Aspirin Hydrolysis in Plasma is a Variable Function of Butyrylcholinesterase and PAF Acetylhydrolase 1b2

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*Running title: Plasma aspirin hydrolysis variation

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

Results: Butyrylcholinesterase and PFAH1b2 hydrolyze aspirin in plasma. Aspirin hydrolysis in plasma varies up to 12-fold from non-genetic modulation of butyrylcholinesterase 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.

SUMMARY
Aspirin is rapidly hydrolyzed within erythrocytes by a heterodimer of PFAH1b2/PFAH1b3, but also in plasma by unidentified activity. Hydrolysis in both compartments is variable, with a 12-fold variation in plasma among 2,226 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 (CAD) compared to control subjects (16.5 ± 4.4 vs. 15.1 ± 3.7 nmol/ml/min; p=3.4×10⁻⁸). A genome-wide association study in 2,054 GeneBank subjects identified a single locus immediately adjacent to the butyrylcholinesterase gene (BCHE) 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 hydrolase was identified in plasma, which purification showed to be homomeric PAF acetylhydrolase 1b2. This is distinct from the erythrocyte PFAH1b2/PFAH1b3 heterodimer. Inhibitors showed both BChE and PFAH1b2 contribute to aspirin hydrolysis in plasma, with variation primarily reflecting non-genetic variation of butyrylcholinesterase activity. Therefore aspirin is...
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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 serine 530 (4,5). The cyclooxygenase product prostaglandin H₂ is rapidly converted in platelets to unstable thromboxane A₂ that aids 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, while platelets cannot synthesize new cyclooxygenase-1, the half-life of circulating aspirin is just ~20 minutes (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 is 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 both within erythrocytes (19) by a heterodimer of PAFAH1b2 and PAFHA1b3 (20), but also is hydrolyzed in plasma. The rate of aspirin hydrolysis by erythrocytes varies several fold (20), with a larger variation in the rate of plasma hydrolysis (vide infra), 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), 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)(27) (pseudocholinesterase (21)), and paraoxonase-1 (PON1) that 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 account for aspirin hydrolysis to find BChE and a new extracellular form of PAF acetylhydrolase 1b 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 butyrylcholinesterase, 5,5’-dithiobis(2-nitrobenzoic acid), and butyrylthiocholine iodide were from...
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Sigma (St. Louis, MO). Salicylic acid and high performance liquid chromatography grade solvents (acetonitrile, formic acid, and water) were from JT Baker Inc. (Phillipsburg, NJ). ECL kits were from Amersham Biosciences (Piscataway, NJ). Polyclonal antibodies for butyrylcholinesterase and paraoxonase-1 were from Santa Cruz Biotechnology, (Santa Cruz, CA), antibody against apo A-I was from R&D systems, antibody against PAFAH1b2 was from Sigma and that against PAFAH1b3 was from Proteintech Group (Chicago, IL).

Aspirin hydrolysis—Salicylic acid from aspirin hydrolysis was isolated by RP-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 PBS (pH 7.2) buffer for 2 h before stopping the reaction with 150 µl acetonitrile containing 0.1% formic acid and acetaminophen (20 µg/ml) 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 ODS (Phenomenex, 5 micron, 150 × 2 mm) with acetonitrile: water (40:60, 0.1% formic acid, v/v) at a 0.4 ml/min and quantified at OD280. Data were analyzed by converting the area under the curve relative to salicylic acid standards minus non-enzymatic hydrolysis.

Plasma aspirin hydrolases—Soluble material and lipoproteins were separated by gradient density centrifugation by adjusting the density of plasma to 1.3 g/ml with potassium bromide and then layering over saline (9 ml onto 27 ml) followed by ultracentrifugation (50,000 × g, 3h). Recovered fractions were pooled and dialyzed against 20 mM Tris-Cl, pH 7.4 and then passed through conditioned Cibacron Blue gel to remove albumin.

Expression and Purification of Recombinant PON1—His-tagged rabbit recombinant PON1 (rPON1-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 (50 mM Tris-Cl, 1 mM CaCl2, 0.1 mM dithiothreitol, 1 µM pepstatin A, 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, 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 × 10^5 units/mg protein against phenyl acetate with protein determined with bicinchoninic acid.

Enzymatic determinations—Butyrylcholinesterase 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 DTNB (31) to detect thiocholine at OD412 (13,260 M⁻¹cm⁻¹). The aryl esterase 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 the molar extinction coefficient at 270 nm (using differences in the absorbance of phenol versus phenyl acetate) of 1,310 M⁻¹cm⁻¹. One unit of aryl esterase activity was defined as a micromole of phenyl acetate hydrolyzed per minute.
Potential inhibition of BChE (0.6 µg) or PAFAH (0.5 µg) hydrolysis of 4 mM aspirin by plasma (10 µl) or human serum albumin (50 mg) was assayed in a final volume of 50 µl PBS for 2h.

Aggregation of washed human platelets (4×10^8/ml) was initiated by stimulation by collagen (5 µg/ml) with stirring in a Chrono-log aggregometer. Platelets were incubated, or not, with the stated amount of aspirin for 10 min prior to aggregometry.

**Western blot**—Apo A-I, BChE, type I PAF acetylhydrolase (PAFAH1b2, PAFAH1b3), PON1 were visualized by immunoblotting after electrophoretic separation by SDS-PAGE. The resolved proteins were transferred to PVDF membranes (Millipore Corp., Bedford, MA), probed with primary and secondary antibodies at the manufacturer’s suggested dilution, and visualized with 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 by 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 (MI) (adjudicated definition based on defined electrocardiographic changes or elevated cardiac enzymes), angiographic evidence of ≥ 50% stenosis of one or more major epicardial vessel, and/or a history of known CAD (documented MI, 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 the present study, aspirin hydrolase activity was determined in plasma from 2,226 subjects. Activity was obtained from a single measurement, which was validated by re-analysis 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**—Genome-wide genotyping of single nucleotide polymorphisms (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 (QC) filters. QC filters for the imputed dataset excluded SNPs with HWE p-values < 0.0001, call rate less than 97% or minor allele frequencies < 1%, and individuals with less than 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 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 less than $5 \times 10^{-8}$ 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 sevenfold from 3.67 to 24.70 nmol/ml/min among 11 healthy donors (Fig. 1A).

Aspirin irreversibly inactivates platelet cyclooxygenase and thromboxane A2 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, only able to hydrolyze aspirin at 3.75 nmol/ml/min, was less effective in suppressing aggregation by 33 µM aspirin compared to 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 2,226 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 hydrolysis activity among the 2,226 GeneBank subjects was approximately 12-fold and similar to the range observed in healthy donors. Furthermore, aspirin hydrolysis activity was significantly ($p=3.4 \times 10^{-8}$) higher in CAD cases compared to control subjects (Fig. 1E).

Genome-Wide Association Study (GWAS) for plasma aspirin hydrolysis 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 hydrolysis activity using ~2.4 million genotyped and imputed SNPs in 2,054 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 primarily controlled by a single locus on chromosome 3q26.1 containing BCHE, where the lead SNP, rs6445035 (G>A), yielded a highly significant p-value of $9.1 \times 10^{-17}$. BCHE encodes the enzyme butyrylcholinesterase, homozygous mutations of which confer susceptibility to prolonged apnea after administration of the muscle relaxant suxamethonium (succinylcholine) (37).

A regional plot of 1 Mb centered around BCHE also revealed that other SNPs yielding significant p-values in this region are in moderate to high
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linkage disequilibrium with rs6445035 (Fig. 2C).

As shown in Fig. 2D, each copy of the A allele lowered aspirin hydrolytic activity by ~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 10.6kb centromeric to the 3’ terminus of BCHE (Fig. 2C).

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

Aspirin hydrolytic activity in addition to butyrylcholinesterase is present in plasma—Our genetic studies implicate BChE as a plasma aspirin esterase, which is supported by aspirin hydrolysis by purified BChE (27). However, western blotting shows 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 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 frame shift mutation in 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 PAF acetylhydrolase is a candidate plasma aspirin esterase—Aspirin hydrolytic activity is reported (28) to physically associate with high density lipoprotein, potentially reflecting Paraoxonase-1 (PON1) that additionally hydrolyzes Clopidogrel (39). In contrast, we find density gradient separation of plasma showed aspirin esterase activity was a soluble activity, and that apoAI-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 before (20) revealed the presence of PAFAH1b2 in plasma (not shown.) Type I PAF acetylhydrolase (PAFAH) hydrolyzes acetylated phospholipids, but additionally accepts aspirin as a substrate (20). This intracellular enzyme is a trimer 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 PAFAH1b2 (Fig. 3C) was uniformly present in normal plasma. PAFAH1b2 was also present in plasma from the patient with the frame shift mutation in BCHE. Notably, the plasma enzyme was homodimeric PAFAH1b2, while 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 (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 plasmas from seven normal donors, and observed both inhibitors suppressed aspirin hydrolysis (Fig. 4B). The procainamide-resistant activity (PAFAH1b2) was relatively uniform among donors, while the o-ATP resistant activity (BChE) varied among donors in concert with total activity.

We determined whether aspirin hydrolysis of recombinant PAFAH1b2 or BChE was differentially affected by plasma from a donor with higher aspirin hydrolytic activity than plasma from a donor with slower hydrolysis of aspirin (Fig. 4C). We found BChE rapidly responded to plasma so that its velocity was greater in plasma with higher endogenous activity compared to 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, however, is present in *ex vivo* assays (42) and 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 that approximates the maximal plasma concentration after ingestion of a standard 325 mg dose (43) correlated to this intrinsic plasma hydrolytic activity. We found a slight, but significant, higher plasma aspirin hydrolytic activity in patients with coronary artery disease compared to controls.

We used GWAS to determine if 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 2,226 GenBank patients varied by 12-fold, and associated strongly with a single SNP on chromosome 3q26.1. rs6445035 is immediately 3’ of *BCHE*, so this region may either 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 nor PON1 (28). Instead, PAFAH1b2, the α2 subunit of PAF acetylhydrolase I, was present as an extracellular enzyme. The type I PAF acetylhydrolase is an α1 and α2 heterodimer composed of independent genes (*PAFAH1b2, PAFAH1b3*) that are independently regulated and have independent roles (46-48). We previously identified the α1/α2 heterodimer is the aspirin hydrolytic enzyme of erythrocytes (20), so the
novel plasma α2 enzyme shows this extracellular enzyme is not derived from lysed erythrocytes.

Procainamide inhibition of BChE and oxidized ATP inhibition of type 1 PAF acetylhydrolase showed both enzymes contribute to plasma aspirin esterase activity. The total amount of the aspirin hydrolytic activity in plasma and its composition is highly variable, with the majority of the variation associated with epigenetic 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 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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References


**Legends**

Figure 1. Aspirin hydrolytic activity varies among plasma donors and alters the effectiveness of platelet inhibition by aspirin. A, Box and whisker plot of plasma aspirin hydrolytic activity. Aspirin hydrolysis by plasma of 11 normal donors is depicted with maximum (24.72 nmol/ml/min), minimum (3.66 nmol/ml/min), lowest and highest quartile (5.67 nmol/ml/min and 10.54 nmol/ml/min) and the median (7.66 nmol/ml/min). Data were processed as mean ± SD, n=3. B, Aspirin inhibits platelet aggregation in a concentration dependent manner. Washed human platelets (4×10^8/ml) were incubated with the stated amount of aspirin for 10 min prior to stimulation by collagen (5 µg/ml). C, Inhibition of aggregation differs after aspirin incubation in plasma with high and low aspirin hydrolytic activity. Aspirin (33 µM) was pre-incubated in platelet-poor plasma from a donor with faster (8.75 nmol/ml/min) or slower (3.75 nmol/ml/min) aspirin hydrolytic activity for 30 min before mixing with washed platelets for another 10 min prior to stimulation by 5 µg/ml collagen (n = 3). D, High throughput HPLC analysis of aspirin hydrolysis in 2,226 plasma samples from the GeneBank cohort. Activity was obtained from a single measurement, which was validated by re-analysis of 285 randomly selected samples. E, Plasma aspirin hydrolytic activity is significantly higher in patients with coronary artery disease (CAD) compared to control subjects (16.5 ± 4.4 vs. 15.1 ± 3.7 nmol/ml/min, respectively; *p=3.4×10^-9*) in the GeneBank cohort.
**Figure 2.** The *BCHE* locus is a major, but not sole, determinant of plasma aspirin hydrolase activity. *A*, As shown by the Q-Q plot from the GWAS analyses, the obtained p-values deviate from that expected by chance, suggesting that a subset of these signals reflect true associations. *B*, Manhattan plot showing plasma aspirin hydrolase activity is controlled predominantly by a single locus on chromosome 3q26.1 containing *BCHE*, with the lead SNP (rs6445035) yielding a highly significant p-value of $9.1 \times 10^{-17}$. *C*, Regional plot for the 1 Mb region centered around *BCHE* demonstrates that other SNPs yielding significant p-values are in moderate to high LD with rs6445035. *D*, Aspirin hydrolysis as a function of genotype for rs6445035 demonstrates that each copy of the minor A allele lowers activity by $\sim 1.2$ nmol/ml/min. Mean ± SD levels are indicated above each genotype group and the number of subjects are shown in parentheses. *E*, BChE is not the only contributor to plasma aspirin hydrolysis. Plasma was separated by density gradient centrifugation and fraction 1 containing aspirin hydrolytic activity was partially purified by Cibacron® blue chromatography. BChE protein in this fraction was assessed by aspirin hydrolytic activity quantified as above (top) or western blotting (bottom) in comparison to known quantities of BChE purified from human plasma. *F*, Butyrylcholine and aspirin hydrolytic activity were determined in normal human plasma and plasma from an individual with an inactivating null mutation in *BCHE*. Data are presented as mean ± SD, n=3.

**Figure 3.** Type I PAF acetylhydrolase is a plasma aspirin hydrolase. *A*, PON1 fails to localize with plasma aspirin hydrolytic activity. Plasma, which had been anti-coagulated with heparin to avoid PON1 inhibition by EDTA) and lipoproteins were separated by density gradient centrifugation before aspirin hydrolytic activity, arylesterase activity, and PON1 protein and HDL-associated ApoAI were detected by western blotting. *B*, Recombinant PON1 does not hydrolyze aspirin. Arylesterase activity and aspirin hydrolysis by plasma and recombinant PON1 (2.5 µg) were determined as in “Methods”. Data are presented as mean ± SD, n=3. *C*, PAFAH1b2 is present in BChE null plasma and plasma from healthy individuals. Western blot for PAFAH1b2 or PAFAH1b3 were performed with 5 µl of each fraction from a gradient centrifugation (top) or seven different normal donors’ plasma and BChE null plasma (bottom). RBC lysates were the positive control. n = 3.

**Figure 4.** BChE and type I PAF acetylhydrolase contribute to plasma aspirin hydrolysis. *A*, Residual aspirin hydrolytic activity in BChE null plasma behaves as type I PAF acetylhydrolase. The effects of oATP or procainamide on aspirin hydrolysis were examined with recombinant PAFAH1b2, BChE null plasma, and normal plasma. *B*, BChE and type I PAF acetylhydrolase act in normal plasma to consume aspirin. Aspirin hydrolysis in plasma from seven normal donors in the presence of procainamide or oxidized ATP was quantified as above. Data are presented as mean ± SD, n=3. *C*, Plasma alters the aspirin hydrolytic concentration response relationship of BChE. Aspirin hydrolytic activity in a total volume of 50 µl containing purified BChE (4 µg, middle) or recombinant PAFAH1b2 (0.8 µg, right), the stated aspirin concentration, and plasma (10 µl) from a donor with high (plasma 1) or low (plasma 2) endogenous aspirin hydrolytic activity (left).
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Figure 2
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Figure 3
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A

Aspirin hydrolytic activity

Aryl esterase activity

Fractions

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

PON 1

Apo A-I

B

PON activity

Aspirin hydrolytic activity

Percentage of Control (%)

Hydrolyzed Aspirin (mmol/hour)

10μl plasma 2.5μg PON1

Control 2.5μg PON1 10μl plasma

C

Fractions

PAFAH1b2

PAFAH1b3

PAFAH1b2

RBC

null

BChE

RBC

30kD

30kD

30kD

30kD
Figure 4
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A

Percentage of Control (%)

Control
2 mM oATP
20 mM Procainamide

BCHE
BCHE null plasma
rPAFAH1b2

B

Aspirin hydrolytic activity (mmol/min)

Control
2 mM oATP
20 mM Procainamide

Donors

C

Velocity (pmol/hour)

plasma1
plasma2

BCHE
plasma1
plasma2

rPAFAH1b2
plasma1
plasma2
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