (9, 10), but contributes to platelet responsiveness because inhibition is distinctly nonlinear where even small amounts of active cyclooxygenase promote platelet function (11).

Attention has focused on a phenomenon of aspirin ineffectiveness where not all individuals or populations appear to receive the full prophylactic or therapeutic benefits of aspirin (12–16). The physiologic basis for this aspirin resistance is undefined, and even whether the phenomenon is real or documentable is debated (17). Despite these clinical and laboratory issues, variation in the time aspirin circulates will affect its ability to inactivate cyclooxygenase 1 and inhibit platelet function.

Aspirin turnover limits its effectiveness, but the enzyme(s) responsible for this hydrolysis is unknown. Aspirin hydrolysis was not subjected to natural selection, and so must be a property of enzymes selected in other ways. Accordingly, several esterases have the ability to hydrolyze aspirin that include uridine diphosphate glucuronyl transferase (18, 19), carboxylesterase 2 (20, 21), and undefined activities distinct from cholinesterases and nonspecific carboxyesterases (22). However, the enzymes that actually contribute to aspirin turnover in blood,

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rather than merely being capable of aspirin hydrolysis, are undefined.

Aspirin hydrolysis in blood need not occur in plasma because aspirin rapidly enters erythrocytes (23) where it is hydrolyzed by first order kinetics (24). Indeed, the rate of aspirin hydrolysis in blood correlates positively with hematocrit (25). The aspirin hydrolase of erythrocytes was first identified as membrane-bound acetylcholinesterase (26), but was then located to cytosol that is inconsistent with this membrane-bound enzyme (27). Partial purification of this activity revealed a 95-kDa protein as the major aspirin hydrolytic activity that is also inconsistent with the ~66-kDa acetylcholinesterase (27).

Butyrylcholinesterase (28), high density lipoprotein-associated activity (29), and a nonenzymatic activity of albumin (30) all can contribute to aspirin hydrolysis in plasma. Plasma also contains a uniquely selective deacetylase, plasma platelet-activating factor (PAF)[3] acetylhydrolase (PLA2G7), which acts on the phospholipid 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) (31). This phospholipase A_2 circulates in association with low and high density lipoproteins.

Type I PAF acetylhydrolase (PAFAH), a structurally unrelated member of this small family of group VII phospholipases A_2 that hydrolyze the acetyl residue of PAF, is present in erythrocytes as a trimer of a noncatalytic 45-kDa protein subunit associated with two catalytic subunits, either α_1 (29 kDa, PAFAH1B3) or α_2 (30 kDa, PAFAH1B2) homo- or heterodimers (32).

We purified erythrocyte aspirin hydrolase and found that the major hydrolytic activity in blood is type I PAF acetylhydrolase. We also found that erythrocytes alter the suppression of aspirin on platelet function, and that the effectiveness of intracellular hydrolysis of aspirin varied among individuals.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Aspirin, purified human plasma butyrylcholinesterase, and antibodies to PAFAH1B2 and PAFAH1B3 were purchased from Sigma. Salicylic acid and high performance liquid chromatography grade solvents (acetonitrile, formic acid, and water) were from J.T. Baker Inc. (Phillipsburg, NJ). ECL kits were from Amersham Biosciences. DNA plasmids encoding *Homo sapiens* platelet-activating factor acetylhydrolase 1b and recombinant *H. sapiens* platelet-activating factor acetylhydrolase 1b were purchased from OriGene Technologies, Inc. (Rockville, MD). Lipofectamine™ 2000 was purchased from Invitrogen. Purified recombinant plasma PAF acetylhydrolase was from ICOS Corporation (Bothel, WA). DEAE-Sepharose™ FF and HiTrap Q columns were purchased from GE Healthcare. Collagen was from CHRONOLOG Corp. (Havertown, PA). C_{18} SPE columns were from Mallinckrodt Baker (Phillipsburg, PA). PAF was from Biomol Research Laboratories (Plymouth Meeting, PA). Thromboxane B_2 Express EIA Kits were from Cayman.

Aspirin Hydrolysis—An enzyme source was incubated with 4 mM aspirin buffered by PBS, pH 7.2, in a 50-μl total volume at 37 °C for 2 h. The reaction was stopped and proteins were precipitated by adding 150 μl of acetonitrile containing 0.1% formic acid and 400 μg/ml of acetaminophen as an internal standard followed by centrifugation at 4,000 × *g* for 20 min at 4 °C. Aspirin hydrolytic activity was measured by quantifying salicylic acid production by RP-HPLC over a 5-μm 150 × 2-mm ODS column from Phenomenex. The mobile phase was acetonitrile, aqueous 0.1% formic acid (40/60, v/v) at a flow rate of 0.4 ml/min. Salicylic acid, aspirin, and acetaminophen were quantified by UV absorption at 280 nm using the internal standard and previously defined spectra with authentic compounds. Data were analyzed by converting the area under the absorption curve. Nonenzymatic produced salicylic acid was subtracted from the analysis.

Kinetic Constants—Aspirin concentrations varied over a range of 0.2 to 8 mM in incubations with PAFAH1B2 or BChE at 37 °C for 1 h with or without addition of PAF (1 mM). Experiments were performed in triplicate before the data were fitted with Prism 3 software to obtain kinetic constants.

Cell Isolation—Human erythrocytes were isolated from blood (400 ml/donor) freshly drawn from healthy human volunteers in a protocol approved by the Cleveland Clinic Institutional Review Board. Blood was collected in EDTA (25 mM) and then centrifuged for 20 min at 2,000 × *g*. The supernatant was discarded, and the blood cells were suspended in 3 volumes of 0.9% NaCl and centrifuged as above. These erythrocytes were washed twice more to remove contaminating plasma.

DEAE Chromatography—All chromatography was carried out at 4 °C. Erythrocytes (100 ml) were hypotonically lysed with 100 ml of 0.2% NaCl. Membrane debris was removed by centrifugation at 10,000 × *g* for 1 h at 4 °C. Supernatants were diluted to 1 liter with 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and loaded onto a 500-ml DEAE-Sepharose™ FF column. The column was washed with equilibrating buffer (50 mM Tris-HCl, pH 7.4) until protein elution from the column ceased (1–1.5 liters). This removed most of the hemoglobin, which is the major protein in this preparation. Aspirin hydrolytic activity was eluted from the gel with a linear 0 to 0.5 M NaCl gradient in 50 mM Tris-HCl (pH 7.4). Fractions (8 ml) were collected and assayed for protein content and aspirin hydrolytic activity. Most of the aspirin hydrolytic activity was present in fractions 34 to 44 (supplemental Fig. S1). Active fractions were pooled and concentrated to 20 ml using Pierce® concentrators (Thermo Scientific). Concentrated material was diluted 20-fold to 100 ml with 50 mM Tris-HCl, pH 7.4, and loaded onto a second DEAE column (50 ml) and eluted with a linear NaCl gradient as before. Fractions (4 ml) were collected and assayed for protein content and aspirin hydrolytic activity. The bulk of the activity was again contained in fractions 35 to 43 (supplemental Fig. S1). Active fractions were pooled and concentrated to 5 ml using Pierce concentrators (Thermo Scientific).

HiTrap Q Chromatography—Concentrated preparations from DEAE chromatography (5 ml) were diluted to 60 ml and loaded onto a 5-ml HiTrap Q column conditioned with 50 mM Tris-HCl (pH 7.4). A linear 0 to 0.1 M NaCl gradient was used to elute material from the column before as fractions (1 ml) were collected and assayed for protein and aspirin hydrolytic activity. Most of the aspirin hydrolytic activity was recovered in frac-

[3] The abbreviations used are: PAF, platelet-activating factor; PAFAH, platelet-activating factor acetylhydrolase.

tions 40 to 50 (supplemental Fig. S1), which were pooled and concentrated to 1 ml.

Sephacryl S-200 Chromatography—The pooled, concentrated fraction from the HiTrap Q column was fractionated by molecular sieving with a Sephacryl S-200 column (1 × 60 cm) equilibrated in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl (supplemental Fig. S1).

Mass Spectrometry—Proteins purified by chromatography were resolved by SDS-PAGE. The Coomassie Blue-stained bands were cut from the gel as tightly as possible with a punch and washed and destained in 50% ethanol with 5% acetic acid. The gel pieces were dehydrated in acetonitrile, dried in a Speed-Vac, and digested with trypsin by adding 5 μl of trypsin (10 ng/μl) in 50 mM ammonium bicarbonate, and incubated overnight at room temperature. The peptides were extracted from the polyacrylamide in two aliquots of 30 μl of 50% acetonitrile with 5% formic acid.

The LC-MS system was a Finnigan LTQ linear ion trap mass spectrometer equipped with an Eksigant nano-one-dimensional HPLC system. The tryptic peptides were eluted from a self-packed 9 cm × 75-μm Phenomenex Jupiter C18 reversed-phase capillary column with an acetonitrile, 0.1% formic acid gradient flowing at 0.25 μl/min. The peptides were introduced directly into the mass spectrometer and the resulting LC-MS/MS data were analyzed by searching the human reference sequence data base using the search program Mascot. All matching spectra were verified by manual interpretation.

Platelet Aggregation—Homotypic aggregation of washed platelets (2×10^8/ml) was measured optically (CHRONOLOG, model 700) with stirring at 600 rpm at 37 °C. In some cases, washed erythrocytes (8×10^8 in 1 μl) were introduced after incubation with 1.25 mM aspirin for 0 or 30 min before dilution into a preparation of platelets to achieve what would have been 50 μM based on the amount of the initial aspirin. Aggregation was induced with 5 μg/ml of collagen. The rate and extent of aggregation were recorded by monitoring light transmission compared with buffer control.

Cell Culture and PAFAH1B2 Expression—Human embryonic kidney 293 cells (HEK 293T) were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) (Cleveland Clinic Core Facility) supplemented with 10% fetal calf serum (HyClone), and penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C. HEK 293T cells (1×10^5 cells/well) were plated in 6-well plates and 24 h later transfected with DNA plasmids (6 μg/well) encoding *H. sapiens* PAF acetylhydrolase PAFAH1B2 or PAFAH1B3 subunits under control of a CMV promoter using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 32 h, cells were harvested and washed twice by PBS buffer.

PAF Acetylhydrolase Activity—PAF hydrolyzing activity was determined using 100 μM [*acetyl*-^{3}H]PAF as the substrate. Briefly, the enzyme source was incubated with [*acetyl*-^{3}H]PAF before released [^{3}H]acetate was isolated over a C_{18} cartridge and counted by liquid scintillation counting as described (33).

Western Blotting—The protein concentration was measured by the Bradford method. Equal amounts of sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% (v/v) Tween 20 buffer (20 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, and 0.1% Tween 20, TBST) and incubated overnight at 4 °C with antibodies against PAFAH1B2 (1:1000), butyrylcholinesterase (1:1000), PAFAH1B3 (1:1000), or β-actin (1:1000). The membrane was washed with TBST buffer and incubated with appropriate secondary antibodies at the manufacturer's suggested dilution. Antibody staining was captured by chemiluminescence reagents and x-ray film.

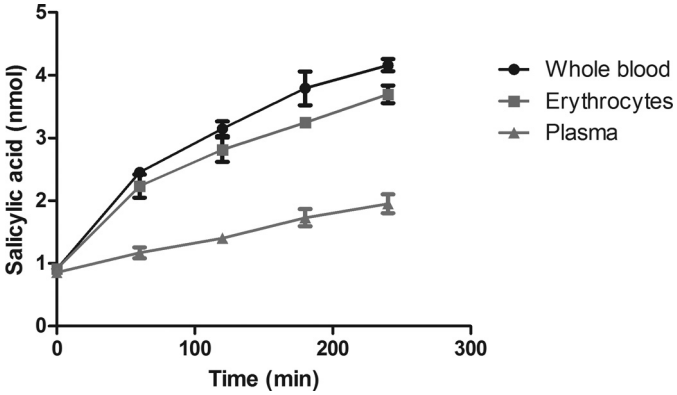

FIGURE 1. **Intact erythrocytes hydrolyze aspirin.** Aspirin (4 mM) hydrolysis by whole human blood (50 μl), washed intact erythrocytes (25 μl), or plasma (25 μl) in a final volume of 500 μl at 37 °C was determined as a function of time. Aliquots of the reaction were removed at the stated time before the salicylic acid product was isolated and quantified by reversed phase HPLC and spectroscopy as described under "Experimental Procedures." Data are presented as mean ± S.D., $n = 6$.

Statistical Analysis—Michaelis-Menten progression curves were fitted using nonlinear regression analyses included in Prism 3. Data are presented as mean ± S.D. of 3 analyses derived from the blood of one donor, with repetition and validation from at least one other donor.

RESULTS

Erythrocytes Contain Aspirin Hydrolase Activity—Aspirin was hydrolyzed when incubated with whole blood, and separation of plasma from erythrocytes showed the bulk of the activity was displayed by intact erythrocytes (Fig. 1). Erythrocytes constitute nearly half the volume of blood, and facile entry of aspirin (23) resulted in greater intracellular hydrolysis than extracellular plasma hydrolysis.

Type I PAF Acetylhydrolase Is the Primary Aspirin Hydrolase of Erythrocytes—We purified erythrocyte aspirin hydrolytic activity to determine whether more than a single activity accounted for intracellular hydrolysis, and to identify the relevant activity(ies). We used a combination of DEAE, HiTrap Q, and Sephacryl S200 size exclusion chromatography (supplemental Fig. S1) to obtain an overall purification of 1,438-fold with a final yield of 24% (Table 1).

Aspirin hydrolytic activity in the final size separating column revealed a single, asymmetric peak of activity with maximal activity eluting in fractions 40 to 43 with an asymmetric abundance in the preceding fractions (Fig. 2*A*). Elution at this position corresponded to an estimated size of 70 kDa for a globular protein. Analysis of the protein in these fractions by denaturing

TABLE 1
Purification of erythrocyte aspirin hydrolytic activity

Step	Total protein	Total activity	Specific activity	Purification	Recovery
	mg	*nmol/h*	*nmol/mg/h*	*-fold*	*%*
RBC lysate	10485.00	129339.65	12.33	1	100.00
DEAE binding	4100.00	99288.60	24.82	2	76.77
DEAE I	91.39	60012.00	656.66	53	46.40
DEAE II	41.56	37083.70	892.29	72	28.67
HiTrap Q FF	12.40	34260.00	2762.90	224	26.49
Sephacryl S-200	1.8	31924.01	17735.6	1438	24.68

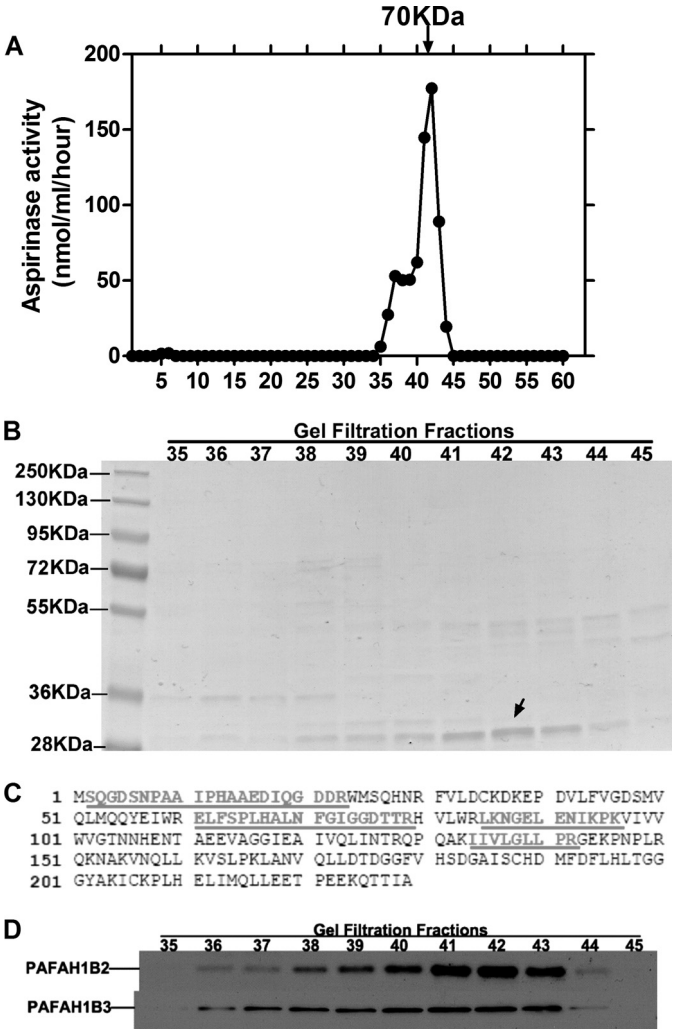

FIGURE 2. **PAF acetylhydrolase type I catalytic subunits PAFAH1B2 and PAFAH1B3 co-localize with purified erythrocyte aspirin hydrolase.** *A,* gel filtration. Aspirin hydrolytic activity in the final purification step was measured in fractions eluting from a Sephacryl S200 gel filtration column as described under "Experimental Procedures." The position of a 70-kDa marker in fraction 42 is shown on the graph. *B,* SDS-PAGE resolution in a 4–15% gradient gel of combined fractions 35–45 eluting from the Sephacryl column with protein detection by Coomassie Blue staining. The *arrow above* the 28-kDa marker shows the band excised for mass spectrometry. *C,* sequence of human PAFAH1B2 with five peptides identified by mass spectrometry identified by underlines. *D,* immunoblot detection of PAFAH1B2 and PAFAH1B3 in fractions eluting from the Sephacryl size separation column.

SDS-PAGE and Coomassie Blue staining, however, detected no protein of this approximate size uniquely migrating in these fractions. Instead, a major band migrating just more slowly than the 28-kDa standard (Fig. 2*B*) was maximal in fractions 41 to 43, with a less abundant and slightly more slowly migrating band foreshadowing this material in fractions 38 to 41.

Elution, tryptic digestion, and separation and analysis of the more rapidly migrating band by nano-liquid chromatography/mass spectrometry showed the preparation was not pure, but we identified 5 peptides, corresponding to 27% coverage, of the protein encoded by *PAFAH1B2* (Fig. 2*C*). This protein is a catalytic α_2 subunit (30 kDa) that together with a homologous α_1 (29 kDa) catalytic subunit and a third noncatalytic subunit LIS1 (45 kDa) form the type I PAF acetylhydrolase trimer (32). Four peptides (12% coverage) corresponding to PAFAH1B3 were also present (not shown). Erythrocytes express PAF acetylhydrolase (34) that has been purified (35) and characterized (36) in erythrocytes. The full physiologic role of this enzyme in erythrocytes remains undefined as targeted deletion of its catalytic subunits primarily reduces spermatid and testis size (37).

We determined by Western blotting whether catalytic PAFAH 1 subunits were present in fractions containing aspirin hydrolytic activity. We found (Fig. 2*D*) immunoreactive PAFAH1B2 heavily concentrated in fractions 41 to 43 of the size exclusion column, and found a wider distribution of PAFAH1B3 that encompassed fractions 36 to 43. The relative distribution of subunits from the human donor differed from the original resolution of the enzyme from pig blood (36).

PAFAH1B2 Is an Effective Aspirin Hydrolase—We verified that erythrocyte PAF acetylhydrolase was an appropriate candidate aspirin hydrolase by testing aspirin hydrolysis by PAFAH1B2 because, at least for PAF at low concentrations, the type I complex is more effective as the α_2 (PAFAH1B2) homodimer or α_1/α_2 heterodimer (36, 38) than the enzyme containing the α_1 PAFAH1B3 homodimer. Recombinant PAFAH1B2 effectively hydrolyzed aspirin, and its rate varied with substrate concentration that was well described by the Michaelis-Menten equation (Fig. 3*A*). The K_m was determined to be 1.13 ± 0.57 mM and its V_{max} was 21.4 ± 2.95 nmol/h/μg. The enzyme purified from erythrocytes displayed an apparent K_m for aspirin of 2.17 ± 0.65 mM with a corresponding V_{max} of 66.05 ± 7.18 nmol/h/μg, suggesting the recombinant material was not fully active. Butyrylcholinesterase is the most completely characterized soluble enzyme to hydrolyze aspirin, which hydrolyzed aspirin with a K_m of 2.78 ± 0.59 mM and a V_{max} of 56.36 ± 4.78 nmol/h/μg.

We determined whether the acetyl group of PAF and aspirin were likely hydrolyzed in the same active site of the type I PAF acetylhydrolase, and tested the effect of PAF on aspirin hydrolysis by the purified erythrocyte enzyme and recombinant PAFAH1B2. We found that the short chain phospholipid inhibited hydrolysis of the xenobiotic aspirin substrate by both enzymes (Fig. 3*B*), and that this inhibition was competitive for both enzymes. The calculated K_i for PAF inhibition of aspirin

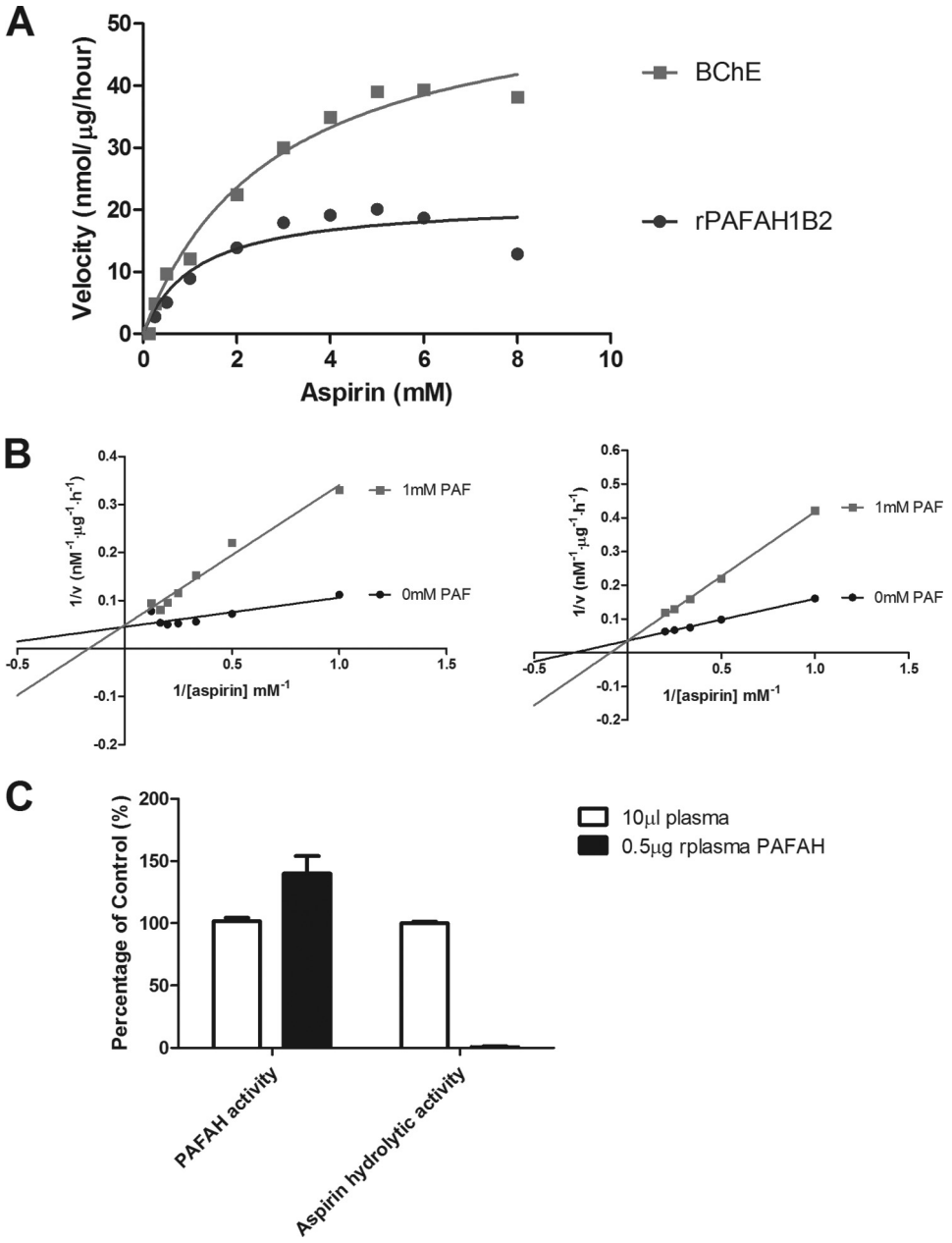

FIGURE 3. **PAFAH1B2 is an effective aspirin hydrolase.** *A,* aspirin hydrolysis (37 °C, 1 h) by purified butyrylcholinesterase, or recombinant PAFAH1B2 fitted to curves generated by Michaelis-Menten kinetics with the aid of nonlinear regression analysis. Data were analyzed by Prism 3. *B,* Lineweaver-Burk plot of aspirin hydrolytic activity of recombinant PAFAH1B2 (*left*) or purified erythrocyte aspirin hydrolase (*right*) with varied concentrations of aspirin in the presence or absence of PAF. *C,* plasma PAF acetylhydrolase (gene name *PLA2G7*) hydrolyzes PAF, not aspirin. Recombinant plasma PAF acetylhydrolase (0.5 μg) or plasma (10 μl) was incubated with 4 mM aspirin or 0.1 mM [*acetyl*-^{3}H]PAF for 15 min before salicylic acid was quantitated by reversed phase HPLC or [^{3}H]acetate quantified by liquid scintillation counting. Data are presented by mean ± S.D., $n = 3$.

hydrolysis for the purified enzyme was 0.47 mM, whereas that for rPAFAH1B2 was 0.29 mM. This indicates a strong preference for PAF compared with aspirin with its 2.1 mM K_m.

The type 1 PAF acetylhydrolase is one of three mammalian family members of the group 7 phospholipase A_2 family (39), a family that is unique in that all members require a short *sn*-2 ester such as the acetyl ester of PAF. The primary PAF degrading activity in blood is the plasma PAF acetylhydrolase (40), and so we tested the ability of purified recombinant human plasma PAF acetylhydrolase to hydrolyze the acetyl ester of aspirin. Recombinant plasma PAF acetylhydrolase (PLA2G7) was active and hydrolyzed PAF as effectively as plasma (Fig. 3*D*). However, the plasma PAF acetylhydrolase was devoid of aspirin hydrolytic activity, and clearly was not the aspirin hydrolytic activity of plasma.

Type I PAF Acetylhydrolase Is an Intracellular Aspirin Hydrolase—The aspirin hydrolase purified from erythrocytes contained type I PAF acetylhydrolase catalytic subunits, and accordingly hydrolyzed PAF. To date, there are no selective inhibitors of the type I PAF acetylhydrolase, but it is reversibly inhibited by sodium fluoride (35). This small molecule phosphatase inhibitor effectively inhibited PAF hydrolysis by recombinant PAFAH1B2, but also by purified erythrocyte aspirin hydrolase (Fig. 4*A*). We used this inhibitor to test whether

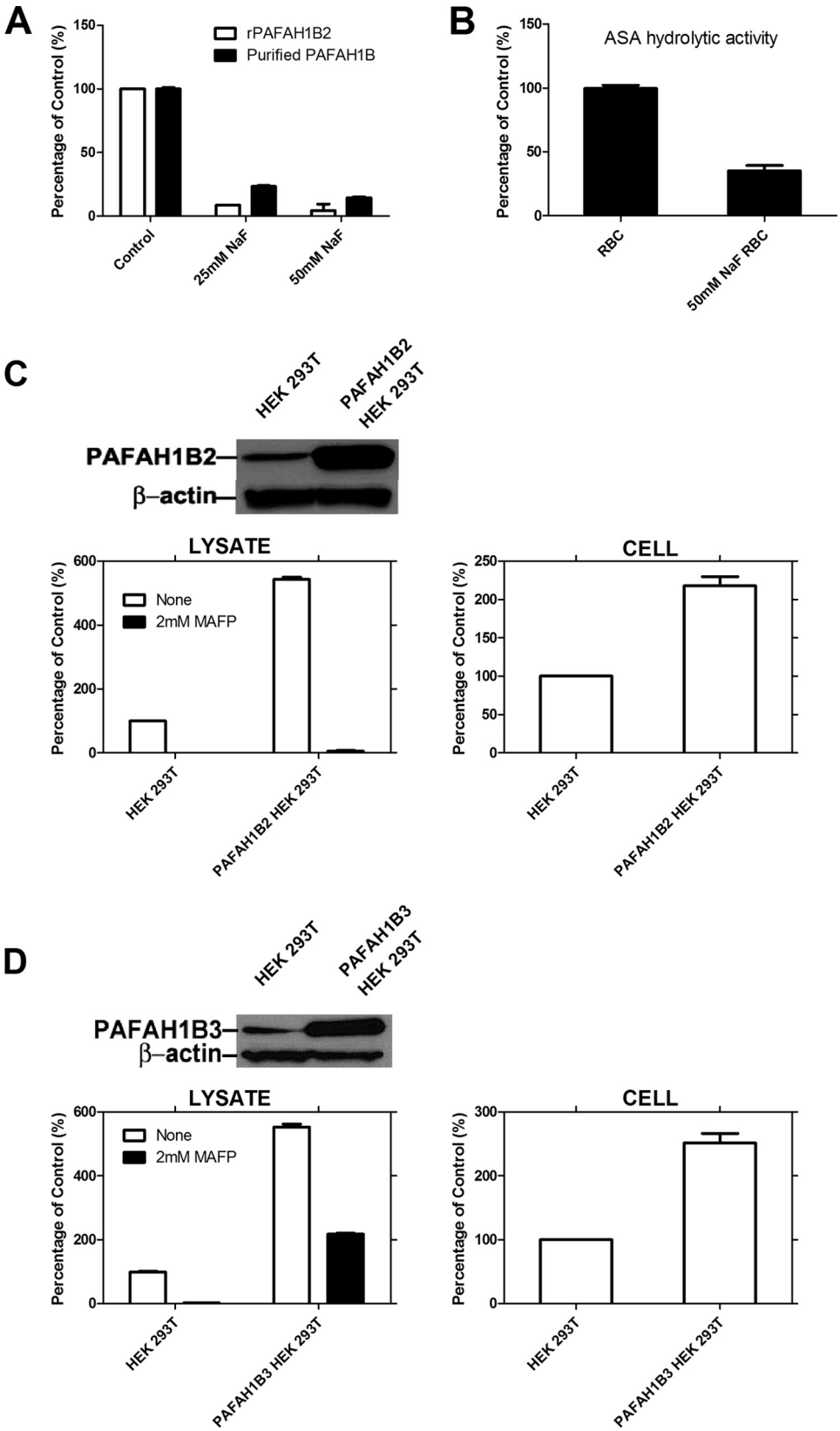

FIGURE 4. **PAFAH1B2 and PAFAH1B3 are aspirin hydrolases.** *A,* recombinant PAFAH1B2 hydrolyzes aspirin, and both recombinant enzyme and enzyme purified from human erythrocytes are inhibited by NaF. Either purified or recombinant enzyme was incubated with the stated concentration of NaF during a 2-h incubation with 4 mM aspirin before salicylic acid production was determined as described in the legend to Fig. 1. *B,* NaF reduces aspirin hydrolysis by intact erythrocytes. Intact washed erythrocytes (10^8) were incubated with 50 mM NaF and 4 mM aspirin in a 50-μl volume for 1 h prior to separation and quantitation of salicylic acid. Overexpression of PAFAH1B2 (*C*) or PAFAH1B3 (*D*) increased aspirin hydrolysis in HEK 293T cells. Western blotting was performed in HEK 293T cells transfected or not with PAFAH1B2 or PAFAH1B3 expression constructs as described under "Experimental Procedures." Aspirin hydrolytic activity was measured in the presence or absence of methyl arachidonyl fluorophosphonate (2 mM). *Left*, cellular lysates. *Right*, intact HEK 293 cells. Data are mean ± S.D., $n = 3$.

the type I PAF acetylhydrolase contributes to aspirin hydrolysis within erythrocytes to find that NaF blocked two-thirds of the aspirin hydrolytic activity of intact erythrocytes (Fig. 4*B*).

We demonstrated that intracellular PAFAH1B2 hydrolyzed exogenously provided aspirin by transiently overexpressing just this α_2 subunit of the enzyme in HEK293 cells. Lysates of cells overexpressing this intracellular PAF acetylhydrolase isoform hydrolyzed aspirin 5 times more effectively than lysates containing just the endogenous amount of the enzyme (Fig. 4*C, left*). Intact cells also were more effective in hydrolyzing exogenously applied aspirin than control cells, although this enhancement was just 2.2-fold (Fig. 4*C, right*). These data show that a rate-limiting step exists prior to hydrolysis, and HEK cells that lack band 3 may not internalize aspirin as effectively as erythrocytes.

We tested homomeric PAFAH1B3 in an identical fashion to find (Fig. 4*D*) that lysates of cells overexpressing just this subunit hydrolyzed aspirin just as effectively as lysates containing the α_2 subunit in the preceding experiment. Intact cells overexpressing PAFAH1B3 also hydrolyzed aspirin more effectively than control cells, but again the level of enhancement was just ~2.7-fold. Unlike PAF hydrolysis, both type I subunits effectively degrade aspirin.

Type 1 PAF acetylhydrolase is irreversibly inhibited by methoxy arachidonyl fluorophosphonate (41). To ensure aspirin hydrolysis was a property of the transfected protein, we treated lysates of cells transfected with PAFAH1B2 (Fig. 4*C*) or PAFAH1B3 (Fig. 4*D*) with this lipid phosphonate to find this irreversible inhibitor abolished aspirin hydrolysis in whole cell lysates.

Erythrocytes Suppress the Anti-platelet Function of Aspirin—We tested the role of erythrocytes in modulating platelet inhibition by aspirin. We exposed washed platelets to varied concentrations of aspirin for 5 min to find little inhibition by 25 μM aspirin, but strong inhibition with 33 μM and complete inhibition of collagen-induced platelet aggregation with 50 μM aspirin (Fig. 5*A*). These data show that platelet responsiveness to aspirin is nonlinear.

We next exposed aspirin to just 1 μl (8×10^8) of washed erythrocytes for 30 min and then added this to washed platelets (450 μl) to achieve what would have been a final concentration of 50 μM aspirin. Aspirin added to erythrocytes and then immediately transferred to washed platelets inhibited about half of the collagen-induced aggregation (Fig. 5*B*, comparison *1*), which was less effective than in the above panel where platelets were preincubated for 5 min with aspirin. However, when aspirin was preincubated for 30 min with erythrocytes and then transferred to the platelet preparation, aspirin failed to inhibit platelet aggregation (Fig. 5*B*, comparison *2*). Inclusion of NaF in the erythrocyte preincubation with aspirin to inhibit type I PAF acetylhydrolase prevented the time-dependent loss of inhibition of aggregation of aspirin (Fig. 5*B*, comparison *3*). This was not the result of a direct action of NaF action on platelets (Fig. 5*B*, comparison *4*).

Platelet production of thromboxane B_2 was reduced by 25 μM, but abolished by 50 μM, aspirin (Fig. 5*C*, comparison *1*). Erythrocytes neither made thromboxane B_2, nor suppressed its production by collagen-activated platelets. However, erythrocytes greatly reduced the effect of aspirin on platelet thromboxane B_2 production: erythrocytes preincubated with aspirin reduced the effectiveness of this drug and allowed platelet synthesis of the prostanoid to recover (Fig. 5*C*, comparison *2*). Aspirin incubated with erythrocytes for 0 time was inhibitory, suggesting rapid metabolism, but aspirin preincubated with erythrocytes for 30 min was significantly less effective in blocking platelet thromboxane B_2 production (Fig. 5*C*, comparison *3*). In fact, aspirin preincubated with erythrocytes was almost completely ineffective as a platelet inhibitor. NaF addition to erythrocytes along with aspirin reduced the inhibitory effect of erythrocytes on aspirin, and again allowed aspirin to inhibit platelet thromboxane B_2 production (Fig. 5*C*, comparison *4*).

Erythrocyte Aspirin Hydrolysis Varies among Individuals—The effectiveness of aspirin in inhibiting platelet function *ex vivo* varies among individuals and populations for unknown reasons (13, 42–44). We found that hydrolysis varied by over 2-fold when erythrocytes were prepared from 10 different donors (Fig. 6*A*). Hydrolysis in lysates from a single donor, however, was reproducible (not shown). We assessed variation of type I PAF acetylhydrolase subunits in the blood of these donors to find that total PAFAH1B2 also varied among donors, whereas the PAFAH1B3 content was uniform (Fig. 6*B*). Moreover, variation of PAFAH1B2, and not PAFAH1B3, content correlated with aspirin hydrolysis, suggesting the PAFAH1B2 containing type I PAF acetylhydrolase is the most effective aspirin hydrolase of erythrocytes, and blood.

DISCUSSION

Erythrocytes readily accumulate aspirin from their environment through band 3 (23), and we found intact erythrocytes hydrolyzed this classic nonsteroidal anti-inflammatory agent, thereby suppressing its inhibition of platelet cyclooxygenase and homotypic aggregation. The identity of the relevant enzyme(s) in blood hydrolyzing aspirin is not established, although a high molecular weight entity has previously been purified from erythrocytes (27).

We isolated the erythrocyte activity that hydrolyzes aspirin and throughout the purification observed the activity primarily behaved as a single entity. Purification of this activity by 1400-fold produced a single, although asymmetric, peak that behaved as a protein of 70 kDa, consistent with a previous partial purification that yielded a protein with an apparent molecular mass of 95 kDa (27). However, when this material was resolved by denaturing SDS-PAGE, the proteins in the active fractions appeared to migrate as much smaller proteins of ~30 kDa. These results are most consistent with a multimeric enzyme, not with simple esterases that are more commonly single chain proteins.

Analysis of the relevant band in the purified preparation by mass spectrometry identified proteins belonging to type I PAF acetylhydrolase. Overexpression of the catalytic PAFAH1B2 or PAFAH1B3 subunits of the type I PAF acetylhydrolase showed each hydrolyzed aspirin. The kinetic constants for PAFAH1B2, whose level varied among donors in common with aspirin hydrolytic capacity, showed this PAF acetylhydrolase was nearly equivalent to the currently best described aspirin esterase of plasma, butyrylcholinesterase (28).

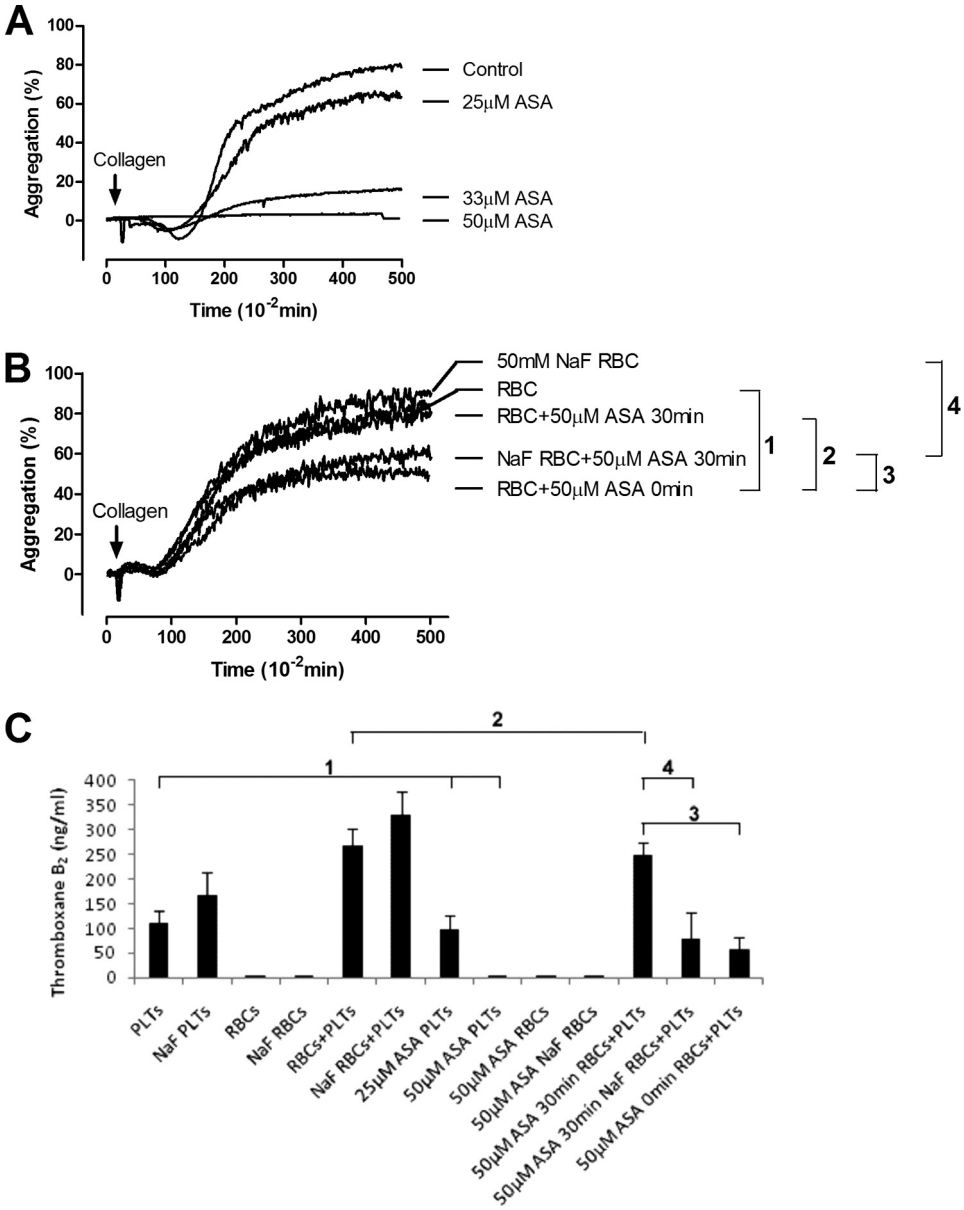

FIGURE 5. **Erythrocytes interfere with aspirin inhibition of platelet thromboxane production and aggregation.** *A,* concentration-response relationship of aspirin with collagen-induced platelet aggregation. Washed human platelets were incubated with the stated concentration of aspirin for 5 min before aggregation was induced with collagen at 5 μg/ml. Aggregation was assessed by optical clearing during stirring ($n = 3$). *B,* aspirin inhibition of platelet aggregation is relieved by prior incubation of aspirin with erythrocytes. Aspirin was incubated with washed erythrocytes (8×10^8 in 1 μl) and incubated for either 0 or 30 min before addition to washed human platelets (10^8) in a total volume of 450 μl to achieve what would have been an original concentration of 50 μM. Collagen was added 5 min later to the stirred cuvette and aggregation was followed in an optical mode ($n = 3$). *C,* erythrocytes reduce aspirin inhibition of platelet thromboxane B_2 production. Platelets were stimulated by 5 μg/ml of collagen after the stated treatments. Supernatants were collected and thromboxane B_2, the stable metabolite of thromboxane A_2, was measured by enzyme-linked immunoassay. Numbered comparisons are defined in the text and the data are present as mean ± S.D., $n = 3$.

Type I PAF acetylhydrolase was initially purified from bovine brain (45) and determined to be a unique trimeric esterase (32, 46) unrelated to the other two members (plasma and type II) of the group VII phospholipase A_2 family (39). The type I PAF acetylhydrolase is a trimer of catalytic α_2 and/or α_1 subunits of 30 or 29 kDa, respectively, and a 45-kDa noncatalytic subunit (45). Subsequently, the PAF acetylhydrolase was purified from erythrocytes (35) that was then discovered to be a rich source of α_1/α_2 type I PAF acetylhydrolase (36). This identification as a trimer is consistent with the lower molecular weight of the activity purified from erythrocytes on denaturing gels and the apparent size discrepancy during size separation as a native protein.

The trimeric type I PAF acetylhydrolase differs from the single chains of other mammalian (47) and *Schizosaccharomyces pombe* (48) type VII family members, and the type I enzyme is strictly limited to hydrolysis of phospholipid acetyl esters (49). This unusually sharp substrate recognition is novel for a phospholipase, but limits understanding of its physiologic role because erythrocytes neither synthesize nor readily accumulate PAF from their environment (50). Genetic ablation of either the α_1 or α_2 gene product has not clarified their physiologic role (37).

The two α catalytic subunits of this enzyme have distinct requirements for glycerophospholipids even when the lipids

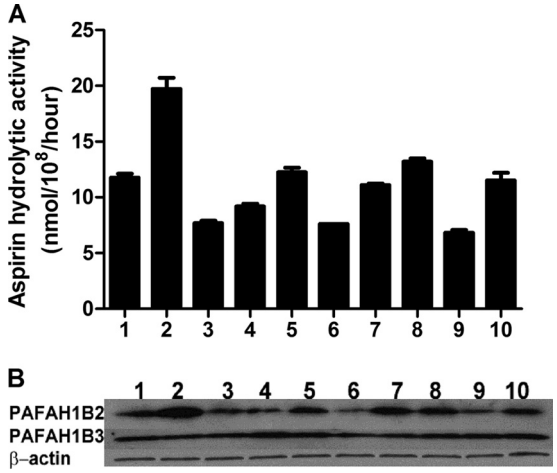

FIGURE 6. **PAFAH1B2 and aspirin hydrolytic activity vary among donors.** *A,* variation in the inter-individual rate of erythrocyte hydrolysis of aspirin. Aspirin hydrolytic activity was measured as described in the legend to Fig. 1 in erythrocytes (8×10^8) obtained from 10 random blood donors. Data are presented by mean ± S.D., $n = 3$. *B,* PAFAH1B2 varied among blood donors. PAFAH1B2 (*upper panel*), PAFAH1B3 (*lower panel*), or β-actin in samples from the donors in *panel A* were determined by immunoblotting as described under "Experimental Procedures."

have S_n-2 acetyl residues in common (38), so acceptance of aspirin must involve more than simple recognition of the acetyl group. Indeed, purification showed that aspirin hydrolysis was not, as might be expected, an activity of the membrane-bound erythrocyte acetylcholinesterase. The importance of the salicylic acid component of the ester in substrate selection is also emphasized by the inability of the plasma PAF acetylhydrolase to hydrolyze aspirin even at high concentrations. Thus, erythrocyte type I PAF acetylhydrolase contributes little to the hydrolysis of PAF in blood (51) that is the domain of plasma PAF acetylhydrolase (40, 52). Conversely, aspirin hydrolysis is exclusively the domain of the erythrocyte type I enzyme, with no contribution by plasma PAF acetylhydrolase.

Erythrocytes, as intact cells, significantly reduced the effectiveness of aspirin when 1 μl of erythrocytes was incubated with aspirin prior to assessing its effect on 450 μl of platelets at their normal abundance. The primary target of the acetyl group of acetylsalicylic acid is the type I cyclooxygenase (prostaglandin H-synthase) (53) of platelets where acetylation irreversibly inhibits production of the prostaglandin H_2 precursor required for platelet thromboxane synthesis. This then sharply reduces platelet function by reducing autostimulation through the thromboxane receptor (54). Accordingly, erythrocytes reduced the ability of aspirin to inhibit platelet thromboxane synthesis, and it inhibited homotypic platelet aggregation in response to the incomplete agonist collagen. This inactivation of thromboxane production and platelet aggregation by aspirin was prevented when erythrocytes were incubated with NaF, indicating the relevant inhibitory activity of erythrocytes on the ability of aspirin to reduce platelet function was likely the type I PAF acetylhydrolase.

Aspirin has been an abundant and effective drug over the past century, with consumption estimated to be over 100 million tons per annum (1). Despite this, the enzyme(s) responsible for its rapid turnover in the circulation has not been identified. Ingested aspirin is present in the circulation for only about 20 min (55, 56), although aspirin effectiveness varies greatly among individuals and is increased in individuals with inflammatory type phenotypes (12- 16) with apparent variation in the effectiveness of aspirin prophylaxis, *i.e.* aspirin resistance, among populations and individuals (17, 57). We found a severalfold variation in the ability of erythrocyte lysates to inactivate aspirin. Although variation in the type I PAF acetylhydrolase has not been reported, only the α_2 PAFAH1B2 varied among donors, and this variation reflected varied aspirin hydrolytic rates.

Aspirin is a xenobiotic agent, and its hydrolysis was not subject to natural selection. We find hydrolysis occurs in two compartments, in plasma and within erythrocytes. Catalysis by both the erythrocyte and plasma (not shown) activities vary severalfold, and the relative effectiveness of the two compartments may not be uniform among individuals. However, a major mechanism of aspirin turnover in blood is intracellular hydrolysis by erythrocyte type I PAF acetylhydrolase.

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