British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01683.x
www.brjpharmacol.org

RESEARCH PAPER

Effects of $P2Y_1$ receptor antagonism on the reactivity of platelets from patients with stable coronary artery disease using aspirin and clopidogrel

B Labarthe[1,*], J Babin[1,*], M Bryckaert[2], P Théroux[1] and A Bonnefoy[1,3]

[1]*Department of Medicine, Montreal Heart Institute and University of Montreal, Montreal, Quebec, Canada,* [2]*INSERM U770, Hôpital Bicêtre, Le Kremlin Bicêtre Cedex, Paris, France, and* [3]*INSERM U743, University of Montreal, Quebec, Canada*

Correspondence
Pierre Théroux, Montreal Heart Institute, 5000 Belanger E, Montreal, QC (Quebec), Canada H1T 1C8. E-mail: Pierre.theroux@icm-mhi.org

*BL and JB contributed equally to this work.

Keywords
platelets; $P2Y_1$; $P2Y_{12}$; clopidogrel; MRS2179; aspirin; coronary artery disease

Received
11 February 2011
Revised
19 July 2011; 13 August 2011
Accepted
18 August 2011

BACKGROUND AND PURPOSE

$P2Y_1$ is a purine receptor that triggers platelet aggregation. Its inhibition was studied in patients with stable coronary artery disease (CAD) receiving standard anti-platelet therapy.

EXPERIMENTAL APPROACH

Blood samples from 10 patients on aspirin therapy (ASA, 80 mg·day^{-1}) were withdrawn before and 24 h after the administration of 450 mg clopidogrel (ASA/C) and were anti-coagulated with citrate or hirudin/PPACK in the presence or absence of the $P2Y_1$ inhibitor MRS2179 (M, 100 μM). Platelet responses to ADP (2.5 μM) and TRAP (2.5 μM), and collagen-induced thrombosis under flow conditions were analysed.

KEY RESULTS

Compared with ASA, ASA + M strongly inhibited ADP-induced peak platelet aggregation (88%), late aggregation (84%), P-selectin expression (85%) and $\alpha_{IIb}\beta_3$ activation (62%) (28%, 65%, 70% and 51% inhibition, respectively, for ASA/C vs. ASA). ASA + M also inhibited platelet/monocyte and platelet/neutrophil conjugate formation by 69% and 71% (57% and 59% for ASA/C vs. ASA). In TRAP-activated blood, ASA + M unexpectedly inhibited $\alpha_{IIb}b_3$ activation by 30%. In blood perfused in collagen-coated glass capillaries (shear rate of 1500 s^{-1}), ASA/C prevented thrombus growth beyond 5 min in relation to thrombus fragments embolization. ASA + M with or without clopidogrel completely prevented thrombus formation. Finally, *ex vivo* addition of MRS2179 and ASA to the blood of healthy donors markedly blocked thrombus formation on collagen in flow conditions, in contrast to ASA plus the $P2Y_{12}$ inhibitor 2-MeSAMP.

CONCLUSIONS AND IMPLICATIONS

Through particularly efficient complementarities with ASA to inhibit platelet activation and thrombus formation, the inhibition of $P2Y_1$ in the blood of patients with CAD appears to play a more important role than previously anticipated.

Abbreviations

ASA, acetylsalicylic acid; C, Clopidogrel; H/*P*, Hirudin/PPACK blood anticoagulant cocktail; MRS2179 (M), N6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate; PAC-1, monoclonal antibody binding activated $\alpha_{IIb}\beta_3$receptors; PMN, polymorphonuclear neutrophils; PPACK, D-phenylalanyl-prolyl-argininechloromethylketone; TRAP, thrombin receptor agonist peptide

Introduction

Upon blood vessel injury, platelets rapidly adhere to sub-endothelial glycoproteins through a number of adhesive receptors present on their surface. Collagen, the most thrombogenic component, supports platelet adhesion through glycoprotein (GP)VI and integrin $\alpha_2\beta_1$. At high shear rates, von Willebrand Factor bound onto the collagen matrix supports platelet arrest through GPIb-IX-V. These interactions trigger intracellular signals that lead to integrin $\alpha_{IIb}\beta_3$ activation, alterations of the plasma membrane converting it into a procoagulant surface and release of soluble mediators that amplify platelet activation and platelet recruitment (Jennings, 2009). Among the secreted mediators, ADP plays a key role in the positive feedback mechanisms of platelet activation through the purine receptors $P2Y_1$ and $P2Y_{12}$ (Gachet, 2008). All these processes are exacerbated in atherothrombotic diseases.

Whereas $P2Y_{12}$ activates $\alpha_{IIb}\beta_3$ and stabilizes platelet aggregates via G(i) proteins (Gachet, 2005; Cattaneo, 2007), $P2Y_1$ increases the intracellular calcium concentration via G(q) proteins stimulating PLC to trigger shape change and the initiation of aggregation in synergy with the platelet agonists collagen and thrombin (Léon *et al.*, 2001; Mangin *et al.*, 2004).

The efficacy of the thienopyridine inhibitors ticlopidine and clopidogrel to prevent acute thrombotic events in patients with CAD and in patients undergoing stent implantation testifies to the major contribution of the $P2Y_{12}$ receptor in thrombosis and haemostasis. These observations have prompted the development of new generations of $P2Y_{12}$ inhibitors, namely prasugrel, a thienopyridine now approved for clinical use, and ticagrelor and elinogrel, two non-thienopyridines (Mousa *et al.*, 2010). However, clopidogrel therapy may be laden by some resistance syndrome in patients with low PON1 and/or cytochrome 2C19 activity (Hulot *et al.*, 2006; Bouman *et al.*, 2011), resulting in a lower inhibitory activity, pro-drugs accumulation and, as a result of NTPDase1 inhibition, an increase in blood clot formation (Lecka *et al.*, 2010). These findings have renewed interest in the development of other platelet nucleotide receptors inhibitors, such as $P2Y_1$.

In contrast to $P2Y_{12}$, $P2Y_1$-based anti-thrombotic therapy has remained at a pre-clinical stage of investigation, although various inhibitors have been identified such as derivatives of adenosine-3′-phosphate-5′-phosphate (A3P5P) and of N6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate (MRS2179) (Boyer *et al.*, 1996; Camaioni *et al.*, 1998). Of clinical interest is the observation that $P2Y_1$-deficient mice are not susceptible to severe bleeding, which is in contrast with $P2Y_{12}$ deficiency (Fabre *et al.*, 1999; Léon *et al.*, 1999; Gachet and Hechler, 2005).

As any new anti-platelet drug will probably be added to standard treatment with ASA in combination with a $P2Y_{12}$ antagonist, this study aimed to evaluate the efficacy of $P2Y_1$ blockade in patients with stable angina on mono (ASA) and dual (ASA and clopidogrel) anti-platelet therapy. The first series of experiments examined the effects of the $P2Y_1$ inhibitor MRS2179 on platelet aggregation and activation following ADP and the thrombin receptor (PAR-1) agonist peptide (TRAP), in both citrated and anti-thrombin anti-coagulated blood. The second series of experiments examined the effect of MRS2179 on platelet adhesion and thrombus formation in collagen-coated glass capillaries under arterial flow condition.

Methods

Study population

The inclusion criteria for the platelet activation and aggregation study were the diagnosis of stable angina associated with coronary artery disease, the daily use of aspirin and the willingness to take one loading dose of clopidogrel and have two blood samples collected 24 h apart. Healthy volunteers not using any drugs were entered in the flow perfusion study. The study was approved by the Montreal Heart Institute Internal Ethics Committee and conducted in compliance with the Declaration of Helsinki's recommendations after having obtained a signed informed consent from all participants.

Study design

Blood samples were obtained from the antecubital vein from each patient in a fasting state before the administration of a loading dose of clopidogrel (450 mg) and 24 h later. Clopidogrel was administered under the direct supervision of a research nurse immediately after the first blood collection (Figure 1). For aggregation and flow cytometry studies, the blood was drawn into one tube containing 0.38% sodium citrate and into a second tube containing a mixture of recombinant Hirudin (2 U·mL^{-1}) and D-phenylalanyl-L-prolyl-L-argininechloromethylketone (PPACK, 40 μM) (Calbiochem,

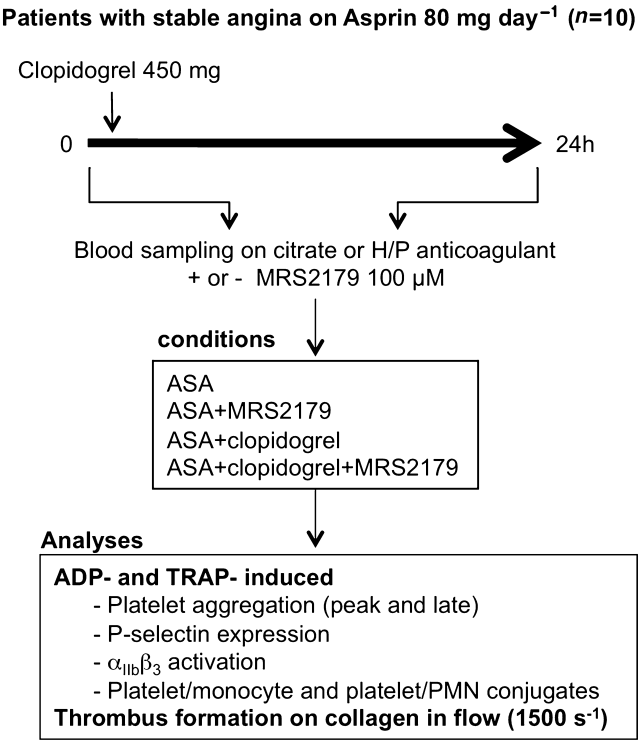

Figure 1
Design of the study.

La Jolla, CA) (H/*P*). For the perfusion experiments, the blood was drawn into a tube containing PPACK (40 μM). MRS2179 (Sigma Chemical Co, St. Louis, MO, USA) was dissolved in Tyrode's buffer and added at a final concentration of 100 μM into the tube immediately before the blood sampling.

Blood samples were processed and activated for the various platelet activation and aggregation experiments within 1.5 h of obtaining the blood.

Platelet aggregation

ADP (2.5 μM)- or thrombin receptor activating peptide (TRAP) SFLLRN (2.5 μM)-induced platelet aggregation (Sigma Chemical Co) was evaluated by turbidometry in platelet-rich plasma (PRP) using a Chronolog 570VS aggregometer (Chronolog Corp with AggroLink Software package, Havertown, PA, USA). Percentages of peak aggregation, corresponding to the maximum of light transmission, and of late aggregation measured at 6 min were quantified as previously described (Labarthe *et al.*, 2005).

Flow cytometry analyses

Flow cytometry measurements were performed on an Epics XL flow cytometer (Beckman Coulter, Miami, FL, USA). Platelet P-selectin expression was measured in unstirred PRP using a phycoerythrin-conjugated (PE) anti-CD62-P murine IgG1 MoAb (Becton Dickinson, San Jose, CA, USA). Activated $\alpha_{IIb}\beta_3$ was measured in unstirred PRP using fluorescein isothiocyanate (FITC)-conjugated murine IgM MoAb PAC-1 (Becton Dickinson). Platelet–monocyte and platelet–PMN co-aggregate formation in response to ADP or TRAP were measured in whole blood after a 30 min incubation period under static conditions at room temperature and in the presence of saturating concentrations of anti-CD14 (CD14-PE; Beckman Coulter) and anti-CD42 (CD42-FITC; Becton Dickinson) MoAbs. Monocytes and neutrophils positive for CD42 were differentiated according to their granularity and CD14 level of expression.

Perfusion studies

PPACK (40 μM) anti-coagulated blood was incubated with rhodamine 6G (10 $\mu g \cdot mL^{-1}$) (EMD Biosciences, Gibbstown, NJ, USA) for 15 min at 37°C and perfused at a wall shear rate of 1500 s^{-1} in glass capillaries (Vitrocom Hollow Rectangle Capillaries, Fiber Optic Center, New Bedford, MA, USA) precoated overnight at 4°C with fibrillar equine type 1 collagen (250 $\mu g \cdot mL^{-1}$) (Chronolog, Havertown, PA, USA) as previously described (Kauskot *et al.*, 2007). Platelet recruitment was monitored in a continuous motion (0.25 frame s^{-1}) during 9 min using a CCD digital Camera (C9300-201, Hamamatsu, Bridgewater, NJ, USA) connected to a Zeiss Observer Z1 inverted microscope. The surface covered by platelets and their fluorescence intensity were measured as indicators of platelet adhesion and thrombus formation respectively. Surface coverage was expressed as the percentage of the observed field covered by platelets, while thrombus growth was expressed in fluorescence intensity units (analogue to digital units, ADU). Image analyses were performed with the Slidebook 4.2 software (Intelligent Imaging Innovation, Inc, Denver, CO, USA).

For all experiments (flow cytometry, aggregometry and flow perfusions), dilutions of PRP or whole blood with the *ex vivo* addition of MRS2179, ASA and 2-MeSAMP were compared with other conditions for which the same dilutions were made using the corresponding vehicle (Tyrode's buffer or saline).

Statistical analysis

Aggregation and flow cytometry data were not normally distributed and were analysed first by Friedman's statistic and, if a statistical significance was found, by Wilcoxon paired tests. Results are expressed as medians and interquartile ranges. The adhesion and aggregation values obtained in blood perfusion chambers were normally distributed and tested by ANOVA and paired *t*-tests, and expressed as means ± SEM. The level of significance for two-sided hypothesis tests was set at a *P*-value less than 0.05, with no correction factors introduced for the multiplicity of analyses. The SPSS 10.0 (Chicago, IL, USA) statistical software was used for all analyses, and graphs were designed with the Prism-5 software (GraphPad Software, La Jolla, CA, USA).

Results

Study population

Valid data were obtained for all experiments from each study. There were one woman and nine men (median age 62 years, 53–75). All patients had a steady-state blood concentration of ASA (80 $mg \cdot day^{-1}$).

Baseline platelet activation markers

The influence of the anticoagulant used was first studied in the basal state before the stimulation studies (Table 1). Irrespective of the drug combination, platelet aggregation, P-selectin expression and activation of $\alpha_{IIb}\beta_3$ were consistently higher in citrate than in hirudin/PPACK (H/*P*), but the formation of platelet–leucocyte was independent of the anticoagulant used.

Platelet aggregation

We then measured the effect of ASA, ASA plus MRS2179 added *ex vivo* (ASA + M), ASA plus Clopidogrel (ASA/C), or ASA in combination with Clopidogrel and MRS2179 (ASA/C + M) on ADP (2.5 μM)- or TRAP (2.5 μM)-induced platelet aggregation and activation. At this concentration of ADP, an 80 $mg \cdot day^{-1}$ dose of ASA is expected to partially inhibit platelet aggregation (Gan *et al.*, 2002).

In the presence of ASA, peak platelet aggregation in response to ADP reached 35% in citrate anti-coagulated blood and was not significantly influenced by the presence of MRS2179 or Clopidogrel (Figure 2A). In contrast, late aggregation significantly decreased in the presence of ASA/C ($P < 0.05$). Similar trends but of smaller magnitude were observed following TRAP stimulation (Figure 2B).

In the presence of ASA, H/*P* anti-coagulated blood had a higher peak aggregation to ADP than citrate (64% vs. 39%) (Figure 2A). A strong inhibition of both peak (92%) and late (90%) aggregation was observed with ASA + M. The use of H/*P* anticoagulant significantly magnified the effect of ASA + M versus ASA and ASA/C + M versus ASA/C in ADP-induced aggregation (Figure 2A). In response to TRAP, ASA + M and

Table 1

Baseline platelet activation markers

		Peak aggr. (%)	Late aggr. (%)	P-selectin (MFI)	PAC-1 (MFI)	Platelet-mono (%)	Platelet-PMN (%)
ASA	H/*P*	3.0 (1.3–4.8)	1.5 (0–3.5)	0.04 (0.02–0.07)	0.05 (0.02–0.13)	2.3 (1.8–3.0)	0.9 (0.8–1.3)
	Citrate	**5.5** (3.5–8.8)	**5.5** (3.3–8.0)	**0.1** (0.1–0.2)	**0.2** (0.1–0.5)	2.0 (1.6–2.8)	1.0 (0.5–1.4)
ASA + MRS2179	H/*P*	3.0 (1.3–4.0)	2.0 (0–3.8)	0.07 (0–0.12)	0.11 (0.04–0.18)	2.5 (2.0–3.1)	1.5 (0.7–1.9)
	Citrate	**7.0** (4.0–12.0)	**7.0** (4.0–11.0)	0.1 (0–0.2)	0.3 (0.1–0.4)	2.4 (1.8–2.6)	1.1 (0.7–1.5)
ASA + clopidogrel	H/*P*	3.0 (1.0–3.5)	3.0 (1.0–3.5)	0.06 (0.02–0.08)	0.08 (0.04–0.14)	2.6 (2.0–3.3)	1.1 (0.9–1.3)
	Citrate	**6.5** (5.3–12.0)	**6.5** (5.0–7.8)	0.1 (0–0.1)	**0.2** (0.1–0.4)	2.3 (1.5–2.9)	1.0 (0.6–1.7)
ASA + clopidogrel + MRS2179	H/*P*	3.0 (2.0–4.3)	2.5 (2.0–3.5)	0.06 (0.04–0.11)	0.1 (0.06–0.15)	2.8 (2.1–3.6)	1.2 (0.9–1.3)
	Citrate	**8.0** (6.0–10.5)	**6.5** (5.8–10.0)	0.1 (0–0.7)	0.2 (0.1–0.3)	2.6 (1.6–3.4)	1.3 (1.1–2.8)

The blood from 10 subjects with stable CAD and taking ASA (80 mg·day^{-1}) was drawn before and 24 h after a loading dose of clopidogrel (450 mg), in tubes containing either hirudin (2 U·mL^{-1})/PPACK (40 μM) (H/*P*) or citrate (0.38%). When indicated, sampling tubes were pre-loaded with MRS2179 (100 μM). Platelet aggregation, P-selectin expression, $\alpha_{IIb}\beta_3$ activation (PAC-1 binding) and platelet/leucocyte conjugates were measured in PRP or whole blood as described in Methods. Peak aggr., maximum aggregation; late aggr., aggregation measured at 6 min.; platelet–mono or platelet–PMN, percentage of monocyte or neutrophils with bound platelets. Values are medians of percentages or mean fluorescence intensity (MFI). The 25th and 75th percentiles are indicated in brackets. Values significantly different ($P < 0.05$) between citrate and hirudin/PPACK conditions are indicated in bold in the citrate panel.

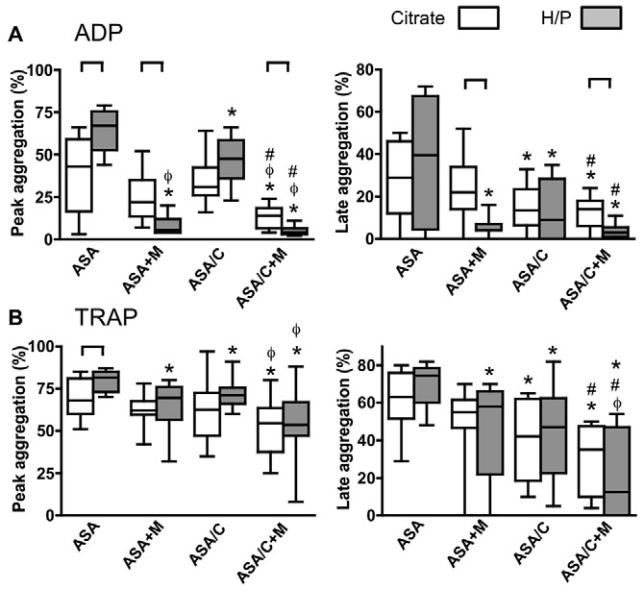

Figure 2

Effect of MRS2179 (100 μM) added *in vitro* to the blood of subjects with stable CAD taking aspirin (80 mg·day^{-1}) before and 24 h after a loading dose (450 mg) of clopidogrel, on platelet aggregation (percentage of peak and late aggregation) in PRP induced by 2.5 μM ADP (A) or 2.5 μM TRAP (B). Results are expressed as box and whisker plots showing median, 25 and 75 percentiles with min and max values for PRP anti-coagulated with citrate or H/*P*. Braces indicate significantly different citrate and H/*P*-values with $P < 0.05$; *$P < 0.05$ versus ASA; $^{\#}P < 0.05$ versus ASA + M; $\phi P < 0.05$ versus ASA/C.

ASA/C had moderate anti-aggregatory effects (Figure 2B). Finally, in both citrate and H/*P* anti-coagulated blood, the combination ASA/C + M significantly increased the inhibitory effects of ASA + M in response to ADP.

Platelet activation and secretion

We next assessed platelet activation using the monoclonal antibody PAC-1, which is specific to the activated form of $\alpha_{IIb}\beta_3$, and platelet degranulation via P-selectin expression. Results obtained in citrate and H/*P* anti-coagulated samples were very similar. However, the latter significantly magnified the effect of ASA + M and ASA/C in almost all conditions (Figure 3A and B).

In H/*P* anti-coagulated blood, ASA + M inhibited ADP-induced $\alpha_{IIb}\beta_3$ activation by 68% compared with ASA (50% for ASA/C vs. ASA). ASA + M was particularly efficient to block P-selectin expression (87% vs. ASA, $P < 0.05$). Moreover, ASA/C + M resulted in an inhibitory potentialization of ADP-induced $\alpha_{IIb}\beta_3$ activation and P-selectin of 90% and 96% respectively (Figure 3A). Surprisingly, ASA + M and ASA/C inhibited TRAP-induced $\alpha_{IIb}\beta_3$ activation by 30% compared with ASA ($P < 0.05$) (40% for ASA/C vs. ASA, $P < 0.05$), with a potentiation when M and C were combined (60% inhibition vs. ASA) (Figure 3B). These results demonstrate that the combination ASA + M interferes with TRAP-induced platelet activation and degranulation.

Platelet/leucocyte conjugates

In H/*P* anti-coagulated blood, both ASA + M and ASA/C strongly inhibited ADP-induced conjugate formation (69% and 57% for monocytes, and 71% and 59% for PMNs vs.

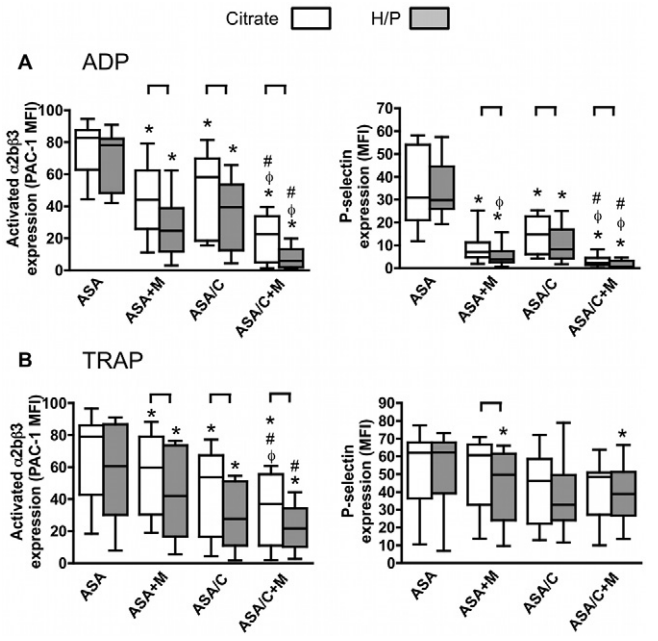

Figure 3

Effect of ASA, ASA + M, ASA/C or ASA/C + M on $\alpha_{IIb}\beta_3$ activation (PAC-1 binding) and P-selectin expression in PRP induced by 2.5 μM ADP (A) or 2.5 μM TRAP (B). Results are expressed as box and whisker plots showing median, 25 and 75 percentiles of mean fluorescence intensities with min and max values for PRP anti-coagulated with citrate or H/*P*. Braces indicate significantly different citrate and H/*P*-values with $P < 0.05$; $^*P < 0.05$ versus ASA; $^{\#}P < 0.05$ versus ASA + M; $\phi P < 0.05$ versus ASA/C.

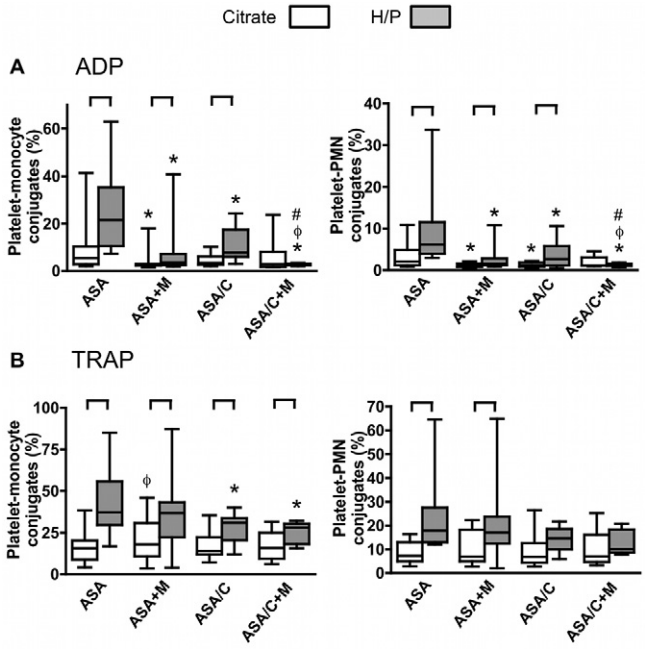

Figure 4

Effect of ASA, ASA + M, ASA/C or ASA/C + M on platelet-monocyte and platelet-PMN conjugate formation (percentage of total monocytes or PMN) induced by 2.5 μM ADP (A) or 2.5 μM TRAP (B) in whole blood anti-coagulated with citrate or H/*P*. Results are expressed as box and whisker plots showing median, 25 and 75 percentiles with min and max values for PRP anti-coagulated with citrate or H/*P*. Braces indicate significantly different citrate and H/*P*-values with $P < 0.05$; $^*P < 0.05$ versus ASA; $^{\#}P < 0.05$ versus ASA + M; $\phi P < 0.05$ versus ASA/C.

ASA), and the combination of ASA/C + M magnified this inhibition (89% for monocytes and 86% for PMN vs. ASA) (Figure 4A). In contrast, ASA + M did not decrease the formation of platelet/leucocyte conjugates upon TRAP activation in H/*P* (vs. ASA), whereas ASA/C inhibited both platelet/monocyte (33%) and platelet/PMN (38%) (vs. ASA, P = NS) conjugate formation (Figure 4B). The combination ASA/C + M resulted in inhibitory values similar to those of ASA/C.

Platelet recruitment in flow

We then analysed the effects of MRS2179 on collagen-induced thrombus formation under flow conditions (1500 s^{-1}). Platelet recruitment and thrombus formation increased continuously during the perfusion of platelets exposed to ASA alone, reaching 86.9 ± 3.7% of surface coverage and 377.5 ± 57.9 ADU of fluorescence intensity after 9 min (Figure 5A and B).

The addition of MRS2179 slightly accelerated the rate of platelet surface coverage after 5 min (79 ± 5% compared with 56 ± 4% with ASA alone; $P < 0.05$) but without modifying the final surface coverage (9 min: 93 ± 2%) (Figure 5A). Platelet deposition with MRS2179 mainly consisted of a monolayer of adherent platelets with sparse, small thrombi (74 ± 10 ADU, $P < 0.001$) (Figure 5B and D).

In contrast to MRS2179, thrombus formation was observed in the presence of clopidogrel (Figure 5B–D). The initial thrombus grew but stabilized after 5 min. Fluorescence intensity was 2.5-fold less than with ASA (151.8 ± 11.2 ADU $P < 0.001$) after 9 min (Figure 5B). Careful examination of the video records revealed that this effect was linked to continuous thrombus formation associated with active shedding and distal embolization of thrombi, a process, only observed with ASA/C.

The combination of ASA/C + M led to an inhibition of thrombus formation (Figure 5B and D), which was greater than that achieved with ASA + M (initial growth rate of 0.45 ADU s^{-1} and final fluorescence intensity of 62.5 ± 5.9 ADU, P = NS vs. ASA + M). Finally, kinetics of the total mass of platelet deposition, estimated by multiplying surface coverage with fluorescence intensity at similar time points, confirmed the strong inhibition of platelet deposition with ASA + M, and an additive effect of drug combination ASA/C + M on platelet mass deposition (Figure 5C). These results indicate that in the blood from atherosclerotic patients under ASA treatment, P2Y$_1$ inhibition with MRS2179 potently inhibits thrombus formation on collagen under arterial flow conditions.

To evaluate the potentiation effect of ASA on P2Y$_1$ receptor inhibition, we designed an additional series of flow experiments (on collagen) in which the blood of healthy donors (n = 3 to 8) was anti-coagulated with PPACK and pre-incubated with 100 μM of MRS2179 or 2-MeSAMP, a specific inhibitor of P2Y$_{12}$, in the absence or presence of exog-

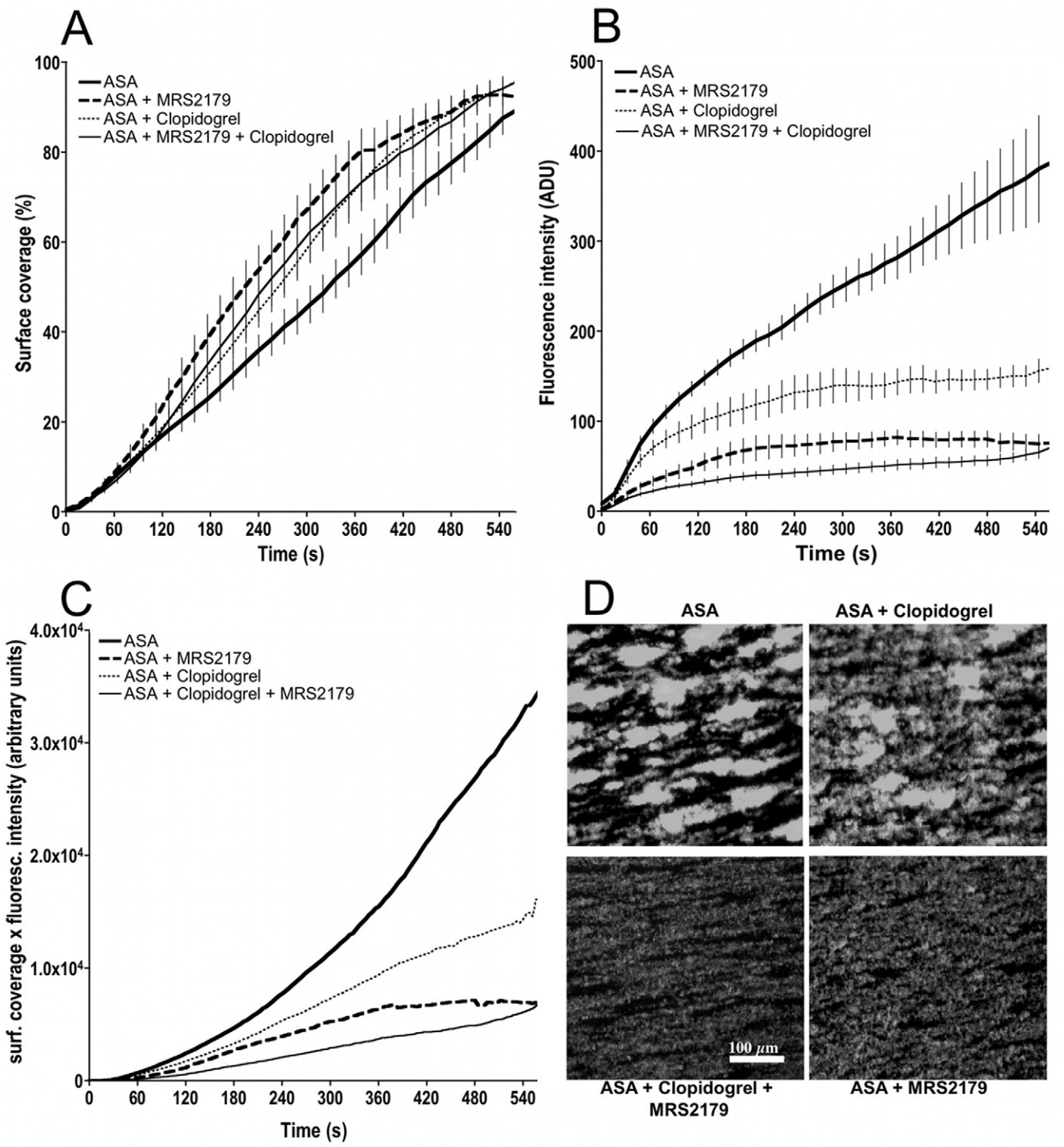

Figure 5

Effects of the *in vitro* addition of MRS2179 (100 μM) on the dynamics of platelet deposition onto collagen fibres at an arterial shear rate (1500 s^{-1}) before and 24 h after a loading dose (450 mg) of clopidogrel. PPACK-anti-coagulated whole blood labelled with rhodamine 6G was perfused for 540 s through rectangular glass capillaries coated with collagen. Real-time deposition of fluorescent platelets was quantified by image analysis. (A) Platelet surface coverage in % as an indicator of platelet adhesion. (B) Fluorescence intensity (ADU) as an indicator of thrombus formation. (C) Product of the surface coverage and fluorescence intensity at similar time points as an indicator of the total mass of platelet deposition. (D) Representative pictures of platelet deposition on the collagen fibres for each condition, taken after 7 to 8 min of blood perfusion.

enous ASA (20 μM) (Figures 6 and 7). A moderate decrease of platelet surface coverage and fluorescence intensity was observed in the presence of ASA (5%, P = NS vs. control) (Figures 6 and 7A and B). A 37% inhibition of thrombus growth was observed at 9 min in the presence of MRS2179 (P = NS vs. control), which was amplified by the addition of ASA (68%, $P < 0.05$ vs. control) (Figure 6C and D). This experiment shows that ASA potentiates the inhibitory effect of MRS2179 on thrombus growth. By contrast, 2-MeSAMP had no inhibitory effect on platelet deposition and thrombus formation, and even tended to increase the kinetics of thrombus growth (+30% at 9 min, Figure 7C). The addition of ASA to 2-MeSAMP increased this tendency (+70% at 9 min, Figure 7C), mainly due to an increase in platelet surface coverage. In sharp contrast, MRS2179 plus 2-MeSAMP strongly inhibited platelet deposition on collagen by 97% at 9 min (Figure 7C).

Discussion

This study was designed to evaluate the effect of P2Y$_1$ inhibition on platelets from CAD patients with a diagnosis of stable angina and taking a daily dose of ASA.

Measurements of basal platelet activation markers in the absence of platelet agonists showed that citrate anticoagulation provided significantly less protection against *ex vivo* spontaneous platelet activation than the H/*P* cocktail. The only exception was the formation of platelet/leucocyte conjugates, which was similar. Interestingly, the presence or absence of clopidogrel or of exogenous MRS2179 did not influence these basal values, suggesting that the platelet activation status in ASA-treated patients was already at a minimal level.

In several aspects, our observations corroborate the existing literature on the effect of P2Y$_1$ blockade on platelet responses to ADP and TRAP in healthy donors (Turner *et al.*, 2001; André *et al.* 2003; Mangin *et al.*, 2004; Ohlmann *et al.*, 2005; Penz *et al.*, 2007; Gachet, 2008) or animal models of thrombosis (Fabre *et al.*, 1999; Léon *et al.*, 1999; Lenain *et al.*, 2003). Thus, in the blood of ASA-treated patients, P2Y$_1$ blockade (i) inhibits peak and late ADP-induced aggregation and, to some extent, by TRAP; (ii) inhibits ADP-induced P-selectin exposure, an indicator of platelet secretion; and, consequently, (iii) inhibits the formation of platelet/leucocyte conjugates. With the exception of platelet aggregation, which was strongly magnified by anti-thrombins, the use of either citrate or H/*P* only induced marginal quantitative and qualitative variations in the effects of MRS2179, save for the inhibition of platelet aggregation that was strongly magnified by anti-thrombins. Such a complementary effect of a P2Y$_1$ inhibitor and thrombin blockade on aggregation has been recently observed by Penz and collaborators (2007), in PRP aggregation induced by atherosclerotic plaque materials. In addition, compared with citrate, we observed that H/*P* anticoagulation significantly increased ADP-induced peak aggregation (Figure 2A, ASA) and platelet/monocyte conjugates (Figure 3A, ASA). We attribute these differences to a better basal protection of the platelet resting state by H/*P* (Table 1) and, therefore, a better platelet reactivity to ADP.

Beyond these findings, our study demonstrates that in the blood of atherosclerotic patients treated with ASA, P2Y$_1$ appears to play a more important role in platelet activation and thrombus formation than previously suggested.

Effect of P2Y$_1$ inhibition on TRAP-induced $\alpha_{IIb}\beta_3$ activation

The MRS2179-mediated 30% reduction in PAC-1 binding following TRAP activation in H/*P* anti-coagulated blood we observed in the present study was similar to the reduction observed in citrated blood (24%, $P < 0.02$), thus excluding a role of anti-thrombins in these inhibitory effects even though PAR-1 receptor mediates TRAP-induced activation of $\alpha_{IIb}\beta_3$. These results are at variance with those of Nylander *et al.* (2003; 2004) obtained in blood of healthy donors not using ASA. Indeed, no reduction of PAC-1 binding was observed with 100 μM MRS2179, which is in contrast with the inhibition of P2Y$_{12}$. In side experiments, we observed that MRS2179 at 100 μM failed to promote the phosphorylation of the platelet vasodilator stimulated phosphoprotein (VASP), a measure of P2Y$_{12}$ activability (PLT VASP/P2Y$_{12}$ assay, Biocytex, Marseille, France; personal data). It is therefore unlikely that, in the present study, MRS2179 inhibited $\alpha_{IIb}\beta_3$ activation through residual P2Y$_{12}$ inhibition.

These results therefore suggest that P2Y$_1$ antagonism acts in synergy with aspirin to inhibit TRAP-induced $\alpha_{IIb}\beta_3$ activation in blood of stable atherosclerotic patients.

Aspirin and P2Y$_1$ inhibition

Redundancy between P2Y$_{12}$ blockade and COX-1 inhibition versus complementarities between P2Y$_1$ blockade and ASA has been reported in the process of platelet activation by thrombin. Indeed, the P2Y$_{12}$ receptor antagonist AR-C69931MX, but not MRS2179, can prevent the production of cytosolic phospholipase A2 (cPLA2), the release of arachidonic acid from membrane-bound phospholipids, and TXA2 generation in human platelets following the activation of PAR-1 and/or PAR-4 (Shankar *et al.*, 2006). Consistently, ASA was more effective in potentiating the effect of P2Y$_1$ inhibition on platelet activation and thrombus formation than P2Y$_{12}$ blockade.

Inhibition of thrombus formation in flow by MRS2179

In contrast to clopidogrel, which was associated with continuous shedding and embolization of the thrombus, we found that MRS2179 could drastically inhibit thrombus formation onto fibrillar collagen. These results extend the observations of Mangin *et al.* (2004) of a complete inhibition of platelet shape change together with a profound decrease in aggregation and secretion upon collagen activation with the combination of MRS2179 and ASA in the blood of healthy donors under flow conditions. In contrast, the combination of ARC69931MX and ASA did not affect platelet shape change and only displayed a moderate decrease in aggregation (Mangin *et al.*, 2004).

We confirmed the complementary action of ASA and MRS2179 by performing perfusion experiments with the blood from healthy donors with both drugs added exogenously Under these conditions, ASA potentiated the inhibi-

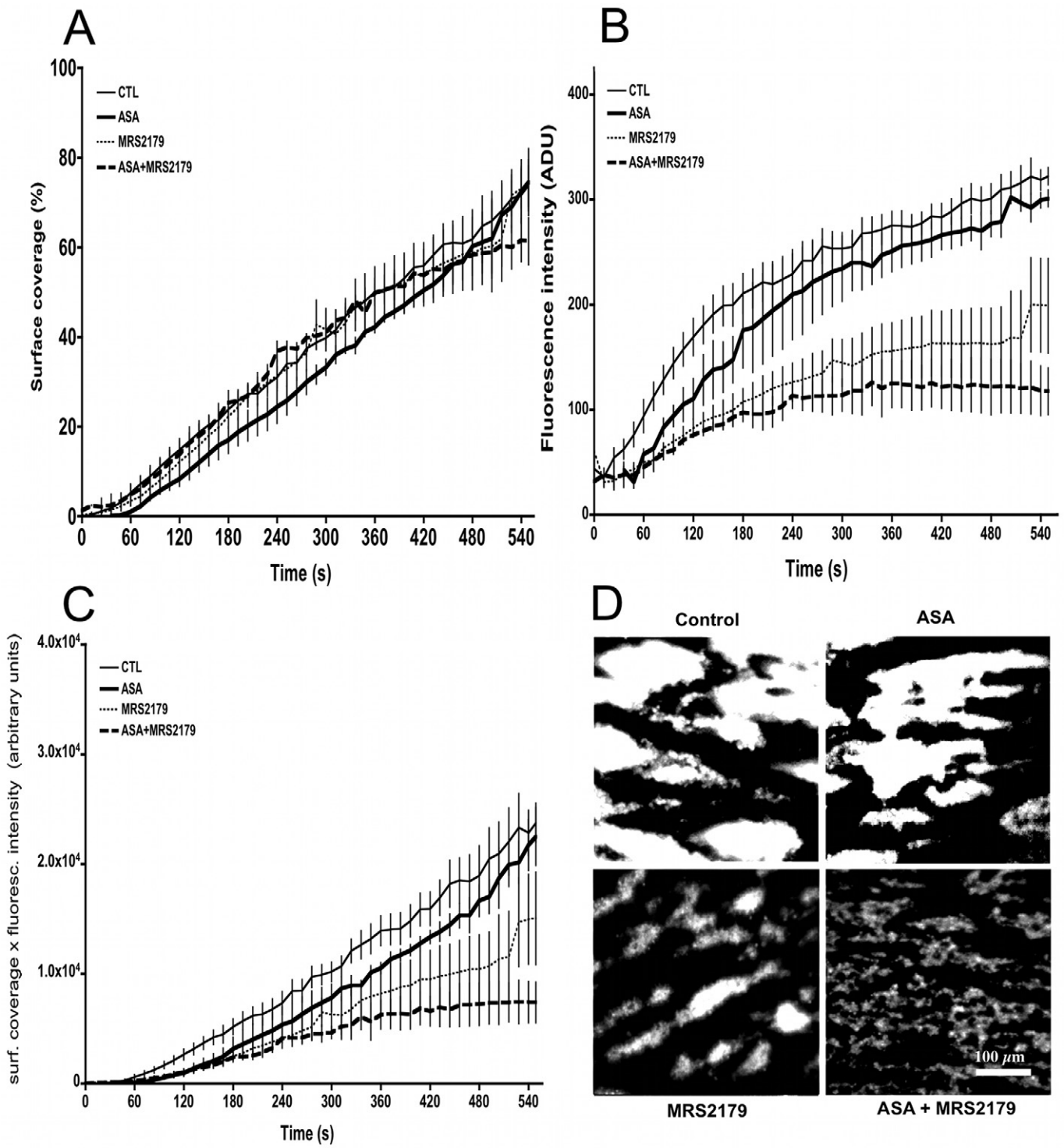

Figure 6

Effects of the combination of MRS2179 and ASA on collagen-induced thrombus formation at an arterial shear rate (1500 s^{-1}). The blood from healthy donors (n = 3–8) was anti-coagulated with PPACK and pre-incubated with different drug combinations of MRS2179 (100 μM) and ASA (20 μM) and was labelled with rhodamine 6G and perfused during 540 s through rectangular glass capillaries coated with collagen. Real-time deposition of fluorescent platelets was quantified by image analysis. (A) Platelet surface coverage in % as an indicator of platelet adhesion. (B) Fluorescence intensity (ADU) as an indicator of thrombus formation. (C) Product of the surface coverage and fluorescence intensity at similar time points as an indicator of the total mass of platelet deposition. (D) Representative pictures of platelet deposition on the collagen fibres for each condition taken after 7 to 8 min of blood perfusion.

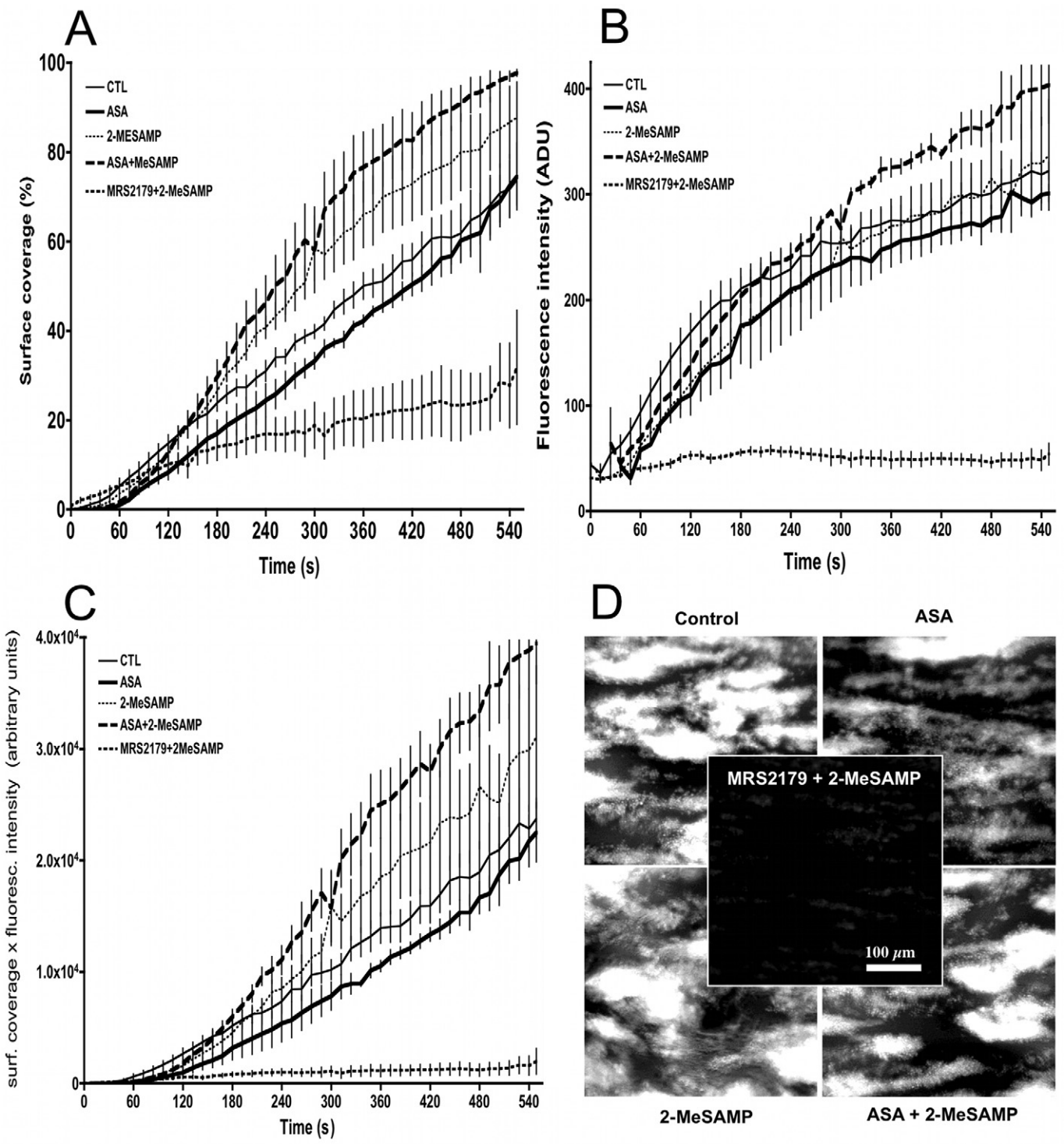

Figure 7

Effects of the combination of 2-MeSAMP, MRS2179 and ASA on collagen-induced thrombus formation at an arterial shear rate (1500 s^{-1}). The blood from healthy donors (n = 3–8) was anti-coagulated with PPACK and pre-incubated with different drug combinations of 2-MeSAMP (100 μM), MRS2179 (100 μM) and ASA (20 μM). Thrombus formation was measured as indicated in the legend of Figure 6. The control (CTL) and ASA results are those from Figure 6, all perfusions were performed with the blood of the same donors. (A) Platelet surface coverage in % as an indicator of platelet adhesion. (B) Fluorescence intensity (ADU) as an indicator of thrombus formation. (C) Product of the surface coverage and fluorescence intensity at similar time points as an indicator of the total mass of platelet deposition. (D) Representative pictures of platelet deposition on the collagen fibres for each condition taken after 7 to 8 min of blood perfusion.

tory effect of MRS2179 on thrombus growth (fluorescence intensity) without, however, reducing platelet surface coverage. In sharp contrast, no potentiating effect of ASA on $P2Y_{12}$ blockade using 2-MeSAMP could be detected.

The paradoxical increase in platelet surface coverage with $P2Y_1$ blockade in the blood of CAD patients and with ASA plus 2-MeSAMP in healthy donors has been previously reported with COX-1 and $P2Y_{12}$ inhibition, but the mechanisms are not well understood. It was suggested by Roald and Sakariassen (1995) that the inhibition of platelet thrombi growth by ASA or clopidogrel was associated with an increase in platelet concentration at the surface and, therefore, greater platelet recruitment on the surface. Savion *et al.* (2001) alternatively suggested that sub-threshold ADP concentrations generated in experimental flow models induced a refractoriness state in platelets together with some platelet activation and soluble microaggregate formation resulting in decreased adhesion. This reduction of platelet adhesion, as reflected by reduced surface coverage, was attenuated in samples from patients treated with clopidogrel (Shenkman *et al.*, 2008). Consistently, we observed this paradoxical increase in platelet adhesion in the blood of stable CAD patients, where platelets are in a sub-activation state (Fuchs *et al.*, 1987; Furman *et al.*, 1998; Osmancik *et al.*, 2007), but not in the blood from healthy donors treated with MRS2179 or ASA + MRS2179.

Because $P2Y_1$ is expressed in many cells and tissues such as the vascular wall, its systemic inhibition may have both positive and negative side effects. Indeed, a recent study (Zerr *et al.* 2011) demonstrated that the *in vivo* inhibition of endothelial $P2Y_1$ inhibited TNFα-induced vascular inflammation. On the other hand, $P2Y_1$ plays an important role in the regulation of neuronal and glial functions in the nervous system and is essential for sensory neuron functions (Nakamura and Strittmatter, 1996).

In conclusion, this study unravels a potent anti-platelet effect of $P2Y_1$ blockade in patients with coronary artery disease that complements the effects of current anti-platelet therapy with ASA, an effect that was not anticipated from previous works. These findings support further clinical investigation with this new class of anti-platelet agents.

Study limitation

The fact that MRS2179, in contrast to clopidogrel, had to be added *ex vivo* to the blood of patients because the drug is not approved for human use is a limitation of the study.

Acknowledgement

We thank Pr Marc F Hoylaerts for his insightful discussions during the preparation of the manuscript, Dr Frédéric Adam for his assistance in the glass capillary perfusion model and M Ghitescu and J Rivard for their technical assistance. This work was supported by the Agence Nationale de la Recherche (grant ANR-05-PCOD-031-02).

Conflict of interest

There exist no conflicts of interest with any of the authors in this manuscript.

References

Andre P, LaRocca T, Delaney SM, Lin PH, Vincent D, Sinha U *et al.* (2003). Anticoagulants (thrombin inhibitors) and aspirin synergize with $P2Y_{12}$ receptor antagonism in thrombosis. Circulation 108: 2697–2703.

Bouman HJ, Schömig E, van Werkum JW, Velder J, Hackeng CM, Hirschhäuser C *et al.* (2011). Paraoxonase-1 is a major determinant of clopidogrel efficacy. Nat Med 17: 110–116.

Boyer JL, Romero-Avila T, Schachter JB, Harden TK (1996). Identification of competitive antagonists of the $P2Y_1$ receptor. Mol Pharmacol 50: 1323–1329.

Camaioni E, Boyer JL, Mohanram A, Harden TK, Jacobson KA (1998). Deoxyadenosine bisphosphate derivatives as potent antagonists at $P2Y_1$ receptors. J Med Chem 41: 183–190.

Cattaneo M (2007). Platelet P2 receptors: old and new targets for antithrombotic drugs. Expert Rev Cardiovasc Ther 5: 45–55.

Fabre JE, Nguyen M, Latour A, Keifer JA, Audoly LP, Coffman TM *et al.* (1999). Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in $P2Y_1$-deficient mice. Nat Med 5: 1199–1202.

Fuchs J, Weinberger I, Rotenberg Z, Joshua H, Almozlino A, Agmon J (1987). Circulating aggregated platelets in coronary artery disease. Am J Cardiol 60: 534–537.

Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC *et al.* (1998). Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. J Am Coll Cardiol 31: 352–358.

Gachet C (2005). The platelet P2 receptors as molecular targets for old and new antiplatelet drugs. Pharmacol Ther 108: 180–192.

Gachet C (2008). P2 receptors, platelet function and pharmacological implications. Thromb Haemost 99: 466–472.

Gachet C, Hechler B (2005). The platelet P2 receptors in thrombosis. Semin Thromb Hemost 31: 162–176.

Gan R, Teleg RA, Florento L, Bitanga ES (2002). Effect of increasing doses of aspirin on platelet aggregation among stroke patients. Cerebrovasc Dis 14: 252–255.

Hulot JS, Bura A, Villard E, Azizi M, Remones V, Goyenvalle C *et al.* (2006). Cytochrome P450 2C19 loss-of-function polymorphism is a major determinant of clopidogrel responsiveness in healthy subjects. Blood 108: 2244–2247.

Jennings LK (2009). Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis. Thromb Haemost 102: 248–257.

Kauskot A, Adam F, Mazharian A, Ajzenberg N, Berrou E, Bonnefoy A *et al.* (2007). Involvement of the mitogen-activated protein kinase c-Jun NH2-terminal kinase 1 in thrombus formation. J Biol Chem 282: 31990–31999.

Labarthe B, Theroux P, Angioi M, Ghitescu M (2005). Matching the evaluation of the clinical efficacy of clopidogrel to platelet function tests relevant to the biological properties of the drug. J Am Coll Cardiol 46: 638–645.

Lecka J, Rana MS, Sévigny J (2010). Inhibition of vascular ectonucleotidase activities by the pro-drugs ticlopidine and clopidogrel favours platelet aggregation. Br J Pharmacol 161: 1150–1160.

Lenain N, Freund M, Léon C, Cazenave JP, Gachet C (2003). Inhibition of localized thrombosis in $P2Y_1$-deficient mice and rodents treated with MRS2179, a $P2Y_1$ receptor antagonist. J Thromb Haemost 1: 1144–1149.

Léon C, Hechler B, Freund M, Eckly A, Vial C, Ohlmann P *et al*. (1999). Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. J Clin Invest 104: 1731–1737.

Léon C, Freund M, Ravanat C, Baurand A, Cazenave JP, Gachet C (2001). Key role of the P2Y(1) receptor in tissue factor-induced thrombin-dependent acute thromboembolism: studies in P2Y(1)-knockout mice and mice treated with a P2Y(1) antagonist. Circulation 103: 718–723.

Mangin P, Ohlmann P, Eckly A, Cazenave JP, Lanza F, Gachet C (2004). The $P2Y_1$ receptor plays an essential role in the platelet shape change induced by collagen when TxA2 formation is prevented. J Thromb Haemost 2: 969–977.

Mousa SA, Jeske WP, Fareed J (2010). Prasugrel: a novel platelet ADP P2Y(12) receptor antagonist. Methods Mol Biol 663: 221–228.

Nakamura F, Strittmatter SM (1996). P2Y1 purinergic receptors in sensory neurons: contribution to touch-induced impulse generation. Proc Natl Acad Sci USA 93: 10465–10470.

Nylander S, Mattsson C, Ramstrom S, Lindahl TL (2003). The relative importance of the ADP receptors, $P2Y_{12}$ and $P2Y_1$, in thrombin-induced platelet activation. Thromb Res 111: 65–73.

Nylander S, Mattsson C, Ramstrom S, Lindahl TL (2004). Synergistic action between inhibition of $P2Y_{12}$/$P2Y_1$ and $P2Y_{12}$/thrombin in ADP- and thrombin-induced human platelet activation. Br J Pharmacol 142: 1325–1331.

Ohlmann P, Eckly A, Mangin P, Lanza F, Gachet C (2005). Further evidence that fibrillar collagen is unable to promote platelet shape change and aggregation in the absence of secondary agonists. J Thromb Haemost 3: 2119–2121.

Osmancik PP, Bednar F, Móciková H (2007). Glycemia, triglycerides and disease severity are best associated with higher platelet activity in patients with stable coronary artery disease. J Thromb Thrombolysis 24: 105–107.

Penz SM, Reininger AJ, Toth O, Deckmyn H, Brandl R, Siess W (2007). Glycoprotein Ibalpha inhibition and ADP receptor antagonists, but not aspirin, reduce platelet thrombus formation in flowing blood exposed to atherosclerotic plaques. Thromb Haemost 97: 435–443.

Roald HE, Sakariassen KS (1995). Axial dependence of collagen-induced thrombus formation in flowing non-anticoagulated human blood. Anti-platelet drugs impair thrombus growth and increase platelet-collagen adhesion. Thromb Haemost 73: 126–131.

Savion N, Shenkman B, Tamarin I, Dardik R, Frojmovic M, Varon D (2001). Transient adhesion refractoriness of circulating platelets under shear stress: the role of partial activation and microaggregate formation by suboptimal ADP concentration. Br J Haematol 112: 1055–1061.

Shankar H, Garcia A, Prabhakar J, Kim S, Kunapuli SP (2006). $P2Y_{12}$ receptor-mediated potentiation of thrombin-induced thromboxane A2 generation in platelets occurs through regulation of Erk1/2 activation. J Thromb Haemost 4: 638–647.

Shenkman B, Matetzky S, Fefer P, Hod H, Einav Y, Lubetsky A *et al*. (2008). Variable responsiveness to clopidogrel and aspirin among patients with acute coronary syndrome as assessed by platelet function tests. Thromb Res 122: 336–345.

Turner NA, Moake JL, McIntire LV (2001). Blockade of adenosine diphosphate receptors P2Y(12) and P2Y(1) is required to inhibit platelet aggregation in whole blood under flow. Blood 98: 3340–3345.

Zerr M, Hechler B, Freund M, Magnenat S, Lanois I, Cazenave JP *et al*. (2011). Major contribution of the P2Y1 receptor in purinergic regulation of TNF{alpha}-induced vascular inflammation. Circulation 123: 2404–2413.