

Ticagrelor, but not clopidogrel, reduces arterial thrombosis via endothelial tissue factor suppression

Martin F. Reiner^{1,2,3}, Alexander Akhmedov⁴, Simona Stivala^{1,3}, Stephan Keller^{2,4}, Daniel S. Gaul⁵, Nicole R. Bonetti^{1,2}, Gianluigi Savarese⁶, Martina Glanzmann⁴, Cuicui Zhu⁷, Wolfram Ruf⁸, Zhihong Yang⁷, Christian M. Matter⁵, Thomas F. Lüscher^{4,9}, Giovanni G. Camici^{1,2,9}, and Juerg H. Beer^{1,3*}

¹Center for Molecular Cardiology, Laboratory for Platelet Research, University of Zurich, Wagistrasse 12, 8952 Schlieren, Switzerland; ²Laboratory of Aging and Stroke, University of Zurich, Wagistrasse 12, 8952 Schlieren, Switzerland; ³Department of Internal Medicine, Cantonal Hospital of Baden, Im Ergel 1, 5404 Baden, Switzerland; ⁴Laboratory for Endothelial Research, University of Zurich, Wagistrasse 12, 8952 Schlieren, Switzerland; ⁵Laboratory for Atherosclerosis Research, University of Zurich, Wagistrasse 12, 8952 Schlieren, Switzerland; ⁶Division of Cardiology, Department of Medicine, Karolinska Institute, Solna (MedS), K2, ZS:00, 171 76 Stockholm, Sweden; ⁷Department of Medicine/Physiology, University of Fribourg, Chemin du Musée 5, 1700 Fribourg, Switzerland; ⁸Center for Thrombosis and Hemostasis, University Medical Center, Johannes Gutenberg-University Mainz, Langenbeckstraße 1, 55131 Mainz, Germany; and ⁹Department of Cardiology, University Heart Center, University Hospital Zurich, Rämistrasse 100, 8091 Zurich, Switzerland

Received 28 June 2016; revised 2 September 2016; editorial decision 10 October 2016; accepted 9 November 2016; online publish-ahead-of-print 15 November 2016

Time of primary review: 18 days

Aims

The P2Y₁₂ antagonist ticagrelor reduces mortality in patients with acute coronary syndrome (ACS), compared with clopidogrel, and the mechanisms underlying this effect are not clearly understood. Arterial thrombosis is the key event in ACS; however, direct vascular effects of either ticagrelor or clopidogrel with focus on arterial thrombosis and its key trigger tissue factor have not been previously investigated.

Methods and results

Human aortic endothelial cells were treated with ticagrelor or clopidogrel active metabolite (CAM) and stimulated with tumour necrosis factor- α (TNF- α); effects on procoagulant tissue factor (TF) expression and activity, its counter-player TF pathway inhibitor (TFPI) and the underlying mechanisms were determined. Further, arterial thrombosis by photochemical injury of the common carotid artery, and TF expression in the murine endothelium were examined in C57BL/6 mice treated with ticagrelor or clopidogrel. Ticagrelor, but not CAM, reduced TNF- α -induced TF expression via proteasomal degradation and TF activity, independently of the P2Y₁₂ receptor and the equilibrative nucleoside transporter 1 (ENT1), an additional target of ticagrelor. In C57BL/6 mice, ticagrelor prolonged time to arterial occlusion, compared with clopidogrel, despite comparable antiplatelet effects. In line with our *in vitro* results, ticagrelor, but not clopidogrel, reduced TF expression in the endothelium of murine arteries.

Conclusion

Ticagrelor, unlike clopidogrel, exhibits endothelial-specific antithrombotic properties and blunts arterial thrombus formation. The additional antithrombotic properties displayed by ticagrelor may explain its greater efficacy in reducing thrombotic events in clinical trials. These findings may provide the basis for new indications for ticagrelor.

Keywords

Thrombosis • Clopidogrel • Coagulation • Endothelium • Ticagrelor • Tissue factor

1. Introduction

Cardiovascular disease is the leading cause of death worldwide.¹ Platelets play a crucial role in arterial thrombus formation, the key event in cardiovascular complications, such as myocardial infarction and stroke. Consequently, platelet antagonists targeting the adenosine diphosphate (ADP) receptor P2Y₁₂ (i.e. clopidogrel, prasugrel, or ticagrelor) in

combination with acetylsalicylic acid is the treatment of choice for patients suffering from acute coronary syndrome (ACS) and for secondary prevention after stent implantation or coronary artery bypass grafting.² In clinical trials, ticagrelor and prasugrel proved to be superior over clopidogrel;^{3,4} in particular, ticagrelor reduced the composite end point mortality due to vascular causes, myocardial infarction, or stroke in addition to overall mortality in patients with ACS, compared with

* Corresponding author. Tel: +41 56 486 25 02; fax: +41 56 486 25 09; E-mail: hansjuerg.beer@ksb.ch

clopidogrel.³ The cyclopentyl-triazolo-pyrimidine ticagrelor displays a different pharmacokinetic and pharmacodynamic profile, compared with the thienopyridines clopidogrel and prasugrel. Specifically, ticagrelor is direct acting and reversibly binding the P2Y₁₂ receptor, whereas the thienopyridines are pro-drugs requiring hepatic metabolism to generate an irreversibly binding active metabolite. Higher levels of platelet inhibition complemented by an additional mechanism of action, the inhibition of cellular adenosine uptake via the equilibrative nucleoside transporter 1 (ENT1)^{5–8} leading to increased adenosine plasma levels,⁹ may in part account for the superior effects of ticagrelor.

A growing body of evidence suggests off-target effects of P2Y₁₂ antagonists; however, effects of ticagrelor and clopidogrel on the endothelium, their underlying molecular mechanisms and potential effects on arterial thrombosis remain elusive.

Tissue factor (TF) is the key trigger of the extrinsic coagulation cascade¹⁰ and plays a major role in the development of arterial thrombotic complications such as myocardial infarction and stroke.¹¹ Correspondingly, anti-TF antibody treatment blocks arterial thrombus formation.¹² Increased levels of TF have been observed in patients with classical cardiovascular risk factors, such as hypertension,¹³ diabetes,¹⁴ and dyslipidaemia,¹⁵ as well as in ACS.¹⁶ TF is expressed in various cell types including endothelial cells and different cytokines such as tumour necrosis factor- α (TNF- α) are known to induce TF expression and activity.¹⁷ Correspondingly, elevated levels of TNF- α have also been reported in patients with ACS¹⁸ and atrial fibrillation¹⁹ and are further associated with cardiovascular disease progression and severity.²⁰

In the present study, we investigate pleiotropic effects of ticagrelor and clopidogrel on TNF- α -stimulated primary human aortic endothelial cells (HAECs), assess the underlying mechanisms and their potential relevance in a mouse model of photochemical injury-induced arterial thrombosis.

2. Methods

2.1 Drugs

Clopidogrel active metabolite (CAM) and ticagrelor were provided by Sanofi-Aventis, Germany and AstraZeneca, Mölndal, Sweden, respectively. Clopidogrel was purchased from Santa Cruz Biotechnology.

2.2 Animals and treatment

Twelve-week-old bodyweight-matched male C57BL/6 mice were treated with clopidogrel (48 mg/kg/day or 32 mg/kg/day) or ticagrelor (120 mg/kg/day or 80 mg/kg/day) given in the chow (clopidogrel, 0.06% w/w or 0.04 w/w; ticagrelor, 0.15% w/w or 0.1% w/w), for 2 weeks to evaluate dosages that provide full and comparable platelet inhibition in mice. Dosages of clopidogrel and ticagrelor differed 2.5-fold corresponding to dosages used in humans² and dosages known to provide comparable platelet inhibitory effects in rodents.²¹ Experimental procedures involving mice were reviewed and approved by the institutional animal care committee (licence number TVA 165/2013; Kommission für Tierversuche des Kantons Zürich, Zurich, Switzerland).

2.3 Plasma concentrations of ticagrelor

In addition to pharmacodynamic evaluation of P2Y₁₂ receptor antagonist treatment, ticagrelor plasma concentrations were determined by protein precipitation and liquid chromatography mass spectrometry as described previously.²²

2.4 Whole blood aggregometry

Sodium-citrate (3.8%) anticoagulated blood was collected by cardiac puncture using a 24-gauge needle after euthanasia of mice by isoflurane. Finally, whole blood (diluted 1–3 in 0.9% saline) aggregometry was performed in response to ADP (10 μ M; 384, chrono-log), thrombin (1 U/mL; T7009, Sigma Aldrich) and collagen (10 μ g/mL; Kollagenreagens Horm, Takeda) by impedance aggregometer (Chrono-Log).

2.5 Arterial thrombosis

After 2 weeks of treatment with ticagrelor, clopidogrel, or control chow, C57BL6 mice were exposed to photochemical injury of the common carotid artery (CCA) as previously described.²³ Briefly, mice were anaesthetized using pentobarbital (87 mg/kg body weight); after midline neck incision, the right CCA was exposed under an operating microscope. To induce photochemical injury of the endothelium, bengal rose (50 mg/kg body weight) was injected into the tail vein and the CCA was exposed to a laser light beam (1.5 mW, 540 nm, Mellesgriot Inc.) up to 120 min. Blood flow and heart rate were monitored (Doppler flow probe carotid artery Transonic Systems Inc., 0.5VB) until occlusion occurred or for a maximum of 120 min, in case arterial thrombosis was not detected.

2.6 Plasma thrombin generation

Plasma thrombin generation was assessed by calibrated automated thrombogram as previously described.²⁴ Sodium-citrate (3.8%) anticoagulated murine whole blood was drawn by cardiac puncture and centrifuged (4000g, 10 min, 4 °C) to receive platelet-poor plasma, which was then mixed with either PPP-Reagent (Thrombinoscope BV) containing TF (f.c. 6 pM) and phospholipids (f.c. 4.8 μ M), or thrombin calibrator (Thrombinoscope BV). Next, fluorogenic thrombin substrate (Thrombinoscope BV), Fluo-Buffer (Hepes buffer, pH 7.35, 20 mM Hepes, with BSA 60 mg/mL, Thrombinoscope BV), and CaCl₂ (246 mM) were added. Thrombin generation was measured over time by Fluoroskan[®] Ascent reader (Thermo Labsystems) and thrombin generation curve was calculated by Thrombinoscope software (Thrombinoscope BV) to finally receive endogenous thrombin potential (ETP) (nmol thrombin \times min).

2.7 Endothelial tissue factor expression

CCAs of mice were embedded in Optimal Cutting Temperature medium, snap frozen and cryosectioned. Sections were incubated with the primary antibodies rat anti-murine CD31 (BD 553370, 1:2500), used as an endothelial cell marker, and rabbit anti-TF (R8084,²⁵ 1:200) for 45 min at room temperature. After washing, the secondary antibodies donkey anti-rat Cy3 (Jackson 712-166-153, 1:250) and donkey anti-rabbit Alexa488 (Jackson 711-545-15, 1:250) were applied for 45 min at room temperature. DNA was counterstained using UltraCruz Hard-set Mounting medium containing 4',6-diamidino-2-phenylindole (Santa Cruz, sc-359850). Images were acquired using a Zeiss Axiovert inverted wide-field fluorescence microscope with Axiovision software. Composite images were generated using Photoshop (CC2015, Adobe) and endothelial TF mean fluorescent intensity was quantified and normalized to endothelial cell area.

2.8 Isolation of human platelets

Human platelets were used as positive control of P2Y₁₂ receptor mRNA and protein expression. Venous blood was drawn from a healthy volunteer not receiving any medication for 10 days and collected in

sodium-citrate (3.8%) tubes (Becton Dickinson). Blood was centrifuged twice at 100g for 10 min at room temperature to obtain platelet-rich plasma, which was centrifuged at 400g for 15 min at room temperature. Finally, the platelet pellet was washed with 1 mL phosphate-buffered saline and after a final centrifugation step (400g, 10 min, room temperature) exposed to either protein lysis buffer or TRIzol for Western blot or real-time polymerase chain reaction (RT-PCR), respectively.

2.9 Cell culture experiments

HAECs (Lonza) were used for experiments between Passages 5 and 8, derived from four individual batches. Endothelial cells were cultured in endothelial growth basal medium-2, supplemented with endothelial growth basal medium-2 bullet kit (Lonza) and 10% foetal bovine serum. After 24 h of growth, cells underwent starvation for 24 h using endothelial basal medium (Lonza) supplied with 0.5% foetal bovine serum. Cells were treated with concentration ranges of ticagrelor (10^{-7} , 10^{-6} , 10^{-5} M)²⁶ or CAM (1.5×10^{-8} , 1.5×10^{-7} , 1.5×10^{-6} M), which are in line with plasma concentrations found in humans,²⁶ and stimulated with TNF- α (10 ng/mL) for various time points. Drugs were dissolved in dimethyl sulfoxide (f.c. 0.1%). Correspondingly, unstimulated cells and TNF- α -stimulated cells were treated with dimethyl sulfoxide (f.c. 0.1%) to exclude vehicle-dependent effects.

2.10 Western blotting

Protein expression was determined by Western blot analysis. Endothelial cells and human platelets were incubated with lysis buffer (NaCl 150 mM, EDTA 1 mM, NaF 1 mM, DTT 1 mM, aprotinin 10 mg/mL, leupeptin 10 mg/mL, Na₃VO₄ 0.1 mM, PMSF 1 mM, and NP-40 0.5%); protein concentration was determined, according to the recommendations of the manufacturer (Bio-Rad); 20–30 μ g of protein lysates were separated on an 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis before being transferred to a polyvinylidene fluoride membrane by semi-dry transfer. Membranes were cut, according to the size of proteins of interest and incubated with primary antibodies overnight at 4 °C on a shaker. Secondary antibodies were applied for 1 h at room temperature. Densitometric analyses were performed and protein expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Antibodies against TF (ADG4507 and 4503; 1:2000) and tissue factor pathway inhibitor (TFPI) (ADG72; 1:8000) were purchased from American Diagnostica; anti-P2Y₁₂ (ab86195; 1:2000) antibody from Abcam; anti-GAPDH antibody (MAB374; 1:40 000) from Merck Millipore. Secondary anti-mouse (1031-05) and anti-rabbit (4050-05) antibodies were obtained from SouthernBiotech; recombinant human TNF- α (210-TA) was purchased from R&D Systems; actinomycin D (A1410), MG132 (C2211), rapamycin (R0395), wortmannin (W1628), SB203580 (S8307), SP600125 (S5567), dipyrindamole (D9766), adenosine receptor antagonist against A1 (8-cyclopentyl-1,3-dipropylxanthine), A2a (SCH 442416), A2b (MRS 1754), and A3 (VUF 5574) as well as adenosine (A4036) from Sigma Aldrich; cycloheximide (239765) from Merck Millipore; PD 98059 (9900) from Cell Signaling Technology.

2.11 Real-time PCR

Total RNA was extracted from HAECs or human platelets using TRI reagent (Sigma Aldrich), according to the recommendations of the manufacturer. Conversion of the total cellular RNA to cDNA was performed with Moloney murine leukaemia virus reverse transcriptase and random hexamers (GE Healthcare) in a final volume of 35 μ L, using 2 μ g of total RNA, according to recommendations of the manufacturer. RT-PCR was

performed in a QuantStudio 7 Flex RT-PCR cycler (Applied Biosystems), according to the instructions of the manufacturer. All RT-PCR experiments were performed using the SYBR Select Master Mix provided by Applied Biosystems (Life Technologies). Each reaction (20 μ L) contained 2 μ L cDNA, 400 fmol of each primer and 10 μ L of Master Mix. The following primers were used: P2Y₁₂ (1), P2Y₁₂ (2), TF, human L28, and human β -actin (ACTB). The amplification program consisted of 1 cycle at 95 °C for 10 min, followed by 40 cycles with a denaturing phase at 95 °C for 15 s, an annealing/elongation phase at 60 °C for 1 min. A melting curve analysis was performed after amplification to verify the accuracy of the amplicon. For verification of the correct amplification, PCR products were analysed on an ethidium bromide stained 1.5% agarose gel. Cycle threshold (C_T) values for each gene were obtained for each sample and differences in C_T values between a test gene and endogenous controls (Δ C_T) were calculated and used for statistical analyses.

For quantitative RT-PCR, the following primers were used; for human P2Y₁₂: sense primer (1) 5'-CTTTCTCATGTCCAGGGT-3', antisense primer (1) 5'-GTTGCCAAACCTCTTTGT-3'; sense primer (2) 5'-TTTCTCATGTCCAGGGTC-3', antisense primer (2) 5'-CTGCAGAGTGGCATCTGG-3; for human TF: sense primer: 5'-CCAAACCCG TCAATCAAGTC-3', antisense primer: 5'-TGCCAAGTACGTCTGCT TCA-3'; for human L28: sense primer: 5'-GCATCTGCAATGGATG GT-3', antisense primer: 5'-TGTTCTTGCGGATCATGTGT-3'; for ACTB: sense primer: 5'-GCACAGAGCCTCGCCTT-3', antisense primer: 5'-GTTGTCGACGACGAGCG-3'. All primers were ordered from Microsynth.

2.12 Tissue factor activity assay

TF activity was determined as previously described²³ in cell lysates of HAECs and murine plasma by ELISA, according to the recommendations of the manufacturer (Sekisui Diagnostics, ACTICHROME[®] TF, 846). Endothelial cells were lysed (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.1% Triton X-100, pH 7.4), diluted 1:15 in assay buffer, and mixed with human factor VIIa and X, which leads to the conversion of factor X to Xa; factor Xa subsequently cleaves the chromogenic substrate SPECTROZYME[®] FXa. Finally, absorbance was measured at 405 nm and after background subtraction, optical density was normalized to protein concentration as determined by Nanodrop 2000 Spectrophotometer (Thermo Scientific). Sodium-citrate (3.8%) anticoagulated plasma was mixed with factor VIIa and X and optical density of cleaved SPECTROZYME[®] FXa was determined at 490 nm and subtracted from absorbance at 405 nm. Finally, plasma TF (pM) was calculated, according to a standard curve.

2.13 Tissue factor mRNA stability

Stability of TF mRNA was investigated as previously described.²⁷ Endothelial cells were stimulated with TNF- α for 1 h to induce TF gene expression and transcription was subsequently terminated using actinomycin D (10 μ g/mL). Next, cells were incubated with ticagrelor (10^{-5} M) for 1, 2, or 3 h before cells were lysed with TRIzol. Messenger RNA was isolated and RT-PCR was performed. Values were plotted as per cent of time 0 against time (min) and half-life was calculated by non-linear regression using Prism 6 (GraphPad software).

2.14 Statistical analysis

Data are expressed as mean \pm SEM. Statistical analysis was performed using one-way ANOVA with *Tukey post hoc* test or unpaired two-tailed Student's *t*-test as appropriate. A probability value below or equal to

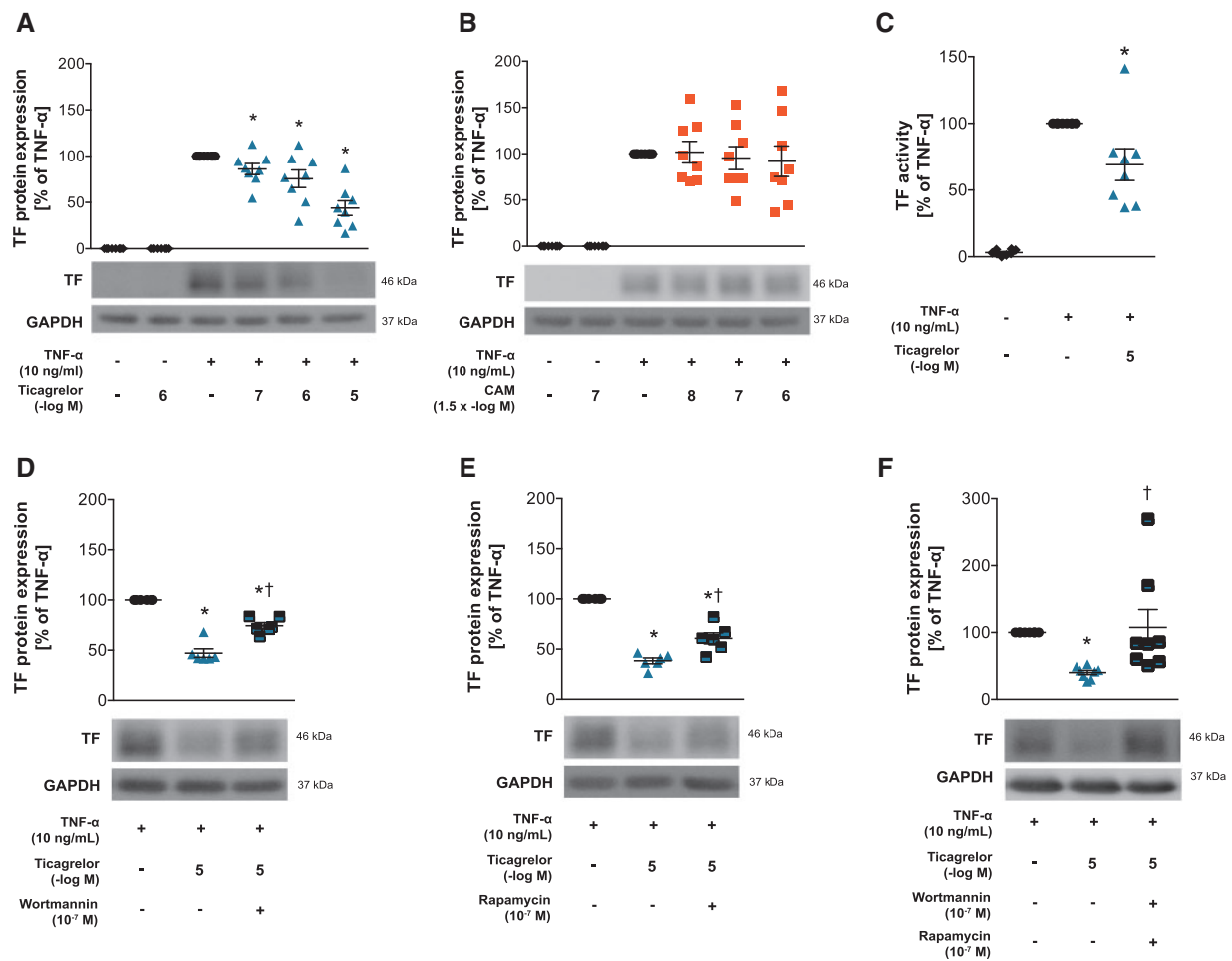


Figure 1 Effects of ticagrelor and CAM on TF expression and activity in HAECs. TF protein expression in ticagrelor-pretreated HAECs ($n = 8$) (A), or CAM-pretreated HAECs ($n = 8$) (B) 1 h before stimulation with TNF- α for 5 h. (C) TF activity in ticagrelor-pretreated HAECs 1 h before TNF- α stimulation for 5 h ($n = 8$). TF protein expression in HAECs pretreated with the PI3 kinase inhibitor wortmannin ($n = 6$) (D), the p70s6 kinase inhibitor rapamycin ($n = 6$) (E) or both ($n = 8$) (F) 1 h prior to ticagrelor treatment for 1 h and subsequent TNF- α stimulation for 5 h. * $P < 0.05$ vs. TNF- α treatment; † $P < 0.05$ vs. TNF- α + ticagrelor treatment. CAM, clopidogrel active metabolite; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HAECs, human aortic endothelial cells; TF, tissue factor; TNF- α , tumour necrosis factor-alpha.

0.05 was considered as statistically significant and calculated by Prism 6 (GraphPad software).

3. Results

3.1 Ticagrelor, but not CAM, reduces TF expression and activity

Ticagrelor concentration dependently decreased TNF- α -induced TF expression in HAECs (Figure 1A), whereas no effect was observed with increasing concentrations of CAM (Figure 1B). In addition to protein expression, TF activity was reduced by ticagrelor, compared with vehicle-treated cells (Figure 1C). On the other hand, protein levels of TFPI were not affected by ticagrelor or CAM (see Supplementary material online, Figure S1). Preincubation with both the PI3 kinase inhibitor wortmannin and the mTOR inhibitor rapamycin partially reversed ticagrelor-mediated TF reduction (Figure 1D and E), whereas co-incubation with both kinase inhibitors abolished the effect of ticagrelor (Figure 1F).

Preincubation using the MAP kinase inhibitors for JNK (SP600125, 10⁻⁶ M), ERK (PD98059, 10⁻⁷ M), or p38 (SB203580, 10⁻⁶ M) did not reverse ticagrelor-mediated reduction of TF expression (data not shown).

3.2 Ticagrelor decreases endothelial TF by proteasomal degradation

To investigate whether the reduction of TF protein expression was due to a decrease in TF gene expression, RT-PCR was performed. Contrary to protein expression, mRNA levels were increased by ticagrelor, compared with vehicle-treated cells (Figure 2A), suggesting a compensatory mechanism for the decrease of tissue factor protein expression. Additionally, potential alterations of mRNA half-life were examined. To this end, actinomycin D was added to stop transcription 1 h after TNF- α stimulation. Next, cells were incubated with ticagrelor or vehicle for additional 1, 2, or 3 h. No differences in mRNA levels between the groups were observed at any time point examined (Figure 2B). To further

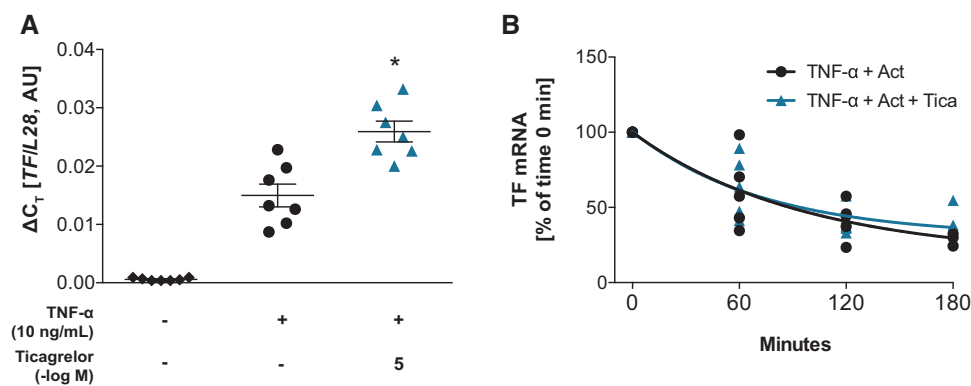


Figure 2 Ticagrelor augmented TF transcription but did not alter mRNA half-life in HAECs. (A) TF mRNA levels in HAECs after preincubation with ticagrelor 1 h before TNF- α -stimulation for 3 h ($n = 7$). (B) TF mRNA stability after treatment with TNF- α (10 ng/mL) for 1 h and subsequent simultaneous incubation with actinomycin D (10 μ g/mL) and ticagrelor ($n = 4-6$) for time points between 0 and 180 min. * $P < 0.05$ vs. TNF- α treatment. Act, actinomycin D; AU, arbitrary unit; HAECs, human aortic endothelial cells; TF, tissue factor; Tica, ticagrelor; TNF- α , tumour necrosis factor-alpha.

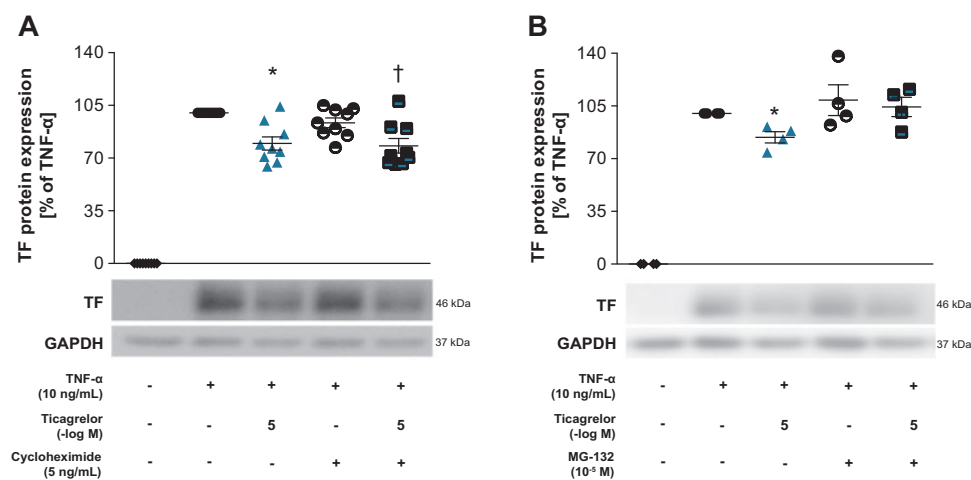


Figure 3 Ticagrelor reduces TF by proteasomal degradation in HAECs. (A) TF expression in HAECs stimulated with TNF- α for 3 h followed by simultaneous treatment with the protein translation inhibitor cycloheximide and ticagrelor for additional 2 h ($n = 9$). (B) TF protein expression in HAECs stimulated with TNF- α for 3 h and treated with the proteasome inhibitor MG-132 and ticagrelor simultaneously for additional 2 h ($n = 4$). * $P < 0.05$ vs. TNF- α treatment. † $P < 0.05$ vs. TNF- α + cycloheximide treatment. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TF, tissue factor; TNF- α , tumour necrosis factor-alpha.

evaluate the underlying mechanism on TF reduction, post-translational mechanisms were investigated, as previously described.²⁸ HAECs were stimulated with TNF- α for 3 h, after which TF protein expression has been reported to reach a steady state.²⁸ Next, translation was arrested with cycloheximide and simultaneously, cells were incubated with ticagrelor or vehicle for additional 2 h. TF protein expression was not altered by cycloheximide, compared with TNF- α . However, ticagrelor significantly reduced TF protein expression, compared with vehicle-treated cells (Figure 3A) suggesting that ticagrelor reduced TF protein stability. In line with this, the proteasome inhibitor MG-132 rescued the effect of ticagrelor on TF reduction (Figure 3B) suggesting that ticagrelor reduces TF by proteasomal degradation.

3.3 Effects of ticagrelor are mediated independently of P2Y₁₂ or ENT1

To investigate the presence of P2Y₁₂ receptor in various endothelial cells, RT-PCR as well as Western blot experiments were performed using human platelets as positive control. Interestingly, P2Y₁₂ mRNA was not detected in human endothelial cells of aortic, cerebral, or cardiac origin by using two different pairs of primers for human P2Y₁₂, whereas it was clearly found in human platelets (Figure 4A). Next, expression of P2Y₁₂ protein was investigated, which was detected in human platelets, but, excluding unspecific bands, could not be detected in any types of endothelial cells (Figure 4B). The absence of P2Y₁₂ mRNA and

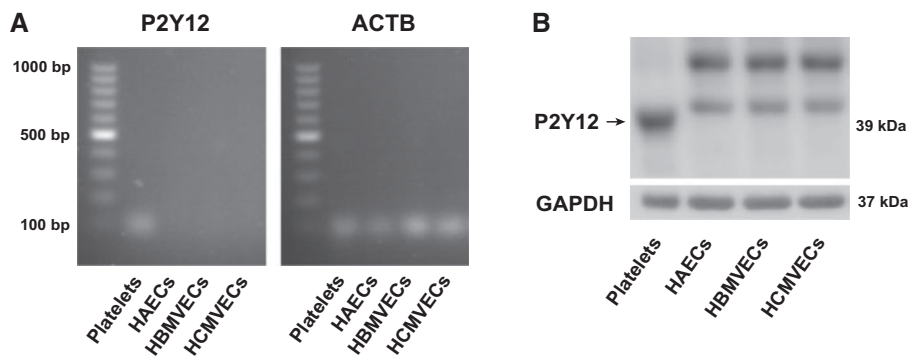


Figure 4 Presence of P2Y₁₂ receptor in human platelets but not in endothelial cells. (A) P2Y₁₂ RNA in human platelets compared with HAECs, HBMVECs and HCMVECs ($n = 3$). (B) Protein expression of the P2Y₁₂ receptor in human platelets, compared with HAECs, HBMVECs, or HCMVECs ($n = 3$). ACTB, human β -actin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HAECs, human aortic endothelial cells; HBMVECs, human brain microvascular endothelial cells; HCMVECs, human cardiac microvascular endothelial cells.

protein suggests that the observed effects of ticagrelor were mediated independently of the P2Y₁₂ receptor.

Next, we investigated whether the observed effects of ticagrelor were mediated by ENT1 inhibition and subsequent increase of adenosine, as recently discussed.^{8,21} Therefore, TNF- α -stimulated cells were treated with increasing concentrations of adenosine to mimic the effects of ticagrelor. Indeed, we observed a concentration-dependent decrease of TF expression in HAECs (Figure 5A). However, contrary to ticagrelor, adenosine decreased TF RNA expression suggesting a transcriptional regulation of TF (Figure 5B). Further, we did not observe similar effects using the ENT1 inhibitor dipyridamole (Figure 5C). Correspondingly, pre-incubation of HAECs using adenosine receptor antagonists against A₁ (8-cyclopentyl-1,3-dipropylxanthine, 10⁻⁷ M), A_{2A} (SCH 442416, 10⁻⁷ M), A_{2b} (MRS 1754, 10⁻⁷ M), and A₃ (VUF 5574, 10⁻⁷ M)^{29,30} receptors individually (data not shown) or in combination did not reverse the effects of ticagrelor (Figure 5D).

3.4 Ticagrelor decreases arterial thrombosis and endothelial TF expression in mice

Whole blood aggregometry after 2 weeks of treatment with ticagrelor 0.15% and clopidogrel 0.06% revealed equal and close to full (>97%) inhibition of ADP-induced platelet aggregation, as compared with animals treated with control chow (Figure 6A); lower concentration, i.e. ticagrelor 0.1% and clopidogrel 0.04% provided subtotal platelet inhibitory effects (Figure 6A). Treatment of rodents using ticagrelor 0.15% resulted in plasma concentration of $2.7 \pm 0.7 \mu\text{M}$ (Figure 6B). Also, platelet aggregation in response to thrombin (1 U/mL) and collagen (10 $\mu\text{g/mL}$) did not differ between ticagrelor- and clopidogrel-treated mice (data not shown). Mice treated with ticagrelor or clopidogrel showed prolonged arterial occlusion times, compared with animals treated with control chow. Moreover, despite equal inhibition of platelet aggregation, ticagrelor prolonged time to arterial occlusion, compared with clopidogrel-treated animals (Figure 6C). In line with our *in vitro* finding, animals treated with ticagrelor, but not clopidogrel, revealed decreased endothelial TF expression in murine CCAs, as compared with rodents treated with control chow (Figure 6D). Instead, plasma TF activity (Figure 6E) and

plasma thrombin generation (Figure 6F) were unchanged between the groups suggesting similar plasma coagulation properties.

4. Discussion

Different P2Y₁₂ receptor antagonists have been shown to differentially affect cardiovascular outcome in several clinical trials.^{3,4} Particularly, the Study of Platelet Inhibition and Patient Outcomes (PLATO) trial showed superior effects of ticagrelor, compared with clopidogrel, in reducing mortality in patients with ACS.³ Stronger antiplatelet effects and reversible binding, which further allows inhibition of newly formed platelets and micro particles, may partly account for the greater efficacy of ticagrelor. Nonetheless, increased platelet inhibition achieved by prasugrel, compared with clopidogrel, did not translate into a similar reduction of overall mortality suggesting the possible involvement of platelet-independent effects.⁴ In line with this, we hypothesized that ticagrelor may prevent endothelial activation with a specific effect on arterial thrombus formation and its key player TF; this indeed could provide a plausible explanation for the reduction in thrombotic events observed in PLATO.

Indeed, in TNF- α -stimulated HAECs, we found that ticagrelor, but not CAM, reduced TF expression via proteasomal degradation and TF activity without affecting TFPI. Interestingly, we found that these effects were mediated independently of both the P2Y₁₂ receptor and the adenosine transporter ENT1. To test the physiological relevance of our *in vitro* findings, we induced arterial thrombosis in C57BL/6 mice treated with ticagrelor or clopidogrel. Dosages were chosen to mimic human plasma concentrations²¹ and to induce comparable platelet inhibition in both groups and, thus, to exclude platelet-dependent effects. Notably, we found that time to arterial occlusion was significantly delayed in ticagrelor-treated mice, compared with clopidogrel-treated rodents. In line with our *in vitro* findings ticagrelor, but not clopidogrel, reduced protein expression of endothelial TF in murine arteries. Plasma coagulation parameters including thrombin generation and tissue factor activity on the other hand remained unchanged, which further supports the hypothesis of local vascular antithrombotic mechanisms.

The herein reported findings support our hypothesis of pleiotropic effects of ticagrelor on endothelial cells and expand previous studies

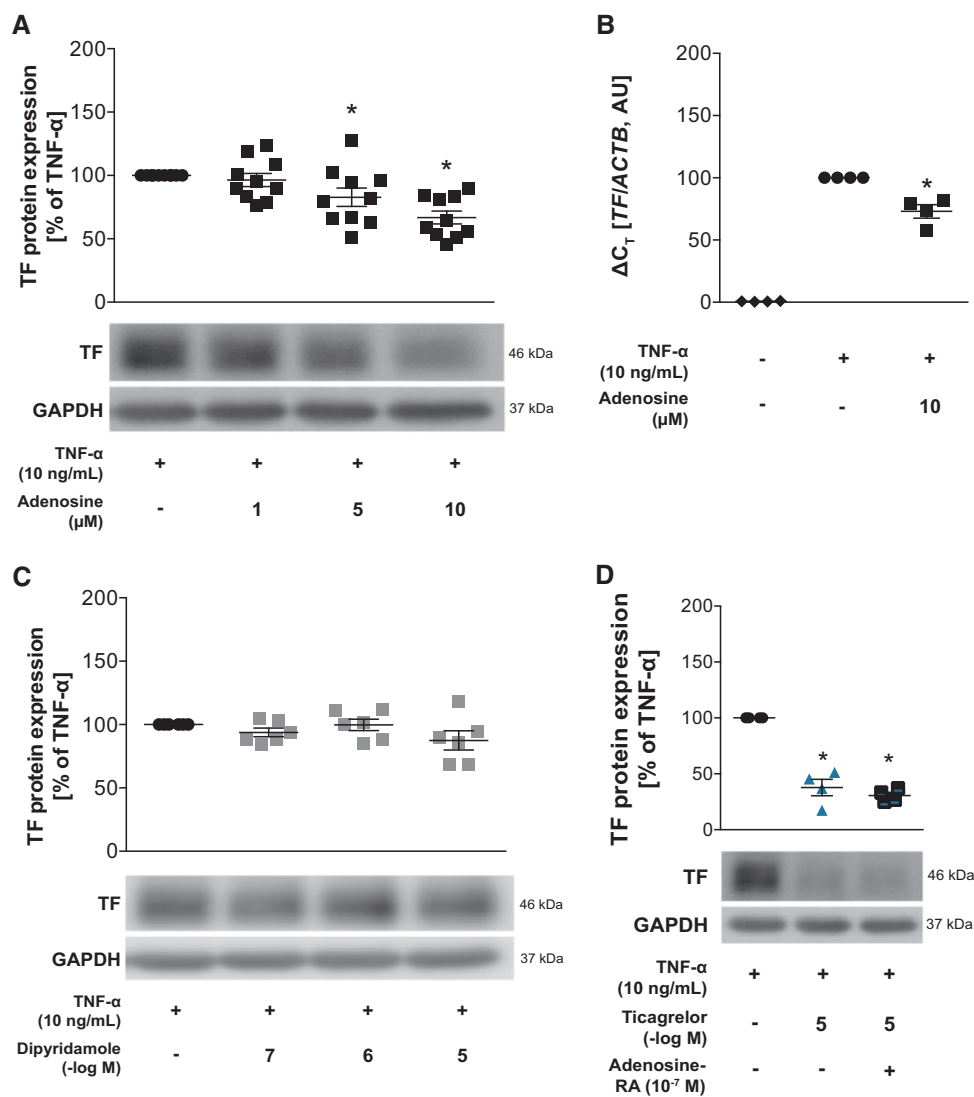


Figure 5 Effects of adenosine, dipyrindamole and adenosine receptor antagonists on TF expression. (A) Effects of adenosine on TF protein ($n = 10$) and (B) RNA expression ($n = 4$) after pretreatment for 1 h and subsequent stimulation with TNF- α for 5 and 3 h, respectively. (C) Effects of the ENT1 inhibitor dipyrindamole on TF expression in HAECs after pretreatment for 1 h and subsequent stimulation with TNF- α for 5 h ($n = 6$). (D) Pretreatment of HAECs with adenosine receptor antagonists for 1 h prior to ticagrelor treatment for 1 additional hour and subsequent TNF- α stimulation for 5 h ($n = 4$). * $P < 0.05$ vs. TNF- α treatment. ACTB, human β -actin; ENT1, equilibrative nucleoside transporter 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HAECs, human aortic endothelial cells; RA, receptor antagonists; TF, tissue factor; TNF- α , tumour necrosis factor-alpha.

describing P2Y₁₂-independent effects of ticagrelor.^{7,8} The inhibitory effect on TF displayed by ticagrelor may be particularly favourable in patients at risk of cardiovascular complications, as both activation of platelets and activation of the coagulation cascade represent key mechanisms in the initiation and propagation of arterial thrombus formation as it occurs in ACS. This notion may contribute to the observed decrease in arterial thrombotic events.³

Unlike previous observations,^{8,21} the herein reported mechanisms cannot be explained by increased adenosine levels following ENT1 inhibition. Although adenosine mimics the effect of ticagrelor, we did not observe similar results using the ENT1 inhibitor dipyrindamole. Additionally, adenosine receptor antagonists could not reverse ticagrelor's effect; thus, implying a different mechanism. Correspondingly, patients receiving ticagrelor or adenosine show similar side effects, such as the induction of

dyspnea,³ and the recent finding of increased adenosine levels⁸ through ENT1 inhibition⁷ provides evidence to link these observations. Nevertheless, potent ENT1 inhibitors such as dipyrindamole also increase adenosine levels,⁸ but do not induce dyspnea.³¹ Therefore, exclusive ENT1 in addition to P2Y₁₂ inhibition may not explain the entire mode of action of ticagrelor and further research is needed to determine its exact mechanisms.

Because of the chemical similarity of ticagrelor and adenosine, direct interactions with adenosine receptors appear to be likely and have been investigated by others; however, such interactions were considered unlikely to be clinically relevant because of the low affinity of ticagrelor.⁷ The P2Y₁₂ receptor belongs to the family of P2 purine and pyrimidine G protein-coupled receptors and seven further P2Y receptors have been characterized, some of which are expressed in the endothelium.³²

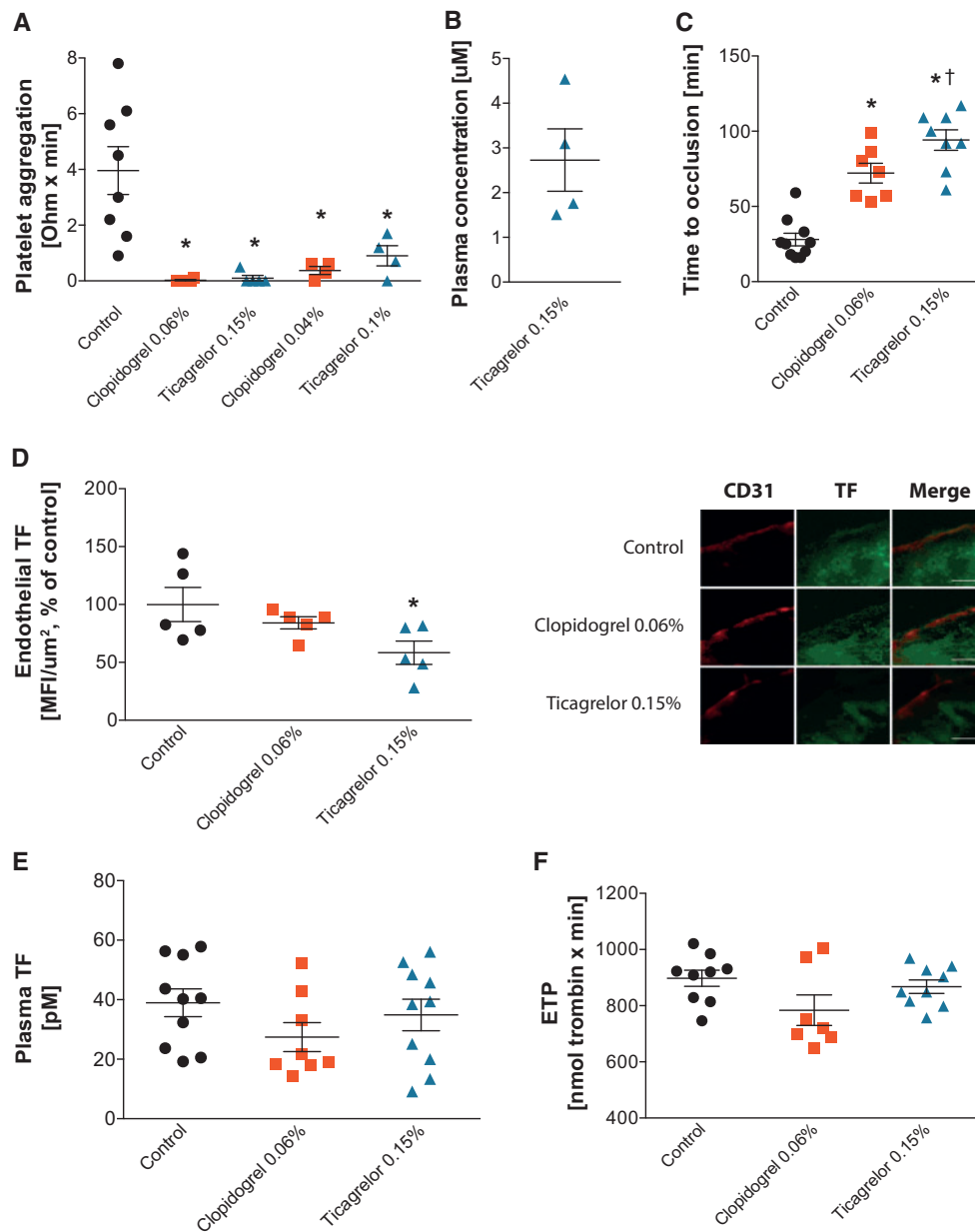


Figure 6 Platelet aggregation, endothelial TF expression, time to arterial occlusion, and thrombin potential. (A) Platelet aggregation in response to ADP (10 μM) in mice treated with control chow, clopidogrel, or ticagrelor for 2 weeks. (B) Plasma concentration of ticagrelor in mice treated with ticagrelor 0.15% added to chow. (C) Time to arterial thrombosis in mice treated with control chow ($n = 10$), clopidogrel ($n = 7$), or ticagrelor ($n = 8$). (D) Endothelial TF expression in CCAs of mice treated with control chow, clopidogrel, or ticagrelor ($n = 5$) and representative transverse sections of CCA stained for endothelium (CD31, red, scale bar 5 μm) and TF (green, scale bar 5 μm) (E). Plasma TF activity and (F) plasma thrombin potential clopidogrel-, ticagrelor-, and control chow-treated animals at baseline. * $P < 0.05$ vs. control; † $P < 0.05$ vs. clopidogrel. ADP, adenosine diphosphate; CCA, common carotid artery; ETP, endogenous thrombin potential; MFI, mean fluorescence intensity; TF, tissue factor.

However, whether ticagrelor shows binding affinity to other purine and pyrimidine receptors and whether these receptors are responsible for the observed effects on endothelial cells remains to be determined.

Here we for the first time provide evidence for endothelium-dependent antithrombotic properties, which indicates that ticagrelor can affect the coagulation system in addition to its well-known antiplatelet effects. Dual antiplatelet therapy is the standard of care in patients suffering from ACS²; however, given the high prevalence of this disease,¹ there is also a great number of patients with comorbidities requiring

anticoagulant treatment, such as deep vein thrombosis, atrial fibrillation, or mechanical heart valves.³³ Although anticoagulants in combination with dual antiplatelet therapy lower the risk of thrombotic events, disproportionately increased bleeding rates have been observed.³⁴ While single antiplatelet therapy using clopidogrel in combination with anticoagulants reduced the risk of bleedings,³⁵ such an approach may not be sufficient to sustain the low rate of thrombotic events. Thus, further studies are needed to determine the effectiveness and safety of new oral anticoagulants in combination with more potent platelet antagonists

such as ticagrelor.³³ Because of its dual antithrombotic effects, ticagrelor may be a more appropriate choice than clopidogrel in patients with ACS with comorbidities requiring additional antithrombotic therapy. Along these lines, ticagrelor showed better efficacy than clopidogrel in the PLATO trial.³ On the other hand, safety in terms of bleeding may be of particular concern in these patients as ticagrelor increased non-procedure-related bleeding rates in the PLATO trial.³

In conclusion, the pleiotropic effects of ticagrelor on the endothelium may in part explain its greater efficacy in reducing thrombotic events in patients with ACS and mortality in clinical trials; its antithrombotic properties may be particularly promising in patients requiring anticoagulant in addition to antiplatelet therapy. Nonetheless, additional antithrombotic properties may increase bleeding risk and special attention may be required in patients at high risk.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Funding

This work was supported by the Swiss National Science Foundation (310030_144152/1 to J.H.B., 310030_147017 to G.G.C., and 310030_166576 to T.F.L.), the Foundation of Cardiovascular Research Zurich, Switzerland, the Foundation Kardio, Baden, Switzerland, and a research grant from Hartmann Müller-Stiftung, Zurich, Switzerland.

Conflict of interest: Ticagrelor and a research grant were provided by AstraZeneca, Mölndal, Sweden and clopidogrel active metabolite by Sanofi-Aventis, Germany GmbH. T.F.L. and J.H.B. have received educational and research grants as well as honoraria from AstraZeneca, Zug, Switzerland.

References

- Nichols M, Townsend N, Scarborough P, Rayner M. Cardiovascular disease in Europe 2014: epidemiological update. *Eur Heart J* 2014;**35**:2950–2959.
- Windecker S, Kolh P, Alfonso F, Collet JP, Cremer J, Falk V, Filippatos G, Hamm C, Head SJ, Juni P, Kappetein AP, Kastrati A, Knuuti J, Landmesser U, Laufer G, Neumann FJ, Richter DJ, Schauerte P, Sousa Uva M, Stefanini GG, Taggart DP, Torracca L, Valgimigli M, Wijns W, Witkowski A. 2014 ESC/EACTS Guidelines on myocardial revascularization: The Task Force on Myocardial Revascularization of the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS) Developed with the special contribution of the European Association of Percutaneous Cardiovascular Interventions (EAPCI). *Eur Heart J* 2014;**35**:2541–2619.
- Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, Horrow J, Husted S, James S, Katus H, Mahaffey KW, Scirica BM, Skene A, Steg PG, Storey RF, Harrington RA, Freij A, Thorsen M. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med* 2009;**361**:1045–1057.
- Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, Neumann FJ, Ardissino D, De Servi S, Murphy SA, Riesmeyer J, Weerakkody G, Gibson CM, Antman EM. Prasugrel versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med* 2007;**357**:2001–2015.
- Cattaneo M, Schulz R, Nylander S. Adenosine-mediated effects of ticagrelor: *evidence and potential clinical relevance. *J Am Coll Cardiol* 2014;**63**:2503–2509.
- Adamski P, Kozinski M, Ostrowska M, Fabiszak T, Navarese EP, Paciork P, Grzesk G, Kubica J. Overview of pleiotropic effects of platelet P2Y₁₂receptor inhibitors. *Thromb Haemost* 2014;**112**:224–242.
- Armstrong D, Summers C, Ewart L, Nylander S, Sidaway JE, van Giezen JJ. Characterization of the adenosine pharmacology of ticagrelor reveals therapeutically relevant inhibition of equilibrative nucleoside transporter 1. *J Cardiovasc Pharmacol Ther* 2014;**19**:209–219.
- Nylander S, Femia EA, Scavone M, Berntsson P, Asztely AK, Nelander K, Lofgren L, Nilsson RG, Cattaneo M. Ticagrelor inhibits human platelet aggregation via adenosine in addition to P2Y₁₂ antagonism. *J Thromb Haemost* 2013;**11**:1867–1876.
- Bonello L, Laine M, Kipson N, Mancini J, Helal O, Fromonot J, Gariboldi V, Condo J, Thuny F, Frere C, Camoin-Jau L, Paganelli F, Dignat-George F, Guieu R. Ticagrelor increases adenosine plasma concentration in patients with an acute coronary syndrome. *J Am Coll Cardiol* 2014;**63**:872–877.
- Breitenstein A, Camici GG, Tanner FC. Tissue factor: beyond coagulation in the cardiovascular system. *Clin Sci (Lond)* 2010;**118**:159–172.
- Steffel J, Luscher TF, Tanner FC. Tissue factor in cardiovascular diseases: molecular mechanisms and clinical implications. *Circulation* 2006;**113**:722–731.
- Pawashe AB, Golino P, Ambrosio G, Migliaccio F, Ragni M, Pascucci I, Chiariello M, Bach R, Garen A, Konigsberg WK, Ezekowitz MD. A monoclonal antibody against rabbit tissue factor inhibits thrombus formation in stenotic injured rabbit carotid arteries. *Circ Res* 1994;**74**:56–63.
- Felmeden DC, Spencer CG, Chung NA, Belgore FM, Blann AD, Beevers DG, Lip GY. Relation of thrombogenesis in systemic hypertension to angiogenesis and endothelial damage/dysfunction (a substudy of the Anglo-Scandinavian Cardiac Outcomes Trial [ASCOT]). *Am J Cardiol* 2003;**92**:400–405.
- Lim HS, Blann AD, Lip GY. Soluble CD40 ligand, soluble P-selectin, interleukin-6, and tissue factor in diabetes mellitus: relationships to cardiovascular disease and risk factor intervention. *Circulation* 2004;**109**:2524–2528.
- Sambola A, Osende J, Hathcock J, Degen M, Nemerson Y, Fuster V, Crandall J, Badimon JJ. Role of risk factors in the modulation of tissue factor activity and blood thrombogenicity. *Circulation* 2003;**107**:973–977.
- Suefuji H, Ogawa H, Yasue H, Kaikita K, Soejima H, Motoyama T, Mizuno Y, Oshima S, Saito T, Tsuji I, Kumeda K, Kamikubo Y, Nakamura S. Increased plasma tissue factor levels in acute myocardial infarction. *Am Heart J* 1997;**134**:253–259.
- Liu Y, Pelekanakis K, Woolkalis MJ. Thrombin and tumor necrosis factor alpha synergistically stimulate tissue factor expression in human endothelial cells: regulation through c-Fos and c-Jun. *J Biol Chem* 2004;**279**:36142–36147.
- Brueckmann M, Bertsch T, Lang S, Sueselbeck T, Wolpert C, Kaden JJ, Jaramillo C, Huhle G, Borggrete M, Haase KK. Time course of systemic markers of inflammation in patients presenting with acute coronary syndromes. *Clin Chem Lab Med* 2004;**42**:1132–1139.
- Guo Y, Lip GY, Apostolakis S. Inflammation in atrial fibrillation. *J Am Coll Cardiol* 2012;**60**:2263–2270.
- Feldman AM, Combes A, Wagner D, Kadakomi T, Kubota T, Li YY, McTiernan C. The role of tumor necrosis factor in the pathophysiology of heart failure. *J Am Coll Cardiol* 2000;**35**:537–544.
- Nanhwan MK, Ling S, Kodakandla M, Nylander S, Ye Y, Birnbaum Y. Chronic treatment with ticagrelor limits myocardial infarct size: an adenosine and cyclooxygenase-2-dependent effect. *Arterioscler Thromb Vasc Biol* 2014;**34**:2078–2085.
- Sillen H, Cook M, Davis P. Determination of ticagrelor and two metabolites in plasma samples by liquid chromatography and mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2010;**878**:2299–2306.
- Holy EW, Besler C, Reiner MF, Camici GG, Manz J, Beer JH, Luscher TF, Landmesser U, Tanner FC. High-density lipoprotein from patients with coronary heart disease loses anti-thrombotic effects on endothelial cells: impact on arterial thrombus formation. *Thromb Haemost* 2014;**112**.
- Reiner MF, Martinod K, Stivala S, Savarese G, Camici GG, Luscher TF, Wagner DD, Beer JH. Dietary omega-3 alpha-linolenic acid does not prevent venous thrombosis in mice. *Thromb Haemost* 2014;**113**.
- Furlan-Freguia C, Marchese P, Gruber A, Ruggeri ZM, Ruf W. P2X7 receptor signaling contributes to tissue factor-dependent thrombosis in mice. *J Clin Invest* 2011;**121**:2932–2944.
- Wallentin L. P2Y₁₂ inhibitors: differences in properties and mechanisms of action and potential consequences for clinical use. *Eur Heart J* 2009;**30**:1964–1977.
- Holy EW, Akhmedov A, Luscher TF, Tanner FC. Berberine, a natural lipid-lowering drug, exerts prothrombotic effects on vascular cells. *J Mol Cell Cardiol* 2009;**46**:234–240.
- Breitenstein A, Stampfli SF, Camici GG, Akhmedov A, Ha HR, Follath F, Bogdanova A, Luscher TF, Tanner FC. Amiodarone inhibits arterial thrombus formation and tissue factor translation. *Arterioscler Thromb Vasc Biol* 2008;**28**:2231–2238.
- Shen J, Halenda SP, Sturek M, Wilden PA. Cell-signaling evidence for adenosine stimulation of coronary smooth muscle proliferation via the A1 adenosine receptor. *Circ Res* 2005;**97**:574–582.
- Fredholm BB, AP IJ, Jacobson KA, Linden J, Muller CE. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors—an update. *Pharmacol Rev* 2011;**63**:1–34.
- Biaggioni I, Onrot J, Hollister AS, Robertson D. Cardiovascular effects of adenosine infusion in man and their modulation by dipyrindamole. *Life Sci* 1986;**39**:2229–2236.
- Burnstock G. Purine and pyrimidine receptors. *Cell Mol Life Sci* 2007;**64**:1471–1483.
- Fox KA. Dual or single antiplatelet therapy with anticoagulation? *Lancet* 2013;**381**:1080–1081.
- Mega JL, Braunwald E, Wiviott SD, Bassand JP, Bhatt DL, Bode C, Burton P, Cohen M, Cook-Brunns N, Fox KA, Goto S, Murphy SA, Plotnikov AN, Schneider D, Sun X, Verheugt FW, Gibson CM, Investigators AAT. Rivaroxaban in patients with a recent acute coronary syndrome. *N Engl J Med* 2012;**366**:9–19.
- Dewilde WJ, Oirbans T, Verheugt FW, Kelder JC, De Smet BJ, Herrman JP, Adriaenssens T, Vrolix M, Heestermaas AA, Vis MM, Tijssen JG, van 't Hof AW, ten Berg JM. *investigators Ws. Use of clopidogrel with or without aspirin in patients taking oral anticoagulant therapy and undergoing percutaneous coronary intervention: an open-label, randomised, controlled trial. *Lancet* 2013;**381**:1107–1115.