Thrombosis Research 130 (2012) 65–69

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

# Ticagrelor, but not clopidogrel and prasugrel, prevents ADP-induced vascular smooth muscle cell contraction: A placebo-controlled study in rats

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ARTICLE INFO

*Article history:*
Received 18 September 2011
Received in revised form 11 December 2011
Accepted 22 December 2011
Available online 21 January 2012

*Keywords:*
ticagrelor
prasugrel
clopidogrel
off-target effect
vascular smooth muscle cells
platelets

ABSTRACT

*Introduction:* Off-target effects of novel antiplatelet agents due to their potential clinical benefits are currently an area of intensive investigation. We aimed to compare the effects of different $P2Y_{12}$ antagonists on the reactivity of vascular smooth muscle cells.
*Materials and methods:* Wistar rats (n = 30) were pretreated with an investigated drug or placebo. Clopidogrel (50 mg/kg, n = 7), prasugrel (10 mg/kg, n = 7), ticagrelor (10 mg/kg, n = 7) or placebo (n = 9) were administered orally 12 and 2 hours before experiments. Constrictions of rat tail arteries induced with a stable analogue of adenosine diphosphate (2-MeS-ADP), phenylephrine and arginine vasopressin were measured as an increase in perfusion pressure. Effects of ticagrelor were assessed in the presence of ticagrelor (1 μM/L) added to the perfusion solution as this drug reversibly inhibits the $P2Y_{12}$ receptor.
*Results:* Pretreatment with clopidogrel and prasugrel did not inhibit 2-MeS-ADP-induced contraction while ticagrelor did. Experiments employing endothelium-deprived arteries provided similar results. Clopidogrel and prasugrel did not influence concentration-response curves in the presence of neither phenylephrine nor arginine vasopressin. The curves obtained for both vasopressors in the presence of ticagrelor and 2-MeS-ADP were shifted to the right with a significant reduction in the maximal response.
*Conclusions:* Oral administration of ticagrelor, in contrast to clopidogrel and prasugrel, prevents adenosine diphosphate-induced contraction of vascular smooth muscle cells in a rat model. Both the clinical significance and detailed mechanism of our findings warrant further investigation.


## Introduction

Antiplatelet agents are the mainstay of treatment to prevent and manage atherothrombotic events [1,2]. Furthermore, new antiplatelet regimens overcoming many limitations of standard-dose clopidogrel were proven to further improve clinical outcomes [3–5].

Prasugrel was introduced into clinical practise as a third generation thienopyridine with a rapid and effective metabolic activation that is associated with a faster onset of action and an increased inhibition of platelet aggregation when compared to clopidogrel [6]. On the other hand, ticagrelor, a cyclopentyl-triazolo-pyrimidine, constitutes a first non-thienopyridine direct $P2Y_{12}$ blocker with a faster onset and offset of action and significantly higher inhibition of platelet aggregation as compared to clopidogrel [7]. Furthermore, ticagrelor, in contrast to thienopyridines, reversibly blocks the $P2Y_{12}$ receptor therefore conferring a great advantage when considering the substantial number of patients with acute coronary syndromes pretreated with antiplatelet drugs who require urgent coronary artery bypass grafting as well as the high incidence of bleeding complications in this population. Both prasugrel and ticagrelor posses similar antiplatelet potency and more effectively than clopidogrel reduce the risk of subsequent myocardial infarction and stent thrombosis [4,5].

However, unexpected mortality benefits observed in the PLATO trial, but not in the TRITON-TIMI 38 study, led to a speculation that benefits of ticagrelor therapy may exceed its antiplatelet properties [5,8–10]. Therefore off-target effects of novel antiplatelet agents are currently an area of intensive investigation.

Since $P2Y_{12}$ receptors were identified on vascular smooth muscle cells (VSMC) [11], they might represent a potential therapeutic target. In the present study we aimed to compare vascular effects of different $P2Y_{12}$ antagonists on the reactivity of VSMC in a rat model.

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doi:10.1016/j.thromres.2011.12.029

## Materials and methods

### Animals

Experiments were performed on isolated, perfused Wistar rat tail arteries. Animals were housed under a 12 h light/12 h dark cycle and had unlimited access to food and water. Rats (n = 7/per group) were pretreated with an investigated drug or placebo (n = 9). Animals, weighing 250–350 g, were narcotized by intraperitoneal injection of 120 mg urethane per 1 kg of body mass. Rats were killed by stunning and cervical dislocation. The study protocol was approved by the Local Ethics Committee. All studies were carried out in accordance with the United StatesNIH guidelines [Guide for the Care and Use of Laboratory Animals (1985), DHEW Publication No. (NIH) 85–23: Office of Science and Health Reports, DRR/NIH, Bethesda, MD, U.S.A.].

### Drugs and solutions

Clopidogrel (50 mg/kg), prasugrel (10 mg/kg), ticagrelor (10 mg/kg) or placebo (normal saline) were administered orally 12 and 2 hours before the experiment. Effects of ticagrelor were assessed in the presence of ticagrelor (1 μM/L) added to the perfusion solution as this drug reversibly inhibits the $P2Y_{12}$ receptor. Doses of investigated drugs were similar to those used in previous studies [12]. Krebs solution contained NaCl (71.8 mmol/L), KCl (4.7 mmol/L), $CaCl_2$ (1.7 mmol/L), $NaHCO_3$ (28.4 mmol/L), $MgSO_4$ (2.4 mmol/L), $KH_2PO_4$ (1.2 mmol/L), and glucose (11.1 mmol/L). All reagents were purchased from Sigma Aldrich Chemical Company (Poznan, Poland).

### Study design and conduction

After dissection from surrounding tissues, 2.5 to 3.0 cm long segment of a rat tail artery was cannulated and connected to a perfusion device. The distal part was weighted with a 500 mg weight and the tail was placed in a 20-mL container filled with oxygenated Krebs solution at 37 °C. The perfusion pressure was continuously measured. We gradually increased perfusion solution flow using a peristaltic pump up to 1 mL/min. Vessel contractions induced with phenylephrine (an adrenergic α1 receptor agonist; PHE), arginine vasopressin (a vasopressin receptor agonist, AVP) and 2-MeS-ADP (a stable analogue of adenosine diphosphate - ADP) were measured as an increase in perfusion pressure. Effects were assessed in the absence and presence of ticagrelor (1 μM/L) added to the perfusion solution as this drug reversibly inhibits the $P2Y_{12}$ receptor. Experiments were performed separately on arteries with and without vascular endothelium to assess the role of the vascular endothelium in regulation of vascular tone in arteries derived from rats pretreated with investigated drugs. Endothelium was removed mechanically for experiments performed on arteries without vascular endothelium. Successful endothelium removal was confirmed by vessel contraction in the response to acetylcholine.

### Data analysis and statistical procedures

Concentration-response curves (CRCs) were calculated according to the van Rossum method. Maximal response of tissue (Emax) was calculated as a percent of maximal response for PHE or AVP administered in KCl solution. Half maximal effective concentration ($EC_{50}$) was estimated using classical pharmacologic methods with pD2 the negative logarithm of the EC50. We used the number of the CRC and Emax in all calculations estimating the statistical significance (data are presented in Tables 1 and 2).

The Kolmogorov-Smirnov test was used to check normal distribution of data. Results are presented as mean values ± standard deviation. Statistical analysis was performed using the Newman-Keuls test for multiple comparison of means. Value of p below 0.05 were considered statistically significant.

**Table 1**
Maximal relative response for 2-MeS-ADP in the presence of antiplatelet agents in arteries with and without vascular endothelium. AWE – artery without vascular endothelium; PHE – phenylephrine; 2-MeS-ADP – stable analogue of adenosine diphosphate; [1] – number of concentration-response curves used for calculations; [2] – Emax calculated as a percent of maximal response for KCl; [3] – p calculated in comparison to control values; [a] – p calculated in comparison to Emax for phenylephrine and KCl.

| | n[1] | Emax [%][2] | p[3] |
|---|---|---|---|
| Phenylephrine (10 μM/L) | 12 | 99.0 ± 7.1 | |
| KCl (30 mM/L) | 12 | 100.0 ± 6.5 | |
| 2-MeS-ADP (10 μM/L) – Control | 12 | 60.0 ± 9.0 | p<0.0001[a] |
| 2-MeS-ADP (10 μM/L) – clopidogrel pretreated rats | 12 | 56.0 ± 10.0 | ns |
| 2-MeS-ADP (10 μM/L) – prasugrel pretreated rats | 12 | 53.0 ± 10.5 | ns |
| 2-MeS-ADP (10 μM/L) – ticagrelor pretreated rats + ticagrelor (1 μM/L) | 12 | 22.0 ± 5.0 | p<0.0001 |
| AWE→2-MeS-ADP (10 μM/L) – Control | 12 | 66.0 ± 12.1 | p<0.0001[a] |
| AWE→2-MeS-ADP (10 μM/L) – clopidogrel pretreated rats | 12 | 58.0 ± 7.5 | ns |
| AWE→2-MeS-ADP (10 μM/L) – prasugrel pretreated rats | 12 | 57.0 ± 10.7 | ns |
| AWE→2-MeS-ADP (10 μM/L) – ticagrelor pretreated rats + ticagrelor (1 μM/L) | 12 | 31.0 ± 4.5 | p<0.0001 |

## Results

### Effect of antiplatelet drugs on the contractility of VSMC

The reactivity of VSMC to the stable analog of ADP – 2-MeS-ADP (10 μM/L) in the control group arteries and arteries taken from clopidogrel, prasugrel and ticagrelor pretreated rats was analyzed. Response to stimulation was analyzed in arteries with and without

**Table 2**
Maximal relative response for phenylephrine and arginine vasopressin in the presence of 2-MeS-ADP and antiplatelet agents. AVP – arginine vasopressin; PHE – phenylephrine; 2-MeS-ADP – stable analogue of adenosine diphosphate; [1] – number of concentration-response curves used for calculations; [2] – Emax calculated as a percent of maximal response for KCl; [3] – p calculated in comparison to control values, [a] – p calculated in comparison to Emax for PHE or AVP.

| | n[1] | Emax [%][2] | p[3] |
|---|---|---|---|
| PHE (10 μM/L) | 12 | 99.0 ± 7.1 | |
| AVP (30 mM/L) | 12 | 100.0 ± 6.7 | |
| PHE (10 μM/L) + ticagrelor (1 μM/L) | 12 | 98.6 ± 4.2 | ns [a] |
| AVP (30 mM/L) + ticagrelor (1 μM/L) | 12 | 99.4 ± 5.5 | ns [a] |
| PHE + 2-MeS-ADP (10 μM/L) – control | 12 | 112.0 ± 13.0 | p<0.0001[a] |
| PHE + 2-MeS-ADP (10 μM/L) – clopidogrel pretreated rats | 12 | 102.0 ± 14.0 | ns |
| PHE + 2-MeS-ADP (10 μM/L) – prasugrel pretreated rats | 12 | 101.0 ± 14.5 | ns |
| PHE + 2-MeS-ADP (10 μM/L) – ticagrelor pretreated rats + ticagrelor (1 μM/L) | 12 | 65.0 ± 12.7 | p<0.0001 |
| AVP + 2-MeS-ADP (10 μM/L) – control | 12 | 118.0 ± 15.0 | p<0.0001[a] |
| AVP + 2-MeS-ADP (10 μM/L) – clopidogrel pretreated rats | 12 | 106.0 ± 14.0 | ns |
| AVP + 2-MeS-ADP (10 μM/L) – prasugrel pretreated rats | 12 | 106.0 ± 14.2 | ns |
| AVP + 2-MeS-ADP (10 μM/L) – ticagrelor pretreated rats + ticagrelor (1 μM/L) | 12 | 60.0 ± 13.8 | p<0.0001 |

vascular endothelium. Pretreatment with clopidogrel and prasugrel did not inhibit contraction by 2-MeS-ADP (Table 1). However, in the presence of ticagrelor a substantial reduction in contraction, calculated as a percentage of maximal response to PHE ($10^{-5}$ M/L), was found. Experiments utilizing arteries without vascular endothelium indicated similar results. Clopidogrel and prasugrel pretreatment did not change the contractility of VSMC but again in the presence of ticagrelor a significant reduction was present (Table 1).

*Effect of PHE and AVP*

In the second part of our CRCs for PHE ($10^{-9}$ - $10^{-3}$), a preferential $\alpha_1$-adrenoceptor agonist, and AVP ($10^{-10}$ – $10^{-4}$), a non-selective vasopressin receptor agonist, were compared in the absence and in the presence of 2-MeS-ADP (10 μM/L) and in the presence of $P2Y_{12}$ receptor antagonists: clopidogrel, prasugrel, and ticagrelor. $EC_{50}$ values calculated for PHE and AVP were $7.54 \pm 0.98 \times 10^{-8}$ M/L and $1.80 \pm 0.80 \times 10^{-8}$ M/L, respectively. EC50 values calculated for PHE ($7.22 \pm 0.98 \times 10^{-8}$ M/L) and AVP ($1.92 \pm 0.94 \times 10^{-8}$ M/L) in the presence of ticagrelor (1 μM/L) did not significantly differ with controls. The CRCs obtained for PHE and AVP in the presence of 2-MeS-ADP were shifted to the leftward with an increase in maximal responses (Figs. 1 and 2). Under these conditions $EC_{50}$ values for PHE and AVP were 2.60 ($\pm 1.65$)$\times 10^{-8}$ M/L ($p<0.0001$) and 5.10 ($\pm 1.79$)$\times 10^{-9}$ M/L ($p<0.0001$), respectively. Using arteries pretreated with clopidogrel and prasugrel there were no significant changes in the CRCs, but in case of ticagrelol CRCs obtained for both PHE and AVP were shifted to the right with a significant reduction in maximal response (Figs. 1, Fig. 2, and Table 2). $EC_{50}$ values calculated for PHE in the presence of clopidogrel, prasugrel and ticagrelor were $6.85 \pm 1.30 \times 10^{-8}$ M/L (ns), $6.92 \pm 1.10 \times 10^{-8}$ M/L (ns) and $6.22 \pm 1.45 \times 10^{-7}$ M/L ($p<0.0001$), respectively. On the other hand, $EC_{50}$ values calculated for AVP in the presence of clopidogrel, prasugrel and ticagrelor were $1.95 \pm 0.95 \times 10^{-8}$ M/L (ns) $2.14 \pm 1.18 \times 10^{-8}$ M/L (ns) and $2.12 \pm 0.75 \times 10^{-7}$ M/L ($p<0.0001$), respectively.

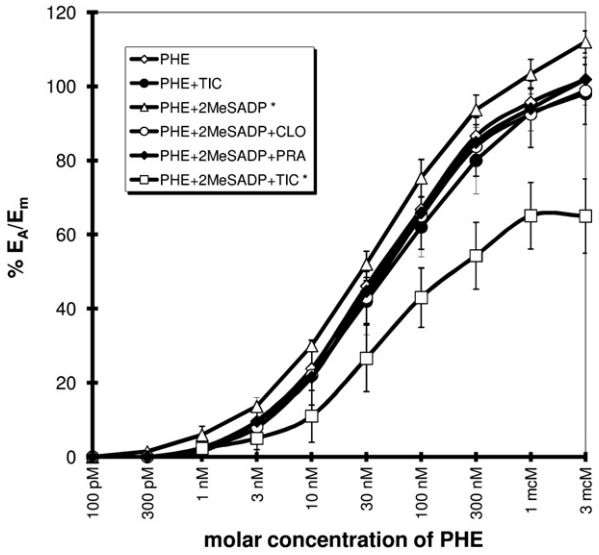


**Fig. 1.** CRCs obtained for PHE in the absence and presence of 2-MeS-ADP and antiplatelet agents. Points and whiskers display mean values ± standard deviations. A curve for PHE represents a control curve for PHE + TIC and PHE + 2MeSADP while a curve for PHE + 2MeSADP is a control curve for PHE + 2MeSADP + CLO, PHE + 2MeSADP + PRA and PHE + 2MeSADP + TIC. ADP –adenosine diphosphate; CLO – clopidogrel; CRC – concentration-response curve; Ea/Em – % of maximal response; PHE – phenylephrine; PRA – prasugrel; TIC – ticagrelor; * – a value of $p<0.05$ when compared the control curve for points of effect between 20% and 80% of the maximal response.

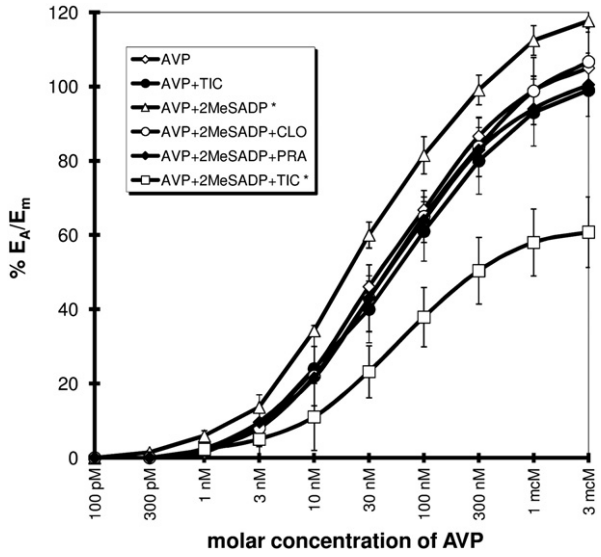


**Fig. 2.** CRCs obtained for AVP in the absence and presence of 2-MeS-ADP and antiplatelet agents. Points and whiskers display mean values ± standard deviations. A curve for AVP represents a control curve for AVP + TIC and AVP + 2MeSADP while a curve for AVP + 2MeSADP is a control curve for AVP + 2MeSADP + CLO, AVP + 2MeSADP + PRA and AVP + 2MeSADP + TIC. ADP –adenosine diphosphate; AVP – arginine vasopressin; CLO – clopidogrel; CRC – concentration-response curve; Ea/Em - % of maximal response; PRA – prasugrel; TIC – ticagrelor; * – a value of $p<0.05$ when compared the control curve for points of effect between 20% and 80% of the maximal response.

## Discussion

The main finding of the present study is that oral ticagrelor, in contrast to clopidogrel and prasugrel, prevents ADP-induced VSMC contraction in a rat model. Vasorelaxant properties of ticagrelor in the presence of 2-MeS-ADP were also pronounced after endothelium removal as well as when the tail arteries were perfused with PHE and AVP.

Inhibition of ADP receptors by antiplatelet drugs became the forefront of therapy in coronary artery disease treated with and without stenting [2,4,5]. ADP like other extracellular nucleotides acts through receptors: P2X and P2Y [13]. P2X receptors are ligand-gated ion channels, whereas P2Y receptors belong to G-protein coupled receptors. There are two different types of P2Y receptors on platelets: $P2Y_1$ which couples to both $G_{q/11}$ and $G_s$ and $P2Y_{12}$ which couples to $G_i$. After platelet activation, ADP released from granula augments aggregation by stimulation of $P2Y_1$ and $P2Y_{12}$ platelet surface receptors. In contrast to $P2Y_1$, $P2Y_{12}$ receptor has very selective tissue distribution. Northern blot experiments conducted by Wihlborg *et al* indicated that $P2Y_{12}$ receptors are present not only in platelets and brain but also in VSMC [11].

Lack of impact of thienopyridines on vessel reactivity in our study may be explained by high instability of their active metabolites that do not reach the systemic circulation in sufficient concentrations [14,15]. Our observations are in line with the results of two other studies demonstrating a neutral effect of clopidogrel pretreatment on VSMC contractility under 2-MeS-ADP stimulation [11,12]. On the other hand, Froldi *et al* observed a direct off-target effect of clopidogrel which administered into the investigated vessel without prior hepatic bioactivation caused relaxation of the rat tail artery [16]. Interestingly, André *et al* demonstrated in $P2Y_{12}$ knockout mice a direct impact of clopidogrel and prasugrel on the vessel wall contributing to bleeding complications which was not mimicked by elinogrel, a direct and reversible $P2Y_{12}$ antagonist [17].

We demonstrated a potentiating effect of 2-MeS-ADP on the maximal contraction induced by PHE or AVP. The presence of ticagrelor

did not change the maximal contraction. However, a significant inhibitory effect was observed in the presence of ticagrelor and 2-MeS-ADP. These results suggest that 2-MeS-ADP may induce contraction via $P2Y_{12}$ receptor and relaxation via another ADP-dependent mechanism. Our observations are in line with experiments performed by Bender *et al* who showed relaxation of coronary VSMC mediated by activation of the endothelial $P2Y_1$ receptors [18]. Similar mechanism was also reported by Winter and Dora who were analysing effects of ATP and ADP on the contractility of perfused isolated mesenteric arteries [19]. This effect in our study was also present in arteries without vascular endothelium that was not assessed in previous experiments [18,19].

Despite the lack of benefits associated with inhibition of the vascular $P2Y_{12}$ receptors by clopidogrel, treatment with a high clopidogrel maintenance dose of 150 mg led to significantly better flow-mediated vasodilation in comparison with a standard therapy with 75 mg of clopidogrel in the recently published ARMYDA-150 mg trial [20]. Those results are concordant with findings of another small randomized study in which therapy with clopidogrel dose-dependently improved endothelial dysfunction in patients with coronary artery disease [21]. Investigating ADP signaling pathways in endothelial cells, Hess *et al* showed that ADP elicits multiple phosphorylation responses, including striking alterations in the phosphorylation state of endothelial nitric oxide synthase [22]. Therefore, based on experimental data [23–25], it is believed that clopidogrel effect on the vascular tone is predominantly attributed to modulation of nitric oxide bioavailability.

Our findings correspond with results obtained by Högberg *et al* who for the first time reported on the ability of ticagrelor, but not clopidogrel, to inhibit ADP-induced contractions of VSMC in an *ex vivo* study in denuded mouse aortic rings as well as in human left internal mammary arteries [12]. We confirmed their observations in a rat model and, for the first time, we have demonstrated that another novel antiplatelet agent, prasugrel, behaves like clopidogrel when assessing its vasoreactive potential under ADP stimulation.

Possible implications of the vascular $P2Y_{12}$ receptor inhibition were shown in a dog thrombosis model [26]. Adjunctive infusion of ticagrelor, when compared to clopidogrel on top of tissue-type plasminogen activator and heparin, was associated with a significantly lower reocclusion rate, lower cyclic flow variation, longer reflow duration, and greater reductions in infarct sizes despite a complete blockade of ADP-induced platelet aggregation with both regimens.

Although potential off-target effects of antiplatelet drugs are being extensively discussed in the literature, our knowledge in this area remains sparse. Previous studies suggested that clopidogrel exerts pro- [27] and anti-inflammatory [28,29] activities as well as modulates vascular reactivity [23–25] as presented above. A direct comparison of clopidogrel and ticagrelor in the randomized DISPERSE 2 trial did not reveal any differences between these two drugs with respect to the inflammatory biomarkers: C-reactive protein, interleukin 6, myeloperoxidase, and soluble CD40 ligand in patients with non-ST-segment elevation acute coronary syndromes [30]. In another study, ticagrelor inhibited the uptake of adenosine by human erythrocytes in a canine model and significantly enhanced the adenosine-induced increase in coronary blood flow [31]. Furthermore, Serebruary speculates that ticagrelor may be transformed to adenosine by degrading oxygenases and/or cyclopropylcarbonyl radical fragmentation pathways [32]. Numerous features related to inhibition of adenosine uptake by ticagrelor and probably chronically increasing adenosine blood levels on ticagrelor therapy, such as promotion of preconditioning, prevention of sudden cardiac death, reduction of infarct size, inhibition of tumor growth, bronchoconstriction, neurocardiogenic syncope, and up-regulation of purine metabolism, were postulated [32].

In conclusion, ticagrelor, in contrast clopidogrel and prasugrel, when administered orally, prevents ADP-induced contraction of VSMC in a rat model. Both the clinical significance and detailed mechanism of our findings warrant further investigation.

## Conflict of interest

The authors do not have any conflict of interest.

## Funding

The study was funded from departmental sources.

## Acknowledgments

The authors are grateful to Tomasz Fabiszak for his outstanding English language assistance.

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