Antiplatelet Therapy

Aspirin Extrusion From Human Platelets Through Multidrug Resistance Protein-4–Mediated Transport

Evidence of a Reduced Drug Action in Patients After Coronary Artery Bypass Grafting

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Objectives	In this study we investigate: 1) the role of multidrug resistance protein-4 (MRP4), an organic anion unidirectional transporter, in modulating aspirin action on human platelet cyclooxygenase (COX)-1; and 2) whether the impairment of aspirin–COX-1 interaction, found in coronary artery bypass grafting (CABG) patients, could be dependent on MRP4-mediated transport.
Background	Platelets of CABG patients present a reduced sensitivity to aspirin despite in vivo and in vitro drug treatment. Aspirin is an organic anion and could be a substrate for MRP4.
Methods	Intracellular aspirin concentration and drug COX-1 activity, measured by thrombin-induced thromboxane B2 (TxB2) production, were evaluated in platelets obtained from healthy volunteers (HV) and hematopoietic-progenitor cell cultures reducing or not reducing MRP4-mediated transport. Platelet MRP4 expression was evaluated, in platelets from HV and CABG patients, by dot-blot or by immunogold-electromicrographs or immunofluorescence-microscopy analysis.
Results	Inhibition of MRP4-mediated transport by dipyridamole or Mk-571 increases aspirin entrapment and its in vitro effect on COX-1 activity (142.7 ± 34.6 pg/10^8 cells vs. 343.7 ± 169.3 pg/10^8 cells TxB2-production). Platelets derived from megakaryocytes transfected with MRP4 small interfering ribonucleic acid have a higher aspirin entrapment and drug COX-1 activity. Platelets from CABG patients showed a high expression of MRP4 whose in vitro inhibition enhanced aspirin effect on COX-1 (349 ± 141 pg/10^8 cells vs. $1,670 \pm 646$ pg/10^8 cells TxB2-production).
Conclusions	Aspirin is a substrate for MRP4 and can be extruded from platelet through its transportation. Aspirin effect on COX-1 is little-related to MRP4-mediated aspirin transport in HV, but in CABG patients with MRP4 over-expression, its pharmacological inhibition enhances aspirin action in an efficient way. (J Am Coll Cardiol 2011;58:752–61) © 2011 by the American College of Cardiology Foundation

The ability of aspirin to act as an antiplatelet agent, reducing cardiovascular complication in high-risk patients, is well-documented (1). However, it was evidenced that aspirin does not exert an equal antiplatelet action on all subjects (2–4) and that platelet sensitivity to the inhibitory effect of aspirin progressively decreases with time (5). Clinical and biochemical evidence support that a persistent thromboxane A2 (TxA2) production is the most likely cause of the residual platelet function, despite aspirin treatment. Particularly, persistent thromboxane generation predicts the risk of the composite outcome of myocardial infarction, stroke,

or cardiovascular death (6–8); patients under chronic aspirin treatment display an interindividual variability of TxA2 production that is functionally important in determining platelet activation (9).

Importantly, 5 days after coronary artery bypass grafting (CABG), patients had a high incidence of transitory aspirin resistance, due to a disturbed inhibition of platelet cyclooxy-

genase (COX)-1 by aspirin (10), because a residual TxA2 production was found despite in vivo plus in vitro aspirin treatment. The molecular mechanisms are still unclear.

Multidrug resistance protein-4 (MRP4) is a member of the MRP/ABCC subfamily of adenosine triphosphate (ATP)-binding cassette transporters, which are capable of pumping a wide variety of structurally diverse endogenous and xenobiotic organic anions out of the cell (11). Over-expression of MRP4 severely impaired the antiviral efficacy of nucleoside analogs in the human T-lymphoid cell line CEM-r1 (12). It has been recently demonstrated that human platelets express MRP4 and drive active transport of nucleotides into dense granules (13). Aspirin is a weak acid (pKa4.4) and can be rapidly converted into organic anion, acetyl-salicylate, becoming a possible MRP4 target.

Hence, the main aim of this study was to verify whether aspirin is a substrate for MRP4 in platelets and whether it reduces drug activity by aspirin extrusion. Moreover, we studied whether the impairment of aspirin–COX-1 interaction, found in CABG patients, could be dependent on MRP4-mediated transport.

Our studies were performed in platelets from healthy volunteer (HV) human hematopoietic progenitor cell (HPC) cultures and CABG patients.

Methods

Sources of materials. Monoclonal antibodies anti-CD34, anti-CD61, anti-CD62, and anti-CD42b were from Becton Dickinson (Franklin Lakes, New Jersey); anti-CD41a was from AdB Serotec, (Kidlington, United Kingdom). Arachidonic acid (AA) was from Cayman-Chem (Ann Arbor, Michigan). All nonspecified chemicals were from Sigma Chemicals Company (St. Louis, Missouri).

Platelet preparation. Platelets were obtained from healthy Caucasian volunteers (HV) (50% women and 50% men; age range 25 to 58 years), who had not been taking any medication for the previous 2 weeks; CABG patient platelets were obtained both at 5 (CABG5 patients) and 10 (CABG10 patients) days after surgery (for characteristics, see Table 1). None of the patients received additional COX inhibitors. Informed written consent was obtained from each patient.

Table 1	Baseline Characteristics of CABG Patients (N = 26)
Age (yrs)	55 ± 8
Men	15 (58%)
Medications	
Aspirin 100 mg/day*	26 (100%)
Calcium antagonist†	26 (100%)
ACE inhibitors/ARB†	26 (100%)
Beta-blocking agents†	26 (100%)
Statins†	26 (100%)

Values are mean ± SD or n (%). *From Day 1 after surgery, previous aspirin treatment was terminated 10 days before CABG. †From Day 2 after surgery.

ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; CABG = coronary artery bypass graft surgery.

Platelet-rich plasma and plasma-free platelets (PFP) preparation were obtained as previously reported (9). In all experiments platelets were resuspended to a final concentration of 3×10^8/ml. **Aspirin and salicylic acid intra-platelet concentration.** The PFP were treated with aspirin (50 μmol/l; 10°C, 15 min) and then kept at 37°C or 4°C (15 min) and washed twice. Two of the most efficient MRP4 inhibitors (11), dipyridamole and MK-571 (0.1 mmol/l both), were added to the platelet suspension before aspirin (15 min, RT). Aspirin and salicylic acid (SA) intraplatelet concentration was evaluated as described (14).

Dense granules rich preparation. Dense granules rich preparation (DGP) was performed as described (15). Dense granules free preparation was performed according to Niessen et al. (16). After the addition of ATP (5 mmol/l) or apyrase (0.1 U/ml), aspirin was added to the DGP and kept either at 4°C or 37°C. Dipyridamole or MK-571 were added as before.

Fluo-cyclic adenosine monophosphate MRP4-mediated transport. The MRP4-dependent 8-Fluo-cyclic adenosine monophosphate (cAMP) uptake in platelet granules was analyzed as previously described (17). Briefly DGP was incubated with 8-Fluo-cAMP, aspirin, and SA (all 20 μmol/l). After 10 min, DGP was washed and fluorescence was measured in Victor3-fluorometer (PerkinElmer, Waltham, Massachusetts). The fluorescence observed in ATP-free DGP was used as blank. **^{14}C-acetylsalicylic acid platelet incorporation and secretion.** After dipyridamole treatment, the PFP suspension was incubated with ^{14}C-labeled aspirin (American Radiolabeled Chemicals, St. Louis, Missouri) for 30 min at 37°C (18). The cells were washed to remove extra-cellular ^{14}C-labeled aspirin.

Secretion was evaluated by measurement of the ^{14}C-radioactivity in a supernatant of thrombin (0.2 U/ml)

activated PFP. The ATP released was measured with a commercial kit (ATPlite, PerkinElmer).

Thromboxane B2 production. Thromboxane B2 (TxB2) production, a stable TxA2 metabolite, was studied in PFP, activated with thrombin 1 U/ml for 1 h at 37°C, and evaluated with a commercial kit (Cayman-Chem).

Measurement of prostaglandin H synthase activity. prostaglandin H synthase activity was performed as previously reported (19). Briefly, platelets were pre-loaded with 2 μmol/l 5-(and-6)-carboxyl-2′,7′-dichlorodihydrofluorescein (CDCF) (MolecularProbes, Eugene, Oregon), which serves as a reducing substrate for the peroxidase activities of COX-1. The platelet COX activity was measured as AA-induced (5 μmol/l) fluorescence enhancement in a spectro-fluorimeter Kontron-SFM25 (Zurich, Switzerland), thermostatically regulated (37°C). Nicotinamide adenine dinucleotide phosphate oxidase inhibitor (diphenyliodonium) (5 μmol/l) was added to avoid reactive oxygen species production interference. The results are reported as the rise in CDCF fluorescence (Arbitrary Units) recorded for 1 min.

Platelet aggregation. Platelet aggregation was evaluated in PFP with the Born method with collagen (0.5 to 10 μg/ml) as agonist (5).

HPC purification and megakaryocyte unilineage cultures. Adult peripheral blood was obtained from HV. Low-density mononuclear cells were isolated by Ficoll-Hypaque density-gradient centrifugation, and CD34$^+$ HPCs were then purified with the MiniMACS columns (Miltenyi Biotech, Paris, France). Purified cells were more than 90% CD34$^+$, evaluated by fluorescence-activated cell sorting analysis.

Purified HPCs (1 $\times$ 10^5 cells/ml) were grown in unilineage megakaryocyte (MK) liquid culture (20) for 13 days. The cells were collected at different days of differentiation, and platelets—produced at the end of the culture—were isolated as reported in Guerriero et al. (21).

Transfection of small interfering ribonucleic acid. At Day 9 of culture, differentiated MKs were seeded in antibiotic-free media and transfected with MRP4 small interfering ribonucleic acid (siRNA) (160 nmol/l) (SmartPool-siRNA human ABCC4, Dharmacon, Chicago, Illinois) in presence of Lipofectamine 2000 (Invitrogen, Carlsbad, California). An equivalent amount of transfection reagent (Lipofectamine 2000, Invitrogen), without MRP4-siRNA, was added as mock control. After transfection the cells were reincubated in MK liquid culture for terminal differentiation. Four days later, mature MKs were analyzed and derived platelets were aspirin-treated and thrombin-stimulated.

Immunoblot analysis. Platelets or cell protein extract were prepared as previously reported (22). Protein expression was evaluated by Western blot with polyclonal antibodies against MRP4 (Alexis, Plymouth Meeting, Pennsylvania). Monoclonal antibody anti−beta-actin was used as control. Scalar concentration of each sample has been spotted onto nitrocellulose membrane, and dot-blot analysis has been performed as previously reported (23), with the same polyclonal antibody as reported in the preceding text. The densitometric analysis was performed with the National Institutes of Health ImageG analyzer program.

Immunoelectron microscopy and immunofluorescence. Platelets were fixed and processed for ultrathin cryosectioning and immunogold labeling as previously described (24). The primary antibody was omitted in control subjects. For cryosection immunoelectron microscopy, platelets were labeled with rabbit anti-MRP4 polyclonal followed by protein A−10 nm gold (G. Posthuma and J. Slot, Utrecht, the Netherlands). For immunofluorescence, platelets were incubated with anti-MRP4 polyclonal followed by FITC-anti-rabbit (Cappel, Cochranville, Pennsylvania). In double labeling experiments, platelets were subsequently incubated with anti-LAMP2 (Santa Cruz Biotechnology, Santa Cruz, California) or anti-CD42b (AlphaDiagnostic International, San Antonio, Texas) monoclonals followed by Texas-Red-conjugated anti-mouse (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania). Images at 40× and 60× were taken with an AxioCam with a Zeiss Apotome Axio Observer Z1 inverted microscope (Carl Zeiss, Thornwood, New York).

Measurement of MRP4 transport activity. The MRP4 transport activity was evaluated according to Bai et al. (25), which takes advantage of bimane to form a fluorescent adduct with glutathione that is a target for MRP4. Briefly, PFP was incubated (6 min, 10°C) with 100 μmol/l MonoChloroBimane (Sigma Chemical Company) and divided into 2 aliquots; 1 was kept in ice, and 1 was incubated at 37°C (7.5 min). Both aliquots were washed, and supernatants fluorescence was read in a Victor3-fluorimeter (PerkinElmer). The MRP4-mediated transport activity was expressed as the Delta bimane-glutathione (bimane-GS) efflux between incubation at 37°C and ice, quantified as previously reported (25).

We performed experiments with carbonylcyanidem-chlorophenylhydrasone, 1-chloro-2,4-dinitrobenzene, and MK571 treated platelets (25).

Prostaglandin E2 production. Arachidonic acid-induced prostaglandin E2 (PGE2) production measured aspirin action in human embryonic kidney (HEK)-293 cells (HEK-Co) and in transiently transfected cells over-expressing recombinant ABCC4 (HEK-ABCC4) according to (26). Briefly, cells, pretreated with aspirin (100 and 200 μmol/l for 30 min) were activated with AA (5 min). The PGE2 secreted was measured by ELISA assay kit (CaymanChem).

Statistical analysis. Results are reported as mean ± SD performed on different platelet samples. Significance was evaluated by Student t test for paired data in in vitro experiments and for unpaired data with unequal variance (Welch t test) for in vivo dosages.

Results

Role of MRP4-mediated transport in human platelet aspirin intake. To investigate whether aspirin is a substrate and can be transported by MRP4, we used DGP, obtained from HV human platelets, because MRP4 is predominantly
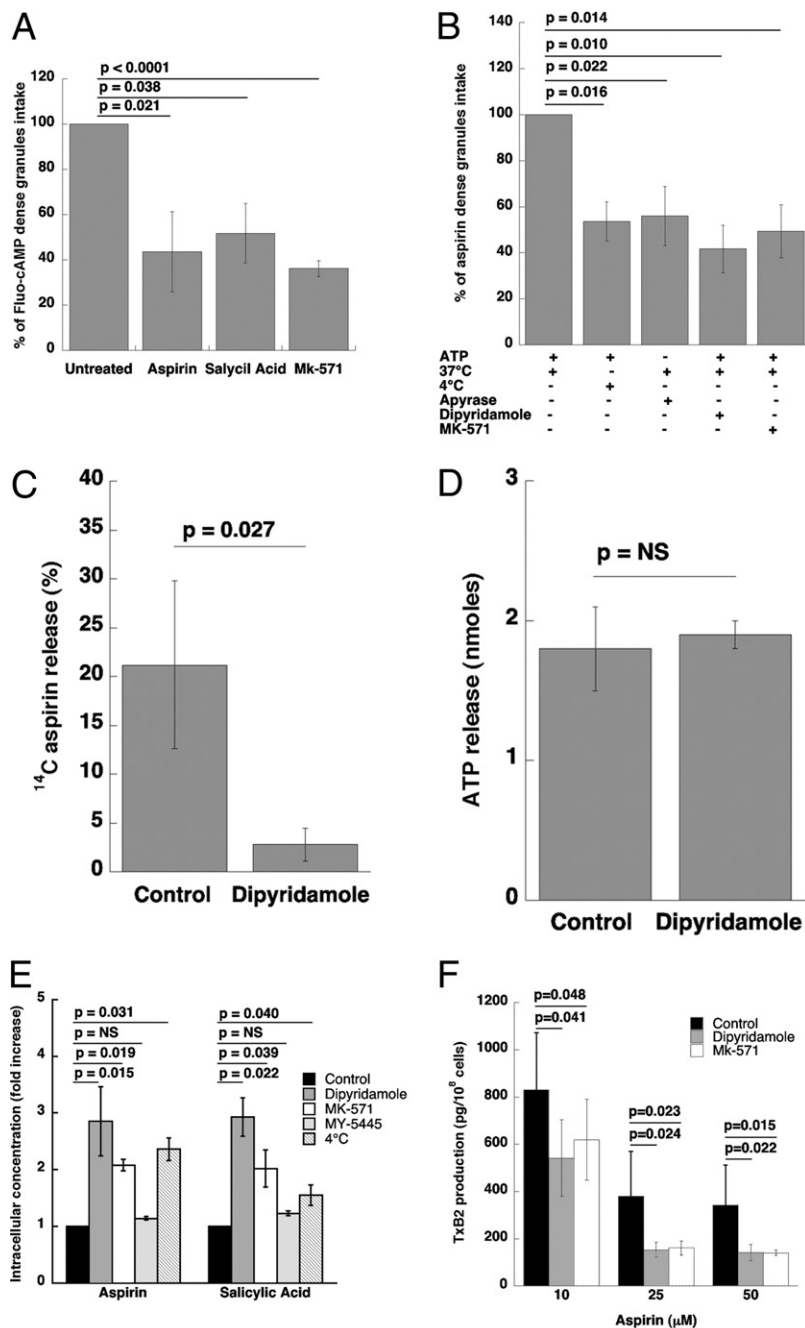
Figure 1   Aspirin Interaction Assay With MRP4 in DGP and Platelets

(A) Percentage of intake Fluo-cyclic adenosine monophosphate (cAMP) evaluated in dense granules rich preparation (DGP) pre-treated with aspirin, salicylic acid (SA), and MK571. Results are the mean ± SD of 4 experiments. **(B)** Percentage of aspirin intake in DGP. Adenosine triphosphate (ATP) at 37°C condition, assumed as control (100%). The DGP were treated at 4°C or with apyrase, dipyridamole, or MK-571. Results are shown as the mean ± SD of 7 experiments. Aspirin concentration found in DGP was 53 ± 23 pg/mg proteins. **(C, D)** Percentage of ^{14}C-aspirin and ATP release, after thrombin stimulation, from platelets labeled with ^{14}C-aspirin (Control) or with dipyridamole. Percentage of ^{14}C-aspirin release versus total radioactivity intake and nmoles of ATP secreted, respectively. Mean ± SD of 4 experiments. **(E)** Aspirin and SA cytosolic concentration, in platelets pre-treated with dipyridamole, MK-571, or MY- 5445 or incubated at 4°C. The results were expressed as fold increase versus aspirin-treated platelets (Control). Mean ± SD of 8 experiments. Aspirin and SA concentrations found in control were 18.3 ± 5.2 pg/10^{9}cells and 13.1 ± 8.5 pg/10^{9}cells, respectively. **(F)** Thromboxane B2 (TxB2) production in aspirinated platelets dipyridamole or MK-571 pretreated or not. Mean ± SEM of 4 experiments. The TxB2 production in non-aspirinated platelets was 78,645 ± 11,317 pg/10^{8}cells.

localized in dense granule membranes (12). To demonstrate that aspirin is a substrate for MRP4, we used Fluo-cAMP, a fluorimetric analog of cAMP (17). In DGP both aspirin and SA reduced the intake of Fluo-cAMP, suggesting that both aspirin and SA interact with MRP4 solely and specifically (Fig. 1A).

To verify whether aspirin can be transported by MRP4, its DGP intake was evaluated in the presence of ATP at 37°C and compared with that obtained by reducing active transport (i.e., by aspirin treatments at 4°C or with an ATP-scavenger agent, apyrase) or inhibiting MRP4 by dipyridamole or MK-571. The results reported in Figure 1B show that the highest aspirin entrapment in dense granules was obtained in the presence of ATP at 37°C (control). With incubation at 4°C or platelet pre-treatment either apyrase or dipyridamole or Mk-571, it was observed a reduced aspirin intake.

In dense bodies-free mixture of platelet organelles, we found no changes in aspirin intake between 4°C and 37°C treatment in the presence of ATP (data not shown).
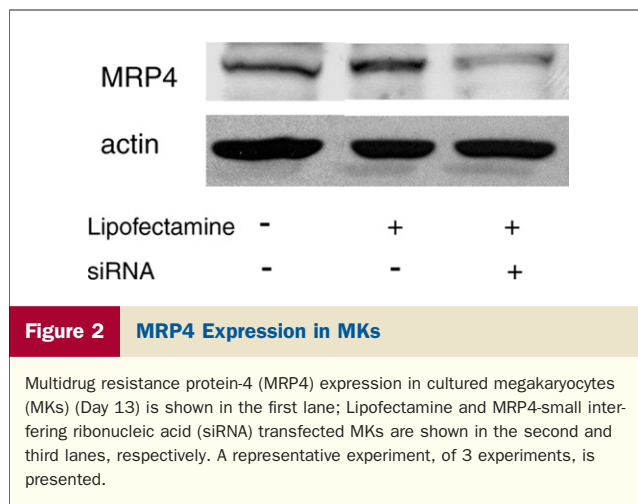
After activation platelets release dense granule contents; thus, we measured the secretion of ^{14}C-aspirin from thrombin-activated platelets to confirm that aspirin is able to enter into dense granules. ^{14}C-aspirin loaded platelets release the drug after thrombin activation that is markedly reduced by dipyridamole pre-treatment (Fig. 1C), whereas ATP release—analyzed to exclude nonspecific dipyridamole action—was unaffected (Fig. 1D).

To investigate whether aspirin can be extruded from platelets through the MRP4-mediated transport, we measured the intracellular concentration of aspirin and SA, in platelets pre-treated or not with MRP4 inhibitors (dipyridamole or MK571). Our results showed that inhibition of MRP4-mediated transport enhanced aspirin and SA platelet accumulation (Fig. 1E).

To exclude that this effect could be consequent to the inhibitory effect on phosphodiesterase type V (PDE-V), we use another PDE-V inhibitor, MY-5445. The results clearly demonstrated that the inhibition of PDE-V has no effect on enhancing aspirin and SA cytosolic concentrations (Fig. 1E).

Active MRP4-mediated transport requires a temperature of 37°C for maximal efficiency. An increased aspirin and SA intracellular concentration was found in platelets treated at 4°C, compared with treatment at 37°C (Fig. 1E). The lower increase of SA concentration could be due to reduced aspirin de-acetylation at 4°C.

To evaluate the MRP4 role in aspirin-dependent platelet COX-1 inhibition, we measured TxB2 production—a marker of the COX-1 pathway—in aspirin-treated platelets in the presence of MRP4 inhibitors. The TxB2 production was lower when dipyridamole or MK-571 was added to the platelet suspension before aspirin treatment (10, 25, and 50 μmol/l), as compared with platelets treated with aspirin alone (Fig. 1F). No statistical differences were found when dipyridamole and Mk-571 were added after aspirin (data not shown), thus suggesting a role of MRP4 in enhancing

Figure 2   **MRP4 Expression in MKs**

Multidrug resistance protein-4 (MRP4) expression in cultured megakaryocytes (MKs) (Day 13) is shown in the first lane; Lipofectamine and MRP4-small interfering ribonucleic acid (siRNA) transfected MKs are shown in the second and third lanes, respectively. A representative experiment, of 3 experiments, is presented.

aspirin effect on COX-1 without any important effect on platelet prostanoid efflux, found in other cell lines (27).

To confirm that the reduced TxB2 production induced by dipyridamole treatment is due to enhanced aspirin action, we performed experiments to evaluate COX-1 activity in CDCF-loaded platelets. The results showed that the COX-1–dependent prostaglandin H synthase activity is reduced in platelet treated with aspirin plus dipyridamole (66.4 ± 15.9% of inhibition vs. untreated platelets) in comparison with aspirin alone (49.7 ± 17.1% of inhibition vs. untreated platelets); no reduction was obtained when platelets were treated with dipyridamole alone (97.6 ± 1.9% of inhibition vs. untreated platelets). The MK-571 was not used, because it interferes with CDCF-fluorescence.

The reduction of TxB2 slightly decreases platelet activation; in fact, the EC50 of platelets pre-incubated with dipyridamole or MK-571 plus aspirin (3.5 and 2.5 μg/ml, respectively) show a reduction of collagen-induced platelet aggregation, in comparison with platelets treated with aspirin alone (1.8 μg/ml).

Inhibition of MRP4 expression by siRNA transfection in MK cultures. In our previous studies we optimized an in vitro unilineage serum-free liquid culture system for a virtually pure and complete MK differentiation, including pro-platelets and platelet formation (20). To evaluate MRP4 expression in MKs, CD34$^+$ HPCs purified from human peripheral blood were grown in MK liquid suspension culture, and differentiated cells were analyzed by Western blotting. The MK MRP4 expression is shown in Figure 2, first lane.

In an effort to confirm the MRP4 involvement in aspirin transport, we used an alternative approach of inhibition by siRNA experiments. Differentiated MKs from liquid suspension culture were transfected on day 9 with an MRP4 siRNA. Analysis of MRP4 protein expression was performed 4 days after transfection. Western blot analysis showed a strong decrease (up to 65%) of MRP4 protein levels in siRNA-transfected cells, as compared with untransfected cells treated only with Lipofectamine (Fig. 2).

After aspirin treatment, platelets derived from transfected MKs showed both a higher aspirin entrapment and greater aspirin effect on COX-1, in comparison with aspirin-treated platelets obtained from untransfected MK culture. Particularly, higher aspirin and SA cytosolic concentrations (1.64 ± 0.4-fold and 1.44 ± 0.3-fold increase, respectively) and lower thrombin-induced TxB2 production (73 ± 18 pg/10^8cells vs. 144 ± 21 pg/10^8cells) were found in aspirinated platelets derived from transfected MKs.
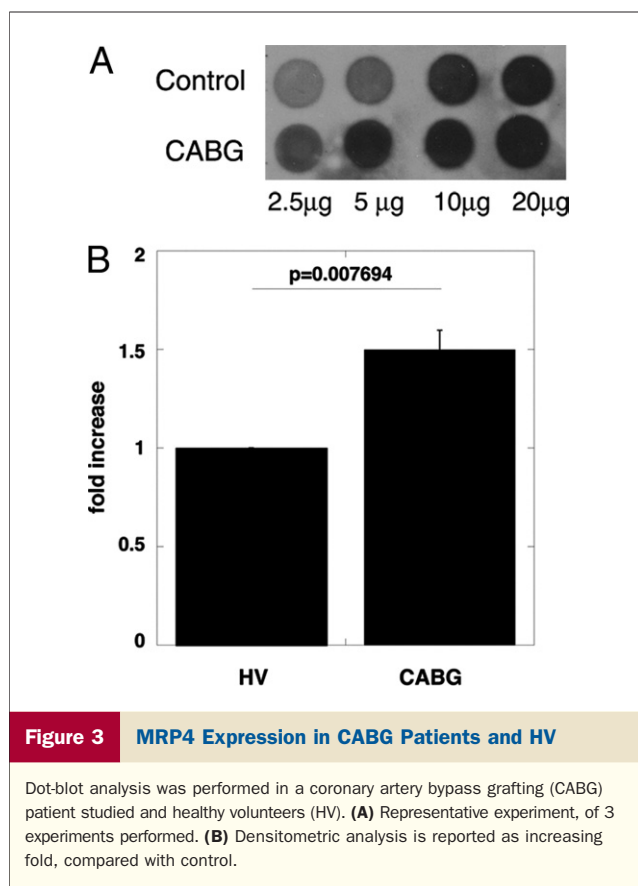
MRP4 expression in platelets from CABG patients. Our experiments confirm an insufficient inhibition of COX-1 in platelets of CABG5-patients under in vivo aspirin treatment; in fact, TxB2 production is approximately $7,431 \pm 3,260$ pg/10^8cells. Additional, in vitro aspirin treatment further reduced TxB2 production in CABG5-patient platelets to a value of $1,670 \pm 646$ pg/10^8cells (i.e., higher than that obtained in HV platelets after the in vitro same treatment [343.7 ± 169.3 pg/10^8cells]). Thus, to verify whether MRP4 inhibition can enhance the in vitro aspirin efficiency in these patients, we measured TxB2 production in CABG5-patient platelets treated in vitro with dipyridamole before aspirin. This treatment significantly reduces TxB2 production from $1,670 \pm 646$ pg/10^8cells to 349 ± 141 pg/10^8cells, thus reaching values similar to those obtained in HV (142.7 ± 34.6 pg/10^8cells). No reduction in TxB2 production relative to untreated platelets was observed with dipyridamole only (data not shown).

To investigate whether the insufficient response of the CABG5-patients to aspirin could be correlated with MRP4 expression, the same CABG5-patients analyzed for TxB2 production were studied for MRP4 expression in comparison with HV, with 3 different experimental approaches: dot-blot analysis, immunoelectron microscopy, and immunofluorescence.

Dot-blot analysis revealed that the MRP4 expression was significantly higher in these patients, as compared with HV (Fig. 3).

Immunoelectron microscopy studies were performed to investigate MRP4 intracellular distribution. In HV platelets MRP4 was mainly localized in intracellular dense granules and cytoplasmic vesicular elements (Fig. 4A, a), consistent with immunofluorescence data showing intracellular colocalization of MRP4 and LAMP-2 (Fig. 4B, d to f, j to l). In platelets from CABG5-patients (Fig. 4A, b), MRP4 staining appeared more intense and preferentially localized at/near the plasma membrane (Fig. 4A, b and c, arrows), whereas intracellular labeling was more disperse and mostly associated with vesicular elements. These observations were supported by double-immunofluorescence that showed MRP4 colocalizing mostly with the plasma membrane marker CD42 and less with the delta-granules marker LAMP-2 (Fig. 4B, a to e).

The MRP4 plasma membrane transport activity has been demonstrated, by analyzing the efflux of bimane-GS (25). In cells over-expressing MRP4 the efflux of the bimane-GS is facilitated (25). We performed experi-

Figure 3 **MRP4 Expression in CABG Patients and HV**

Dot-blot analysis was performed in a coronary artery bypass grafting (CABG) patient studied and healthy volunteers (HV). **(A)** Representative experiment, of 3 experiments performed. **(B)** Densitometric analysis is reported as increasing fold, compared with control.

ments with specific inhibitors to demonstrate that, also in platelets, the efflux of the bimane-GS is due to MRP4-mediated transport. Platelets were treated with 1-chloro-2,4-dinitrobenzene, to limit bimane-GS adduct, or carbonylcyanidem-chlorophenylhydrasone, to inhibit active transport, or MK571, to reduce MRP4-mediated transport. In all treatments the bimane-GS efflux was reduced (Fig. 5A), demonstrating the specificity of this method to study MRP4 transport activity in platelets too, according to Bai et al (25).

In CABG5-patients, efflux of the bimane-GS is strongly higher than HV (Fig. 5B).

In some patients MRP4 expression was analyzed in platelets from the same patients—both at 5 and 10 days after surgery. Although MRP4 expression was quantitatively similar, as assessed by dot-blot assays (data not shown), it appeared preferentially localized at the plasma membrane in CABG5-patient platelets, compared with those obtained in CABG10-patient platelets (Fig. 5C, a to f).

The MRP4 plasma membrane transport activity was reduced in CABG10-patients, compared with CABG5-patients (Fig. 5B). With regard to aspirin action, TxB2 production is reduced in CABG10-patient platelets ($1,377 \pm 491$ pg/10^8cells) versus CABG5-patients. In vitro aspirin addition further reduced TxB2 production (462 ± 194 pg/10^8cells). Dipyridamole treatment before aspirin addi-
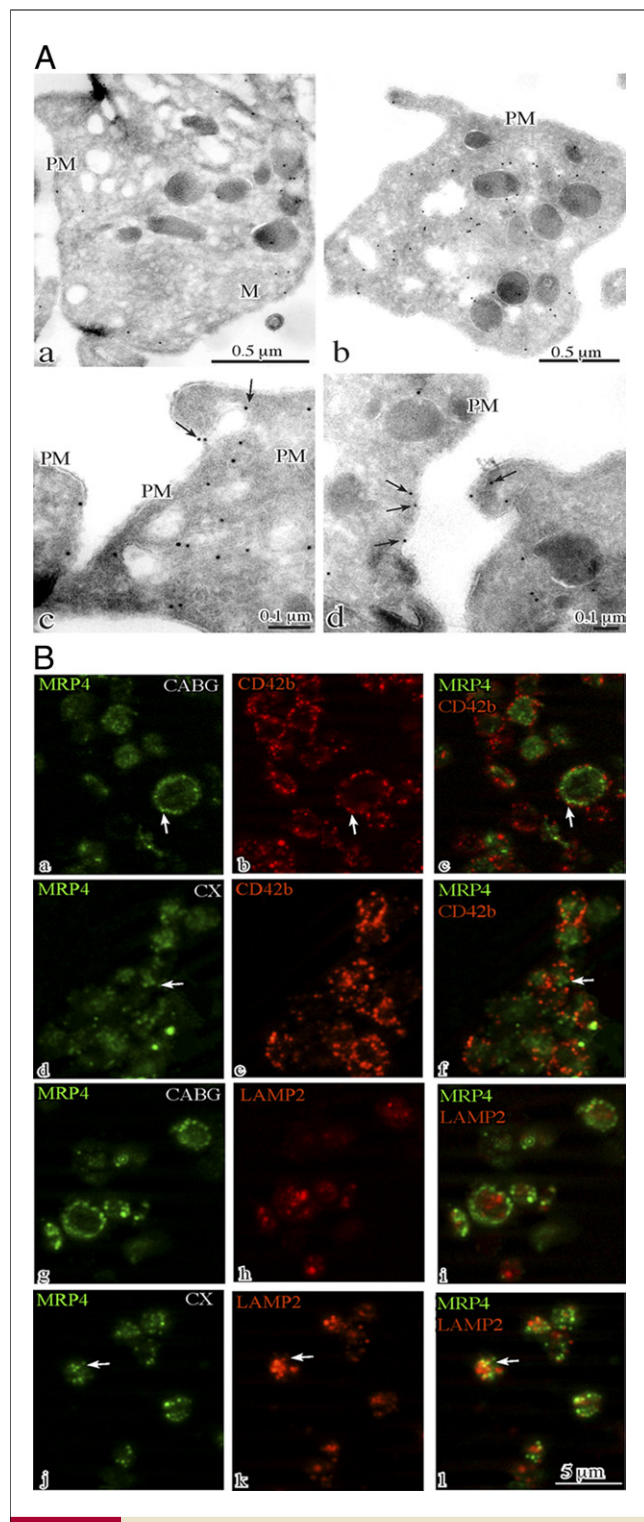
Figure 4   Platelet MRP4-Localization in HV and CABG Patients

(A) MRP4 subcellular localization in platelet ultrathin cryosections from HV **(a)** and CABG5 patients **(b)**. **(c, d)** High magnification evidences MRP4 localization in plasma membrane from CABG5 patient platelets. A representative experiment, of 5 experiments performed. **(B)** Double-labeled immunofluorescence microscopy of HV **(d to f, j to l)** and CABG5 patients **(a to c, g to i)** platelets. Platelets incubated with antibodies against MRP4 (green fluorescence) or CD42b or LAMP-2 (both red fluorescence). In **c, f, i, l**, colocalizing images are reported. Representative experiment, of 5 experiments performed. M = mitochondria; PM = plasma membrane; other abbreviations as in Figure 3.

tion caused the lowest thromboxane production (266 ± 215 pg/10^8cells).

Finally, to confirm that MRP4 over-expression reduces aspirin action on COX-1, we performed studies in HEK-ABCC4 (over-expressing MRP4). The PGE2 production, a COX-1 activation marker, is higher in HEK-ABCC4 cells than HEK-Co cells after aspirin treatment, thus indicating that MRP4 acts as a negative regulator of aspirin by limiting its action on COX-1 enzymes (Fig. 6). Such PGE2 effect is not due to the higher prostaglandin secretion, because MK571 treatment added after aspirin does not induce further reduction of the PGE2 (65% vs. 63%).

Discussion

The molecular mechanisms responsible for reduced aspirin action on COX-1 activity in CABG patients remain largely unclear. In this study, we investigated the role of MRP4-mediated transport in aspirin extrusion from human platelets and analyzed its modulation in platelets from CABG patients.

Our first series of experiments, performed in platelets from HV, demonstrated the role of MRP4 as aspirin transporter.

The MRP4 is highly concentrated in platelet delta granules (12). Therefore, we assessed that aspirin is a substrate for MRP4 and can be transported into dense granules. This study, carried out with DGP, provides evidence that aspirin reduces uptake of Fluo-cAMP, fluorescent substrate that is helpful in analyzing MRP4 function and regulation, and gets into dense granules through MRP4-mediated transport (17). The last observation was confirmed because we obtained a release of aspirin in thrombin-activated platelets.

Specific inhibition of MRP4 transport, such as low temperature and MRP4 inhibitors, increases intraplatelet entrapment of aspirin and SA. The MRP4 inhibitors markedly reduced such aspirin release, whereas ATP secretion was not affected.

The enhancement of the intraplatelet aspirin concentration, due to the MRP4 inhibitors dipyridamole or MK-571, leads to greater aspirin efficiency. In fact, in HV, when platelets were pre-treated with MRP4 inhibitors before aspirin addition, TxB2 production was lower than platelets treated with aspirin only.

The MRP4 is also a prostanoid efflux pump (27); we can exclude that the TxB2 reduction observed is dependent on a direct effect of MRP4-mediated thromboxane release. In fact, only a slight reduction is observed when aspirin is added before the MRP4 inhibitors versus aspirin alone. Furthermore, the DCFH fluorescence, useful to study platelet COX-1 activation, demonstrates that aspirin is more efficient in reducing COX-1 activation in platelets pretreated with MRP4 inhibitors. These data are in agreement with a previous observation (28) showing that in vivo treatment with the combination of dipyridamole plus aspirin (ASAsantin) is more efficient in reducing serum-TxB2
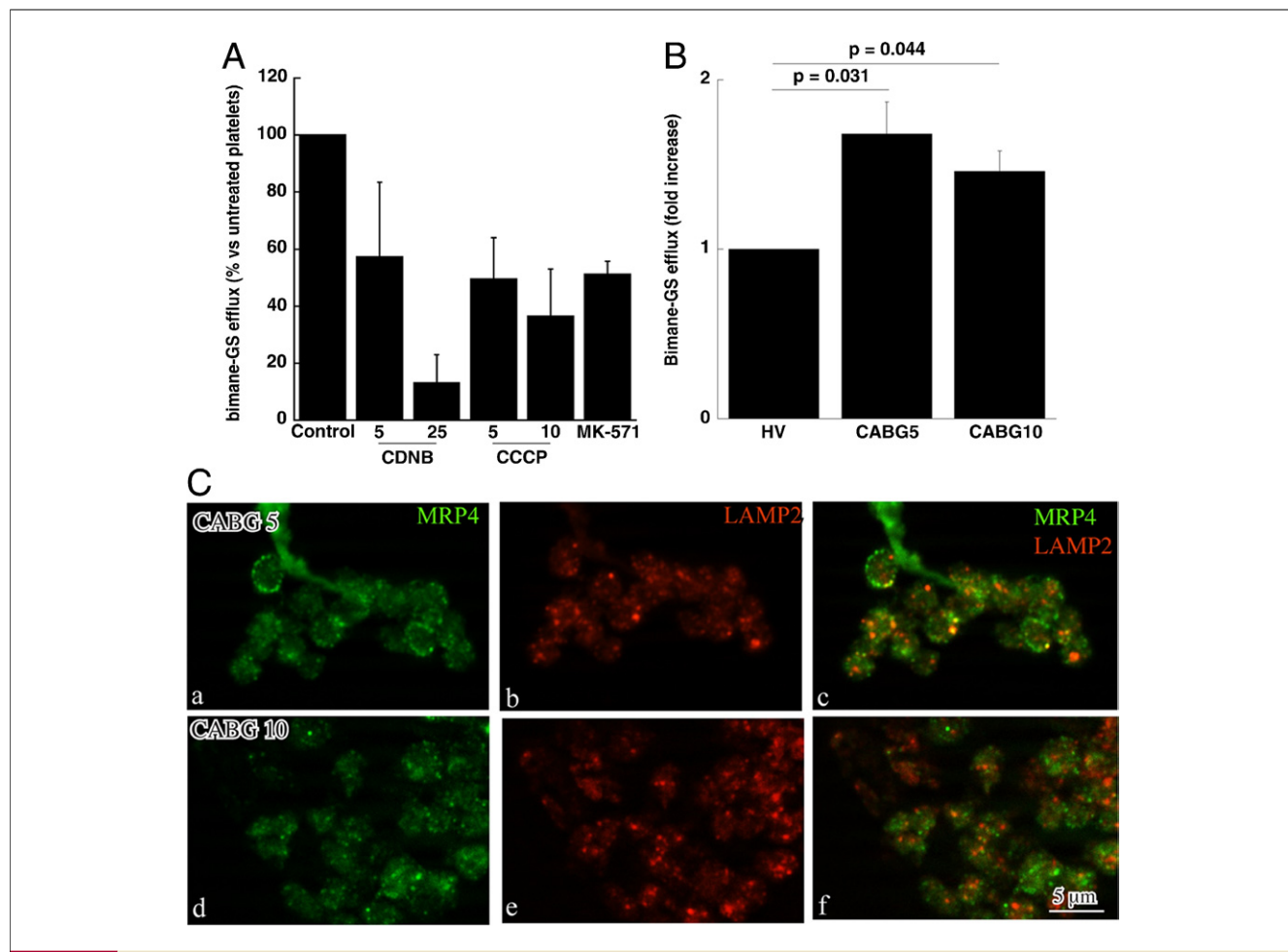
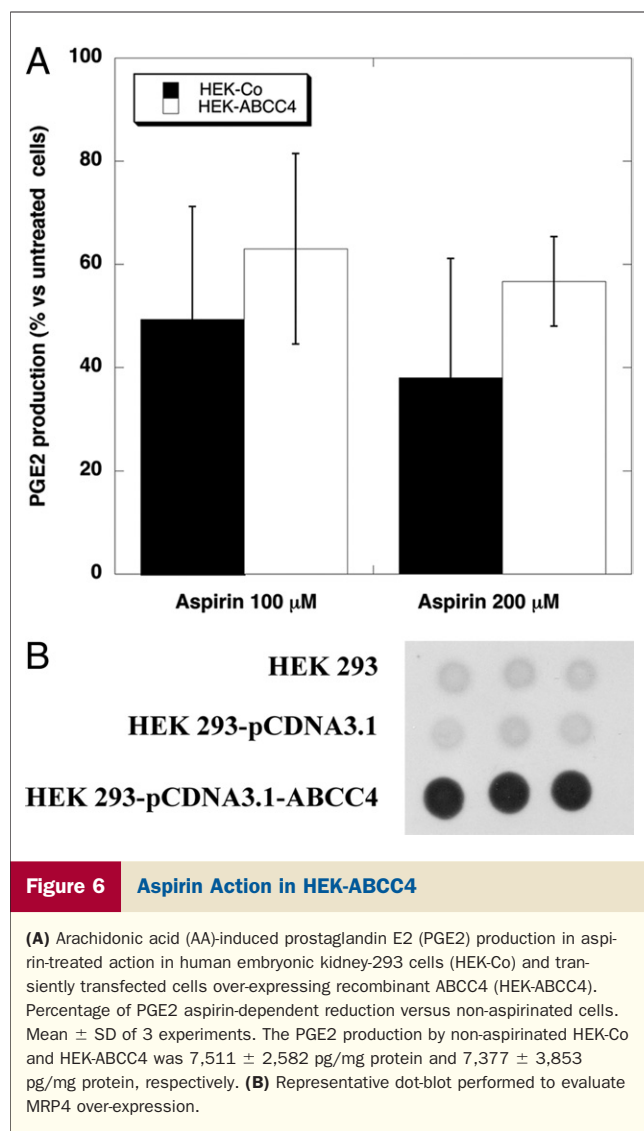**Figure 5**   **Platelet MRP4 Transport Activity and Localization in CABG5- and CABG10 Patients**

(A) Inhibition percentage of Bimane-glutathione (bimane-GS) efflux in HV platelets pre-treated with 1-chloro-2,4-dinitrobenzene (CDNB), carbonylcyanidem-chlorophenylhy-drasone (CCCP), and MK571. Results are the mean ± SD of 4 experiments. Control represents efflux of bimane-GS without inhibitors (0.87 ± 0.12 nmol/mg protein). **(B)** Bimane-GS efflux from HV and CABG patient platelets. Mean ± SD of fold increase of bimane-GS efflux in platelets obtained from patients 5 days after coronary artery bypass grafting surgery (CABG5) (n = 20) and platelets obtained from patients 10 days after coronary artery bypass grafting surgery (CABG10) (n = 5) versus HV (n = 50). **(C)** Double-labeled immunofluorescence microscopy of CABG5 **(a to c)** and CABG10 patients **(d to f)**. Platelets incubated with antibodies against MRP4 (green fluorescence) or LAMP-2 (red fluorescence). Representative experiment, of 3. Abbreviations as in Figure 3.

production in comparison with enteric-coated aspirin alone, despite the one-half amount of aspirin present in the combined formula. Inhibition of MRP4-mediated transport slightly reduces aspirinated platelet activation; in fact, both MK-571 and dipyridamole enhance collagen EC50.

Furthermore, we carried out experiments on aspirin-treated platelets derived from cultured MKs transfected with MRP4-siRNA. An increased aspirin and SA concentration with high activity and, consequently, a lower TxB2 production was observed in platelets derived from transfected MKs in comparison with their counterpart from untransfected MKs. Therefore, the fundamental role of MRP4 in aspirin transport is confirmed also in an in vitro cell culture system.

A previous report (10) shows residual platelet TxB2 production in CABG5 patients that was completely inhibited in healthy subjects (29). As a consequence of

our results, we postulated a correlation of MRP4 function and the disturbed inhibition of platelet COX-1 by aspirin in CABG5 patients. In agreement with Zimmermann et al. (10), we observed that in CABG5 patients in vitro aspirin platelet treatment is less efficient in reducing TxB2 production. Immunoblot, immunogold, and immunofluorescence analysis evidenced a higher MRP4 platelet expression in CABG5 patients versus HV and, importantly, revealed that this higher expression is particularly localized in plasma membrane. This MRP4 over-expression is also confirmed by its higher transport activity (Bimane-GS secretion). Therefore, the high incidence of aspirin resistance in such patients might be ascribed to MRP4 over-expression, responsible for aspirin intracellular concentration reduction and its disturbed inhibition of COX-1. In fact, as expected, patient platelets treated in vitro with dipyridamole (MRP4-mediated

Figure 6   Aspirin Action in HEK-ABCC4

(A) Arachidonic acid (AA)-induced prostaglandin E2 (PGE2) production in aspirin-treated action in human embryonic kidney-293 cells (HEK-Co) and transiently transfected cells over-expressing recombinant ABCC4 (HEK-ABCC4). Percentage of PGE2 aspirin-dependent reduction versus non-aspirinated cells. Mean ± SD of 3 experiments. The PGE2 production by non-aspirinated HEK-Co and HEK-ABCC4 was 7,511 ± 2,582 pg/mg protein and 7,377 ± 3,853 pg/mg protein, respectively. **(B)** Representative dot-blot performed to evaluate MRP4 over-expression.

transport inhibitor) before aspirin showed increased aspirin effect on COX-1, leading to a reduced TxB2 production.

Platelets of the same CABG patients, obtained at 10 days after surgery, are more sensitive to in vivo aspirin treatment, according to Zimmermann et al. (10) and present reduced MRP4 plasma membrane expression and lower transport activity. This leads us to hypothesize that aspirin effect on COX-1 is little related to MRP4-mediated aspirin transport in normal conditions, but in patients with MRP4 over-expression, its inhibition enhances aspirin action in an efficient way.

Similarly a reduced aspirin action on COX-1 activity was found in HEK293-ABCC4 over-expressing MRP4 (22), versus HEK293-Co.

Conclusions

To our best knowledge, this is the first study to identify MRP4 as a modulator of aspirin action in platelets. Our results indicate that direct elimination of aspirin from cytosol through MRP4-mediated transport limits drug capacity in reducing platelet function.

The MRP4 over-expression is directly linked to an aspirin-reduced cell entrapment that leads to increased TxB2 production, as found in CABG patients, with residual platelet activation despite aspirin treatment. Moreover, in vitro inhibition of MRP4-mediated transport enhances aspirin action in platelets.

Notably, our results pave the way to further studies of the possible correlation between MRP4 expression and a residual platelet function also in high-risk cardiovascular patients under chronic aspirin treatment.

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