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Aspirin Induces Platelet Receptor Shedding via ADAM17 (TACE)*

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Aspirin is effective in the therapy of cardiovascular diseases, because it causes acetylation of cyclooxygenase 1 (COX-1) leading to irreversible inhibition of platelets. Additional mechanisms can be suspected, because patients treated with other platelet COX inhibitors such as indomethacin do not display an increased bleeding tendency as observed for aspirin-treated patients. Recently, aspirin and other anti-inflammatory drugs were shown to induce shedding of L-selectin in neutrophils in a metalloproteinase-dependent manner. Therefore, we investigated the effects of aspirin on the von Willebrand Factor receptor complex glycoprotein (GP) Ib-V-IX, whose lack or dysfunction causes bleeding in patients. As quantified by fluorescence-activated cell sorting analysis in whole blood, aspirin, but not its metabolite salicylic acid, induced dose-dependent shedding of human and murine GPIbα and GPV from the platelet surface, whereas other glycoproteins remained unaffected by this treatment. Biotinylated fragments of GPV were detected by immunoprecipitation in the supernatant of washed mouse platelets, and the expression level of GPIbα was decreased in these platelets as measured by Western blot analysis. Although shedding occurred normally in COX-1-deficient murine platelets, shedding was completely blocked by a broad-range metalloproteinase inhibitor and, more importantly, in mouse platelets expressing an inactive form of ADAM17. Shed fragments of GPIbα and GPV were elevated in the plasma of aspirin-injected mice compared with animals injected with control buffer. These data demonstrate that aspirin at high concentrations induces shedding of GPIbα and GPV by an ADAM17-dependent mechanism and that this process can occur *in vivo*.

The activation of platelets at sites of vascular injury, followed by controlled aggregate formation, is required for normal hemostasis, but can cause thrombosis in pathologically altered arteries leading to myocardial infarction or stroke (1). Under conditions of elevated shear, platelet adhesion is initiated by the von Willebrand Factor receptor glycoprotein (GP)[2] Ib–V–IX, a structurally unique receptor complex expressed in platelets and megakaryocytes. The interaction of GPIbα with von Willebrand factor bound to subendothelial collagen or on the surface of activated adherent platelets is essential for platelet adhesion and thrombus formation (1, 2). In patients, a reduced expression or malfunction of GPIbα determines the Bernard-Soulier syndrome, a congenital bleeding disorder, which is characterized by a macrothrombocytopenia, an inability of platelets to adhere to subendothelial matrices, and a dramatically prolonged bleeding time (3).

The expression levels of GPIb and GPV are physiologically regulated by internalization processes and/or by proteolytic ectodomain cleavage ("shedding"), which might play a role in the clearance of aged platelets from the circulation (4). In contrast to the well known protease activity of the platelet activator thrombin that directly releases a 69-kDa fragment from GPV (GPV f1), the membrane-bound metalloproteinase a disintegrin and metalloprotease 17 (ADAM17, a tumor necrosis factor-α-converting enzyme, or TACE) was identified recently to be essentially involved in agonist-induced shedding of GPIbα (5) and of GPV (6) resulting in 130- and 80-kDa soluble fragments of the receptors, respectively.

Receptor shedding from other blood cells such as neutrophils appears to occur in a similar manner. L-selectin, which plays a crucial role in the extravasation of leukocytes and a correct inflammatory response is enzymatically cleaved by ADAM17 (7). This mechanism appears to be a major component contributing to the anti-inflammatory action of the non-steroidal anti-inflammatory drugs (NSAIDs), as treatment with NSAIDs, including acetylsalicylic acid (aspirin) down-regulates L-selectin in these cells in an ADAM17-dependent manner (8, 9). Although the effect of NSAIDs on L-selectin expression seems to be linked to the uncoupling capability of these compounds (10), the precise molecular mechanism(s) involved remain unclear.

It is well established that aspirin at therapeutically relevant concentrations (up to 2 mM) (11, 12) causes irreversible acetylation of cyclooxygenase-1 (COX-1) thereby inhibiting the generation of the platelet activator thromboxane A2 (13). This mechanism is thought to account for the effect of aspirin in the prevention of coronary artery and cerebrovascular thrombosis (14, 15). However, not all clinical observations can be explained by this mechanism. Aspirin treatment causes an increased bleeding tendency in uremic patients compared with healthy volunteers, although COX was completely inhibited in all subjects tested (16). In addition, ibuprofen, a member of the NSAID family, at a dose that fully inhibits COX-1 activity did not significantly prolong bleeding time in these patients indicating a second anti-platelet mechanism of aspirin distinct from COX-1 inhibition (16). This is supported by *in vivo* findings demonstrating an increase in bleeding time of high dose aspirin-treated rabbits, which was not related to COX-1 blockage (17).

In the present study, we investigated the effects of aspirin on platelet function at low and high concentrations. In whole blood flow adhesion studies on immobilized collagen, virtually no thrombus formation was

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[2] The abbreviations used are: GP, glycoprotein; ADAM, a disintegrin and metalloproteinase; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; COX, cyclooxygenase; NSAID, non-steroidal anti-inflammatory drug; PMA, phorbol 12-myristate 13-acetate; ELISA, enzyme-linked immunosorbent assay.

observed in platelets treated with high aspirin concentrations, whereas low dose aspirin caused a moderately reduced thrombus size and surface coverage under high shear flow conditions. This severe effect of high dose aspirin was due to shedding of GPIbα and of GPV, as shown by flow cytometry and Western blot in both human and murine platelets. Aspirin-induced shedding occurred in COX-1 but not in mouse platelets expressing functionally inactive ADAM17, and injection of mice with high doses of aspirin resulted in an increased level of GPIbα and GPV fragments in the plasma. These data demonstrate that aspirin at high concentrations induces shedding of surface receptors by an ADAM17-dependent mechanism and that this process can occur *in vivo*.

EXPERIMENTAL PROCEDURES

Animals—All animal experiments and care were approved by the local Animal Care and Use Committee. ADAM17 mutant mice (7) were kindly provided by Amgen. Both mutant and wild-type control animals as well as COX-1-deficient mice were of 129/Sv x C57BL/6 genetic backgrounds and were used at the ages of 10–16 weeks. To obtain platelets lacking functional ADAM17 (ADAM17 lacking zinc-binding domain; $ADAM17^{\Delta Zn/\Delta Zn}$), bone marrow chimeras were produced (5).

Platelet Preparation and Aggregation Studies—Whole blood was taken from anesthetized mice from the retro-orbital plexus and collected in TBS containing 20 units/ml heparin and washed platelets were obtained as described previously (18). Briefly, whole blood was drawn from the retro-orbital plexus of anesthetized mice and collected in Tris-buffered saline containing 20 units/ml heparin. After washing the platelets twice with HEPES-Tyrode's buffer (137 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 5.5 mM glucose, 5 mM HEPES, 0.35% bovine serum albumin), platelets were resuspended in Tyrode's buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ to 2×10^6 platelets/μl. To determine platelet aggregation, light transmission was measured using platelet-rich plasma. Transmission was recorded in a Fibrintimer 4 channel aggregometer (APACT Laborgeräte und Analysensysteme, Hamburg, Germany) over 10 min and was expressed as arbitrary units with 100% transmission adjusted with plasma.

Antibodies and Chemicals—Acetylsalicylic acid (Aspisol®) was purchased via a local pharmacy. JON/A-PE, which binds to activated integrin αIIbβ3 (19), and all other anti-mouse antibodies were from Emfret Analytics, Würzburg, Germany. Anti-human GPIbα and anti-human GPV antibodies were from Immunotools, Friesoythe, Germany. All other chemicals were from Sigma-Aldrich.

Flow Cytometry—Whole blood was taken from healthy volunteers that did not take any platelet-affecting drugs 2 weeks prior the experiment and was collected in a citrate buffer (100 μM sodium citrate, 7 μM citric acid, 140 μM glucose, pH 6.5). Whole human or murine blood samples were left untreated or were incubated with acetylsalicylic acid at 37 °C for the indicated times. The samples were diluted 1:20 in phosphate-buffered saline and then stained with fluorophore-conjugated monoclonal antibodies at saturating concentrations for 15 min at room temperature and analyzed directly on a FACSCalibur (BD Biosciences). Platelets were identified by forward scatter/SSC characteristics.

Adhesion under Flow Conditions—Heparinized human blood (1 vol) was collected into 0.5 vol of HEPES buffer, pH 7.45, containing 137 mM NaCl, 5.6 mM glucose, 5 mM HEPES, 2.7 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 0.1% bovine serum albumin, 120 μM D-Phe-Pro-Arg-chloromethylketone, and 15 units/ml heparin. Rectangular coverslips (24 × 60 mm) were coated with 0.25 mg/ml "Horm type" fibrillar collagen (Nycomed, Munich) for 1 h at 37 °C and blocked with 1% bovine serum albumin. Perfusion of whole blood was performed as described (20). Briefly, transparent flow chambers with a slit depth of 50 μm, equipped with the collagen-coated coverslips, were rinsed with HEPES buffer supplemented with 2 mM $CaCl_2$ and 1 unit/ml heparin and connected to a syringe filled with the anti-coagulated blood. Perfusion was performed at room temperature with a pulse-free pump at high shear stress (4 min, flow rate of 7.53 ml/h, equivalent to a wall shear rate of 1000 s^{-1}). During perfusion, microscopic phase-contrast images were recorded in real-time. Thereafter, the chambers were rinsed by a 4-min perfusion with HEPES buffer, pH 7.45 (2 mM $CaCl_2$ and 1 unit/ml heparin), at the same shear stress. Phase-contrast images were recorded (×63 objectives).

Western Blot Analysis—For immunoblotting, washed platelets were lysed, separated by SDS-PAGE (12.5%) under reducing conditions, and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with horseradish-conjugated anti-GPIbα monoclonal antibody for 1 h, and proteins were visualized by enhanced chemiluminescence.

Immunoprecipitation—Washed mouse platelets were surface-labeled with EZ-Link sulfo-NHS-LC-biotin (25 μg/ml in phosphate-buffered saline, from Pierce) and subsequently incubated with acetylsalicylic acid for the indicated time points and then centrifuged (2000 × *g*, 10 min). Supernatants were then collected and incubated with 10 μg of anti-GPV monoclonal antibody and 25 μl of protein G-Sepharose (Amersham Biosciences) overnight at 4 °C. Samples were separated by 12% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with streptavidin-horseradish peroxidase (1 μg/ml) for 1 h after blocking. After extensive washing, biotinylated proteins were visualized by ECL.

ELISA—To detect cleaved GPV (GPV f1) or GPIbα (glycocalicin) by ELISA (6, 21), washed platelets were treated as indicated, and supernatants (from 300 μl of 0.5×10^9 platelets) were cleared by 10-min centrifugation at 15,000 × *g*. Alternatively, blood was collected retro-orbitally from mice treated with aspirin at the indicated doses, and plasma was produced by centrifugation. The samples were transferred to anti-GPV (20 μg/ml)- or anti-GPIbα (pOp3, 20 μg/ml)- coated ELISA plates, incubated for 1 h at 37 °C, and washed three times with buffer (phosphate-buffered saline containing 0.1% Tween). Horseradish peroxidase-conjugated secondary antibody was added at a concentration of 2.5 μg/ml followed by 1-h incubation at 37 °C and detected with tetramethylbenzidine. The reaction was stopped with 0.5 M sulfonic acid and analyzed by WinRead.

RESULTS

Aspirin Selectively Induces Down-regulation of Human GPIbα and GPV—Acetylsalicylic acid (aspirin) prevents myocardial infarction and stroke in patients. To examine the effect of aspirin on thrombus formation under high shear flow conditions *in vitro*, we incubated human blood with increasing concentrations of aspirin for 2 h and studied platelet adhesion to collagen. Although untreated platelets adhered and formed stable and large thrombi, therapeutically relevant concentrations of aspirin sufficient to completely inhibit COX-1 (2 mM) (22, 23) caused a moderate decrease in thrombus formation (Fig. 1*A*). Surface coverage was modestly lower (Fig. 1*B*), and thrombus size and stability were slightly reduced under these conditions confirming previous observations (24–27). Surprisingly, higher concentrations of aspirin (20 mM) dramatically reduced the number of adherent platelets and virtually abrogated thrombus formation. This indicated an anti-platelet mechanism of high dose aspirin that is different from the inhibition of COX-1 and directly interferes with platelet adhesion on collagen under conditions of elevated shear (Fig. 1, *A* and *B*).

It is known that a number of platelet receptors that are involved in the initial adhesion process, including GPV and GPIbα, are proteolytically

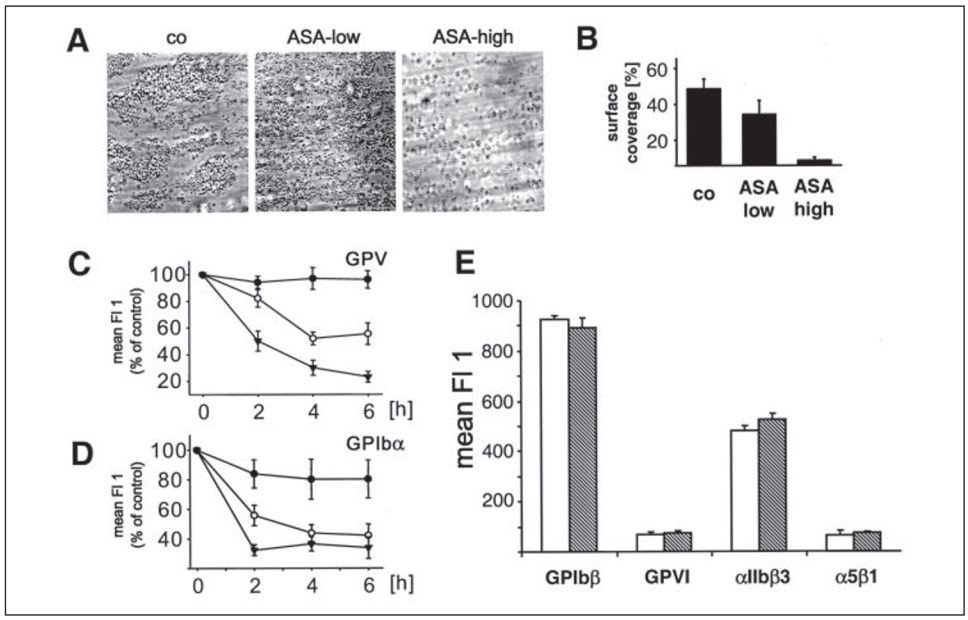

FIGURE 1. **Aspirin impairs human platelet adhesion to collagen by down-regulation of GPIbα and GPV.** *A*, heparinized whole blood was perfused over fibrillar collagen-coated coverslips at high shear (1000 s^{-1}). Whole blood was incubated at 37 °C for 2 h with control buffer (*co*), 2 mM aspirin (*ASA-low*), or with 10 mM aspirin (*ASA-high*). Pictures shown are representative phase contrast images taken at the end of one experiment. *B*, adhesion was quantitatively determined by the surface area that was covered by thrombi or single adhering cells. At the end of experiments performed with six individual blood donors, surface coverage of each sample was assessed and is given as mean ± S.D. *C* and *D*, the time course of GPIbα (*D*) and GPV (*C*) down-regulation was measured by flow cytometry. Blood was incubated with buffer (*black circles*), 20 mM (*open circles*), and 50 mM aspirin (*black triangles*) for 2, 4, and 6 h. *E*, effects of aspirin on the expression of surface glycoproteins on human platelets. Blood was incubated with buffer (*open bars*) or 20 mM aspirin (*hatched bars*) for 6 h and the expression of glycoproteins was measured by flow cytometry ($n = 6$).

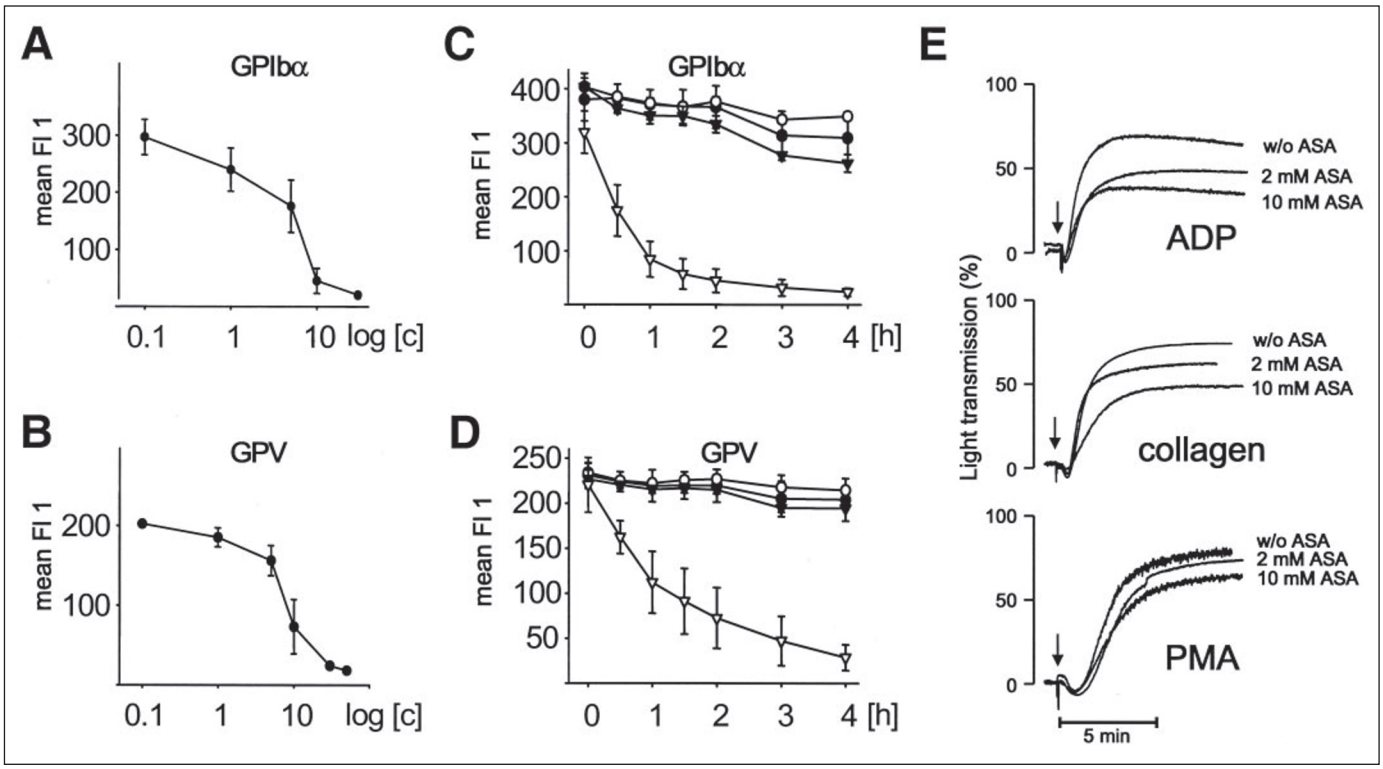

FIGURE 2. **Aspirin causes a dose-dependent down-regulation of murine GPIbα and GPV.** Whole blood was incubated with 100 μM, 1, 5, 10, 30, and 50 mM of aspirin for 2 h. After that, GPIbα (*A*) and GPV (*B*) expression on the platelet surface was quantitatively determined by flow cytometry. The kinetics of GPIbα (*C*) and GPV (*D*) down-regulation was assessed using 10 mM aspirin (*open triangles*). At the same concentration and conditions, lysine (*black circles*) or salicylic acid (*black triangles*) were incubated in parallel ($n = 9$) and samples were measured by flow cytometry. *E*, after incubation of platelet-rich plasma with aspirin for 2 h at indicated concentrations (2 mM and 10 mM), platelet aggregation was stimulated with ADP (10 μM), collagen (3 μg/ml), or PMA (500 ng/ml) (indicated by the *arrows*) and light transmission was recorded. Data shown are representative for three different experiments.

down-regulated from the cell surface in response to various agonists (6, 28). In addition, NSAIDs, including aspirin, have been shown to induce shedding of L-selectin in neutrophils (8, 9). Therefore, we suspected that high concentrations of aspirin could affect the surface expression of platelet receptors. To test this hypothesis, we incubated whole blood with different concentrations of aspirin and measured the surface expression of prominent glycoproteins on the platelet surface by flow cytometry. Indeed, aspirin caused a dose-dependent down-regulation of GPV and GPIbα (Fig. 1, *C* and *D*), whereas the expression levels of other glycoproteins, including GPVI, β3-, or α5β1 integrin remained largely unaffected (Fig. 1*E*). Importantly, the surface levels of GPIbβ were also virtually unchanged, suggesting that the down-regulation of GPV and GPIbα had occurred through ectodomain shedding rather than internalization (21).

Aspirin Causes Down-regulation of GPIbα and GPV in Murine Platelets—To characterize the mechanisms underlying this unexpected aspirin effect in detail and to identify the responsible metalloproteinase

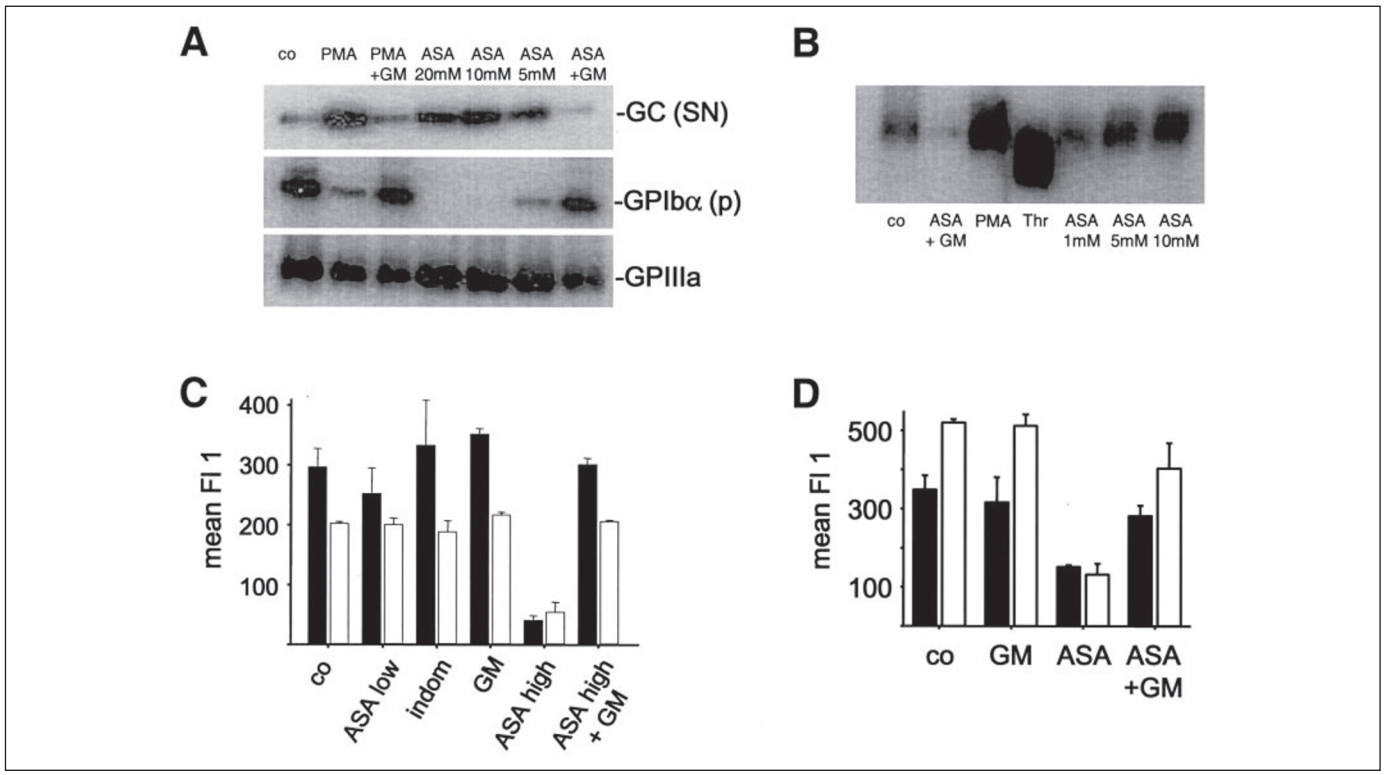

FIGURE 3. **Aspirin causes shedding of murine and human GPIbα and GPV in a metalloproteinase-dependent manner.** *A*, washed mouse platelets were incubated at 37 °C for 2 h with buffer (*co*), GM-6001 (*GM*, 100 μM), and aspirin (*ASA*) at the concentrations given above, whereas PMA (500 ng/ml) was incubated for 15 min at 37 °C. After centrifugation, platelets (*p*) and the supernatant (*SN*) were separated and GPIbα was detected by Western blot. To control loading, GPIIIa (80 kDa) was detected. *B*, biotinylated, washed platelets were incubated at 37 °C with buffer (*co*), aspirin (*ASA*) at different concentrations in the presence or absence of GM-6001 (*GM*, 100 μM). PMA (500 ng/ml) was incubated for 15 min and thrombin (*Thr*, 0.01 unit/ml) for 5 min at 37 °C. After centrifugation, cleaved fragments of GPIbα (glycocalicin, *GC*) and GPV were detected in the supernatant by immunoprecipitation. *C*, whole mouse blood was incubated with buffer (*co*), 1 mM aspirin (*ASA low*), indomethacin (*indom*, 200 μM), GM-6001 (*GM*, 100 μM), 10 mM aspirin (*ASA high*) for 2 h at 37 °C. The expression of GPIbα (*filled bars*) and GPV (*open bars*) were quantitatively determined by flow cytometry ($n = 7$). *D*, whole human blood was incubated with buffer (*co*), GM-6001 (*GM*, 100 μM), and aspirin (*ASA*, 20 mM) for 2 h at 37 °C. The expression of GPIbα (*filled bars*) and GPV (open bars) were quantitatively determined by flow cytometry ($n = 5$).

involved, we used the mouse as a model for further experiments. To test whether the observed effect can also be detected in the mouse system, murine whole blood was incubated with aspirin at different concentrations, and the expression of glycoproteins on the platelet surface was measured by flow cytometry at different time points. In these experiments, aspirin caused a dose-dependent down-regulation of GPIbα and GPV (Fig. 2, *A–D*), whereas no such effect was seen for other glycoproteins, including GPIbβ, GPVI, or αIIbβ3 or α5β1-integrins (Fig. 1*E*).

Aggregation experiments revealed that the cells were still responsive to different agonists. As shown in Fig. 2*E*, maximal aggregation induced by ADP, thrombin, phorbol 12-myristate 13-acetate (PMA), or collagen was slightly reduced in washed platelets incubated with 2 mM aspirin for 2 h. In the case of PMA and collagen, the onset of aggregation was moderately delayed reflecting lack of thromboxane A2 release due to COX-1 inhibition. Similar results were obtained in platelets that were incubated with high dose aspirin (10 mM). Maximal aggregation was reduced compared with untreated platelets, and the delay in the onset of aggregation in PMA- or collagen-stimulated samples was increased to a similar extent as observed for platelets that were incubated with low dose aspirin (2 mM). This demonstrated that incubation of platelets with high doses of aspirin induces shedding of GPV and GPIbα but largely preserves the signaling machinery required for aggregation.

In all experiments (in mice or humans), we used lysine-coupled aspirin, because acetylsalicylic acid alone is very poorly water-soluble. Therefore, to exclude any lysine-mediated artificial effects, lysine was tested in parallel (10 mM). As shown in Fig. 2 (*C* and *D*), lysine alone, at concentrations at which aspirin induced shedding, did not alter GPIbα or GPV expression demonstrating that a specific aspirin-related mechanism was responsible for the observed shedding.

Under *in vitro* and *in vivo* conditions, aspirin is rapidly deacetylated to release salicylic acid. To delineate which compound is involved in aspirin-induced shedding, whole blood was incubated with salicylic acid (10 mM). As shown in Fig. 2 (*C* and *D*), salicylic acid did not cause down-regulation of murine GPIbα or GPV strongly suggesting that aspirin causes shedding via an acetylation-dependent mechanism. Similar results using lysine or salicylic acid were obtained with human platelets (not shown).

Aspirin Induces Shedding via Metalloproteinase Activation in Human and Mouse Platelets—Recent studies suggest an involvement of metalloproteinases in receptor shedding in platelets as well as in leukocytes (9, 28). To test an involvement of sheddases in our system, we incubated whole blood either with vehicle or aspirin in the presence or absence of the broad-range metalloproteinase inhibitor GM-6001 (100 μM). The protein kinase C activator PMA (500 ng/ml) is a well known activator of metalloproteinase-dependent shedding and therefore was used in our control experiments. After incubation of washed platelets with aspirin for 2 h, platelets were centrifuged and the supernatant was collected to detect the shed fragments of GPIbα (glycocalicin) and GPV. As shown in Fig. 3 (*A* and *B*), GPIbα and GPV were found in the supernatant of PMA- and aspirin-treated samples. Release of both glycoproteins was blocked completely in the presence of 100 μM GM-6001 showing that metalloproteinases are involved in this process. Whereas aspirin and PMA induced the release of an 80-kDa fragment of GPV, thrombin directly cleaved GPV at a different site resulting in a smaller fragment

size (69 kDa). Accordingly, remaining fragments of GPIbα after aspirin treatment were found in the platelet membrane. In parallel experiments, shedding of murine and human GPIbα and GPV was quantitatively determined by fluorescence-activated cell sorting analysis. In the presence of the broad-range metalloproteinase inhibitor GM-6001, shedding was blocked comparably in both murine (Fig. 3*C*) and human platelets (Fig. 3*D*). In parallel experiments, the effect of ibuprofen and indomethacin on GPIbα and GPV levels was tested in dose-response experiments. For up to 4 h, neither ibuprofen (10 μM to 20 mM, not shown) nor indomethacin (1–200 μM) induced significant shedding of the two receptors. The results for the highest concentration of indomethacin (200 μM) are shown in Fig. 3*C*.

Aspirin-induced Shedding Is COX-independent but Essentially Involves ADAM17 Activation—It is well established that aspirin irreversibly inhibits platelet function by acetylating COX-1 at serine 530 (13, 29) thereby inhibiting binding of arachidonic acid that serves as a substrate for the generation of the platelet agonist thromboxane A2 (30). In our experiments, inhibition of COX-1 by established inhibitors such as indomethacin and aspirin at low dose (2 mM) did not alter the expression levels of GPIbα or GPV (Fig. 3*C*) suggesting that shedding occurs independently of COX inhibition. To test this directly, we analyzed platelets from COX-1-deficient mice. In these platelets, aspirin induced shedding of GPIbα and GPV to a similar extent and with similar kinetics as in wild-type mice (Fig. 4) demonstrating that COX-1 is not essentially involved in aspirin-induced shedding of these receptors.

In neutrophils, ectodomain shedding of L-selectin occurs in an ADAM17-dependent manner (9), and recent studies have shown that ADAM17 is the metalloproteinase that mediates GPIbα and GPV shedding from the platelet surface (5, 6). To test directly whether the shedding of these receptors in response to aspirin is mediated by ADAM17, we used chimeric mice that express inactive ADAM17 in blood cells only. For that purpose, we transplanted irradiated wild-type recipient mice with fetal liver cells isolated from ADAM17$^{+/+}$ embryos or embryos carrying a targeted deletion of the Zn^{2+}-binding domain in mice (ADAM17$\Delta^{Zn/\Delta Zn}$) (7). Although normal shedding occurred in control platelets in response to aspirin (150 mM), this process was completely abrogated in ADAM17$\Delta^{Zn/\Delta Zn}$ platelets (Fig. 4). This finding demonstrated that aspirin stimulates shedding of GPIbα and GPV in an ADAM17-dependent manner.

Aspirin-induced Shedding Occurs in Vivo—To test whether aspirin can induce shedding of GPIbα and GPV *in vivo*, we intravenously injected mice with vehicle, low (80 mg/kg mouse), or high concentrations of aspirin (150 mg/kg mouse), and GPIbα and GPV fragment distribution in the blood was analyzed after 2 h. Although aspirin (high or low dose) had no significant effect on the number of circulating platelets and the surface levels of GPV and GPIbα on circulating platelets (Fig. 5, *A*, *B* and *C*), plasma levels of shed GPIbα and GPV were increased 1.5- and 3-fold, respectively, as compared with vehicle-treated control mice (Fig. 5*A*). This result demonstrated that high doses of aspirin induced increased plasma levels of soluble GPIbα and GPV clearly indicating that aspirin-induced shedding of both receptors occurs *in vivo*.

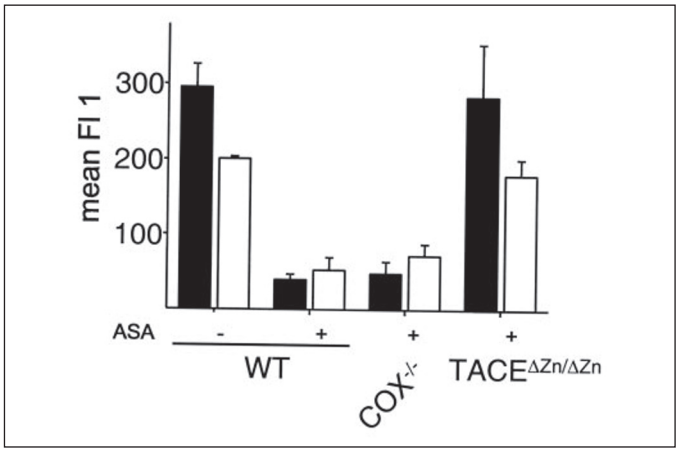

FIGURE 4. **Aspirin-induced shedding of GPIbα and GPV is independent of COX-1 inhibition or absence but essentially involves ADAM17 activation.** Whole blood was incubated with 10 mM aspirin (*ASA*) for 2 h at 37 °C and expression of GPIbα (*filled bars*) and GPV (*open bars*) were measured by flow cytometry ($n = 5$). Both mutant and wild-type animals were of 129/Sv x C57BL/6 genetic background.

DISCUSSION

Aspirin is widely used as a painkiller, as an anti-inflammatory drug, and to prevent myocardial infarction or stroke. Its use is frequently accompanied by a massively increased bleeding tendency in some patients, a side-effect that is not observed with other COX-1 inhibitors such as ibuprofen indicating other mechanism(s) to be involved. Here, we report that aspirin induces the proteolytic cleavage of human and murine platelet surface receptors by activating the metalloproteinase ADAM17. This process is relevant *in vivo*, because mice injected with high doses of aspirin have increased levels of shed GPIbα and GPV in their plasma.

The mechanisms by which aspirin and other NSAIDs exert their action are mainly attributed to their interference with the formation of prostaglandins that mediate inflammation, swelling, pain, and fever. As members of the prostaglandin family also protect the stomach mucosa from damage by hydrochloric acid and lead to platelet aggregation, a unifying explanation is given to affirm the shared side-effects of this class of drugs such as gastrointestinal bleeding (for review see Ref. 31). Recently, a novel mechanism has been added to the known mode of action of NSAIDs as in leukocytes NSAIDs, including aspirin at therapeutically relevant concentrations caused shedding of L-selectin in a ADAM17-dependent manner *in vitro* (8, 9), possibly explaining the anti-inflammatory action of these compounds in patients. The signaling

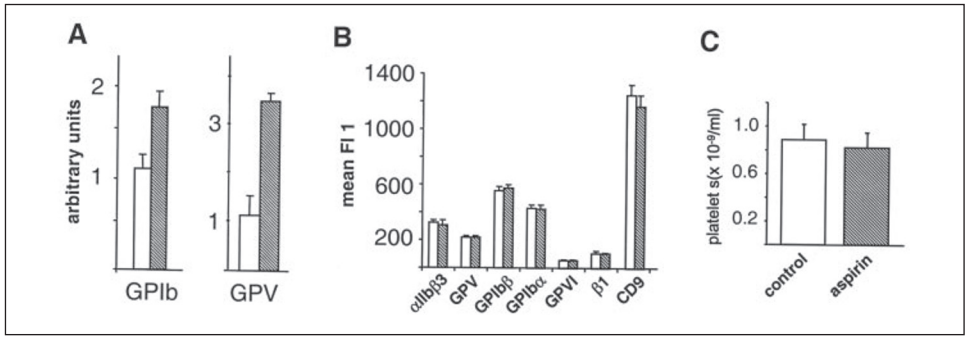

FIGURE 5. **Aspirin-induced shedding of GPIbα and GPV occurs *in vivo*.** *A*, mice were injected with buffer (*white bars*) or high-dose aspirin (150 mg/kg mouse, *shaded bars*). After 2 h, plasma was isolated and GPIbα and GPV fragments were measured by ELISA ($n = 5$). In these treated animals, the expression of prominent glycoproteins on the platelet surface was measured by flow cytometry (*B*) and platelet counts were determined (*C*).

cascade underlying this process might involve the uncoupling of the mitochondrial oxidative phosphorylation and a reduction in the intracellular ATP concentration (32–34). Similar mechanisms appear to underlie shedding of glycoproteins in platelets. By the use of the non-physiological, lipid-soluble amphiphatic molecule CCCP (carbonylcyanide *m*-chlorophenylhydrazone), it has been shown that the induction of mitochondrial damage followed by an uncoupling of the oxidative phosphorylation leads to the down-regulation of prominent glycoproteins such as GPIbα, GPV, and GPVI (5, 6, 28). Comparable to L-selectin shedding in leukocytes, GPIbα and GPV shedding in this experimental system occurs in an ADAM17-dependent manner, and this pathway is also triggered by aspirin. However, mechanistic differences appear to exist between CCCP and aspirin-induced metalloproteinase activation, because CCCP also induces shedding of the collagen receptor GPVI, whereas aspirin has no such effect (Fig. 1*E*). Similarly, stimulation of mouse platelets with agonists such as collagen-related peptide or PMA results in ADAM17-dependent down-regulation of both GPIbα and GPV but not GPVI (5, 6, 28). Possibly, signaling pathways similar to those induced by physiological agonists underlie aspirin-caused shedding of GPIbα and GPV converging in the activation of ADAM17, whereas by strong mitochondrial damage additional or other pathways might become activated leading to GPVI down-regulation by other metalloproteinases, such as ADAM10 (35, 36). However, the possible involvement of other metalloproteinases cannot be investigated at present as there are no specific inhibitors or gene-deficient mice available.

Although ADAM17 has been recognized as an essential sheddase, the mechanisms underlying its activation are still unclear. Previously, it has been reported that apoptotic pathways might participate in this shedding process (37) similar to those occurring in aged or damaged cells supporting the idea that shedding plays a role in the clearance of platelets (4). Recent studies suggest a regulation of shedding events by calmodulin binding to membrane-proximal sequences of target receptors (38, 39). Accordingly, down-regulation of GPVI was shown to be induced by incubation of platelets with calmodulin inhibitors suggesting that shedding might be triggered by disruption of calmodulin from the cytoplasmic tail of GPVI (40). It seems possible that aspirin might interfere with these suggested pathways by acetylation of one or more substrates. But, clearly further work will be required to test this hypothesis.

Although ectodomain shedding has been recognized as a key mechanism in the regulation of platelet surface receptors, its physiological role is not fully understood. Recent studies indicate that it might participate in the clearance of aged platelets from the circulation, because GPIbα shedding correlates with the removal of damaged cells (4). Interestingly, CCCP-induced shedding of mouse or human GPIbα, GPV, and GPVI is accompanied by morphological alterations of the platelets such as shape change and disruption of membrane asymmetry that are similar to *in vitro* aged mouse or human platelets. Supportive *in vivo* data are derived from canine platelets, in which GPVI function was found to decline progressively with age (41). These data are substantially fostered by the observation that platelets deficient in functional ADAM17 display a significantly prolonged survival *in vivo* after CCCP treatment as compared with wild-type platelets after the same treatment. Therefore, it is feasible to speculate that metalloproteinase-dependent shedding occurs under physiological conditions, thereby controlling the clearance of aged platelets.

Shedding might also influence the hemostatic function of platelets as most of the receptors affected are adhesion receptors, such as P-selectin (42), CD40 ligand (43), GPVI (28), GPIbα (5), and GPV (6). This hypothesis is supported by the observation that *in vivo* model of arterial thrombosis ADAM17$^{\Delta Zn/\Delta Zn}$ platelets showed enhanced adhesion as compared with wild-type controls (5). Interestingly, the platelet count in the mutant mice was normal although the life-time of the cells after the induction of mitochondrial injury is increased.

We detected increased plasma levels of shed GPIbα and GPVI in aspirin-treated mice demonstrating that shedding processes occur *in vivo* (Fig. 5*A*). Interestingly, this was not accompanied by a significant decrease in the circulating platelet count or the surface expression levels of both receptors on the circulating cells (Fig. 5*B*). This suggests that either those platelets that had shed their receptors were removed from the circulation or, alternatively, the loss of receptors is compensated by mechanisms to regulate the expression of these receptors. Possibly, the remaining fragments of GPIbα and GPV on the platelet get internalized and an intact GPIb–V–IX receptor complex comes to the surface instead as the complex is stored in the surface-connected open canalicular system (SCCS) inside the cell. Alternatively, both mechanisms may contribute to reconstitute a normal platelet population in aspirin-treated animals.

Here, we have shown that aspirin causes shedding of human and murine GPIbα and GPV *in vitro*. This process is acetylation-dependent and essentially involves activation of the metalloproteinase ADAM17. Although this novel anti-platelet mechanism of aspirin can be observed *in vivo* in the mouse, it is unlikely that receptor shedding occurs in healthy humans, because after intake of aspirin plasma levels of about 2 mM are reached and shedding takes place in our experiments at concentrations of 20 mM. However, in some patients with impaired renal clearance (16) or patients that display massive bleeding after aspirin intake (hyper responders (44)) shedding may occur at concentrations of aspirin lower than 20 mM. Although we do not have evidence for this, these data might suggest that effective compensatory mechanism may exist to prevent shedding under therapeutically relevant conditions in humans. Further experiments will be required to identify the physiological regulators of receptor shedding.

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