Aspirin Hydrolysis in Plasma Is a Variable Function of Butyrylcholinesterase and Platelet-activating Factor Acetylhydrolase 1b2 (PAFAH1b2)*

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Background: Aspirin use is extensive, but its short half-life limits bioavailability.

Results: Butyrylcholinesterase (BChE) and PAFAH1b2 hydrolyze aspirin in plasma. Aspirin hydrolysis in plasma varies by up to 12-fold from non-genetic modulation of BChE activity.

Conclusion: Two enzymes hydrolyze aspirin in plasma, and their contribution varies among individuals.

Significance: Aspirin hydrolysis in plasma is variable, affecting platelet inhibition by aspirin.

Aspirin is rapidly hydrolyzed within erythrocytes by a heterodimer of PAFAH1b2/PAFAH1b3 but also in plasma by an unidentified activity. Hydrolysis in both compartments was variable, with a 12-fold variation in plasma among 2226 Cleveland Clinic GeneBank patients. Platelet inhibition by aspirin was suppressed in plasma that rapidly hydrolyzed aspirin. Plasma aspirin hydrolysis was significantly higher in patients with coronary artery disease compared with control subjects (16.5 ± 4.4 versus 15.1 ± 3.7 nmol/ml/min; p = 3.4 × 10⁻⁵). A genome-wide association study of 2054 GeneBank subjects identified a single locus immediately adjacent to the BCHE (butyrylcholinesterase) gene associated with plasma aspirin hydrolytic activity (lead SNP, rs6445035; p = 9.1 × 10⁻¹⁷). However, its penetrance was low, and plasma from an individual with an inactivating mutation in BCHE still effectively hydrolyzed aspirin. A second aspirin hydrolyase was identified in plasma, the purification of which showed it to be homomeric PAFAH1b2. This is distinct from the erythrocyte PAFAH1b2/PAFAH1b3 heterodimer. Inhibitors showed that both butyrylcholinesterase (BChE) and PAFAH1b2 contribute to aspirin hydrolysis in plasma, with variation primarily reflecting non-genetic variation of BChE activity. Therefore, aspirin is hydrolyzed in plasma by two enzymes, BChE and a new extracellular form of platelet-activating factor acetylhydrolase, PAFAH1b2. Hydrolytic effectiveness varies widely primarily from non-genetic variation of BChE activity that affects aspirin bioavailability in blood and the ability of aspirin to inhibit platelet aggregation.

Aspirin (acetylsalicylic acid) use is estimated at 100 billion tablets annually (1) as an analgesic, to reduce mortality and re-infarction in individuals with unstable angina, and in secondary prevention in a range of cardiovascular diseases (2, 3). The primary effect of aspirin is irreversible inhibition of cyclooxygenase (prostaglandin H synthase)-1 by acetylation of Ser-530 (4, 5). The cyclooxygenase product prostaglandin H₂ is rapidly converted in platelets to unstable thromboxane A₂, which aids in platelet responsiveness to ADP and collagen through the platelet thromboxane A₂ receptor (6).

Aspirin inhibition of platelet thromboxane synthesis is irreversible (2, 7, 8), and although platelets cannot synthesize new cyclooxygenase-1, the half-life of circulating aspirin is just ~20 min (9, 10), so newly released platelets are not inhibited by the transient bolus of aspirin. Certain individuals or groups of individuals, e.g. diabetics or stroke survivors (11–13), may not receive the full benefit of aspirin, although defining, measuring, and assessing such resistance to the therapeutic effects of aspirin are complex and incomplete (14–17). A single low dose of coated enteric aspirin fails to inhibit platelet function in half of those studied, reflecting varied bioavailability that is not genetically encoded (18).

Aspirin is hydrolyzed in blood within erythrocytes (19) by a heterodimer of PAFAH1b2 and PAFHA1b3 (20) and also in plasma. The rate of aspirin hydrolysis by erythrocytes varies severalfold (20), with a larger variation in the rate of plasma hydrolysis (see below), so the relative contribution of the two compartments varies but is approximately similar. The identity of the enzyme in plasma that hydrolyzes aspirin remains unknown. Aspirin hydrolysis in plasma is not normally distributed (21) and is increased in patients with type 2 diabetes (22, 23), atherosclerosis (24), and aspirin-sensitive asthma or cold urticaria (25) or after surgery (26).

Aspirin hydrolysis is not an evolutionarily selected trait and so reflects the action of an existing esterase able to accept it as a substrate. Esterases able to accept aspirin as a substrate include
butyrylcholinesterase (BChE; also known as pseudocho- linesterase (21)) (27) and PON1 (paraoxonase-1), which is additionally proposed to also hydrolyze aspirin nitrate, a novel anti-inflammatory agent (28). The actual contribution of these candidate enzymes to aspirin hydrolysis in plasma is undefined.

We identified enzymes in plasma that hydrolyze aspirin and found that BChE and a new extracellular form of PAFAH1b together account for aspirin hydrolysis in plasma. The rate of aspirin hydrolysis varied widely among donors, primarily from epigenetic BChE variation, and was sufficient to alter platelet sensitivity to aspirin inhibition.

EXPERIMENTAL PROCEDURES

Materials—Aspirin, acetaminophen, Cibacron blue 3GA- agarose (type 3000-CL), potassium bromide, phenyl acetate, purified human plasma BChE, 5,5'-dithiobis(2-nitrobenzoic acid), and butyrylthiocholine iodide were from Sigma. Salicylic acid and HPLC-grade solvents (acetonitrile, formic acid, and water) were from Mallinkrodt Baker. ECL kits were from Amersham Biosciences. Polyclonal antibodies against BChE and PON1 were from Santa Cruz Biotechnology (Santa Cruz, CA), antibody against apoA-I was from R&D Systems, antibody against PAFAH1b2 was from Sigma, and antibody against PAFAH1b3 was from Proteintech Group (Chicago, IL).

Aspirin Hydrolysis—Salicylic acid from aspirin hydrolysis was isolated by reversed-phase HPLC and quantified by absorption with recovery corrected by an acetaminophen internal standard. Plasma (10 µl) was added to aspirin (4 mM) in 40 µl of PBS (37 °C, pH 7.2) for 2 h before stopping the reaction with 150 µl of acetonitrile containing 0.1% formic acid and 20 µg/ml acetaminophen, followed by centrifugation to remove precipitated proteins. The assay was linear over the plasma volumes used, and plasma aspirin hydrolysis was stable to freezing and storage at −80 °C for 24 months (p = 0.039). Aspirin and salicylic acid were separated by reversed-phase chromatography over Phenomenex ODS columns (150 × 2 mm, 5 µm) with 40:60 (v/v) acetonitrile/water (0.1% formic acid) at 0.4 ml/min and quantified at A280. Data were analyzed by converting the area under the curve relative to salicylic acid standards minus non-enzymatic hydrolysis.

Plasma Aspirin Hydrolyses—Soluble material and lipoproteins were separated by gradient density centrifugation by adjusting the density of the plasma to 1.3 g/ml with potassium bromide and then layering over saline (9 ml onto 27 ml), followed by ultracentrifugation at 50,000 × g for 3 h. Recovered fractions were pooled and dialyzed against 20 mM Tris-Cl and then passed through conditioned Cibacron blue gel to remove albumin.

Expression and Purification of Recombinant PON1—Histagged rabbit recombinant PON1 (clone G3C9; a gift of Dan S. Tawfik, Weizmann Institute of Science, Rehovot, Israel), highly similar to its human counterpart (29), was expressed in Escherichia coli (30). Lysate protein was precipitated by 55% (w/v) ammonium sulfate; recovered by centrifugation; dissolved in 50 mM Tris-Cl, 1 mM CaCl2, 0.1 mM dithiothreitol, 1 µM pepstatin A, and 0.1% Tergitol (pH 8); and dialyzed overnight at 4 °C against this buffer and then against 50 mM Tris-Cl, 50 mM NaCl, 1 mM CaCl2, and 0.1% Tergitol (pH 8) for 4 h. The dialysate was passed over a nickel-nitrilotriacetic acid column, and bound protein was recovered with the first dialysis buffer and stored at 4 °C. Recombinant PON1 had a specific activity of (5.0 ± 0.1) × 105 units/mg of protein against phenyl acetate with protein determined with bicinchoninic acid.

Enzymatic Determinations—BChE activity was measured by hydrolysis of 2 mm butyrylthiocholine iodide in phosphate buffer (pH 7.2) over 2 min at room temperature using 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (31) to detect thiocholine at A412 (13,260 M−1 cm−1). The arylesterase activity of PON1 was determined spectrophotometrically using phenyl acetate as the substrate (32). The assay contained 1 mM phenyl acetate, 20 mM Tris-Cl (pH 8.0), and 1 mM CaCl2. Blanks without enzyme were used to correct for spontaneous hydrolysis. Activity was calculated from a molar extinction coefficient at 270 nm (using differences in the absorbance of phenol versus phenyl acetate) of 1310 M−1 cm−1. One unit of arylesterase activity is defined as 1 µmol of phenyl acetate hydrolyzed per min.

Potential inhibition of BChE (0.6 µg) or platelet-activating factor acetylhydrolase (PAFAH; 0.5 µg) hydrolysis of 4 mM aspirin in plasma (10 µl) or by human serum albumin (50 mg) was assayed in a final volume of 50 µl of PBS for 2 h. Aggregation of washed human platelets (4 × 108/ml) was initiated by stimulation with collagen (5 µg/ml) after aggregometry. Platelets were incubated or not with the indicated amount of aspirin for 10 min prior to aggregometry.

Western Blotting—ApoA-I, BChE, type I PAFAH (PAFAH1b2 and PAFAH1b3), and PON1 were visualized by immunoblotting after electrophoretic separation by SDS-PAGE. The resolved proteins were transferred to PVDF membranes (Millipore, Bedford, MA), probed with primary and secondary antibodies at the manufacturer’s suggested dilution, and visualized using extended chemiluminescence reagent and x-ray film.

Human Samples—The Cleveland Clinic GeneBank study is a single site sample repository generated from consecutive patients undergoing elective diagnostic coronary angiography or elective cardiac computed tomographic angiography with extensive clinical and laboratory characterization and longitudinal observation. Subject recruitment occurred between 2001 and 2006. Ethnicity was self-reported, and information regarding demographics, medical history, and medication use was obtained in patient interviews and confirmed by chart reviews. All clinical outcome data were verified by source documentation. Coronary artery disease (CAD) was defined as adjudicated diagnoses of stable or unstable angina, myocardial infarction (adjudicated definition based on defined electrocardiographic changes or elevated cardiac enzymes), angiographic evidence of ≥50% stenosis of one or more major epicardial vessels, and/or a history of known CAD (documented myocardial infarction, CAD, or history of revascularization). Control subjects were classified as having <30% stenosis in any vessel and no history of known CAD. The GeneBank cohort has been used previously for discovery and replication of novel genes and risk factors for atherosclerotic disease (33–36). For this study, aspirin hydro-
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Activity was determined in plasma from 2226 subjects. Activity was obtained from a single measurement, which was validated by reanalysis of 285 randomly selected samples. All patients provided written informed consent prior to being enrolled in GeneBank, and the study was approved by the Institutional Review Board of the Cleveland Clinic. BChE null plasma was a gift from Dr. Oksana Lockridge (Nebraska Medical Center).

Genome-wide Association Study (GWAS)—Genome-wide genotyping of SNPs was performed on the Affymetrix genome-wide human array 6.0 chip. Using these data and those from 120 phased chromosomes from the HapMap CEU samples (HapMap r22 release, NCBI build 36), genotypes were imputed for untyped autosomal SNPs across the genome using MACH 1.0 software. All imputations were done on the forward (+)-strand using 562,554 genotyped SNPs that had passed quality control filters. Quality control filters for the imputed data set excluded SNPs with Hardy-Weinberg equilibrium \( p \) values <0.0001, call rates <97%, or minor allele frequencies <1% and individuals with <90% call rates. This resulted in 2,421,770 autosomal SNPs in 2054 subjects in whom plasma aspirin hydrolytic activity was available for analysis.

Statistics—Each experiment was repeated at least three times, and each assay was performed in triplicate. Genome-wide linear regression analyses were used to identify loci associated with plasma aspirin hydrolase activity after adjustment for age and gender under an additive model. Genetic analyses were carried out with PLINK v1.07, and a genome-wide \( p \) value of \( <5 \times 10^{-8} \) was considered significant evidence for association.

RESULTS

Aspirin Is Hydrolyzed in Plasma, Hydrolysis Is Highly Variable, and Extracellular Hydrolysis Affects Platelet Inhibition—Aspirin was hydrolyzed in cell-free plasma but with considerable variation. The rate of hydrolysis ranged by 7-fold from 3.67 to 24.70 nmol/ml/min among 11 healthy donors (Fig. 1A).

Aspirin irreversibly inactivates platelet cyclooxygenase and thromboxane \( \text{A}_2 \) production, thereby inhibiting aggregation induced by weak agonists. Aspirin inhibited platelet aggregation induced by collagen in a concentration-dependent fashion (Fig. 1B). Platelet-poor plasma reduced the effectiveness of aspirin in suppressing this aggregation, but again, the effectiveness of plasma in blocking aspirin inhibition varied among donors. Plasma from donor 6, able to hydrolyze aspirin only at 3.75 nmol/ml/min, was less effective in suppressing platelet aggregation by 33 \( \mu \text{M} \) aspirin compared with plasma from donor 3, which hydrolyzed aspirin at a rate of 8.75 nmol/ml/min (Fig. 1C).

We established a high-throughput assay to examine population variation in plasma aspirin hydrolytic activity. These assays were carried out in plasma from 2226 individuals enrolled in the GeneBank study, with 1928 individuals having CAD and 298 subjects being defined as controls. Among these subjects, the minimum and maximum of aspirin hydrolytic activity were 3.06 and 37.38 nmol/ml/min, respectively, with a mean of 16.33 nmol/ml/min and a standard deviation of 4.36 nmol/ml/min (Fig. 1D). Values from duplicate measurements in 285 randomly selected samples yielded high correlations (data not shown). Thus, variation in aspirin hydrolytic activity among the 2226 GeneBank subjects was ~12-fold and similar to the range observed in healthy donors. Furthermore, aspirin hydrolytic activity was significantly higher \( (p = 3.4 \times 10^{-8}) \) in CAD cases compared with control subjects (Fig. 1E).

GWAS for Plasma Aspirin Hydrolylase Activity—We sought to identify the genetic determinants of variation in plasma aspirin hydrolytic activity using an unbiased approach. We performed a GWAS analysis for aspirin hydrolytic activity using ~2.4 million genotyped and imputed SNPs in 2054 GeneBank subjects (all of Caucasian ancestry) for whom both genotype and phenotype data were available. The Q-Q plot from these analyses is shown in Fig. 2A, and the observed genomic control factor was 1.0, suggesting that the GWAS results were not confounded by underlying population stratification.

As shown by the Manhattan plot in Fig. 2B, plasma aspirin hydrolytic activity was controlled primarily by a single locus on chromosome 3q26.1 containing \( \text{BCHE} \), where the lead SNP, rs6445035 \((\text{G} \rightarrow \text{A})\), yielded a highly significant \( p \) value of 9.1 \( \times 10^{-17} \). \( \text{BCHE} \) encodes the enzyme BChE, homozygous mutations of which confer susceptibility to prolonged apnea after administration of the muscle relaxant suxamethonium (suxcyntholine) (37). A regional plot of 1 megabase centered around \( \text{BCHE} \) also revealed that other SNPs yielding significant \( p \) values in this region are in moderate-to-high linkage disequilibrium with rs6445035 (Fig. 2C).

As shown in Fig. 2D, each copy of the A allele lowered aspirin hydrolytic activity by \( \sim 1.2 \) nmol/ml/min. The minor allele (A) of rs6445035 had a frequency of 0.19, explained 3.3% of the variation in aspirin hydrolytic activity, and was located 10.6 kb centromeric to the 3' terminus of \( \text{BCHE} \) (Fig. 2C).

To determine whether the other SNPs in this region represent independent association signals, we also ran analyses taking into account the effect of the lead SNP (rs6445035). However, these conditional analyses did not reveal other SNPs associated with aspirin hydrolyase activity at the genome-wide threshold for significance (data not shown).

Aspirin Hydrolytic Activity, in Addition to \( \text{BCHE} \), Is Present in Plasma—Our genetic studies implicated BChE as a plasma aspirin esterase, which is supported by aspirin hydrolysis by purified BChE (27). However, Western blotting showed that partially purified material from the soluble faction of plasma contained little of the enzyme, but at least 200 ng of purified BChE was required to achieve aspirin hydrolysis equivalent to that in the purified fraction (Fig. 2E).

These results suggest that human plasma may contain additional aspirin hydrolytic activity. To investigate this, we obtained plasma from an individual homozygous for a frameshift mutation in \( \text{BCHE} \), which leads to a premature stop codon (38). Although plasma from this individual was unable to hydrolyze the BChE substrate butyrylthiocholine (Fig. 2F), this complete loss of BChE activity did not result in a corresponding loss of aspirin hydrolysis.

Type I PAFAH Is a Candidate Plasma Aspirin Esterase—Aspirin hydrolytic activity is reported (28) to physically associate with HDL, potentially reflecting PON1 that additionally hydrolyzes clopidogrel (39). In contrast, density gradient separation
of plasma showed that aspirin esterase activity was a soluble activity and that apoA-I-containing lipoprotein particles were essentially devoid of activity (Fig. 3A). This conclusion was supported by the inability of functional recombinant PON1 to hydrolyze aspirin (Fig. 3B).

Purification of soluble fraction 1 and mass spectrometry of candidate proteins as described (20) revealed the presence of PAFAH1b2 in plasma (data not shown.) Type I PAFAH hydrolyzes acetylated phospholipids but additionally accepts aspirin as a substrate (20). This intracellular enzyme is a trimer con-
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containing a common LIS1 non-catalytic subunit and either or both PAFAH1b2 and PAFAH1b3 catalytically active esterases (40). This is an intracellular enzyme of brain and erythrocytes, but Western blotting showed that PAFAH1b was uniformly present in normal plasma (Fig. 3C). PAFAH1b2 was also present in plasma from the patient with the frameshift mutation in BCHE. Notably, the plasma enzyme was homodimeric PAFAH1b2, whereas the erythrocyte enzyme was the PAFAH1b2/PAFAH1b3 heterodimer. These are therefore distinct enzymes, and the plasma enzyme does not arise from erythrocyte lysis. We examined the genomic regions containing the genes encoding PAFAH1b2 and PAFAH1b3, but SNPs at these loci were not associated with plasma aspirin hydrolytic activity after correcting for multiple testing (data not shown).

Both BCHE and Type I PAFAH Contribute to Plasma Aspirin Hydrolysis—Recombinant PAFAH1b2 hydrolyzed aspirin, and the irreversible P2X7 inhibitor oxidized ATP (oATP) inhibited this activity (Fig. 4A). Purified BCHE was largely unaffected by oATP, but BCHE hydrolysis of aspirin was abolished by the inhibitor procainamide. Conversely, PAFAH was only modestly affected by procainamide. We found that oATP fully abolished aspirin hydrolysis in plasma from the BCHE null donor, implicating PAFAH1b2 as the non-BCHE aspirin hydrolytic activity.

We compared the effectiveness of these two inhibitors in plasma from seven normal donors and observed that both inhibitors suppressed aspirin hydrolysis (Fig. 4B). The procainamide-resistant activity (PAFAH1b2) was relatively uniformly among donors, whereas the oATP-resistant activity (BCHE) varied among donors in concert with total activity.

We determined whether aspirin hydrolysis of recombinant PAFAH1b2 or BCHE was differentially affected in plasma from a donor with higher aspirin hydrolytic activity compared with plasma from a donor with slower hydrolysis of aspirin (Fig. 4C). We found that BCHE readily responded to plasma, so its velocity was greater in plasma with higher endogenous activity compared with hydrolysis in plasma with slower endogenous activity. In contrast, aspirin hydrolysis by PAFAH1b2 was equivalent in the two plasmas.

**DISCUSSION**

Aspirin has been in extensive use for well over 100 years, and its unique properties ensure its continued use (41). Not all individuals or members of groups appear to receive the benefits of aspirin administration (14–17), but whether this phenomenon of “aspirin resistance” is a valid description or even identifiable given difficulties in clinical measurement of platelet function (17) is debated (14, 15). Platelet resistance to aspirin inhibition is, however, present in ex vivo assays (42) in certain in vivo studies (15) and correlates with measures of type 2 diabetes (22, 23).

Aspirin was hydrolyzed in cell-free plasma, and this activity varied widely among normal donors. This is relevant because the effectiveness of aspirin at a dose approximating the maximal plasma concentration after ingestion of a standard 325-mg dose (43) was affected with this intrinsic plasma hydrolytic activity. We found a slight but significant higher plasma aspirin hydrolytic activity in patients with CAD compared with control patients.
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We used GWAS to determine whether variation in aspirin consumption included a genetic component and to elucidate candidate loci affecting the undefined hydrolytic activity. The rate of aspirin hydrolysis in plasma of 2226 GeneBank patients varied by 12-fold and was associated strongly with a single SNP on chromosome 3q26.1. rs6445035 is immediately 3′ of BCHE, so this region either may contain enhancer elements that control expression of BCHE or is in linkage disequilibrium with a coding variant(s) that affects enzymatic activity. However, the genetic effects of rs6445035 were relatively weak and explained only 3.3% of the variation in plasma aspirin hydrolytic activity.

BChE circulates as a tetramer with complex kinetics (44) subject to environmental effects (45), and this epigenetic variation is the primary modifier of plasma BChE activity.

Plasma contained a second aspirin hydrolytic activity, which was not HDL-associated or PON1 (28). Instead, PAFAH1b2, the α2 subunit of PAFAH I, was present as an extracellular enzyme. Type I PAFAH is an α1/α2 heterodimer composed of independent genes (PAFAH1b2 and PAFAH1b3) that are independently regulated and have independent roles (46–48). We previously identified the α1/α2 heterodimer as the aspirin hydrolytic enzyme of erythrocytes (20), so the novel plasma α2 enzyme shows that this extracellular enzyme is not derived from lysed erythrocytes.

Procainamide inhibition of BChE and oATP inhibition of type I PAFAH showed that both enzymes contribute to plasma aspirin esterase activity. The total amount of the aspirin hydrolytic activity in plasma and its composition are highly variable, with the majority of the variation associated with non-genetic modulation of BChE activity.

Aspirin inactivation in the vascular compartment modifies the effectiveness of aspirin in inhibiting platelet aggregation, which reflects the contributions of plasma PAFAH1b2, plasma BChE, and erythrocyte PAFAH1b2/PAFAH1b3 heterodimers. All of these enzymes vary among individuals, yielding a complex pattern of aspirin consumption that affects the effectiveness of physiologically relevant doses of aspirin in inhibiting platelet function.

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