Differential impairment of aspirin-dependent platelet cyclooxygenase acetylation by nonsteroidal antiinflammatory drugs

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The cardiovascular safety of nonsteroidal antiinflammatory drugs (NSAIDs) may be influenced by interactions with antiplatelet doses of aspirin. We sought to quantitate precisely the propensity of commonly consumed NSAIDs—ibuprofen, naproxen, and celecoxib—to cause a drug–drug interaction with aspirin in vivo by measuring the target engagement of aspirin directly by MS. We developed a novel assay of cyclooxygenase-1 (COX-1) acetylation in platelets isolated from volunteers who were administered aspirin and used conventional and microfluidic assays to evaluate platelet function. Although ibuprofen, naproxen, and celecoxib all had the potential to compete with the access of aspirin to the substrate binding channel of COX-1 in vitro, exposure of volunteers to a single therapeutic dose of each NSAID followed by 325 mg aspirin revealed a potent drug–drug interaction between ibuprofen and aspirin and between naproxen and aspirin but not between celecoxib and aspirin. The imprecision of estimates of aspirin consumption and the differential impact on the ability of aspirin to inactivate platelet COX-1 will confound head-to-head comparisons of distinct NSAIDs in ongoing clinical studies designed to measure their cardiovascular risk.

Cyclooxygenase acetylation | MS | nonsteroidal antiinflammatory drugs

Chronic pain, most commonly inflammatory musculoskeletal pain, afflicts hundreds of millions worldwide (1). Nonsteroidal antiinflammatory drugs (NSAIDs) consumed chronically or intermittently remain the mainstay of therapy for inflammation-associated pain. These agents inhibit cyclooxygenase (COX)-1 and COX-2, thereby reducing the production of inflammatory prostanooids, lipid mediators that lower the activation threshold of nociceptors and sensory neurons. The prevalence of chronic pain rises in the elderly, coinciding with an increase in concomitant disease (1), which complicates drug treatment. Pain management in patients with preexisting cardiovascular disease is a particular challenge because of the cardiovascular adverse effects of NSAIDs and the risk of drug–drug interactions that might undermine the antiplatelet effects of aspirin prescribed for cardioprotection (2).

Although NSAIDs relieve pain effectively, they can cause serious renal and cardiovascular complications by inhibiting COX-dependent prostanooids with homeostatic functions (3, 4). All NSAIDs have the potential to elevate blood pressure and may cause heart failure. COX-2-selective NSAIDs, which were developed to reduce gastrointestinal toxicity, additionally raised the rate of myocardial infarction and stroke (5, 6), affecting ~1–2% of patients exposed per year (3, 4). This adverse drug reaction may have caused thousands of deaths in the general population. Traditional NSAIDs (tNSAIDs) have also been associated with cardiovascular events, but although pharmacoepidemiological studies and metaanalyses of randomized, controlled trials suggest that not all tNSAIDs carry the same risk, there is considerable heterogeneity across studies in the comparative estimates of risk. It remains uncertain which NSAID to choose for patients at risk for cardiovascular complications. This uncertainty has been the argument in support of two ongoing large randomized, controlled trials comparing the cardiovascular safety of celecoxib with the most commonly prescribed tNSAIDs (7, 8).

Aspirin is a unique COX inhibitor: it acetylates serine-529 in the substrate binding channel of COX-1 and inactivates the enzyme irreversibly, whereas tNSAIDs are reversible COX inhibitors. Experiments with radio-labeled aspirin in vitro and X-ray crystallography suggested that some but not all NSAIDs may compete with aspirin for binding COX-1 (9, 10). This concept was borne out further in clinical studies that exploited the distinct recovery kinetics of platelet function between reversible COX inhibitors and the irreversible inhibitor aspirin to predict whether a drug–drug interaction might occur (11–18). Again, heterogeneity was observed between studies, NSAIDs, doses, and time of dosing.

We devised a direct MS assay with which to quantitate acetylation of platelet COX-1 by aspirin and used this methodology to address its interaction with NSAIDs. Although ibuprofen, naproxen, and celecoxib all have the potential to compete with the access of aspirin to the substrate binding channel of COX-1 in vitro (9, 10), exposure of volunteers to a single therapeutic dose of each NSAID followed by 325 mg aspirin revealed a potent drug–drug interaction between ibuprofen and aspirin and between naproxen and aspirin but not between celecoxib and aspirin. This uncertainty has been the argument in support of two ongoing large randomized, controlled trials comparing the cardiovascular safety of celecoxib with the most commonly prescribed tNSAIDs (7, 8).

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Significance

Painkillers classified as nonsteroidal antiinflammatory drugs (NSAIDs) are among the most commonly consumed drugs. Although they ameliorate pain effectively by inhibiting the enzyme cyclooxygenase, they can cause serious cardiovascular complications, including heart attack and stroke. Additionally, NSAIDs have the potential to render low-dose aspirin taken to reduce the risk of heart attack and stroke ineffective through a drug–drug interaction, and there is great uncertainty in how to manage pain in patients with cardiovascular disease. We developed a MS assay to quantitate precisely the interaction of aspirin with NSAIDs. Exposure of volunteers to aspirin revealed a potent drug–drug interaction with ibuprofen and naproxen but not celecoxib. This observation has relevance to the interpretability of ongoing randomized clinical trials comparing the safety of NSAIDs.


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Results

COX-1 Acetylation by Aspirin. The value of measuring COX-1 acetylation as a quantitative and highly specific drug response marker was assessed. We developed an MS assay to measure precisely the relative abundance of the acetylated vs. the unacetylated form of a proteolytically excised COX-1 peptide: S24-IGAPESaceLKL-531 vs. 524-IGAPFSKLLK-531 (Fig. S1 and S2 and Table S1). Acetylation of purified COX-1 and platelet COX-1 by aspirin was concentration- and time-dependent and inversely correlated with enzyme function (Fig. S3). The relationship between COX-1 acetylation, enzymatic activity, and platelet function was assessed in healthy volunteers who were administered a single oral dose of 325 mg uncoated aspirin (Fig. L4 and Table S2). COX-1 acetylation was maximal 4 h after dosing (median = 72% of total COX-1; interquartile range = 69–74%), reaching levels close to the acetylation achieved by the addition of a supra therapeutic concentration of aspirin (500 μmol/L) to washed platelets ex vivo (Fig. 2). The fraction of acetylated COX-1 declined ~7% per day, which was reflective of the daily release rate of new platelets. The time course of platelet COX-1 acetylation was mirrored by that of serum thromboxane B2 (TxB2) formation, an index of the enzymatic capacity of platelet COX-1 during the clotting process, and arachidonic acid-induced platelet aggregation, which measures primarily COX-1–dependent platelet function. The urinary excretion of 11-dehydro-TxB2 followed with a slight delay, reflecting the time-integrated nature of this index of Tx metabolism and clearance. An analysis of the receiver-operating characteristic suggested that the acetylation of over 62% of the total platelet COX-1 content resulted in more than 95% depression of serum TxB2 (specificity = 0.95; sensitivity = 0.87; area under the receiver-operating characteristics curve = 0.926), a level that is necessary for effective inhibition of platelet function (19).

Fig. 2. The human pharmacology of platelet COX-1 acetylation. Healthy volunteers (n = 8) received a single dose of 325 mg aspirin under supervision and were followed up for 10 d (0–240 h plotted on a log scale). Platelet COX-1 acetylation and established markers of COX-1 pathway inhibition were measured. (A) Platelet COX-1 acetylation in vivo was quantified before and after aspirin administration (orange). The maximally achievable COX-1 acetylation was determined by exposing washed platelets to a supra therapeutic concentration of aspirin (500 μmol/L) ex vivo (gray). (B) The enzymatic activity of platelet COX-1 was assessed by measurement of serum TxB2 ex vivo (green). (C) Urinary excretion of the major Tx metabolite, 11-dehydro-TxB2, was measured as an index of COX function in vivo (blue). (D) COX-1–dependent platelet function was determined by arachidonic acid-induced platelet aggregometry (red). Box and whisker plots show medians (horizontal lines), interquartile ranges (boxes), and ranges (whiskers) of data at each time point.

Quantitation of the Interaction Between NSAIDs and Aspirin in Vitro. Purified COX-1 was exposed to increasing concentrations of ibuprofen, naproxen, or celecoxib before aspirin was added (Fig. S4). Although the NSAIDs themselves did not inhibit COX

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function under the selected experimental conditions, all three compounds—including the COX-2-selective drug celecoxib—dose-dependently prevented COX-1 acetylation. The likelihood of a pharmacodynamic drug–drug interaction at the same target would be expected to depend on their relative concentration. Washed human platelets were exposed to two aspirin concentrations (50 and 250 μmol/L) in vitro (Fig. 3). Again, all NSAIDs, including the COX-2-selective inhibitor celecoxib, reduced the acetylation of platelet COX-1 dose-dependently. The high aspirin dose was less susceptible to interference of the NSAIDs than the lower dose.

Direct Detection of the NSAID–Aspirin Interaction in Humans. We quantitated the potential of celecoxib, naproxen, and ibuprofen to interfere with the target engagement of aspirin in healthy volunteers (Fig. 1B and Table S3). Platelet COX-1 acetylation by aspirin was almost completely prevented—proving that the drug–drug interaction had occurred—in the presence of ibuprofen ($P = 0.016$) and naproxen ($P = 0.016$) but not celecoxib ($P = 0.219$) compared with aspirin alone (Fig. 4A). Similarly, serum TxB$_2$ was insufficiently inhibited by aspirin after consumption of ibuprofen and naproxen, whereas celecoxib did not interfere with platelet TxB$_2$ inhibition (Fig. 4D). Naproxen was associated, on average, with a more pronounced but partial serum TxB$_2$ inhibition than ibuprofen at the time of measurement; serum TxB$_2$ levels were fully depressed in three of seven individuals, despite incomplete COX-1 acetylation, presumably because of the longer half-life of naproxen in these individuals (13). This observation was reflected by arachidonic acid-induced platelet aggregation, which was fully inhibited in the naproxen group 24 h after the administration of aspirin, despite the interaction with aspirin (Fig. 4C). Ibuprofen prevented platelet inhibition by aspirin under flow conditions—a perhaps more physiological assessment of platelet function—whereas celecoxib plus aspirin and naproxen plus aspirin both sustained platelet inhibition (Fig. 4D).

Discussion

There is great uncertainty in how to manage musculoskeletal pain in patients with atherosclerotic disease. Although placebo controlled, randomized trials documented the cardiovascular hazard associated with NSAIDs selective for COX-2, such as celecoxib (3, 4), indirect comparisons between tNSAIDs and placebo across studies conducted during the development of COX-2-selective NSAIDs are the primary source of comparative outcome data for older compounds (5, 6). The most comprehensive metaanalysis indicates that the cardiovascular hazard extends to diclofenac, which behaves much like a COX-2–selective NSAID (20), and with considerably lower confidence, to ibuprofen but to a lesser degree, on average, to naproxen (6). Differences in the degree and duration with which tNSAIDs inhibit COX-1 and COX-2 throughout the dosing interval are thought to contribute to the observed heterogeneity in risk (3, 4). However, the cardiovascular safety estimates may be confounded by drug–drug interactions between antiplatelet doses of aspirin and NSAIDs, which bind to the COX enzymes reversibly and interfere with the capacity of aspirin to inhibit platelet COX-1 irreversibly. It has been estimated that 12.8% of US adults (29.4 million) were regular consumers of NSAIDs and that 19.0% (5.5 million) were taking aspirin chronically for cardioprevention in 2010 (21). Thus, just by chance, roughly 2.4% (5.5 million) would have taken both an NSAID plus aspirin chronically. In reality, this percentage is probably higher given the similar demographic characteristics of patients with musculoskeletal pain and those consuming aspirin for prevention of cardiovascular events. Randomized, controlled trials studying the efficacy and safety of NSAIDs in arthritis patients report rates of simultaneous use of low-dose aspirin that range from 20% to 30% (22–24).

The drug–drug pharmacodynamic interaction has been characterized biochemically and in clinical studies designed to distinguish between a predominantly irreversible inhibition by aspirin and a predominately reversible inhibition by the NSAID (9–18). However, the deconvolution of reversible and irreversible antiplatelet effects can be challenging—particularly with drugs with variable and extended half-lives, such as naproxen (13, 14, 16–18). We hypothesized that direct measurement of COX-1 acetylation by MS using a proteomics approach (25) would enable a quantitative analysis of the propensity of chemically distinct NSAIDs to cause the drug–drug interaction in vivo. We screened for combinations of proteases to excise a peptide containing the target serine-529 of aspirin from COX-1 that was small enough for efficient ionization in the mass spectrometer and long enough to be specific for just this one protein in the human proteome. We devised a specific, sensitive, precise, and reproducible liquid chromatography MS method to quantify the

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**Fig. 3.** Ibuprofen, naproxen, and celecoxib blunt aspirin acetylation of platelet COX-1 dose-dependently in vitro. Washed human platelets were preincubated with (A) ibuprofen, (B) naproxen, or (C) celecoxib at concentrations that inhibited enzymatic function in the higher concentration ranges (gray). Platelet COX-1 acetylation was assessed after the addition of 50 ($n = 7$ donors; dashed orange lines) or 250 μmol/L ($n = 5$ donors; solid orange lines) aspirin. All three NSAIDs abated COX-1 acetylation dose-dependently. The higher concentration of aspirin was less susceptible to the drug–drug interaction indicated by a right shift of the concentration–response curve. The reduction in COX-1 acetylation resulted in increased platelet TxB$_2$ formation compared with aspirin alone (the green curve shows the TxB$_2$ response with 250 μmol/L aspirin). Data are medians and interquartile ranges (quartiles 1 and 3).
Aspirin is thought to acetylate primarily the catalytic but not the allosteric monomer of the homodimeric COX-1 complex (10). Here, we observed maximum acetylation of more than one-half of the COX molecules, suggesting that a fraction of acetylated COX-1 is not assembled into dimers and/or both monomers are acetylated in a fraction of dimers. Inhibition of platelet aggregation induced by arachidonic acid, serum Tx formation, and urinary excretion of a Tx metabolite all reflect quantitatively the action of aspirin in healthy volunteers but are also affected by other drugs (including NSAIDs) and many clinical conditions (26–28). By contrast, platelet COX-1 acetylation is specific to the pharmacological activity of aspirin. It predicted accurately the inhibition of the established indirect aspirin response markers. A reduction of acetylation by just slightly more than 10% resulted in inhibition of the established indirect aspirin response markers.

In vitro studies of isolated COX-1 enzyme incubated with ibuprofen, naproxen, or celecoxib before exposure to aspirin showed that all (even the COX-2–selective compound celecoxib) reduced acetylation of COX-1 by aspirin in vitro with similar potency. This drug–drug interaction occurred at NSAID concentrations that did not inhibit COX-1 function and seems to reflect binding to the allosteric monomer of the dimeric COX-1 complex (10). In isolated platelets, the drug–drug interaction between celecoxib and aspirin was less potent and occurred at concentrations that also inhibited COX enzyme function. This observation suggested that the more dominant mechanism underlying the celecoxib–aspirin interaction in intact platelets was direct competition at the catalytic monomer. However, although therapeutic doses of celecoxib inhibit COX-1 function by roughly 10%, on average, in volunteers (29), this activity did not translate into a functionally relevant reduction of COX-1 acetylation by aspirin. By contrast, administration of ibuprofen and naproxen prevented acetylation of COX-1 almost completely in vivo. Thus, the susceptibility to the drug–drug interaction varied between chemically distinct NSAIDs and would be expected to undermine cardioprotection by aspirin asymmetrically across treatment groups in clinical trials.

Although some studies have reported an interaction between naproxen and aspirin by measuring offset rates of platelet enzyme inhibition (13, 14, 16), others have not (17, 18). This heterogeneity is likely to reflect the comparatively long but highly variable half-life of naproxen ranging from 9 to 25.7 h (30, 31), which might have obscured the functional consequences of the interaction in some individuals. The volunteers enrolled here had apparently long half-lives on average, because Tx formation was still partially suppressed at 24 h, despite the occurrence of the interaction with aspirin resulting in almost complete inhibition of
arachidonic-acid-induced aggregation. Individuals with relatively long half-lives of naproxen might be protected by the extended antiplatelet effects of naproxen, despite the interaction with aspirin. Individuals with relatively short half-lives of naproxen might still subserve an interaction that undermines cardiovascular benefit from aspirin, but inhibition by naproxen in such individuals would not last long enough to result in reliable cardioprotection (13). Similarly, lower naproxen doses might still cause the interaction but be insufficiently long acting for cardioprotection.

The concentration of aspirin also affected the susceptibility to the drug–drug interaction. The NSAID concentrations required to outcompete 50 μM aspirin concentration, which corresponds roughly to systemic peak plasma concentrations after a single tablet of 325 mg plain uncoated aspirin (27), were lower than those required to outcompete 250 μM/L. This observation suggests that aspirin formulations that lead to low systemic aspirin concentrations in plasma, such as the most frequently consumed doses of 81 or 100 mg, would be particularly vulnerable to the drug–drug interaction. Enteric-coated formulations, which result in retarded and further reduced systemic plasma concentrations (28), would be expected to be even more susceptible to the interaction with NSAIDs.

The cardiovascular risks of celecoxib, ibuprofen, and naproxen are currently being prospectively compared in two large trials: the Prospective Randomized Evaluation of Celecoxib Integrated Safety Versus Ibuprofen or Naproxen Trial (PRECISION; clinicaltrials.gov identifier NCT00346216) (7) and the Standard Care Versus Celecoxib Outcome Trial (clinicaltrials.gov identifier NCT00447759) (8). Both trials measure cardiovascular risk as a primary outcome, and both allow for low-dose aspirin cardioprophylaxis at the discretion of the treating physician. The PRECISION is specifically targeting patients at elevated cardiovascular risk at baseline (thus, likely taking aspirin). However, it was not powered to address the comparative safety of the NSAIDs with and without concomitant aspirin. Furthermore, the advice to avoid the interaction by taking aspirin 2 h before the morning dose of the study NSAID stems from a misinterpretation of the single-dosing phase of a study by Catella-Lawson et al. (11). When both drugs were given over multiple days (simulating chronic dosing) and when ibuprofen was started 1 wk after low-dose aspirin (at which time platelet inhibition by the cumulative effect of multiple low-dose aspirin maximally), the order of administration was irrelevant (11). In both cases (ibuprofen given first or aspirin given first), ibuprofen prevented aspirin from maintaining irreversible inhibition over time, presumably because of high trough plasma levels during steady state (11). Thus, one would expect that, under conditions of chronic dosing with both aspirin and ibuprofen, residual ibuprofen plasma levels from the evening dose would sustain the interaction, even when the morning doses of aspirin antedated those of ibuprofen.

We simulated the chronic dosing pharmacokinetics of ibuprofen, naproxen, and celecoxib and compared the expected steady-state plasma concentrations with those observed 2 h after single dosing in our study (Fig. S5). Chronic dosing with ibuprofen and naproxen in the dose range used in the PRECISION and the Standard Care Versus Celecoxib Outcome Trial would result in trough plasma concentrations that are similar to or higher than the concentrations that we found to prevent COX-1 acetylation. Thus, it is likely that platelet inhibition by aspirin will be blocked in the majority of patients on these dosing regimens, irrespective of the order in which aspirin and the NSAID are taken. By contrast, the trough levels simulated with celecoxib are in the range of the plasma concentrations that we observed to have little or no effect on aspirin acetylation.

How would this affect the interpretation of the two largest prospective trials investigating the cardiovascular safety of NSAIDs? Both are noninferiority trials with an intended power of 80% and a noninferiority limit of 1.4. Thus, potential confounding factors, such as NSAID–aspirin interaction, would bias the interpretation toward the null hypothesis. The effect of NSAIDs on cardiovascular risk will result from (i) the direct hazard conferred by the NSAID (3, 4) and (ii) its interaction to undermine the cardioprotective effect of aspirin. Here, we show that the interaction will be distributed asymmetrically across the treatment groups, rendering assessment of the comparative direct effects of the NSAIDs uninterpretable. As was suggested (32), such a situation was avoidable by either performing a study with sufficient power to assess the impact of the aspirin interaction or using an alternative antiplatelet drug, such as cilostazol or ticagrelor, where the interaction is irrelevant. Indeed, selecting an antiplatelet agent that does not target COX-1 may currently be a reasonable therapeutic approach for patients who require both a potent NSAID and a platelet inhibitor.

Methods

A detailed description of the methods is provided in SI Methods.

NSAID–Aspirin Interaction Studies with Purified COX-1 Protein. Purified ovine COX-1 (Cayman Chemicals) was used to assess the potential of NSAIDs to compete with acetylation by aspirin with incubating ibuprofen, naproxen, or celecoxib before aspirin was added (Fig. S3). Prostaglandin E2 was measured by MS as previously described (29).

Study Design. The study protocol was approved by the Institutional Review Board of the University of Pennsylvania and conducted in the university’s Clinical Translational Research Center. Healthy nonsmoking volunteers (18–55 y old) who provided written informed consent were enrolled and abstained from all medications and nutritional supplements for 1 mo before beginning the study and for 3 mo without its duration.

Study 1: COX-1 acetylation as a marker of the antiplatelet effect of aspirin in healthy volunteers. Healthy volunteers (n = 8) were administered a single tablet of 325 mg plain uncoated aspirin, and the on- and offset of platelet COX-1 acetylation were determined (Fig. 1). Classical markers of the platelet inhibitory effect of aspirin (serum TxB2, urinary 11-dehydro TxB2, and arachidonic acid-induced platelet aggregation) were measured (Table S1).

Study 2: NSAID–aspirin interaction studied ex vivo. Platelets isolated from healthy volunteers (n = 5) were preincubated with ibuprofen, naproxen, or celecoxib for 10 min before they were treated with aspirin (50 or 250 μM) for 1 h at 37 °C. Platelets were activated with 100 μM arachidonic acid for 10 min. COX-1 acetylation and TxB2 formation were measured by MS (SI Methods).

Study 3: NSAID–aspirin interaction studied in vivo. In the first study period, fasted healthy volunteers received a single dose of uncoated aspirin (325 mg) to assess their aspirin-responsiveness (Fig. 1). Blood was drawn 2 h and immediately before aspirin administration and 24 h thereafter. In the second study period, after a washout period of at least 2 wk, subjects received a single dose of 600 mg ibuprofen (n = 7), 500 mg naproxen (n = 7), or 200 mg celecoxib (n = 7) in a sequential treatment group with an open-label design. Two hours after the NSAID, a single dose of aspirin (325 mg) was administered. COX-1 acetylation, arachidonic acid-induced platelet aggregation, platelet TxB2, and urinary 11-dehydro TxB2 were measured before NSAID administration, before aspirin administration, and 24 h thereafter. Microfluidic platelet deposition on collagen was measured at baseline and 24 h after aspirin as described (34). Plasma drug concentrations were determined by MS (SI Methods).

Statistical Considerations. Data were graphed in GraphPad Prism (version 4.0; GraphPad Software), and statistics were calculated in R (version 3.0.2; cran.r-project.org). Results were expressed as medians ± interquartile
ranges. \( P \) values below 0.05 were considered statistically significant. Receiver-operating characteristics analyses were performed posthoc (R package ROCR) using an inhibition of serum TxB2 of less than 95% as an experimentally supported threshold for the effectiveness of aspirin. In study 3, six subjects within each treatment sequence (aspirin alone followed by NSAID plus aspirin) were estimated to provide 80% power at a two-sided significance level of 0.05 to detect an effect size of 1.5 SDs in the change from 24 h in the first study period (aspirin alone) to the same value in the second period (NSAID plus aspirin). One additional subject per NSAID treatment group was tested to allow for missing evaluations.

Thus, the total sample size was \( n = 21 \). The Wilcoxon matched pairs signed rank test was used for comparisons within each arm. The Kruskal–Wallis test followed by the Nemenyi–Damo–Wolfe–Dunn posthoc test (R package coin) were used for comparisons of treatment groups.

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Supporting Information

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SI Methods

Platelet Isolation. Fresh peripheral blood was treated with anti-coagulant acid–citrate–dextrose [14% (vol/vol) final concentration] and aliquoted into 15-mL polypropylene centrifuge tubes with ∼5 mL in each tube. The blood samples were centrifuged at 180 × g for 10 min at 21 °C using a swing bucket centrifuge. Platelet-rich plasma (PRP) was removed and spiked with 2 μL 100 μmol/L prostaglandin E2 (PGE2) stock for each 1 mL PRP, and it was further diluted with Tyrode Hepes-EDTA buffer (50 mM Hepes, 140 mM NaCl, 3 mM KCl, 12 mM NaHCO3, 0.4 mM NaH2PO4, H2O, 1.5 mM MgCl2·6H2O, 5 mM EDTA, pH 6.4) in the ratio of 1:1. The diluted PRP solution was centrifuged at 430 × g for 10 min at 21 °C. The pellet was resuspended in the same volume of Tyrode Hepes-EDTA buffer. The washed platelets were recovered by centrifugation at 740 × g for 10 min at 21 °C.

Protein Separation. Washed platelets were dissolved in RIPA buffer (Sigma) supplemented with protease inhibitor mixture (Roche) at room temperature for 30 min. Proteins were separated and digested as described (1, 2). Briefly, the supernatant proteins were collected by centrifugation at 12,000 × g for 10 min at 4 °C. Platelet protein (around 150 μg) mixed with a sample loading buffer was run on NuPAGE precast gels (Invitrogen). Gels were stained with a colloidal blue staining kit according to the manufacturer’s instructions (Invitrogen). The bands from the platelet sample in the molecular weight range corresponding to purified ovine COX-1 were excised from the 1D SDS/PAGE gel and cut into small pieces, which were then destained in 40% acetonitrile (ACN) solution at 37 °C, washed in 100% ACN, and dried in a Speed-Vac concentrator. The gel pellet was rehydrated in 15 μL 20 ng/μL protease mixture with trypsin (10 ng/μL; Promega,) and Glu-C (10 ng/μL; Roche) in 0.01% ProteaseMAX surfactant (Promega) and 50 mM NH4HCO3 for 10 min. The gel was then covered with 30 μL 0.01% ProteaseMAX Surfactant and 50 mM NH4HCO3 in the presence of the stable isotope-labeled internal standards (IGAPFSLK and IGAPFS[+ace]LK, each 125 fmol; AQUA Ultimate; Thermo). The digestion was performed at 37 °C for 2 h and then stopped by adding 2 μL 10% (vol/vol) formic acid. The supernatant peptide solution was collected by centrifugation at 12,000 × g for 10 min at 4 °C and desalted using MiniSpin columns (The Nest Group, Inc.) before isotope dilution MS analysis.

MS Analysis of Peptides. The targeted MS analysis of peptides was performed on a Waters ACQUITY Ultra Performance Liquid Chromatography System coupled with a Thermo Scientific TSQ Vantage Triple Quadrupole Mass Spectrometer. Chromatographic separations were performed on a 150 μm × 100 mm ACQUITY 1.7-μm BEH130 C18 column (Waters Corp). Mobile phases A and B were 0.5% ACN/0.1% formic acid and 98% (vol/vol) ACN/0.1% formic acid, respectively. A linear gradient of mobile phase B was developed to elute peptides from 5% to 30% (vol/vol) at 1–20 min. After gradient elution, the column was washed with 90% (vol/vol) buffer B for 10 min and then equilibrated with 95% (vol/vol) buffer A for 10 min. The flow rate was 1.5 μL min−1.

Nanoelectrospray ionization was performed in the positive ion mode. The mass spectrometer was operated in MRM mode, including 3 transitions for each peptide in a single liquid chromatography (LC)–isotope dilution MS analysis for 12 transitions in total (Table S1). For ionization, a 1,888-V spray voltage and 230 °C capillary temperature were used. The resolutions for both quadrupole Q1 and Q3 were set to 0.7 (full width half-maximum). The collision gas pressure of Q2 was set at 1.4 mtorr argon. The collision energy for all of the targeted peptide transitions was 12. The cycle time of all transitions was 1 s. The peptides peak areas were extracted and analyzed using Thermo’s Qual Browser.

The full-scan MS analysis for sequence confirmation was performed on an Eksigent HPLC coupled with a Thermo LTQ-FT MS (Thermo Fisher Scientific). The HPLC separation was done on an Eksigent NanoLC-2D System (Eksigent Technologies) equipped with an online Eksigent Autosampler. Solvents used were 0.5% ACN/0.1% formic acid (mobile phase A) and 98% (vol/vol) ACN/0.1% formic acid (mobile phase B). Peptides picked up by the autosampler were first loaded and cleaned on an IntegraFrit trap column (10 mm × 75 μm; New Objective) at 5 μL/min for ∼10 min with 95% (vol/vol) mobile phase A in channel 1. Then, the LC gradient in channel 2 at 1 μL/min was used to separate peptides on a Magic C18 Column (50 × 0.2 mm; Michrom BioResources). A linear gradient of mobile phase B was developed to elute peptides from 2% (vol/vol) to 30% (vol/vol) B at 1–60 min. After gradient elution, the column was washed using 95% (vol/vol) mobile phase B for 10 min and then equilibrated with 98% (vol/vol) mobile phase A for 10 min.

MS analyses were performed on an LTQ-FT mass spectrometer (Thermo). The nano-LC system was connected to an LTQ (Thermo) equipped with a nanoelectrospray interface operated in the positive ion mode (3). The linear ion trap mass spectrometer was operated in a data-dependent MS/MS mode (m/z 300–2,000), in which each full MS scan in the Fourier transform ion cyclotron resonance MS was followed by seven MS/MS scans in the ion trap. The seven most intense precursor ions were selected for collision-induced dissociation with normalized collision energy of 35%. The mass resolution was set at 100,000.

MS Analysis of Prostanoids and NSAIDs. MS analysis of lipids (PGE2, TxB2, and 11-dehydro TxB2) and NSAIDs (ibuprofen, naproxen, and celecoxib) was performed on a TSQ Quantum Ultra Tandem Quadrupole Instrument (Thermo) coupled to an Accela Ultra Performance Liquid Chromatography System (Thermo) using a Hypersil GOLD C18 Column (200 × 2.1 mm; Thermo). The mobile phase consisted of water (solvent A) and ACN/methanol [95%/5% (vol/vol); solvent B], both of which had 0.005% acetic acid and were adjusted to pH 5.7 with ammonium hydroxide. The flow rate was 350 μL/min. A linear solvent gradient of 20–40% B in 20 min was used. The transitions for PGE2 were 351→271 and 355→275 for the endogenous material and the tetradeuterated internal standard, respectively. For the plasma concentrations of ibuprofen, naproxen, and celecoxib, samples were spiked with internal standards and purified by solid-phase extraction. The transitions for ibuprofen and (±)-ibuprofen-d3 were 205.00→161.15 and 208.00→164.15, respectively. The transitions for naproxen and naproxen-d3 were 231.00→185.15 and 234.00→188.15, respectively. The transitions for celecoxib and celecoxib-d2 were 381.90→362.20 and 388.90→369.20, respectively.

Quality Control. Standards and quality control (QC) solutions were prepared using certified Hamilton microsyringes. For standard curve preparation, the stock solution of synthesized endogenous peptides at 100 fmol/μL (IGAPFSLK and IGAPFS[+ace]LK; AQUA Ultimate; Thermo) was used to perform serial dilutions to cover the expected range of COX-1 in platelets. The endogenous peptides were mixed with the same volume (10 μL) of stock heavy isotopic-labeled peptides (IGAPFS[13C6;15N]
K and IGA[12]C3;[15]N][PF6][\text{\textasciitilde}ace]–LK, each with 12.5 fmol/μL; AQUA Ultimate; Thermo). The peak area ratio between endogenous peptide and heavy-labeled peptide was plotted against the amount of endogenous peptide to calculate the linearity. For QC validation, three concentrations of synthesized endogenous peptides were selected, which included 50 fmol/μL for high QC, 12.5 fmol/μL for middle QC, and 3.125 fmol/μL for low QC. The QC samples were made by mixing the same volume (10 μL) of stock heavy isotopic-labeled peptides and each of the three QC peptides. The calculated amount of the QC samples from the standard curve was compared with the theoretical amount to calculate the precision and accuracy of the method. The limit of detection was determined as the lowest concentration that produced a response three times greater than the noise level. The lower limit of quantification was established as the lowest concentration that could be quantitated with a precision less than 20%.

To check the intrasample variability for human platelet samples, the COX-1 acetylation in platelets at 96 h after aspirin in vivo was measured at 5 times independently. The average coefficient of variability was calculated. To test the assay stability, washed platelets from three healthy volunteers were treated with 250 μM aspirin in vitro. Three aliquots of freshly prepared platelet protein were kept at −80 °C for 24 h and then thawed unassisted at room temperature. When completely thawed after 30 min, the protein was re-frozen at −80 °C for another 24 h. The platelet COX-1 acetylation was measured at 24 h after three freeze-thaw cycles over 2 wk.

**Data Analysis for Peptide Quantification.** Comparing the time-resolved area under the curve of the analyte peaks with the stable isotope-labeled standard peaks allows absolute quantitation of both the unacetylated and the acetylated peptides (Fig. S1 D and E). The peptides peak areas were extracted using Quan Browser (Thermo), and the results expressed the average ratio of acetylated to total COX-1, \( R_{COX}^{acyt} \), based on the individual quantitation of the three transitions, \( i \),

\[
R_{COX}^{acyt} = \frac{y_{i}^{\text{ion}+ace}}{y_{i}^{\text{ion}}} \quad \text{and} \quad \frac{y_{i}^{\text{ion}+ace}}{y_{i}^{\text{ion}+ace}} = \frac{1}{3} \sum_{i=5}^{9} R_{i}^{acyt},
\]

where \( y_{i} \) represents the quantity of the unacetylated form, and \( y_{i}^{\text{ion}+ace} \) represents the quantity of the acetylated form.

**Microfluidic Assay of Platelet Deposition on Collagen.** Platelet function was evaluated in a microfluidic platelet function test under venous flow conditions as previously described (4). Briefly, blood samples were drawn into a tube prefilled with the thrombin inhibitor H-D-Phe-Pro-Arg-chloromethylketone (100 μmol/L final concentration; Hematologic Technologies). Platelets were fluorescently labeled with a phycoerythrin-conjugated mouse anti-human CD61 (\( \text{mAbB2} \)) antibody (0.125 μmol/L final concentration; BD Biosciences). H-D-Phe-Pro-Arg-chloromethylketone–treated whole blood was then perfused over collagen (Equine fibrillar collagen type 1; Chrono, Chronofiber; Chronolog) at a wall shear rate of 200 s\(^{-1}\) using an eight-channel microfluidic device with perfusion by withdrawal into a single syringe pump (PHD 2000; Harvard Apparatus). A custom-stage insert held three microfluidic devices, allowing 24 simultaneous thrombi to be imaged in 15-s intervals. Clotting was imaged with an inverted fluorescence microscope (IX81; Olympus America) equipped with a CCD (Hamamatsu). Platelet deposition rates on collagen were quantified with background-corrected fluorescence values between the time intervals 60–150 and 150–300 s. Normalization of late-stage platelet deposition rate (150–300 s) to early-stage platelet deposition rate (60–150 s) represented TxA\(_2\)-dependent secondary platelet aggregation expressed as an \( R \) score, which was scaled to one. An \( R < 1 \) indicated that secondary aggregation was inhibited.

**NSAID–Aspirin Interaction Studies with Purified COX-1 Protein.** Purified ovine COX-1 (Cayman Chemicals) was used to assess the potential of NSAIDs to compete with acetylation by aspirin. COX-1 (final working concentration of 0.5 μmol/L) dissolved in 500 μL reaction buffer (0.1 mol/L Tris-HCl, pH 8.0 containing 5 mmol/L EDTA and 2 mmol/L phenol) was preincubated with 25 μmol/L ibuprofen, naproxen, or celecoxib at 37 °C for 10 min. Aspirin (250 μmol/L) was added to each reaction for 1 h at 37 °C, and 400 μL reaction buffer was heat-denatured (10 min at 90 °C) and concentrated in a Speed-Vac Concentrator (Savant Instruments, Inc.) for subsequent analysis of COX-1 acetylation. The remaining portions of the reactions were stimulated with 10 μmol/L arachidonic acid for 10 min (37 °C), and the catalysis was stopped by 5 μL 1 mol/L hydrochloric acid. The samples were spiked with 5 ng tetradecanoyl internal standard. PGE\(_2\) was liquid/liquid extracted by adding 300 μL methyl-tert-buty ether, dried under nitrogen, and measured by MS as previously described (5).

**Statistical Considerations.** Data were graphed in GraphPad Prism (version 4.0; GraphPad Software), and statistics were calculated in R (version 3.0.2; cran.r-project.org). Results were expressed as medians ± interquartile ranges. \( P \) values below 0.05 were considered statistically significant. Study 1 was designed as an exploratory experiment to assess whether the measured levels of COX-1 acetylation in healthy volunteers were mechanistically plausible. No sample size justification was given, and no formal statistical tests were conducted. Nine subjects were enrolled, and eight subjects were evaluable. Receiver-operating characteristics analyses were performed posthoc (R package ROC) using an inhibition of serum TxB\(_2\) of less than 95% as an experimentally supported threshold for the effectiveness of aspirin (7). Study 2 was also designed as an exploratory experiment without sample size justification and formal statistical testing. In study 3, six subjects within each treatment sequence (aspirin alone followed by NSAID plus aspirin) were estimated to provide 80% power at a two-sided significance level of 0.05 to detect an effect size of 1.5 SDs in the change from 24 h in the first study period (aspirin alone) to the same value in the second period (NSAID plus aspirin). Assuming a value for COX acetylation of ~70.6% ± 3.9% in the first period as seen in study 1, this effect size corresponded to a difference of 5.9%. One additional subject was tested to allow for loss to follow-up or other missing evaluations. Thus, the total sample size was \( n = 21 \) (\( n = 7 \) per NSAID treatment sequence). The Wilcoxon matched pairs signed rank test was used for these comparisons within each arm. The Kruskal–Wallis test followed by the Nemenyi–Damico–Wolfe–Dunn posthoc test (R package coin) (8) were used for comparisons of treatment groups.


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**Fig. S1.** An MS assay to quantify COX-1 acetylation. (A) Sequence alignment of human COX-1 and COX-2 protein fragments, which contain the aspirin acetylation site serine-529. A protease digestion regimen using Glu-C and trypsin was developed to excise an 8-aa peptide containing serine-529 (524-IGAPFSLK-531) from COX-1. (B and C) Typical product ion spectra generated by collision-induced dissociation of the (α) unacetylated and (C) acetylated forms of 524-IGAPFSLK-531 isolated from human platelet COX-1. Acetylation is reflected by a shift of 42 Da of the relative molecular mass (Mr) from COX-1. (B) Typical product ion spectra generated by collision-induced dissociation of the (α) unacetylated and (C) acetylated forms of 524-IGAPFSLK-531 isolated from human platelet COX-1. Acetylation is reflected by a shift of 42 Da of the relative molecular mass (Mr) from COX-1. (D and E) A multiple reaction monitoring MS assay was developed to measure the \( y_3 \), \( y_5 \), and \( y_7 \) transitions in their (D) unacetylated and (E) acetylated forms. Known quantities of synthesized heavy isotope-labeled standard peptides (internal standard) were spiked into the samples, and their \( y_3 \), \( y_5 \), and \( y_7 \) transitions were monitored simultaneously with the analyte. The comparison of the areas under the peaks between analyte and internal standard allows quantitation of the analyte. The average of the area under the peak ratios of the \( y_3 \), \( y_5 \), and \( y_7 \) transitions was calculated to quantify the unacetylated and the acetylated forms of 524-IGAPFSLK-531. D and E show typical spectra with 12 simultaneously monitored transitions of platelet COX-1 isolated from a volunteer after the administration of 325 mg aspirin. Area refers to peak area in arbitrary units.
Fig. S2. QC of the MS COX-1 acetylation assay. Standard curves of the unacetylated and the acetylated forms of the synthetic 524-IGAPFSLK-531 were generated. Increasing amounts of the synthesized analyte (0.5–125 fmol) were mixed with 31.25 fmol heavy isotope-labeled internal standard peptides, and the logarithm of their peak area ratios was plotted as a function of analyte amount. (A, C, and E) Unacetylated y5, y6, and y7 transitions. (B, D, and F) Acetylated y5, y6, and y7 transitions. Data of three independent standard curves are shown (×), and linear regression curves are plotted. $r^2$ denotes the goodness of fit in the regression analyses.
**Fig. S3.** Time- and dose-dependent acetylation of (A) purified and (B and C) platelet COX-1. Increasing COX-1 acetylation coincides with reduction in COX function as quantified as prostanoid formation. COX-1 acetylation does not reach 100%, because the functional enzyme forms dimers, of which only one monomer is accessible for aspirin acetylation (1). (A) Ovine COX-1 (0.5 μmol/L) was incubated with 250 μmol/L aspirin. COX-1 acetylation and PGE\(_2\) formation were measured by MS. Data are medians and ranges (n = 3). (B) Washed human platelets from three donors were incubated with 50 μmol/L aspirin, and COX-1 acetylation and TxB\(_2\) formation were quantitated mass spectrometrically. Data are medians and ranges (n = 3). (C) Dose-dependent COX-1 acetylation. Washed human platelets were incubated with increasing concentrations of aspirin for 1 h. Data are medians and ranges (n = 3).

Ibuprofen, naproxen, and celecoxib blunt aspirin acetylation of purified COX-1 dose-dependently in vitro. Purified COX-1 was preincubated with (A) ibuprofen, (B) naproxen, or (C) celecoxib at concentrations that did not inhibit enzymatic function alone (gray) followed by the addition of aspirin. All NSAIDs decreased the acetylation of COX-1 by aspirin dose-dependently (orange). The inhibition of enzyme function by aspirin was dose-dependently blunted by the NSAIDs (green). Data are medians and interquartile ranges (quartiles 1 and 3; n = 3).

Fig. S4.
Plasma concentration (µmol/L)

A

Ibuprofen

Observed: 600 mg, single dose
Color: COX-1 Ace (%):

Simulated: 800 mg every 8 hrs
Simulated: 600 mg every 8 hrs

B

Naproxen

Observed: 500 mg single dose
Color: COX-1 Ace (%):

Simulated: 800 mg every 8 hrs
Simulated: 375 mg every 12 hrs
Simulated: 500 mg every 12 hrs

C

Celecoxib

Observed: 200 mg single dose
Color: COX-1 Ace (%):

Simulated: 800 mg every 8 hrs
Simulated: 200 mg every 12 hrs

Legend continued on following page

Fig. S5. The relationship between NSAID plasma concentrations and the drug–drug interaction with aspirin. Simulated chronic dosing plasma concentration profiles are shown for (A) ibuprofen (600 and 800 mg administered every 8 h), (B) naproxen (375 and 500 mg administered every 12 h), and (C) celecoxib (100 and 200 mg administered every 12 h). NSAID plasma concentrations measured 2 h after administration in this study were plotted. This time was the time at which 325 mg aspirin was given. The plasma concentration data points are color-coded by the degree of platelet COX-1 acetylation (COX-1 Ace in percentage) measured 24 h later. The color scale is centered on 62% acetylation (green, above 62% acetylation; red, below 62% acetylation), which is the threshold
determined in study 1 that segregated best functionally relevant from irrelevant acetylation based on the inhibition of platelet Tx formation. The simulations were based on published two-compartment models to describe the kinetics of ibuprofen and naproxen and a two-compartment model developed for celecoxib to fit plasma concentration data collected previously (1). Simulations were conducted in R (www.cran.org) using the PKfit package. Median plasma concentrations were calculated from 10,000 simulations varying the model parameters randomly within the constraints of the error model. Model parameters are given: ibuprofen ($k_a = 1.5 \text{ h}^{-1}$, $V_d = 6.3 \text{ L}$, $k_{12} = 0.4 \text{ h}^{-1}$, $k_{13} = 1.41 \text{ h}^{-1}$, and $k_{21} = 2.43 \text{ h}^{-1}$; error model: $e_{k_a} = 0.12 k_a$, $e_{V_d} = 0.29 V_d$, $e_{k_{12}} = 0.37 k_{12}$, $e_{k_{13}} = 1.51 k_{13}$, and $e_{k_{21}} = 1.40 k_{21}$), naproxen ($k_a = 1.5 \text{ h}^{-1}$, $V_d = 8 \text{ L}$, $k_{12} = 0.037 \text{ h}^{-1}$, $k_{13} = 0.547 \text{ h}^{-1}$, and $k_{21} = 0.598 \text{ h}^{-1}$; error model: $e_{k_a} = 0.12 k_a$, $e_{V_d} = 0.29 V_d$, $e_{k_{12}} = 0.15 k_{12}$, $e_{k_{13}} = 1.51 k_{13}$, and $e_{k_{21}} = 1.40 k_{21}$), and celecoxib ($k_a = 0.75 \text{ h}^{-1}$, $V_d = 50 \text{ L}$, $k_{12} = 0.075 \text{ h}^{-1}$, $k_{13} = 0.08 \text{ h}^{-1}$, and $k_{21} = 0.03 \text{ h}^{-1}$; error model: $e_{k_a} = 0.8 k_a$, $e_{V_d} = 0.3 V_d$, $e_{k_{12}} = 0.3 k_{12}$, and $e_{k_{21}} = 0.3 k_{21}$).


### Table S1. Parent → product ion transitions monitored by MS

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### Table S2. Demographics of the study population in which the use of the COX-1 acetylation assay to assess the pharmacological response to aspirin was studied

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Table S3. Demographics of the study population in which the NSAID–aspirin interaction was assessed

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