Epinephrine restores platelet functions inhibited by ticagrelor: A mechanistic approach

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Abstract

Ticagrelor, an antagonist of the platelet adenosine diphosphate (ADP)-$P2Y_{12}$ receptor is recommended for patients with acute coronary syndromes. However, ticagrelor exposes to a risk of bleeding, the management of which is challenging because platelet transfusion is ineffective, and no antidote is yet available. We hypothesized that the vasopressor drug epinephrine could counter the antiplatelet effects of ticagrelor and restore platelet functions. We assessed *in vitro* the efficiency of epinephrine in restoring platelet aggregation inhibited by ticagrelor and investigate the underlying mechanisms. Washed platelet aggregation and secretion were measured upon stimulation by epinephrine alone or in combination with ADP, in the presence or absence of ticagrelor. Mechanistic investigations used $P2Y_1$ and phosphoinositide 3-kinase (PI3K) inhibitors and included vasodilator-stimulated phosphoprotein (VASP) and Akt phosphorylation assays as well as measurement of Ca^{2+} mobilisation. We found that epinephrine restored ADP-induced platelet aggregation, but not dense granule release. Epinephrine alone failed to induce aggregation whereas it fully induced VASP dephosphorylation and Akt phosphorylation regardless of the presence of ticagrelor. In the presence of ticagrelor, blockage of the $P2Y_1$ receptor prevented restoration of platelet aggregation by the combination of epinephrine and ADP, as well as intracellular Ca^{2+} mobilisation. In combination with ADP, epinephrine induced platelet aggregation of ticagrelor-treated platelets through inhibition of the cAMP pathway and activation of the PI3K pathway, thus enabling the $P2Y_1$ receptor signalling and subsequent Ca^{2+} mobilisation. This proof-of-concept study needs to be challenged *in vivo* for the management of bleeding in ticagrelor-treated patients.

Keywords antiplatelet agent; ticagrelor; reversal; epinephrine; signalling pathway.

1. Introduction

Ticagrelor is a potent direct-acting, selective and reversible antagonist of the platelet adenosine diphosphate (ADP) $P2Y_{12}$ receptor, approved for the treatment of acute coronary syndromes (Valgimigli et al, 2018). Like other antiplatelet agents, ticagrelor exposes to a bleeding risk, which management is challenging because platelet transfusion, usually recommended to reverse antiplatelet agents, is inefficient (Martin et al, 2016; Godier et al, 2015; Rossaint et al, 2016). In addition, ticagrelor is not dialyzable and haemostatic agents, including recombinant activated factor VII, tranexamic acid, and desmopressin, are unlikely to fully control ticagrelor-associated bleeding complications (Calmette et al, 2017; Pehrsson et al, 2016; Teng et al, 2014). PB2452 is a specific antidote under development but not available yet (Buchanan et al, 2015; Bhatt et al, 2019). Therefore, there is still a critical need for an effective strategy to restore platelet functions in ticagrelor-treated patients facing bleeding events. Our rationale was to evaluate the potential of bypassing $P2Y_{12}$ pathway, and epinephrine was an interesting candidate. This physiological neurohormone is released from adrenal chromaffin cells or neurons in response to stress. Epinephrine binds to various adrenergic receptors (α_1, α_2, β_1, β_2) selectively present in different organs leading to activation of their specific pathways and adaptive responses. As a therapy, epinephrine is used for its hemodynamic properties to treat cardiogenic and anaphylactic shock. In addition, epinephrine is an important mediator of platelet activation acting through the stimulation of α_{2A}-adrenergic receptors coupled to G_z-protein on the platelet membrane surface (Kaywin et al, 1978; Newman et al, 1978; Motulsky and Insel, 1982; Jakobs et al, 1978). G_z-protein belongs to the G_i-protein family and shares functional and structural similarities with other members, including the G_i-protein coupled with the $P2Y_{12}$ receptor. In platelets, signalling pathways downstream ADP activation have been more thoroughly explored than those resulting from epinephrine activation. ADP is a weak agonist acting via the two receptors $P2Y_{12}$ and $P2Y_1$. The α subunit of G_i-protein ($G_{i\alpha}$) coupled to $P2Y_{12}$ binds to adenylate cyclase and inhibits the synthesis of the platelet inhibitory second messenger cyclic

adenosine monophosphate (cAMP) (Li et al, 2010). cAMP stimulates cAMP-dependent protein kinase (PKA) leading to phosphorylation of downstream targets, critical for maintaining platelets in a resting state, including the vasodilator-stimulated phosphoprotein (VASP). Therefore, $G_{i\alpha}$ stimulation leading to VASP dephosphorylation participates in platelet activation. Moreover, the dissociated βγ subunit of the G_i-protein activates phosphoinositide 3-kinase (PI3K) and generates phosphatidylinositol 3,4,5-triphosphate, which mediates membrane translocation of the protein kinase Akt, allowing its subsequent phosphorylation and activation. Eventually, stimulation of PI3K pathway promotes sustained activation, granule secretion, and stable thrombus formation. On the other hand, the $P2Y_1$ ADP receptor coupled to its G_q-protein stimulates the phospholipase C (PLC), leading to intracellular Ca^{2+} increase and protein kinase C (PKC) activation. Ca^{2+} mediates several responses, including Rap1 activation and thromboxane synthesis via phospholipase A2. Although epinephrine had been shown to share part of its signalling pathways with ADP, its effects on platelet functions are not completely characterized. We investigated whether epinephrine could restore platelet functions inhibited by ticagrelor. We hypothesized that platelet activation induced by epinephrine in a milieu containing ADP translates into platelet aggregation despite the presence of ticagrelor. We performed an *in vitro* study to assess efficacy of epinephrine in restoring platelet aggregation in the presence of ticagrelor and to decipher the signalling pathways involved in this process.

2. Materials and Methods

Blood samples were obtained from healthy donors at the French Blood Bank Institute (Etablissement Français du Sang, EFS) after written informed consent, according to the agreement between the EFS and Université Paris Descartes (convention ref. C CPSL UNT n°12/EFS/038). Blood samples were collected in 0.105 M sodium citrate tubes (BD Vacutainer, Franklin Lakes, New Jersey, USA) or in acid citrate dextrose (ACD) tubes (BD Vacutainer, citric acid 5.7 mM, trisodium citrate 11.2 mM and dextrose 20 mM, final concentrations) to prepare washed platelet suspension.

2.1 Isolation and preparation of washed platelet suspension

Blood samples collected into ACD tubes were mixed with wash buffer (citric acid 36 mM, glucose 5 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM and NaCl 103 mM; pH=6.5) containing 0.2 µM prostaglandin E1 (PGE1, Sigma Aldrich, St. Louis, Missouri, USA) and 0.03 IU/ml apyrase (Agro-Bio, La Ferté-Saint-Aubin, France) to avoid platelet activation during preparation. Platelet-rich plasma was obtained after a first spin (216 g for 11 min at room temperature). Platelets were washed twice with wash buffer, containing PGE1 and apyrase, and centrifuged (1200 g for 11 min). The last pellet was resuspended in suspension buffer (Hepes 10 mM, NaCl 140 mM, KCl 3 mM, $NaHCO_3$ 5 mM, $MgCl_2$ 0.5 mM, and glucose 10 mM; pH=7.35) to a final concentration of $3x10^8$ platelets/ml. Apyrase (0.03 IU/ml) was added to prevent ADP receptor desensitization, then, $CaCl_2$ 2 mM was added (Ardlie et al, 1971).

2.2 Agonists and inhibitors

Ticagrelor was extracted from tablets (Brilique® 90 mg, AstraZeneca). Briefly, tablets were dispersed in 10 mM HCl and the water-soluble excipient extracted by centrifugation (5 min, 5000 g, 20°C). Pellet was washed 3 times with distilled water followed by centrifugation (5 min, 5000 g, 20°C). Pellet was resuspended in distilled water, snap-frozen in liquid nitrogen, then lyophilized. Ticagrelor was extracted from the lyophilized material by incubation in pure DMSO. Insoluble material in the ticagrelor-saturated DMSO was removed by two centrifugations (5 min, 5000 g, 20°C). The concentration of ticagrelor-saturated DMSO solution was around 100 mg/ml, and was diluted 1/10 in pure DMSO prior storage at -80°C. Further dilutions were performed in suspension buffer containing 1% DMSO. The final DMSO concentration in all experiments was 0.001%. Final concentration of ticagrelor was assessed by reference to ticagrelor raw material provided by AstraZeneca (Mölndal, Sweden).[2] ADP (Roche Molecular Biochemicals, Meylan, France) diluted in suspension buffer was used at 10 µM final concentration. The proteinase-activated receptor-1 activating peptide (PAR-1-AP, Bachem, Basel, Switzerland) was used as a primary agonist at 2 µM

final concentration to assess ADP released from the dense granules following platelet activation or at 10 µM final concentration to provide maximal activation. The specific $P2Y_1$ inhibitor MRS2179 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted in 0.9% saline and used at 10 µM final concentration. The non-selective PI3K inhibitor wortmannin (Sigma-Aldrich, St. Louis, MO, USA) was diluted in suspension buffer containing 0.2% dimethyl sulfoxide (DMSO) and used at 0.5 µM final concentration. Washed platelets were incubated with ticagrelor for 10 min at 37°C prior to use. A final concentration of 150 nM ticagrelor was chosen as it inhibited at least 80% of platelet aggregation, according to a dose-ranging study (Fig. S1). In whole blood experiments, a final concentration of 3.25 µM ticagrelor was used to obtain at least 80% of inhibition of platelet aggregation as previously described.[2] Epinephrine (EPI, Aguettant, Lyon Gerland, France) was diluted in suspension buffer and used at a final concentration of 10 µM, selected after a dose-ranging study as the lowest concentration increasing ADP-induced platelet aggregation to a similar level than 10 µM PAR-1-AP.

2.3 Platelet aggregation

Platelet aggregation was measured using light transmission aggregometry (LTA, ChronoLog Aggregometer Model 700, Chrono-log Corporation, Havertown, PA, USA): washed platelets (290 µl; $3x10^8$/ml) pre-treated with ticagrelor or vehicle were incubated for 2 min at 37°C under stirring in the presence of fibrinogen (300 µg/ml, Hyphen Biomed, Neuville-sur-Oise, France). Aggregation was induced by adding the agonist (10 µM ADP, 10 µM epinephrine, same concentrations of ADP and epinephrine combined, or 10 µM PAR-1-AP). For PI3K signalling pathway analysis, washed platelets were first incubated for 2 min at 37°C with wortmannin (0.5 µM final concentration) or vehicle. Aggregation was recorded during 6 min and results were expressed in percentage of the maximal aggregation. Experiments were repeated without adding ADP, using 2 µM PAR-1-AP as the primary agonist, a concentration sufficient to induce ADP release and subsequent aggregation. Percentage of disaggregation reflecting time-course of aggregation and stability of platelet aggregates at three time points

(1, 2 and 3 minutes after maximal aggregation) was computed as: [(maximal aggregation – time point aggregation)/maximal aggregation] x 100. Effect of wortmannin was quantified as the percentage of inhibition relative to the maximal aggregation obtained in its absence.

2.4 Adenosine triphosphate (ATP) secretion

ATP release as a measure of platelet dense granule secretion was assessed by lumi-aggregometer using the luciferin-luciferase reagent (Chrono-Log Corporation). Luciferin-luciferase was incubated for 2 min under stirring with washed platelets pre-incubated with ticagrelor or vehicle (resuspension buffer/DMSO), for 10 min at 37°C before adding the agonists. ATP secretion was expressed in nanomoles (nmol).

2.5 Vasodilator-stimulated phosphoprotein (VASP) phosphorylation analysis

VASP phosphorylation was determined using the immunoassay kit (CY-QUANT VASP/$P2Y_{12}$, BioCytex, Marseille, France) modified to assess the specific effects of epinephrine. Briefly, PGE1 ([PGE1]) or a combination of PGE1 and ADP, with or without 1 µM epinephrine ([sample]) were added to whole blood pre-incubated with ticagrelor or vehicle. Following incubation at room temperature for 10 min and lysis, samples were frozen and stored at –20°C and subsequently thawed, immediately prior to ELISA. Samples were loaded on a 16-well strip coated with a mouse anti-human VASP monoclonal antibody, then the wells were covered, incubated for 30 min at room temperature, and washed 3 times. A specific mouse anti-human phosphorylated-VASP monoclonal antibody coupled to peroxidase was added, wells were covered, incubated for 30 min at room temperature, and washed. 3,3',5,5'-tetramethylbenzidine was added for colour development and incubated for 5 min at room temperature. Reactions were stopped by acidification with H_2SO_4 and the A450 measured using a microplate reader (MGW Discovery HT-R, Winooski, VT, USA). Results were conventionally expressed in platelet reactivity index (PRI), computed as: ((A450 [PGE1] - A450 [sample]) / (A450 [PGE1] - A450 [blank])) x 100, where A450 [blank]

is the absorbance of the dilution buffer. PRI increases as VASP is dephosphorylated, thus reflecting platelet activation.

2.6 Measurement of thromboxane generation

Thromboxane B2, the stable metabolite of thromboxane A2 (TxA2), was used as a readout of TxA2 production and quantified using an enzyme immunoassay kit (Bio-techne, Minneapolis, MN, USA). In brief, 6 min after triggering platelet aggregation, further production of TxA2 was quenched by adding 1 mM EDTA at 4°C. Samples were immediately centrifuged (2 min, 13 000 g, 4°C) and supernatants stored at -80°C until analysis. Results were expressed in ng/ml.

2.7 Measurement of Ca^{2+} mobilisation

Calcium ion (Ca^{2+}) mobilisation measurements were performed as previously described (Ohlmann, 1967). In brief, changes in intracellular Ca^{2+} concentration of stirred platelets ($3x10^8$/ml) loaded with fura-2 at 37 °C were measured by spectrofluorimetry. Fluorescence excitation was performed at 340 nm and 380 nm, and emission recorded at 510 nm. All experiments were performed in the presence of 1 mM extracellular EGTA, to selectively and exclusively detect Ca^{2+} mobilisation from intracellular stores. The concentration of free intracellular Ca^{2+} was quantified using the Grynkiewicz equation: $[Ca^{2+}] = [Kd \times Sfb \times (R - Rmin)]/(Rmax - R)$ where Kd is the Ca^{2+} dissociation constant for fura-2 at 37°C (225 nM), R the ratio of fluorescence through 340 nm excitation to that through 380 nm, Rmax is R obtained in saturating Ca^{2+} conditions, Rmin is R in Ca^{2+}-free conditions, and Sfb the ratio of baseline fluorescences (380 nm excitation) in Ca^{2+}-free to that in Ca^{2+}-bound conditions. Intracellular Ca^{2+} mobilisation was calculated as the difference between Ca^{2+} concentration after and before agonist stimulation, and expressed in nmol/L.

2.8 Protein extraction and immunoblotting

Phosphorylation of Akt, one of several down-stream effectors of PI3K, was used as readout of PI3K pathway activation. Platelets were stimulated with agonists in the presence of 0.5 mM RGDS peptide (Sigma-Aldrich), to prevent the amplification process consecutive to fibrinogen binding and “outside-in” signalling. After 3 min activation, the reaction was stopped by the addition of detergent buffer (NuPAGE LDS, Invitrogen) and heating at 70°C for 10 min. Proteins were separated by SDS-PAGE on a 10% acrylamide gradient gel and transferred onto a nitrocellulose membrane. Non-specific binding sites were blocked by incubation in 20 mM Tris, 140 mM NaCl, 0.1% (v/v) Tween 20 (TBST) containing 0.5% (w/v) milk protein for 45 min at room temperature. Membranes were incubated overnight at 4°C with a rabbit primary antibody, anti-phospho-Akt Ser473 (Cell Signalling, #9271) under gentle agitation. Staining was obtained with a Dylight 800 goat anti-rabbit Ig-G antibody (Thermo Fisher Scientific) and image recorded with an Odyssey CLx Imager (LI-COR Biotechnology). Protein loading was controlled by probing the membranes with GAPDH (Abcam, Cambridge, UK). Quantification of Akt phosphorylation was performed with ImageJ software and expressed in arbitrary units.

2.9 Statistical analysis

Quantitative data were expressed as medians [minimum-maximum] or box-and-whisker plots showing median, interquartile range, minimum, and maximum values, or histograms with median and maximum values. Statistical analysis was performed using Prism v6.0 (GraphPad software, CA). Nonparametric tests were used for comparisons. Variables were compared using the Friedman test, followed by the Wilcoxon test (paired samples) or the Mann-Whitney (independent samples). All statistical tests were two-sided and $P<0.05$ was considered statistically significant.

3. Results

3.1 In the absence of ticagrelor, epinephrine potentiates ADP-induced platelet aggregation

As expected, ADP did not induce washed platelet aggregation in the absence of fibrinogen (data not shown). In the presence fibrinogen, ADP induced platelet aggregation, which spontaneously disaggregated over time (Fig. 1A, B, C). In contrast, epinephrine induced very weak aggregation (6 [4-13] vs. 59 [54-73]%, for epinephrine and ADP, respectively, P<0.01). Epinephrine and ADP in combination induced significantly stronger aggregation than ADP (or epinephrine) alone, reaching the aggregation level induced by 10 µM PAR-1-AP (87 [76-94] vs. 84 [76-93]%, P=0.62). Thus, epinephrine and ADP exhibited synergistic effects.

3.2 Epinephrine restores ADP- and PAR-1-AP-induced platelet aggregation inhibited by ticagrelor

Ticagrelor inhibited ADP-induced platelet aggregation (5 [2-17] vs. 59 [54-73]%, P=0.002) (Fig. 1A, B). Whereas epinephrine alone had almost no effect, it fully restored the ADP-induced platelet aggregation abolished by ticagrelor. Indeed, in the presence of ticagrelor, the maximum aggregation in response to the combination was comparable to that observed with ADP alone in the absence of ticagrelor (62 [49-72] vs. 59 [54-73]%, P=0.67). Moreover, aggregation was more stable (Fig. 1A, C), since disaggregation was significantly lower with the combination of epinephrine and ADP in the presence of ticagrelor than with ADP alone in its absence (22% vs. 76%, P=0.002 at 3 min). Owning the role played by ADP as a second agonist released in response to strong platelet stimulation, we repeated the experiments in the absence of exogenous ADP, using PAR-1-AP as the agonist. Ticagrelor also inhibited PAR-1-AP-induced platelet aggregation (16 [5-25] vs. 70 [55-86]%, P<0.01), which was completely restored by adding epinephrine (73 [69-76] vs. 70 [55-86]%, P=0.496) (Fig. 2).

3.3 Restoration of ADP-induced platelet aggregation is independent of dense granule release and of thromboxane formation

We assessed ATP release to reflect dense granule secretion. As expected, in the absence of ticagrelor, neither ADP nor epinephrine alone, which are both weak agonists, induced washed-platelet ATP secretion, compared to 10 µM PAR-1-AP (Fig. 3). In contrast, combination of ADP and epinephrine induced a significant ATP release (0.69 [0.53-0.87] vs. 0 [0-0.13] nmol, for the combination and ADP alone, respectively, P=0.002). However, ATP secretion was far lower with the combination of ADP and epinephrine than with 10 µM PAR-1-AP (0.69 [0.53-0.87] vs. 1.3 [1.1-1.6] nmol, P=0.002), despite a similar level of aggregation (Fig. 1B). In the presence of ticagrelor, combination of epinephrine and ADP, while restoring platelet aggregation, failed to induce detectable ATP release. Therefore, epinephrine restoration of ADP-induced aggregation inhibited by ticagrelor did not involve dense granule release. The potential role of TxA2 formation as an amplifying signal for platelet activation was also assessed. Whatever the presence or the absence of ticagrelor, neither ADP, epinephrine, nor the combination of epinephrine and ADP induced TxA2 synthesis, in contrast to 10 µM PAR-1-AP (149 [100-181] ng/ml).

3.4 Epinephrine restores VASP dephosphorylation in the presence of ticagrelor

Next, we examined potential mechanisms underlying the restoration of platelet aggregation by epinephrine and ADP in the presence of ticagrelor. In ticagrelor-free samples, epinephrine and ADP induced comparable VASP dephosphorylation (PRI=75 [50-90] vs. 90 [87-95]%, NS) (Fig. 4). As expected, ticagrelor strongly decreased ADP-induced VASP dephosphorylation (PRI=15 [0-55] vs. 90 [87-95]%, P<0.05). In contrast, ticagrelor did not modify VASP dephosphorylation in epinephrine-treated samples: levels of phosphorylation were comparable with and without ticagrelor (78 [40-92] vs. 78 [44-94]%, P=0.94). Adding ADP to epinephrine had no incremental effect (83 [55-98] vs. 78 [40-92]%, P=0.15). Thus, whereas epinephrine alone was unable to induce platelet aggregation it was able, as ADP, to fully inhibit cAMP-pathway in this assay and to induced VASP dephosphorylation even in the presence of ticagrelor.

3.5 Epinephrine restores PI3K pathway activation in the presence of ticagrelor

Fig. 5 (A and B) show that ticagrelor inhibited ADP-induced Akt phosphorylation via the inhibition of $P2Y_{12}$ and its downstream PI3K effector. In contrast, we evidenced that epinephrine-induced Akt phosphorylation was not affected by ticagrelor, thus was independent of $P2Y_{12}$ signalling (P=0.34). Therefore, because ticagrelor abolished ADP-induced Akt phosphorylation, the remaining activation of the PI3K pathway would only result from epinephrine stimulation. In the absence of ticagrelor, epinephrine and ADP acted in synergy resulting in a much stronger Akt phosphorylation. In the presence of ticagrelor, Akt phosphorylation induced by the combination of epinephrine and ADP was similar to that induced by epinephrine alone with or without ticagrelor (1898 [1220-5280] vs 1987 [1528-5106] U and 2461 [2306-4930] U, P=0.9 and P=0.8, respectively). Moreover, adding enough wortmannin to fully abolish Akt phosphorylation decreased the ADP-induced platelet aggregation (46 [35-64]% reduction), as well as that induced by a combination of epinephrine and ADP in the presence of ticagrelor (54 [35-69]% reduction): regardless the agonists, PI3K pathway activation contributed to about half of the observed platelet aggregation. Altogether our results support the involvement of the PI3K pathway in the restoration of platelet aggregation by epinephrine.

3.6 $P2Y_1$–induced signalling pathway plays a crucial role in the restoration of platelet aggregation

Next, we investigated the role of $P2Y_1$ receptor signalling pathway using the competitive inhibitor MRS2179. In the absence of ticagrelor, MRS2179 inhibited ADP-induced platelet aggregation, but had only a weak inhibitory effect on platelet aggregation induced by a combination of ADP and epinephrine (71 [54-77] vs. 83 [79-87]%, P=0.04) (Fig. 6A). In contrast in ticagrelor-spiked samples, MRS2179 abolished platelet aggregation previously restored by the combination of epinephrine and ADP (9 [6-12] vs. 55 [51-65]%, P=0.0006). Nevertheless, MRS2179 affected platelet aggregation induced by the combination of ADP and epinephrine to a lesser extent than ticagrelor.

To further explore the role of Gq/$P2Y_1$ receptor pathway, we focused on intracellular Ca^{2+} mobilisation. ADP induced intracellular Ca^{2+} mobilisation (68 [62-70] nmol/L) which was abolished by MRS2179 (6 [6-7] nmol/L), but not by ticagrelor (48 [40-93] nmol/L) (Fig. 6B). Epinephrine alone was unable to induce significant Ca^{2+} mobilisation, but tended to potentiate Ca^{2+} mobilisation induced by ADP alone (93 [73-119] vs. 68 [62-70] nmol/L). As expected, ticagrelor did not alter Ca^{2+} mobilisation induced by a combination of epinephrine and ADP (115 [87-130] nmol/L) unlike MRS2179 (55 [30-60] nmol/L). However, in presence of both inhibitors, combination of epinephrine and ADP only induced a very weak Ca^{2+} mobilisation (15 [15-42] nmol/L). A schematic model of the signalling pathways involved in epinephrine restoration of ADP-induced platelet aggregation inhibited by ticagrelor is proposed Fig. 7.

4. Discussion

Several *in vitro* studies described epinephrine as a weak platelet activator acting synergistically with other agonists such as ADP or thrombin to potentiate platelet aggregation (Mills and Roberts, 1967; Lanza et al, 1988; O'Brien, 1963). However, the synergism of epinephrine and ADP to overcome the inhibitory effect of $P2Y_{12}$ inhibitors, especially ticagrelor, has not been investigated in details. Here, we confirmed that epinephrine alone fully inhibited cAMP signalling pathway and activated the PI3K pathway (Fig. 7), but failed to induce washed platelet aggregation by itself. Mainly, we provided evidence that in combination with ADP, epinephrine counters the antiplatelet effect of ticagrelor on platelet aggregation. We evidenced that aggregation was partly due to epinephrine itself through cAMP pathway inhibition and PI3K pathway activation, as well as ADP-induced $P2Y_1$-receptor related signalling and Ca^{2+} mobilisation.

In our results, epinephrine failed to induce secretion and aggregation by itself, but we established that, far from only potentiating the effects of ADP, epinephrine in combination with ADP triggered functions that no agonist alone initiated, in particular dense granule release (Blair et al, 2015). However, our findings suggested that restoration of ADP-induced

platelet aggregation inhibited by ticagrelor did not involve dense granule secretion nor thromboxane generation. Interestingly, it has been previously reported that epinephrine improves platelet adhesion in a high shear stress model of flow-based adhesion on von Willebrand Factor (VWF) (Goto et al, 1992). Similarly, we observed under flow that, in the presence of ticagrelor, epinephrine in combination with ADP increased platelet adhesion and formation of large aggregates (data not shown). Regarding ADP, Mazzucato et al. demonstrated the early role of $P2Y_1$ in promoting stable platelet adhesion to VWF, which is an absolute requirement for subsequent aggregation, while $P2Y_{12}$ supports the formation of large platelet aggregates (Mazzucato et al, 2004). Therefore, epinephrine might not only improve adhesion by reinforcing the contribution of $P2Y_1$, it could substitute the $P2Y_{12}$ pathway in the amplification of thrombus formation.

Studies in knockout mice have established that $G_{z\alpha}$ plays a critical role in modulating adenylate cyclase activity and platelet aggregation in response to epinephrine (Jantzen et al, 2001). Epinephrine signalling pathway has been investigated mainly through measuring changes in intracellular cAMP level (Yang et al, 2002; Keularts et al, 2000). Herein we investigated the implication of this pathway in the ability of epinephrine to restore ADP-induced platelet aggregation abolished by ticagrelor and assessed the involvement of the downstream VASP phosphorylation. We observed that epinephrine induced full VASP dephosphorylation, regardless the presence of ticagrelor or ADP. This important result confirmed that epinephrine inhibited activation of cAMP pathway, via α_{2A}-adrenergic-coupled G_z, independently of other signalling pathways, especially those induced by $P2Y_{12}$ activation. Epinephrine appeared to substitute the cAMP pathway inhibition, impaired by $P2Y_{12}$ blockage. Therefore, using VASP assay to evaluate recovery of the cAMP pathway signalling following epinephrine administration could represent a clinical option for reversal monitoring in ticagrelor-treated patients.

We then characterized the contribution of the PI3K pathway. Our results supported that epinephrine, via the βγ subunits of G_z-protein, supplied βγ subunits of G_i-protein coupled with $P2Y_{12}$, inhibited by ticagrelor. However, we found that PI3K pathway, while not essential,

contributed to approximately half of platelet aggregation. Our findings are in agreement with those of Blair et al. who showed, in a model of PAR-1-AP-stimulated platelets, that wortmannin partially abolishes the enhancement of aggregation induced by epinephrine in aspirin- and/or AR-C66096-spiked samples (Blair et al, 2015). Also, our study put an end to the debate whether epinephrine alone can or cannot activate the PI3K pathway, independently of $P2Y_{12}$ activation (Lova et al, 2002, Woulfe et al, 2002).

Finally, we investigated the role of $P2Y_1$ and downstream signalling in restoration of platelet activation induced by the combination of epinephrine and ADP, in the presence of ticagrelor. It is known that G_q-mediated activation of phospholipase C-β (PLC-β) is a prerequisite for platelet aggregation with most agonists, and that weak agonists such as ADP require the simultaneous co-activation of G_i-coupled signalling pathways to elicit the aggregation response. In our study, we demonstrated that G_z signalling pathway substituted for the inhibited G_i pathway albeit requiring cross-talking with G_q-protein. In fact, adding $P2Y_1$ inhibitor to ticagrelor-spiked samples suppressed the capacity of combination of epinephrine and ADP to restore platelet aggregation. This was expected, because G_z belongs to G_i-protein family, but had not been previously explored using $P2Y_1$ inhibitor. We confirmed that ticagrelor reduced Ca^{2+} mobilisation in response to ADP. Indeed, as described previously, $P2Y_{12}$ inhibitors alter $P2Y_1$ downstream signalling through cross-talking between G_i and G_q-proteins at the level of PLC (Guidetti et al, 2008). We also found that, in conjunction with G_q-protein activation, epinephrine/G_z overcame the inhibitory effect of ticagrelor on G_i-protein by significantly increasing Ca^{2+} mobilisation to an even higher level than that induced through G_i and G_q-pathways. Ca^{2+} mobilisation was abolished by MRS2179. This highlighted the cross-talk between the G_z and G_q-proteins, and emphasized the crucial role of the $P2Y_1$-coupled G_q pathway for the epinephrine-ADP restoration of platelet aggregation inhibited by ticagrelor. Altogether our results highlight that G_z-protein acts through specific mechanisms of action. Although G_z and G_i-proteins belong to the same family, thus share both structural and functional homologies, they are not redundant (Yang et al, 2002). Indeed, in the presence of ticagrelor, co-stimulation of G_z and G_q with epinephrine and ADP induced a more stable,

although still reversible, platelet aggregation than high concentrations of ADP in the absence of ticagrelor. Overall, G_z-protein ensures or enhances platelet aggregation; it even restores platelets with compromised functions, such as ticagrelor-inhibited $P2Y_{12}$.

Our original perspective was to consider epinephrine as a therapeutic option for rescuing ticagrelor-induced platelet inhibition to improve haemostasis and potentially control bleeding, especially during haemorrhagic shock. Our results are in line with previous studies, showing that elevated level of epinephrine contributes to platelet hyperactivity and thrombus formation. Using a model of artery stenosis, Yao et al. and Samama et al. have evidenced, in canine and pig, respectively, that epinephrine infusion fully restored platelet thrombus formation abolished by clopidogrel (Yao et al, 1992; Samama et al, 1992). Recently, a randomized study demonstrated that infusion of low epinephrine doses (0.05 µg/kg/min) in patients undergoing total hip replacement surgery reduced bleeding without increasing thromboembolic events compared to the standard of care (Liu et al, 2018). Although patients treated with $P2Y_{12}$ inhibitors were excluded from this analysis, these results highlight the feasibility of epinephrine administration to control bleeding during planned surgery in patients without hemodynamic instability.

Our study has inherent weaknesses since there is limited extend and no guarantee to directly translate data obtained *in vitro* into the clinical scenario of severe bleeding in patient receiving ticagrelor. One could question the doses and concentrations used in our experimental study. We carefully selected our *in vitro* conditions as a proof-of-concept to further translate it into a more physiological context. Indeed, we have extended this concept using PAR-1-AP as a primary agonist, a more physiological model than using ADP which acts as a secondary agonist at the site of injury. Here, the limited amount of ADP released from the dense granules following platelet activation appeared sufficient to support the epinephrine restoration of platelet aggregation. We used µM concentrations of epinephrine, whereas nM concentrations are sufficient to fulfil physiological effects. However, we performed a dose-ranging study to select the lowest dose able to potentiate ADP-induced platelet aggregation in our experimental conditions, and this is in line with most *in vitro*

publications (Lanza et al, 1988, O'Brien, 1963; Blair et al, 2015). Actually, Singh et al. recently confirmed that even low clinically relevant doses of epinephrine infusion (0.15 µg/kg/min, leading to epinephrine concentration 20 nM) slightly improved platelet aggregation and clot formation in healthy volunteers receiving ticagrelor (Singh et al, 2019). In this study, potential adverse effects of epinephrine, especially increased blood pressure and heart rate were controlled. Therefore, our *in vitro* conditions do not restrict *per se* the scope of our findings, and this underpins the potential transposition of our *in vitro* "proof-of concept" to clinical practice.

Our *in vitro* study provides evidence that epinephrine in the presence of ADP restores platelet functions inhibited by ticagrelor. Restoration involves multiple steps in several pathways of platelet activation. This proof-of-concept *in vitro* study should be considered as hypothesis generating and requires to be confirmed *in vivo* on bleeding and thrombosis outcomes prior considering its potential benefit in ticagrelor-treated patients.

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Declaration of interests

ACM, DZ, GPB, EB, BD, TBR, BLB, SPC, MCA, PG, AG, CBL declare no conflict of interest in connection with the submitted article.

Author's contributions: ACM designed the study, performed and analysed experiments, wrote and critically reviewed the manuscript. DZ, GPB and EB performed and analysed experiments, and critically reviewed the manuscript. BD, TBR and SPC performed experiments and interpreted experimental data. BLB provided experimental material, and critically reviewed the manuscript. MCA contributed in the concept of the study and critically reviewed the manuscript. AG contributed in the concept of the study, designed and analysed experiments, and critically reviewed the manuscript. PG and CBL designed and analysed experiments, and critically reviewed the manuscript. The final version was approved by all authors.

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Figure legends

Fig. 1

Epinephrine restores ADP-induced platelet aggregation inhibited by ticagrelor

A- Typical time-course of platelet aggregation using light transmission aggregometry. Washed platelets, pre-treated with 150 nM ticagrelor or vehicle, were activated with various agonists (10 µM ADP, 10 µM epinephrine (EPI), or a combination of ADP and EPI) in the presence of fibrinogen (300 µg/ml).

B- Comparisons of percent of the maximal aggregation of washed platelet stimulated with 10 µM ADP, 10 µM epinephrine (EPI), combination of both, or 10 µM PAR-1-AP, in the absence or presence of 150 nM ticagrelor (n=6). Boxes represent the interquartile range and the median value, whiskers the minimum and maximum values observed. All samples were compared to results obtained with ADP alone (* P<0.01 vs ADP alone). Moreover, aggregation induced upon ADP+EPI was compared to that obtained with PAR-1-AP.

C- Kinetic of platelet disaggregation (in percent) reflecting the stability of platelet aggregates 1, 2 and 3 minutes after maximal aggregation was obtained. Comparison in ticagrelor-spiked samples upon stimulation with ADP and EPI compared ticagrelor-free samples upon stimulation with ADP (n=3), * P<0.01. Results are expressed in median and maximum.

Fig. 2

Epinephrine restores PAR-1-AP-induced platelet aggregation altered by ticagrelor

Comparison of the percent of maximal aggregation of washed platelets stimulated with 2 µM PAR-1-AP, 10 µM epinephrine (EPI), or combination of both, in the absence or presence of 150 nM ticagrelor (n=6). Boxes represent the interquartile range and the median value, whiskers the minimum and maximum values observed. * P<0.01 vs PAR-1-AP.

Fig. 3

Epinephrine restores ADP-induced platelet aggregation inhibited by ticagrelor independently of dense granule release

ATP release (nmol) by washed platelets upon 10 µM PAR-1-AP stimulation compared with stimulation by 10 µM ADP, 10 µM epinephrine (EPI), or combination of both in the absence or presence of 150 nM ticagrelor (n=6). Boxes represent the interquartile range and the median value, whiskers the minimum and maximum values observed. * P<0.01 vs PAR-1-AP.

Fig. 4

Epinephrine restores VASP dephosphorylation in the presence of ticagrelor

Comparisons of the platelet reactivity index (PRI, %) of whole blood stimulated with 10 µM ADP, 10 µM epinephrine (EPI), or a combination of both, in the absence or in the presence of 3.25 µM ticagrelor (n=6). Boxes represent the interquartile range and the median value, whiskers the minimum and maximum values observed. All samples were compared to ADP alone (* P<0.01 vs ADP alone) and to ADP + ticagrelor (# P<0.05 vs ADP + ticagrelor).

Fig. 5

Implication of the PI3K pathway in epinephrine restoration of ADP-induced platelet aggregation inhibited by ticagrelor

A- Phosphorylation of Akt, used as readout of PI3K pathway activation, was assessed through immunoblotting following addition of vehicle (-) or stimulation with 10 µM ADP, 10 µM epinephrine (EPI), or combination of both in the absence or presence of 150 nM ticagrelor (n=3). All the activations were performed in presence of RGDS (0.5 mM). Some samples were also pre-incubated with wortmannin, an inhibitor of PI3K, used as control. Quantification of Akt phosphorylation was performed using ImageJ software. For each agonist, samples with wortmannin were compared with those without (* P<0.01 vs without wortmannin). Results are expressed in median and maximum values. Non-significant comparisons were noticed as ns.

B- Representative immunoblotting of P-Akt. Protein loading was controlled by probing the membranes with GAPDH.

Fig. 6

$P2Y_1$ receptor pathway plays a crucial role in epinephrine restoration of ADP-induced platelet aggregation inhibited by ticagrelor

A- Effects of MRS2179 on the maximal aggregation of washed platelets upon stimulation by 10 µM ADP, or a combination of ADP and 10 µM epinephrine (EPI) in the presence or absence of 150 nM ticagrelor (n=6). Boxes represent the interquartile range and the median value, whiskers the minimum and maximum values observed * P<0.01 vs. ADP, ** P<0.01 vs. ADP and epinephrine, # P<0.01 vs ADP and epinephrine in the presence of ticagrelor.

B- Effects of ticagrelor, MRS2179, or a combination of both, on intracellular Ca^{2+} mobilisation upon stimulation by 10 µM ADP, 10 µM epinephrine (EPI) or ADP and EPI (n=3). Results are expressed in median and maximum values. Pair-wise comparisons did not significantly differ.

Fig. 7

Schematic model of the signalling pathways involved in epinephrine restoration of platelet aggregation inhibited by ticagrelor

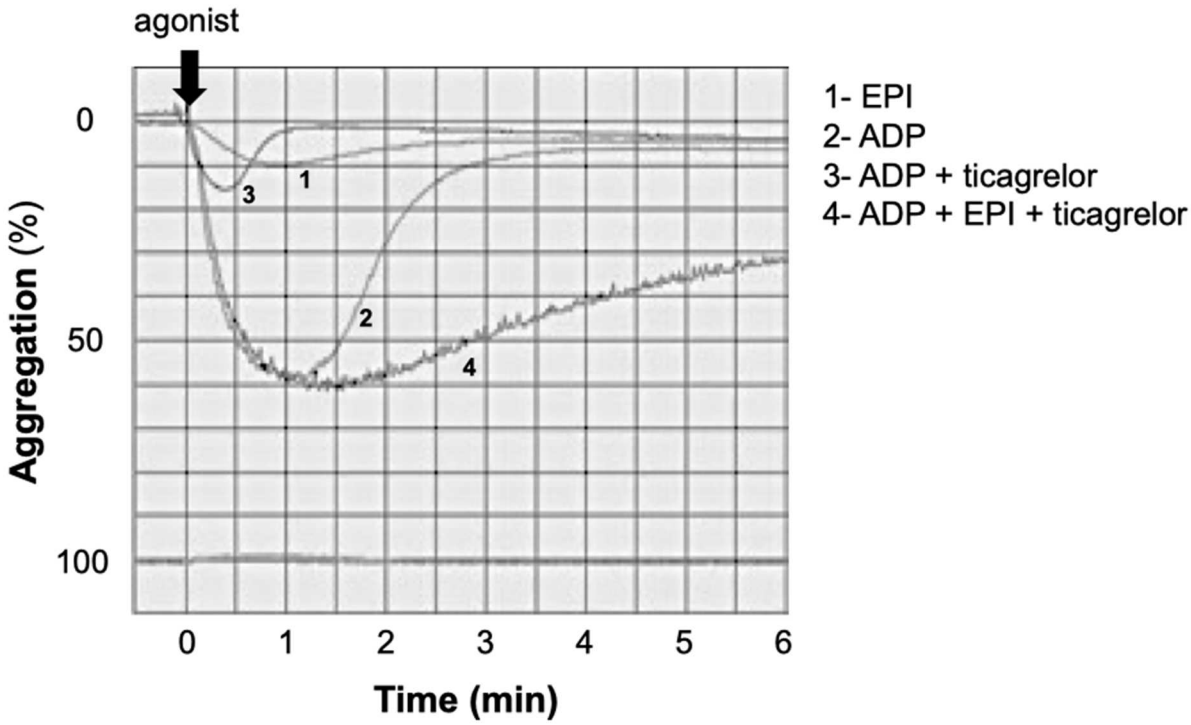
Epinephrine (EPI) combined with ADP restored the antiplatelet effect of ticagrelor blocking $P2Y_{12}$. Restoration involved multiple interlinked mechanisms, at different steps of platelet activation: epinephrine inhibited the cAMP signalling pathway and activated PI3K, but, alone, failed to induce aggregation of washed platelets. When epinephrine was associated to ADP platelet aggregated despite ticagrelor blocking $P2Y_{12}$, through inhibition of the cAMP pathway and activation of the phosphoinositide 3-kinase pathway, thus allowing the $P2Y_1$ receptor signalling and subsequent Ca^{2+} mobilisation. Ca^{2+} elevation mediates several responses, including Rap1 activation and thromboxane synthesis via phospholipase A2.

DAG: diacylglycerol; IP3: inositol triphosphate; PLC: phospholipase C; PLA2: phospholipase A2; PI3K: phosphoinositide 3-kinase; PKA: cAMP-dependent protein kinase; PKC: protein kinase C; TXA2: thromboxane; VASP: vasodilator-stimulated phosphoprotein.

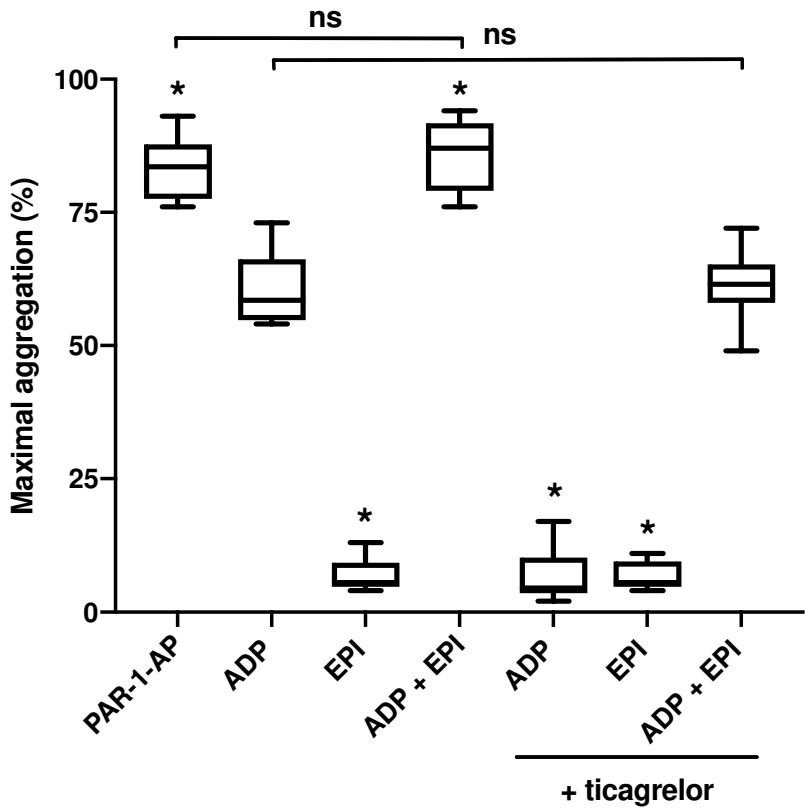
Figures

Figure 1

A



B



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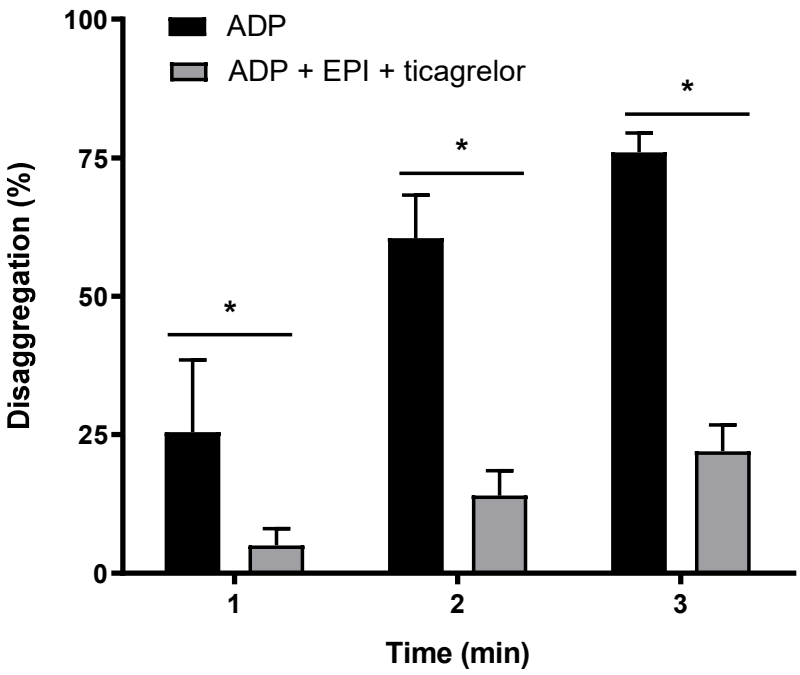

100
75
50
25
0
Disaggregation (%)
ADP
ADP + EPI + ticagrelor
*
*
*
1
2
3
Time (min)

Figure 2

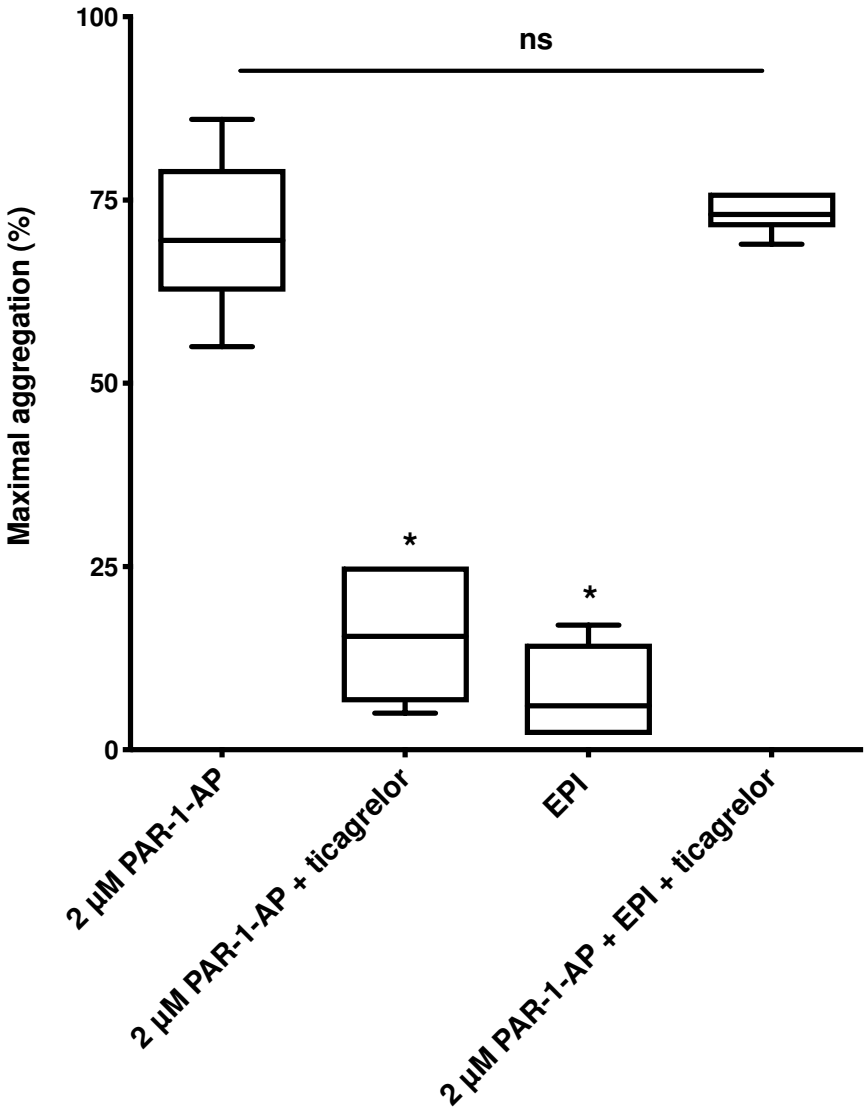

100
75
50
25
0
Maximal aggregation (%)
ns
*
*
2 µM PAR-1-AP
2 µM PAR-1-AP + ticagrelor
EPI
2 µM PAR-1-AP + EPI + ticagrelor

Figure 3

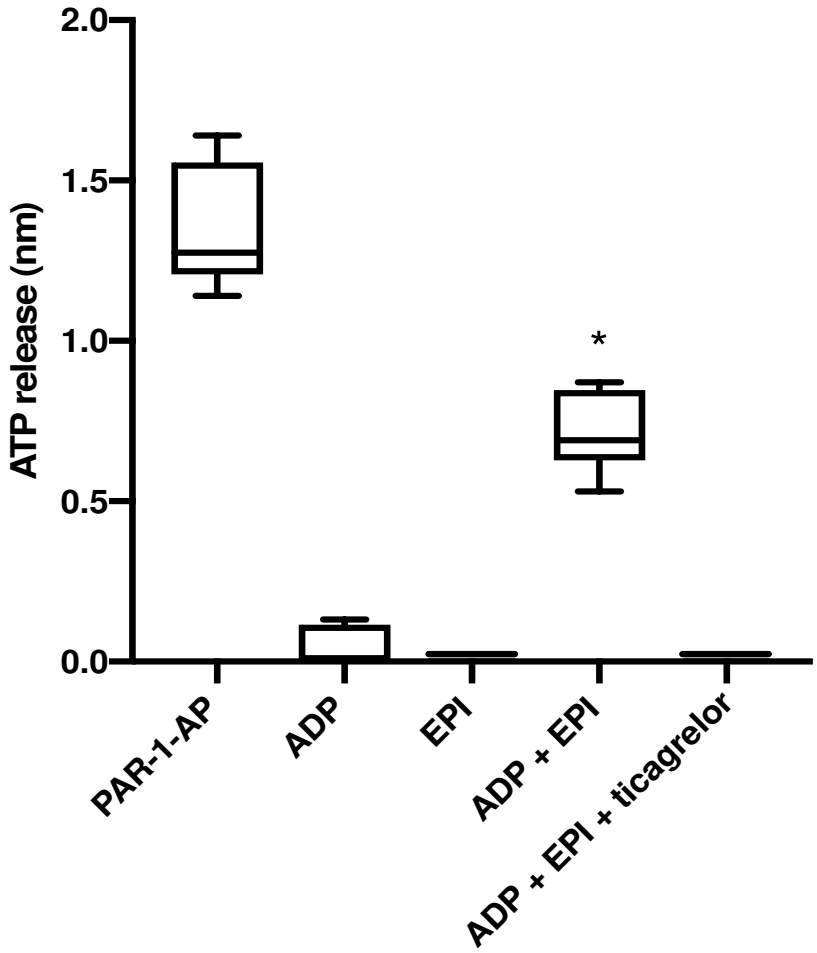

2.0
1.5
1.0
0.5
0.0
ATP release (nm)
*
PAR-1-AP
ADP
EPI
ADP + EPI
ADP + EPI + ticagrelor

Figure 4

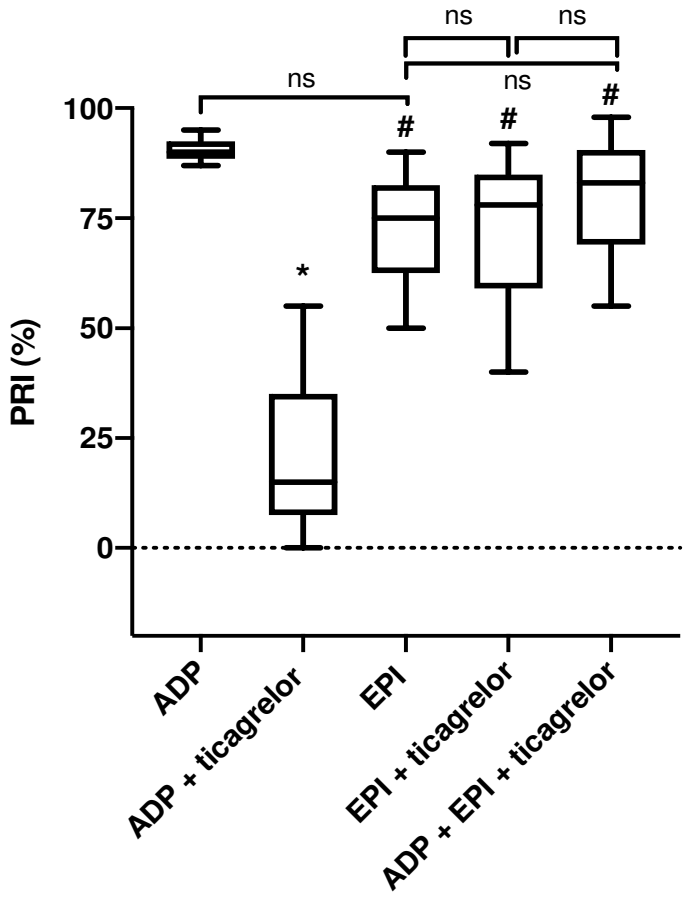
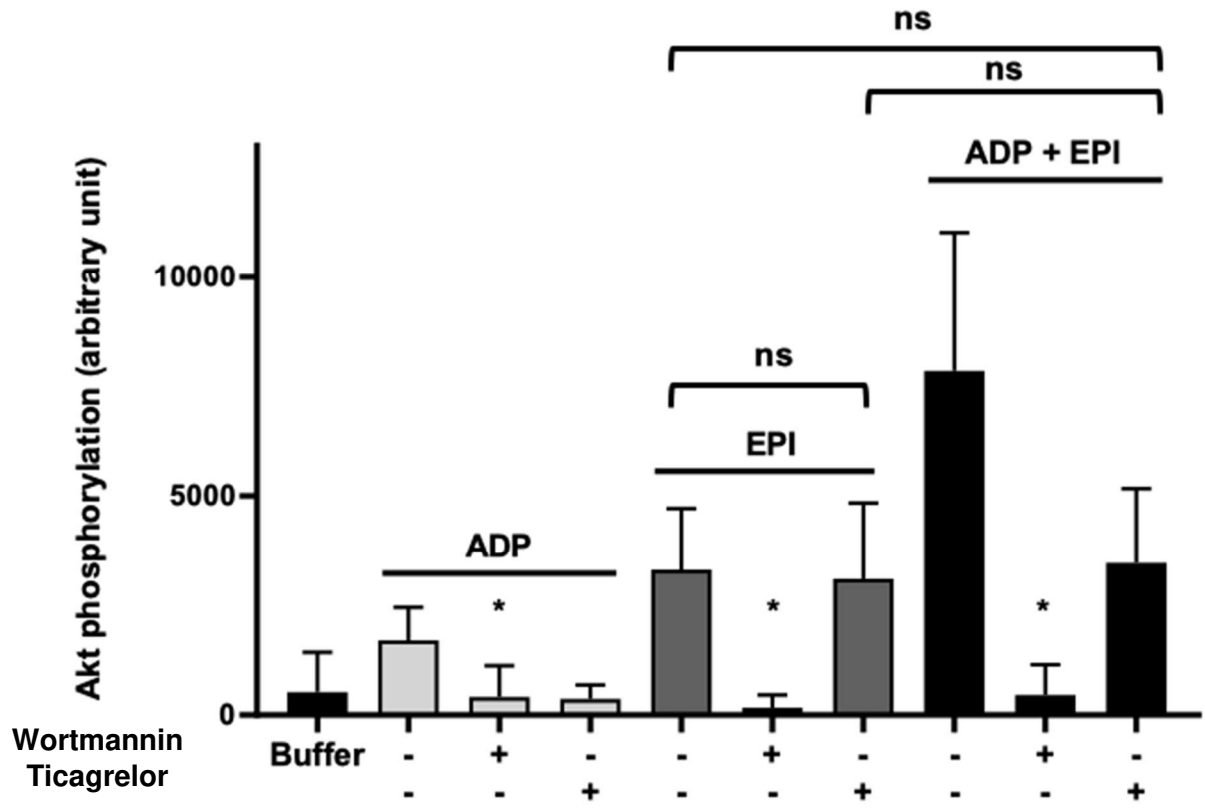

ns
ns
ns
ns
100
75
50
25
0
PRI (%)
*
#
#
#
ADP
ADP + ticagrelor
EPI
EPI + ticagrelor
ADP + EPI + ticagrelor

Figure 5

A


ns
ns
ADP + EPI
ns
EPI
ADP
*
*
*
Akt phosphorylation (arbitrary unit)
10000
5000
0
Wortmannin
Ticagrelor
Buffer
- + - - + - - + -
- - + - - + - - +

B

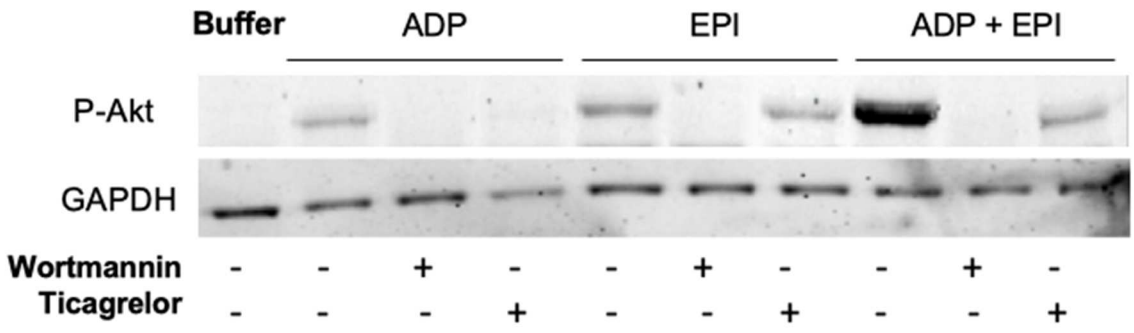
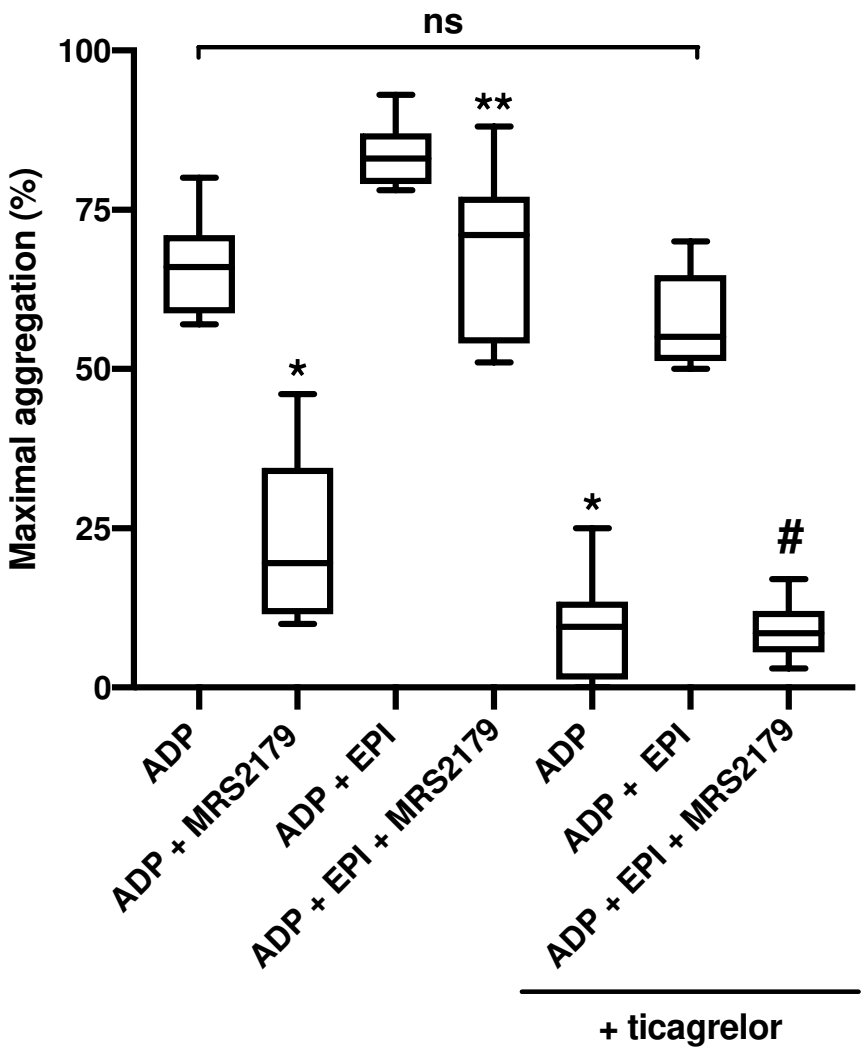

Buffer
ADP
EPI
ADP + EPI
P-Akt
GAPDH
Wortmannin
Ticagrelor
- - + - - + - - + -
- - - + - - + - - +

Figure 6

A


ns
100
75
50
25
0
Maximal aggregation (%)
**
*
*
#
ADP
ADP + MRS2179
ADP + EPI
ADP + EPI + MRS2179
ADP
ADP + EPI
ADP + EPI + MRS2179
+ ticagrelor

B

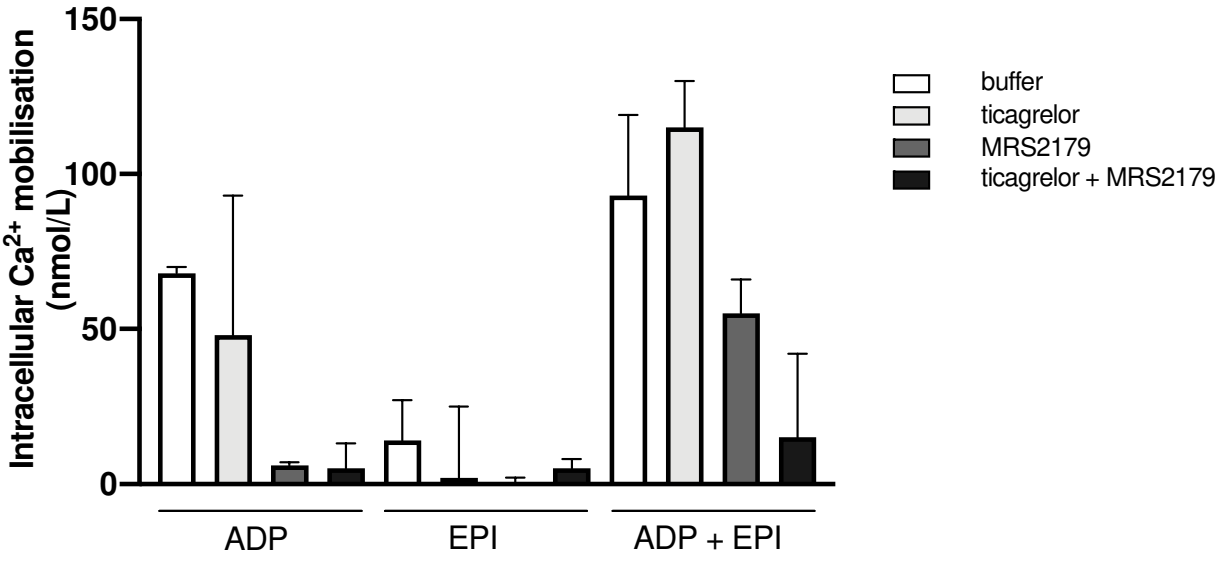
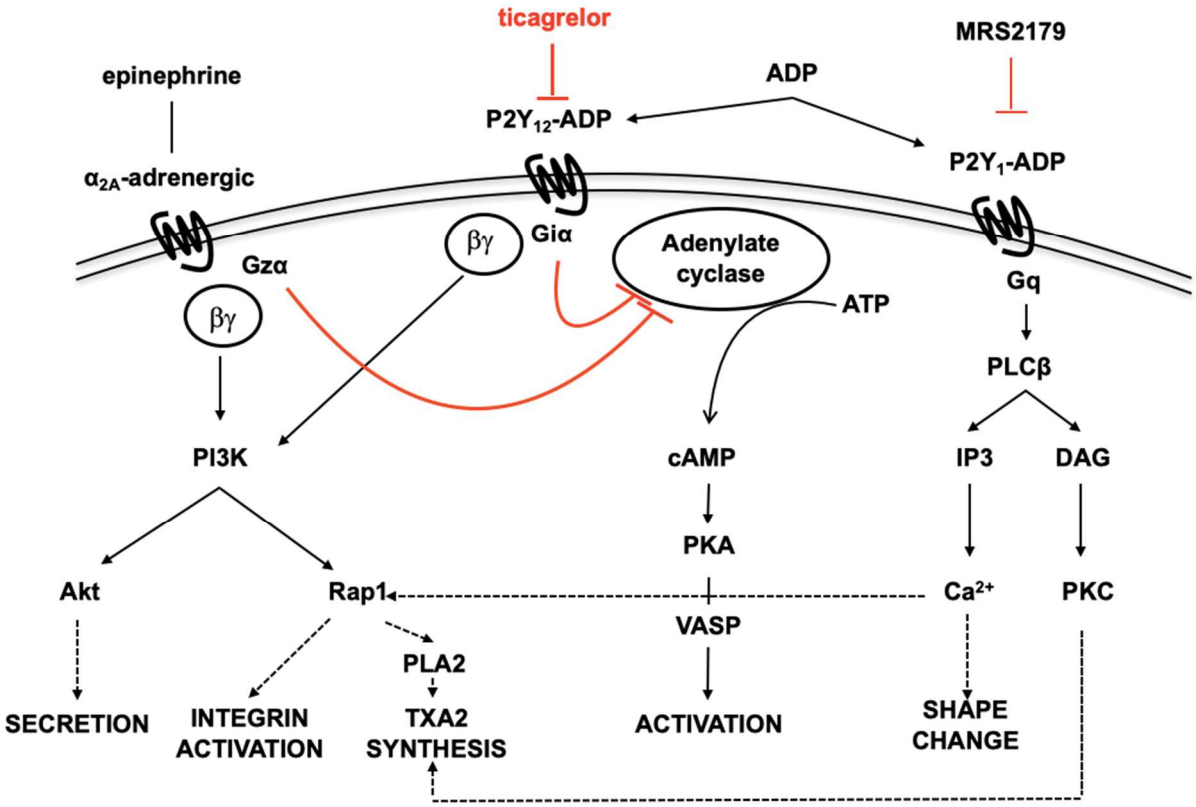

150
100
50
0
Intracellular Ca2+ mobilisation (nmol/L)
buffer
ticagrelor
MRS2179
ticagrelor + MRS2179
ADP
EPI
ADP + EPI

Figure 7


ticagrelor
MRS2179
epinephrine
ADP
P2Y12-ADP
P2Y1-ADP
α2A-adrenergic
βγ
Giα
Adenylate cyclase
Gzα
βγ
ATP
Gq
PLCβ
PI3K
cAMP
IP3
DAG
PKA
Akt
Rap1
Ca2+
PKC
VASP
PLA2
SECRETION
INTEGRIN ACTIVATION
TXA2 SYNTHESIS
ACTIVATION
SHAPE CHANGE

Graphical Abstract