Combined Blockade of ADP Receptors and PI3-Kinase p110β Fully Prevents Platelet and Leukocyte Activation during Hypothermic Extracorporeal Circulation

Stefanie Krajewski¹*, Julia Kurz¹, Tobias Geisler², Karlheinz Peter³, Hans Peter Wendel⁴, Andreas Straub¹

¹ Department of Anesthesiology and Intensive Care Medicine, University of Tübingen, Tübingen, Germany, ² Department of Cardiology, University of Tübingen, Tübingen, Germany, ³ Atherosclerosis and Vascular Biology, Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia, ⁴ Department of Thoracic, Cardiac and Vascular Surgery, University of Tübingen, Tübingen, Germany

Abstract

Extracorporeal circulation (ECC) and hypothermia are used to maintain stable circulatory parameters and improve the ischemia tolerance of patients in cardiac surgery. However, ECC and hypothermia induce activation mechanisms in platelets and leukocytes, which are mediated by the platelet agonist ADP and the phosphoinositide-3-kinase (PI3K) p110β. Under clinical conditions these processes are associated with life-threatening complications including thromboembolism and inflammation. This study analyzes effects of ADP receptor P2Y12 and P2Y1 blockade and PI3K p110β inhibition on platelets and granulocytes during hypothermic ECC. Human blood was treated with the P2Y12 antagonist 2-MeSAMP, the P2Y1 antagonist MR52179, the PI3K p110β inhibitor TGX-221, combinations thereof, or PBS and propylene glycol (controls). Under static in vitro conditions a concentration-dependent effect regarding the inhibition of ADP-induced platelet activation was found using 2-MeSAMP or TGX-221. Further inhibition of ADP-mediated effects was achieved with MR52179. Next, blood was circulated in an ex vivo ECC model at 28°C for 30 minutes and various platelet and granulocyte markers were investigated using flow cytometry, ELISA and platelet count analysis. GPIIb/IIIa activation induced by hypothermic ECC was inhibited using TGX-221 alone or in combination with P2Y blockers (p < 0.05), while no effect of hypothermic ECC on antiplatelet agents on GPIIb/IIIa and GPIb expression and von Willebrand factor binding was observed. Sole P2Y and PI3K blockade or a combination thereof inhibited P-selectin expression on platelets and platelet-derived microparticles during hypothermic ECC (p < 0.05). P2Y blockade alone or combined with TGX-221 prevented ECC-induced platelet-granulocyte aggregate formation (p < 0.05). Platelet adhesion to the ECC surface, platelet loss and Mac-1 expression on granulocytes were inhibited by combined P2Y and PI3K blockade (p < 0.05). Combined blockade of P2Y12, P2Y1, and PI3K p110β completely inhibits hypothermic ECC-induced activation processes. This novel finding warrants further studies and the development of suitable pharmacological agents to decrease ECC- and hypothermia-associated complications in clinical applications.

Introduction

Under physiological conditions, platelets play a fundamental role in hemostasis, prevention of blood loss, and healing of vascular injury. However, dysfunctional platelets can cause serious problems like abnormal thrombus formation and consecutive vessel occlusion as well as severe bleeding complications, which are all feared side effects of extracorporeal circulation (ECC) [1,2].

ECC is employed in many cardiac surgical procedures to ensure gas exchange and to maintain stable circulatory parameters of the patient. In addition, hypothermia ranging between 28°C and 32°C is routinely employed during cardiac operations in addition to ECC to increase the ischemia tolerance of the patient. Shear stress, contact of blood with the artificial surfaces of the ECC circuit as well as hypothermia are all known to be associated with platelet activation, which results in disturbed platelet function and associated complications [1,3,4]. Furthermore, activated platelets can trigger an inflammatory response through interactions with leukocytes [5]. These platelet-leukocyte interactions are mainly mediated by binding of the platelet surface receptor P-selectin to its counter receptor P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes. Subsequently, upregulation and activation of the Mac-1 receptor (CD11b/CD18) on leukocytes is induced as a result of the P-selectin-PSGL-1 interaction [5,6]. Furthermore, it has been shown that CD40 ligand, which is shed from platelets upon activation, also promotes Mac-1 upregulation [7].

Inhibition of platelet activation is a possible approach to inhibit platelet dysfunction and related detrimental effects during ECC. One pharmacological strategy to inhibit platelet activation is blockade of the platelet ADP receptors P2Y12 and P2Y1 [8,9].

We have recently shown that ADP plays a major role in ECC- and hypothermia-induced platelet activation [10]. Inhibition of platelet granule release could be achieved during hypothermic ECC via P2Y12 blockade [11]. Nevertheless, despite effective
platelet protection by P2Y12 blockade, still higher degrees of platelet activation compared to baseline values were observed. Furthermore, platelet adhesion to the ECC surface and therefore platelet loss could not be prevented. Consequently, in addition to ADP other factors obviously activate platelets during ECC. In this regard, shear-induced activation of platelets is another important factor during ECC [4,12]. Shear triggers a signaling pathway, which includes activation of the class Ia phosphoinositide-3-kinase (PI3Ks) p110β isoformal. This results in activation of the platelet fibrinogen receptor GPIIb/IIIa and platelet aggregate formation [13,14,15,16].

On the basis of these data, we hypothesize that substantial platelet protection during ECC and hypothermia may be achieved by combined inhibition of P2Y12, P2Y1 and PI3K p110β. To prove this, we first defined effective doses of the P2Y12 antagonist 2-MeSAMP and the PI3K p110β blocker TGX-221 to achieve substantial inhibition of platelet activation in vivo. Afterwards, the effect of single and combined blockade of ADP receptors and PI3K p110β on platelet activation, platelet adhesion to the ECC surface, their interaction with granulocytes as well as subsequent granulocyte activation was investigated in human whole blood employing an ex vivo ECC model at hypothermia (20°C).

Results

Concentration-dependent Inhibition of ADP-induced P-selectin Expression using 2-MeSAMP and TGX-221 and the Effect of MRS2179

Treatment of whole blood with different concentrations of 2-MeSAMP (10 and 100 μM) showed that ADP-induced (final ADP concentration: 20 μM) P-selectin expression is more potently inhibited with higher antagonist concentrations (Figure 1A). The addition of MRS2179 (100 μM) in the 2-MeSAMP-treated group further decreased the expression of platelet P-selectin expression upon ADP activation (Figure 1A).

Furthermore, a TGX-221 concentration of 2.2 μM is more potently inhibiting ADP-induced P-selectin expression in human whole blood, when compared to a TGX-221 concentration of 0.5 μM (Figure 1B).

PI3K p110β Inhibition Alone or in Combination with ADP Receptor Blockade has no Effect on Expression Levels of GPIIb/IIIa and GPIbα as Well as on vWF Binding of Platelets, but Reduces GPIIb/IIIa Activation during Hypothermic ECC

Platelet activation is accompanied by a conformational change of the platelet GPIIb/IIIa receptor into a high-affinity conformation, which enables binding of ligands including fibrinogen and vWF [19]. Using specific antibodies we analyzed (1) the general expression of GPIIb/IIIa on the platelet surface and (2) the GPIIb/IIIa activation state. Basic GPIIb/IIIa expression was neither influenced by hypothermic ECC nor by antplatelet agents (Figure 2A), while hypothermic ECC induced a significant increase in GPIIb/IIIa activation in controls (p<0.01; Figure 2B). This effect was inhibited by PI3K p110β inhibition using TGX-221 alone (p<0.05) and more profoundly by PI3K p110β inhibition combined with P2Y blockade (2-MeSAMP and MRS2179; p<0.001).

The GPIbα receptor is involved in mediating primary platelet adhesion via binding to its ligand vWF [19]. Upon platelet activation GPIbα surface expression is decreased due to down-regulation [20] or receptor shedding [21,22]. Regarding the expression of the GPIbα receptor on platelets as well as vWF binding to platelets in our experiments, no effect of either hypothermic ECC or P2Y and PI3K p110β inhibition was observed (Figure 2C and D). It has previously been described that the platelet agonist ADP decreases GPIbα surface expression on platelets [20,23,24]. This finding was confirmed in our experiments and indicated intact platelet reactivity before and after hypothermic ECC in the PBS group (Figure 2C).

In addition, vWF binding was investigated on ADP-activated platelets before and after hypothermic ECC in the PBS group. ADP induced a significant increase in vWF binding at both timepoints (Figure 2D). Regarding the fact that GPIbα surface expression was downregulated at the same time, this finding is...
most likely due to binding of vWF to GPIIB/IIIa. In this regard, it has previously been described that platelets have other binding sites for vWF then GPIbα [25] and that vWF is a ligand of the GPIIb/IIIa receptor [19,26].

Hypothermic ECC-induced P-selectin Expression on Platelets and Platelet Microparticles is Profoundly Inhibited by P2Y and PI3K p110b Inhibitors

As an indicator for α-granule release and platelet activation, P-selectin expression on the surface of platelets and platelet-derived microparticles was measured before and after hypothermic ECC. Single platelets and PMPs were identified according to their size and granularity (Figure 3A). As a positive control P-selectin expression on ADP-stimulated platelets was measured to confirm platelet reactivity before and after circulation in the control group (Figure 3B).

P-selectin expression significantly increased during hypothermic ECC in controls (p<0.05, Figure 3C). Both P2Y blockade and PI3K p110b inhibition alone as well as combined inhibition of ADP receptors and PI3K p110b completely inhibited this phenomenon (p<0.05).

PMPs, which express typical platelet surface receptors including P-selectin, are released from platelets upon activation [3,27,28]. In our experiments the total number of PMPs was not affected by hypothermic ECC or antiplatelet agents (data not shown). Nevertheless, P-selectin expression on PMPs was significantly increased by hypothermic ECC (p<0.001; Figure 3D). P2Y receptor blockade, PI3K p110b inhibition as well as the combination of P2Y receptor and PI3K p110b inhibition significantly reduced P-selectin expression on PMPs (p<0.05).
Effects of Hypothermic ECC, P2Y Blockade and PI3K p110β Inhibition on Platelet-ECC Adhesion, Platelet-granulocyte Aggregate Formation and Platelet Loss

Hypothermic ECC induced a significant increase in platelet adhesion to the artificial ECC surface in controls (p<0.001; Figure 4A). This was only prevented by a combination of P2Y blockade and PI3K p110β inhibition (p<0.05), but not by P2Y blockade or PI3K p110β inhibition alone.

Interaction of activated platelets with leukocytes plays a pivotal role in ECC-related pro-inflammatory complications [1,29]. We evaluated the formation of platelet-granulocyte aggregates during hypothermic ECC ex vivo. A significant increase in aggregate formation was found after 30 minutes of circulation at 28°C in controls (p<0.01; Figure 4B). This phenomenon was significantly inhibited by P2Y blockade alone (p<0.05) and by combined treatment with P2Y and PI3K p110β blockers (p<0.05).

A significant decrease of platelet counts was observed during hypothermic ECC (p<0.01; Figure 4C). The combination of P2Y blockers and PI3K p110β inhibitor significantly prevented hypothermic ECC-induced platelet loss (p<0.05).

P2Y Blockade in Combination with a PI3K p110β Inhibitor Prevents Upregulation of the Mac-1 Receptor on Granulocytes

Interaction of activated platelets with leukocytes triggers the upregulation of the Mac-1 receptor on leukocytes. Mac-1 mediates leukocyte adhesion and migration as well as interaction and binding of leukocytes and platelets [30]. To evaluate granulocyte activation, we measured surface CD11b expression on granulocytes. Hypothermic ECC induced a profound increase in CD11b expression on granulocytes in controls (p<0.05; Figure 5). This was significantly decreased by P2Y blockade in combination with PI3K p110β inhibition (p<0.001). P2Y and PI3K p110β inhibition alone had no effect on hypothermic ECC-induced CD11b expression on granulocytes.
Discussion

In this study, we investigate the effects of ADP receptor blockade combined with PI3K inhibition on platelets and granulocytes in an ex vivo model simulating hypothermic ECC. In general, our current findings confirm previously reported effects of hypothermic ECC on platelet activation, which lead to numerous responses in platelets like: platelet adhesion to the ECC surface, granule release associated with upregulation of P-selectin on platelets and PMPs, activation of GPIIb/IIIa and subsequent interaction of platelets and granulocytes as well as granulocyte activation (Figure 6) [1,2,12,31,32,33,34]. In our current study, GPIbα and GPIIb/IIIa expression levels as well as vWF binding to platelets were unaffected by hypothermic ECC. Hypothermic ECC did not result in increased PMP numbers, but in an increase of P-selectin expression on PMPs and single platelets. This contributes to explain the increase in platelet-granulocyte aggregate formation and upregulation of Mac-1 expression on granulocytes as observed in our experiments, since P-selectin expressing platelets and PMPs can mediate binding and consecutive activation of platelets and leukocytes [35]. Furthermore, the observed loss of platelet counts after hypothermic ECC can be explained by the fact that aggregate formation as well as platelet adhesion to the ECC surface occurred during hypothermic ECC.

Based on these data and previous findings [3,10,36], a routine strategy for platelet protection during ECC and hypothermia is warranted and could be very beneficial for many patients in clinical settings where ECC and hypothermia are employed.
life administration of cangrelor only during ECC results in rapid recovery of platelet function after ECC and is not associated with blood loss. Nevertheless, cangrelor had no inhibitory effects on platelet-ECC binding and no sustainable effect on GPIIb/IIIa activation [11]. In the setting of normothermic ex vivo ECC (37°C), platelet-ECC adhesion as well as platelet-platelet and platelet-granulocyte aggregation can be very effectively decreased with the P2Y12 inhibitors cangrelor and TGX-221 [4]. In addition, further inhibition of ADP-mediated effects may be achieved by blocking not only P2Y12, but also P2Y1 on platelets. Therefore, the aim of our current study was to combine full platelet ADP receptor blockade with inhibition of shear-induced platelet activation to completely inhibit platelet activation during ECC and hypothermia.

At first, we employed a combination of experimental P2Y12 and P2Y1 blockers to protect platelets during hypothermic ECC. The P2Y1 receptor has been suggested as a crucial target for inhibition of ADP-mediated effects, since P2Y1 activation is necessary for full platelet aggregation [8]. Nevertheless, until today, no P2Y1 antagonist for clinical applications has been developed [40,41]. We therefore used the experimental and reversible 2-MeSAMP, a specific inhibitor of the platelet P2Y12 receptor [43,44], was used in the current study, because previous ex vivo experiments as well as our current in vitro experiments showed substantial and specific P2Y12 blockade to a similar extent then the effect observed with cangrelor [10,11].

Our results show, that combined P2Y12 and P2Y1 blockade results in significant inhibition of P-selectin expression on platelets and PMPs as well as a reduction in platelet-granulocyte aggregates. Nevertheless, similar to results observed in a previous study [11], no effect of P2Y12 and P2Y1 blockade was observed for GPIIb/IIIa activation and platelet-ECC adhesion.

Jackson et al. have reported the platelet P3K p110β isoform as an important target for new anti-thrombotic agents, since it plays a major role in shear-induced activation of platelets [43]. In order to inhibit the platelet P3K isoform p110β, TGX-221, an experimental compound with a reported 1,000-fold selectivity over the other P3K isoforms p110α and p110γ [45], was used. Only the combined administration of P2Y12 blockade and P3K p110β inhibition prevented platelet-ECC adhesion during hypothermic ECC and provided a better protection of platelet counts and inhibition of GPIIb/IIIa activation then P2Y blockers or TGX-221 alone.

Overall, combined blockade of P2Y12, P2Y1, and P3K p110β during hypothermic ECC in our current experiments resulted in an effective reduction of all investigated platelet activation markers. Furthermore, the effect of combined blockade of P2Y12, P2Y1 and P3K p110β achieved inhibitory effects close to values measured at baseline before ECC and is therefore more profound than the sole inhibition of P2Y12, P2Y1 or P3K p110β. This confirms that activation processes mediated via ADP and shear stress in combination are the major players for the induction of platelet activation during hypothermic ECC. Therefore, inhibition of the respective receptors and activation pathways may be very beneficial for patients undergoing hypothermic ECC. As a consequence the development of respective agents for use in clinical applications is highly desirable. Such innovative substances for administration during ECC and hypothermia should optimally have a short half-life. In this case platelet inhibition can be achieved only during the phase of ECC and hypothermia and platelet function is quickly restored after termination of infusion after ECC. We have recently demonstrated the functionality of this innovative pharmacological principle by employing cangrelor during hypothermic ECC in vivo [11].

In conclusion, our novel findings indicate that hypothermic ECC-induced platelet activation as well as subsequent events including aggregate formation and granulocyte activation are completely inhibited by combined P2Y12, P2Y1, and P3K p110β blockade. This therapeutic strategy carries the potential to very effectively protect platelets and to prevent life-threatening complications like thrombosis, bleeding and systemic inflammation in the setting of ECC and hypothermia. Hence, our findings warrant additional studies to further evaluate this new antiplatelet strategy and to develop innovative reversible antiplatelet agents for clinical use.

Limitations and Outlook

The Chandler loop ECC model, which was used for the ex vivo ECC experiments in this study, is a well-established and routinely used model to analyze effects of ECC and hypothermia on platelets and other blood components. However, the obtained results need to be interpreted with caution, since the Chandler loop model differs in some points from the setting of ECC as employed in cardiac surgery. In the Chandler loop PVC tubings, which contain human whole blood, are circulated in a water bath at a designated temperature. Therefore, human whole blood is circulated exclusively and continuously over artificial surfaces, which causes activation of platelets and leukocytes. However, in the Chandler loop model heart-lung machine components like oxygenator and roller pumps, which are routinely used during cardiac surgery, are omitted and therefore shear stress and other activating effects might be decreased in comparison to what happens under clinical conditions. Furthermore, no priming volume is used in the Chandler loop model, which may result in
augmented activating effects. Nevertheless, it has been shown in previous studies that platelet activation is induced during cardiac operations employing a heart-lung machine and hypothermia to a similar extent compared to what we found in our experiments [1,36,46,47].

Our findings indicate that combined P2Y and PI3K inhibition has the potential to achieve complete platelet inhibition during hypothermic ECC and may therefore be very beneficial in the clinical situation. Therefore, our final aim is to evaluate this antiplatelet strategy in a carefully designed clinical study. However, previous studies employing different animal models revealed that there is a species-dependent variation regarding the effect of TGX-221 on bleeding [45,48]. Therefore, before a clinical investigation may be initiated, the effect of our experimental strategy on the bleeding time needs to be carefully evaluated in human blood. Especially administration of short-acting drugs like cangrelor or drugs that do not influence the bleeding time, will be the agents of choice for the in vivo setting and should be further evaluated in the future.

Methods

Blood Sampling

All blood sampling procedures were approved by the Research and Ethics Unit of the University of Tübingen, Germany (project number 270/2010BO1). Written informed consent was obtained from all subjects before blood sampling.

Blood from healthy donors was collected by venipuncture and anticoagulated with heparin [final concentration (fc): 3 I.U./ml]. All subjects were free of platelet-affecting drugs for at least 14 days.

In vitro Evaluation of Different Concentrations of 2-MeSAMP and TGX-221

Heparinized human whole blood (n = 4) was treated for 5 minutes at 37°C with the following agents: PBS as control (Invitrogen GmbH, Karlsruhe, Germany); 2-MeSAMP (fc: 10 and 100 μM; dissolved in PBS; Sigma-Aldrich Corporation, St. Louis, USA), a combination of 2-MeSAMP and MRS2179 (fc: 100 μM each; dissolved in PBS; Tocris Biosciences, Bristol, UK), propylene glycol (PG; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) as control or TGX-221 (fc: 0.5 or 2.2 μM; dissolved in PG; Merck Chemicals Ltd., Nottingham, UK). Afterwards whole blood was incubated with...
ADP (fc: 20 µM) and a phycocyanin (PE)-labeled anti-P-selectin monoclonal antibody (mAb) (BD Biosciences, Heidelberg, Germany) as well as a fluorescein isothiocyanate (FITC)-labeled anti-GPIIb mAb (Beckman Coulter, Aulnay-sous-Bois, France) according to previously described methods for 20 minutes at 37°C [3]. Next, all samples were fixed using CellFix® (BD Biosciences) and flow cytometric analysis was performed within 6 hours.

**Ex vivo ECC Chandler Loop Model**

In each experiment, baseline values were measured directly after blood sampling in 20 ml of heparinized blood. A well-established closed-loop ECC model (Chandler loop) [17] and PVC tubing (tubing length: 50 cm; inner diameter: 7.6 mm; wall: 2.3 mm) without additional coating (Jostra, Hirrlingen, Germany) were used to simulate ECC at 28°C. For each donor, five tubings were filled with 20 ml of fresh heparinized whole blood and treated with the following agents: the first and second sub-samples were treated with PBS and PG as controls, respectively. In the third sub-sample, both platelet ADP receptors were blocked (“P2Y block”) employing the experimental P2Y12 antagonist 2-MeSAMP (fc: 100 µM) and the experimental P2Y1 antagonist MRS2179 (fc: 100 µM). In the fourth sub-sample, PI3K p110β was inhibited by TGX-221 (fc: 2.2 µM). In the last sub-sample, a combination of P2Y block and PI3K p110β inhibition was employed. Afterwards, each tubing was closed into a circuit and circulated in a water bath (at 30 rpm) at 28°C for 30 minutes.

In this study, blood was anticoagulated with heparin, because heparin is routinely applied during clinical procedures employing ECC as anticoagulant to prevent activation of plasmatic coagulation during ECC. The degree of potential platelet activating effects of heparin on platelets has been analyzed in a previous study and found to be of minor extent in our ex vivo ECC model [3].

**Flow Cytometric Analyses of Blood Samples Before and After ECC**

Analyses of human platelets and granulocytes were performed directly after blood sampling (“before circulation”) and after ex vivo ECC in all treatment groups. Expression of P-selectin, GPIIb as well as binding of the PAC-1 mAb against activated GPIIIa/IIIa on platelets was measured according to previously described methods [3,10].

Expression of the GPIIb/IIIa receptor on platelets as well as platelet von Willebrand factor (vWF) binding was evaluated in 25 µl of diluted whole blood (1:50 dilution in modified Tyrode’s buffer) using 5 µl of an anti-GPIIb-FITC (Beckman Coulter) and 5 µl of an anti-vWF-FITC mAb (Ancell, Cambridge, UK), respectively. P-selectin and GPIIb expression as well as vWF binding were also analyzed on ADP-activated (fc: 20 µM) platelets before and after hypothermic ECC in the PBS group as positive controls.

Analysis of platelet microparticles (PMPs) was performed after flow cytometric calibration and setup using a forward scatter/sideward scatter dot plot and 1 and 3 µm-sized latex beads (Polysciences, Eppelheim, Germany). PMPs are defined as objects with a size of ≤1 µm. The number of PMPs was counted and P-selectin expression was analyzed as described previously [3]. Platelet-granulocyte aggregates were detected in 25 µl of whole blood, which was incubated for 20 minutes with 5 µl of an anti-GPIIb-FITC mAb and 5 µl of an anti-CD15-PE mAb. Afterwards, samples were treated with FACS Lysing Solution (BD Biosciences) to lyse erythrocytes, centrifuged at 200 g for 5 minutes and washed with PBS.

CellFix® (BD Biosciences) was used for sample fixation. Flow cytometry was performed within 6 hours using a FACScan® cytometer (BD Biosciences) according to standard procedures [3,10].

For evaluation of granulocyte activation, 90 µl of whole blood was incubated with 10 µl of an anti-CD11b-PE mAb (Beckman Coulter) for 20 minutes at 37°C, treated with FACS Lysing Solution, centrifuged at 200 g for 5 minutes and afterwards washed with PBS. All samples were fixed using CellFix®. Within 6 hours after antibody staining, granulocytes were identified according to their size and granularity and analyzed using flow cytometry.

For all samples, suitable isotype antibodies were used to adjust fluorescence amplification settings. A total of 10,000 events were acquired in each sample. If not otherwise indicated, geometric mean fluorescence intensities were used for analyses of flow cytometric data.

**Detection of Platelet Adhesion to the ECC Surface**

Platelet adhesion to the ECC surface was detected using a specially designed enzyme-linked immunosorbent assay (ELISA) method as previously described [4]. Briefly, ECC tubing was washed and blocked after circulation and surface-bound GPIIb was detected employing a primary anti-GPIIb antibody (Sigma, Deisenhofen, Germany) and an alkaline phosphatase conjugated secondary antibody (Immunotech/Coulter, Marseille, France). The chromogenic reaction was stopped by addition of NaOH. Light absorbance was determined with an ELISA reader MR 5000 (Dynatech, Denkendorf, Germany) at 405 nm.

**Analysis of Platelet Counts**

Before and after circulation, human whole blood was anticoagulated with EDTA (EDTA-Monovette®, Sarstedt, Numbrecht, Germany) for platelet count analysis using an ABX Micros 60 blood analyzer (Axon Lab AG, Baden-Dättwil, Switzerland).

**Statistical Analysis**

Data are depicted as means with standard deviation (SD). In order to test data sets for normality, the Kolmogorov-Smirnov test was performed. Normally distributed data were then analyzed using repeated measures (RM) ANOVA with Bonferroni’s multiple comparison test to analyze differences between groups, while not normally distributed data were analyzed using a non-parametrical test (Friedman test with Dunn’s multiple comparison test). All analyses were performed using the statistical software program GraphPad Prism (version 5, GraphPad Software, La Jolla, USA). Statistical significance was defined as p<0.05.

**Author Contributions**

Conceived and designed the experiments: KP HW AS. Performed the experiments: SK JK AS. Analyzed the data: SK JK TG HW AS. Wrote the paper: SK AS. Approved the final manuscript version: SK JK TG KP HW AS.

**References**

1. Weerasinghe A, Taylor KM (1998) The platelet in cardiopulmonary bypass. Ann Thorac Surg 66: 2145–2152.
2. Paparella D, Brister SJ, Buchanan MR (2004) Coagulation disorders of cardiopulmonary bypass: a review. Intens Care Med 30: 1050–1057.
3. Straub A, Breuer M, Wendel HP, Peter K, Dietz K, et al. (2007) Critical temperature ranges of hypothermia-induced platelet activation: Possible implications for cooling patients in cardiac surgery. Thromb Haemost 97: 600–616.
4. Straub A, Wendel HP, Dietz K, Schiebold D, Peter K, et al. (2008) Selective expression of glycoprotein IIb/IIIa p110beta as promising new strategy for platelet protection during extracorporal circulation. Thromb Haemost 99: 609–613.

5. May AE, Seager P, Gavaz M (2008) Platelets: inflammatory firebugs of vascular walls. Arterioscler Thromb Vasc Biol 28: s5–10.

6. da Costa Martins PA, van Gils JM, Mol A, Hordijk PL, Zwaginga JJ (2006) Platelet binding to monocytes increases the adhesive properties of monocytes by up-regulating the expression and functionality of beta1a and beta2 integrins. J Leukoc Biol 79: 499–507.

7. Rahman M, Zhang S, Chev M, Ersson A, Jepsson B, et al. (2009) Platelet-derived CD40L (CD154) mediates neutrophil upregulation of Mac-1 and recruitment to sites of lung injury. J Am Coll Surg 209: 783–790.

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

9. Cattano M, Pedda GM (2010) State of the art of new P2Y12 antagonists. In: Antithrombotic therapy: from bench to bedside. InTech, doi: 10.5772/15564.

10. Straub A, Krajewski S, Hohmann JD, Westein E, Jia F, et al. (2011) Evidence of platelet activation at medically used hypothermia and mechanistic data indicating ADP as a key mediator and therapeutic target. Arterioscler Thromb Vasc Biol 31: 1607–1616.

11. Krajewski S, Kurz J, Neumann B, Greiner TO, Stolz A, et al. (2012) Short-acting P2Y12 blockade to reduce platelet dysfunction and coagulopathy during experimental extracorporal circulation and hypothermia. Br J Anaesth doi: 10.1093/bja/ear250.

12. Maxwell MJ, Dopheide SM, Turner SJ, Jackson SP (2006) Shear induces shedding from platelets. J Biomech 39: 385–391.

13. Straub A, Krajewski S, Hohmann JD, Westein E, Jia F, et al. (2011) Continuous signaling via PI3K isoforms beta and gamma is required for platelet ADP receptor function in dynamic thrombus stabilization. Blood 108: 3045–3052.

14. Jackson SF, Schoenwaelder SM (2006) Type I phosphoinositide 3-kinases: potential antithrombotic targets? Cell Mol Life Sci 63: 1085–1099.

15. Andrews RK, Gardiner EE, Shen Y, Whistock JC, Berndt MC (2003) Glycoprotein Ib-IX-V. Int J Biochem Cell Biol 35: 1170–1174.

16. Andrews RK, Gardiner EE, Shen Y, Berndt MC (2004) Platelet interactions in thrombosis. IUBMB life 56: 13–18.

17. Straub A, Schiebold D, Wendel HP, Azevedo SM, Turner SJ, Jackson SP (2006) Shear induces a unique series of morphological changes in translocating platelets: effects of morphology on translocation dynamics. Arterioscler Thromb Vasc Biol 26: 663–669.

18. Cossman JM, Munnix IC, Wetzker R, Heller R, Jackson SP, et al. (2006) Continuous signaling via PI3K isoforms beta and gamma is required for platelet ADP receptor function in dynamic thrombus stabilization. Blood 108: 3045–3052.

19. JacksonSF, Schoenwaelder SM (2006) Type I phosphoinositide 3-kinases: potential antithrombotic targets? Cell Mol Life Sci 63: 1085–1099.

20. May AE, Seizer P, Gawaz M (2008) Platelet: inflammatory firebugs of vascular walls. Arterioscler Thromb Vasc Biol 28: s5–10.

21. Merchant AD, Ellis PA, Barnard MR, Matic GB, Viles AF, et al. (1991) Platelet activation and aggregation in heparinized flowing human blood: Identification of a high responder subpopulation. Ann Biomed Eng 19: 821–833.
Author/s: 
Krajewski, S; Kurz, J; Geisler, T; Peter, K; Wendel, HP; Straub, A

Title: 
Combined Blockade of ADP Receptors and PI3-Kinase p110 beta Fully Prevents Platelet and Leukocyte Activation during Hypothermic Extracorporeal Circulation

Date: 
2012-06-06

Citation: 
Krajewski, S., Kurz, J., Geisler, T., Peter, K., Wendel, H. P. & Straub, A. (2012). Combined Blockade of ADP Receptors and PI3-Kinase p110 beta Fully Prevents Platelet and Leukocyte Activation during Hypothermic Extracorporeal Circulation. PLOS ONE, 7 (6), https://doi.org/10.1371/journal.pone.0038455.

Persistent Link: 
http://hdl.handle.net/11343/244608

File Description: 
published version

License: 
CC BY