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Antithrombotic effects of PAR1 and PAR4 antagonists evaluated under flow and static conditions

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ABSTRACT

Introduction: Thrombin-mediated activation of human platelets involves the G-protein-coupled protease-activated receptors PAR1 and PAR4. Inhibition of PAR1 and/or PAR4 is thought to modulate platelet activation and subsequent procoagulant reactions. However, the antithrombotic effects of PAR1 and PAR4 antagonism have not been fully elucidated, particularly under flow conditions.

Materials and Methods: A microchip-based flow chamber system was used to evaluate the influence of SCH79797 (PAR1 antagonist) and YD-3 (PAR4 antagonist) on thrombus formation mediated by collagen and tissue thromboplastin at shear rates simulating those experienced in small- to medium-sized arteries (600 s^{-1}) and large arteries and small veins (240 s^{-1}).

Results: At a shear rate of 600 s^{-1}, SCH79797 (10 μM) efficiently reduced fibrin-rich platelet thrombi and significantly delayed occlusion of the flow chamber capillary (1.44 fold of control; $P < 0.001$). The inhibitory activity of SCH79797 was diminished at 240 s^{-1}. YD-3 (20 μM) had no significant effect at either shear rate. The antithrombotic effects of SCH79797 were significantly augmented when combined with aspirin and AR-C66096 ($P2Y_{12}$ antagonist), but not with YD-3. In contrast, no significant inhibition of tissue factor-induced clot formation under static conditions was observed in blood treated with SCH79797 and YD-3, although thrombin generation in platelet-rich plasma was weakly delayed by these antagonists.

Conclusions: Our results suggest that the antithrombotic activities of PAR1 and/or PAR4 antagonism is influenced by shear conditions as well as by combined platelet inhibition with aspirin and a $P2Y_{12}$-antagonist.

Introduction

Thrombin activates human platelets via two protease-activated G protein-coupled receptors, protease-activated receptor (PAR) 1 and PAR4 [1,2]. PAR1 is thought to be the main thrombin receptor on platelets due to its high affinity and sensitivity to thrombin, and is therefore regarded as a promising target for antiplatelet therapy [3–7]. Although PAR4 is a low-affinity thrombin receptor, its activation at high thrombin concentrations (>5 nM) is reported to mediate prolonged platelet aggregation [2]. The dual activation of PAR1 and PAR4 collectively promotes platelet morphological changes, granule secretion, and glycoprotein (GP) IIb/IIIa receptor activation, resulting in stable platelet thrombus formation [1,2,8].

PAR1 antagonism has potent antithrombotic and antiplatelet effects in animal models of arterial thrombosis [9–12], as well as in acute coronary syndrome (ACS) patients in phase II clinical trials [13,14]. A recent phase III trial in ACS patients also demonstrated that combined PAR1 blockade with aspirin and/or clopidogrel reduces the incidence of myocardial infarction; however, the combination therapy increased the risk of fatal bleeding, including intracranial hemorrhage [15]. Therefore, further optimization of treatment strategies based on the assessment of total antiplatelet response to combination therapy may be necessary to achieve clinical benefits from PAR1 antagonism.

Platelet aggregometry is commonly used to assess platelet activation in response to a single exogenous agonist, such as collagen, thrombin, or

Abbreviations: ACS, acute coronary syndrome; ADP, adenosine 5'-diphosphate; AP, activating peptide; ASP, aspirin; AUC, area under the aggregation curve; AU, aggregation units; CFT, clot formation time; CT, clotting time; CTI, corn trypsin inhibitor; ETP, endogenous thrombin generation potential; GP, glycoprotein; LT, lag time; MCF, maximum clot firmness; OT, occlusion time; PAR, protease-activated receptor; PH, peak height; PPP, platelet-poor plasma; PRP, platelet-rich plasma; r-TF, recombinant tissue factor; TF, tissue factor; TG, thrombin generation; T-TAS, total thrombus-formation analysis system; TTP, time to peak; TXA_2, thromboxane A_2.

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adenosine 5'-diphosphate (ADP). However, *in-vivo* thrombus formation is a dynamic and complex process that involves multiple platelet activation and coagulation pathways under flow conditions [16–18]. In addition, the species specificity of platelet PARs often limits the direct application of animal models to investigate the roles of PAR1 and PAR4 in physiological haemostasis, and the influence of PAR antagonism on pathologic thrombus formations. To overcome these limitations, a microchip-based flow chamber system capable of evaluating fibrin-rich platelet thrombus formation under adjustable flow conditions is a potential alternative for modeling the pathogenesis of arterial thrombosis after plaque rupture [19].

In the present study, we hypothesized that the antithrombotic mechanisms and efficacies of PAR antagonists can be accurately elucidated by analyzing fibrin-rich platelet thrombus formation patterns under flow conditions. Flow chamber experiments were conducted under shear conditions mimicking mid- to small-sized arteries (600 s^{-1}) and large arteries and small veins (240 s^{-1}) using human blood samples pre-treated with PAR1 and PAR4 antagonists. Coagulation assays, including whole blood viscoelastometry and thrombin generation (TG) assays, were also performed to comparatively evaluate PAR1- and PAR4-mediated platelet procoagulant activity under static conditions.

Materials and methods

Materials

SCH79797 [20] and AR-C66096 [21], specific antagonists of PAR1 and $P2Y_{12}$ receptor, respectively, were purchased from Tocris Bioscience (Bristol, United Kingdom). YD-3, a specific antagonist of PAR4, was synthesized as previously reported [22]. Acetylsalicylic acid was purchased from Wako Pure Chemicals (Osaka, Japan). PAR1-activating peptide (AP), SFLLRN, and PAR4-AP, AYPGKF, were purchased from Verum Diagnostica (Munich, Germany). The microchips used in the flow-chamber system were manufactured by Richell Corp. (Toyama, Japan). Porcine type I collagen was purchased from Nitta Gelatin, Inc. (Osaka, Japan). Tissue thromboplastin was purchased from Sysmex (Hyogo, Japan). Corn derived trypsin inhibitor (CTI), a specific inhibitor of factor XII, was prepared as reported previously [23]. For the TG assay, the tissue factor (TF)-based reagent for PRP, and FluCa-reagent, a fluorogenic substrate (Z-Gly-Gly-Arg-AMC) dissolved in HEPES buffer and calcium chloride, were purchased from Diagnostica Stago (Parsippany, NJ, USA). r-TF was purchased from Mitsubishi Chemical Medience (Tokyo, Japan). All other reagents were obtained from Wako Pure Chemicals (Osaka, Japan).

Blood samples

Whole blood samples were collected in plastic tubes containing either 3.2% sodium citrate (Terumo, Tokyo, Japan) or 25 μg/ml hirudin (Verum Diagnostica, Munich, Germany). The subjects had not taken any medication that might have affected platelet function or coagulation in the two weeks preceding the blood draw, and had no history of coagulation disorders. The study protocol was approved by the institutional ethics committee of Kinki University (Osaka, Japan), and informed written consent was obtained from all subjects.

Effects of SCH79797 and YD-3 on platelet aggregation

Antiplatelet effects of SCH79797 and YD-3 on platelet aggregation induced by SFLLRN and AYPGKF, respectively, were confirmed by whole blood platelet aggregometry using the Multiplate™ analyzer (Verum Diagnostica, Munich, Germany) as directed by the manufacturer. Briefly, 300 μl saline and 300 μl hirudinized blood were pipetted into a single-use cuvette, which was then incubated at 37 °C for 3 min. Either SFLLRN (final concentration, 32 μM) or AYPGKF (final concentration, 662 μM) was then added to the sample to initiate platelet aggregation. Platelet adhesion and aggregation were monitored for 6 min. The impedance change caused by platelet adhesion and aggregation was plotted against time and the area under the aggregation curve (AUC) to measure the aggregation response, which was quantified in arbitrary aggregation units (AU).

Effects of SCH79797 and YD-3 on thrombus formation measured under flow conditions

Microchip-based flow chamber analysis

For the analysis of thrombus formation under various shear rates, we used the Total Thrombus-formation Analysis System (T-TAS; Fujimori Kogyo Co., Ltd., Tokyo, Japan), which is a microchip-based flow chamber system equipped with a pneumatic pump, a flow pressure sensor, and a videomicroscope, as described previously [19] (Fig. S1). Blood samples were used for assay within 1 – 3 hours after collection. Briefly, citrated whole blood (480 μl) was mixed with 20 μl of 0.3 M $CaCl_2$ containing 1.25 mg/ml CTI immediately before the sample was perfused over a microchip coated with collagen and tissue thromboplastin at flow rates of 4 and 10 μl/min, corresponding to initial wall shear rates of 240 and 600 s^{-1}, respectively, as estimated by the FLUENT program (Ansys Co., Ltd., Tokyo, Japan). Flow pressure changes were monitored by the pressure transducer located upstream of the microcapillary during the perfusion experiments. Thrombus formation and breakdown within the microcapillary result in pressure increases and decreases, respectively. Based on the flow pressure pattern, the following parameters are used to analyze thrombus formation process. T_{10} (time to 10 kPa) is the lag time for the flow pressure to increase by 10 kPa from the baseline due to partial occlusion of the capillary. The T_{10} value represents initial thrombi formation. OT (occlusion time) is the lag time for the flow pressure to increase by 80 kPa from baseline owing to near complete occlusion of the capillary by thrombi. The OT value reflects the onset, growth, and stability of thrombi inside the capillary. Blood perfusion and flow pressure monitoring are programmed to stop when the flow pressure increases to 80 kPa.

In addition to the flow pressure analyses, thrombus formation in the capillary was visually inspected using the built-in light microscope. The two-dimensional area covered by thrombi was analyzed using image analysis software (Zia; Fujimori Kogyo Co., Ltd., Tokyo, Japan), as previously described [19] (Fig. S2). The progression of thrombus formation within the microchip was recorded at 8 and 12 min after the start of blood perfusion.

Analysis of thrombi by confocal laser scanning microscopy

Thrombi formed on the coated microchip surface were washed three times with phosphate-buffered saline (PBS) and then incubated with FITC-conjugated mouse anti-human CD41 (platelet GPIIb) IgG (1:5 dilution) for 15 min in the dark. After three washes with Tris-buffered saline containing 0.1% Triton X-100 (TBST), thrombi were immobilized with OptiLyse C (Immunotech, Marseille, France) for 15 min. After three washes with TBST, the sample was blocked for 1 h with Block Ace (Yukijirushi, Osaka, Japan) and then incubated for 30 min in the dark with rabbit anti-human fibrinogen IgG (1:99 dilution) labeled with Alexa 594. To test the immunospecificity of the primary antibodies, control experiments were conducted with isotype-matched IgG for primary antibodies against GPIIb and fibrinogen. Following antibody staining, the sample was visualized using a LSM700 confocal laser microscope (Carl Zeiss Microscopy Co., Ltd., Oberkochen, Germany).

Effects of SCH79797 and YD-3 on thrombus formation measured under static conditions

Thromboelastometry measurements

The ROTEM™ system (TEM International, Munich, Germany) was used to analyze the clot formation process in whole blood under static conditions. All ROTEM™ assays were performed in the presence of