Full Length Article

Paradoxical enhancement of the intrinsic pathway-induced thrombin generation in human plasma by melagatran, a direct thrombin inhibitor, but not edoxaban, a direct factor Xa inhibitor, or heparin

Taketoshi Furugohri *, Yoshiyuki Morishima

Biological Research Laboratories, R&D Division, Daiichi Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan

A R T I C L E   I N F O

Article history:
Received 11 March 2015
Received in revised form 28 May 2015
Accepted 30 June 2015
Available online 4 July 2015

Keywords:
Intrinsic pathway
Direct thrombin inhibitor
Melagatran
Activated protein C
Direct factor Xa inhibitor
Edoxaban

A B S T R A C T

Introduction: The blood coagulation cascade consists of two pathways, the tissue factor (TF)-dependent extrinsic pathway and the contact factor-dependent intrinsic pathway. We have previously shown that a direct thrombin inhibitor, melagatran, paradoxically increased TF-induced thrombin generation (TG) in thrombomodulin (TM)-containing human plasma in vitro. However, the effect of melagatran on the intrinsic pathway-induced TG remains to be investigated. We investigated whether melagatran enhances the intrinsic pathway-induced TG.

Methods and results: TG was induced by kaolin in human plasma and assayed by the calibrated automated thrombography method. Melagatran at 150 and 300 nM significantly increased the peak level (2.40-fold) and endogenous thrombin potential of TG in normal plasma in the presence of 5 nM TM. In the absence of TM or in protein C (PC)-deficient plasma, the paradoxical enhancement of TG by melagatran disappeared. A direct FXa inhibitor, edoxaban, and an antithrombin-dependent anticoagulant, unfractionated heparin (UFH), did not increase, but simply decreased TG under each condition in a concentration-dependent manner.

Conclusion: Melagatran enhanced the intrinsic pathway-induced TG as well as the extrinsic pathway-induced TG in human plasma under the condition where PC system is active. In contrast, edoxaban and UFH showed concentration-dependent decrease of TG, but no enhancement. These results indicated that edoxaban and UFH may have a low risk of the paradoxical enhancement of TG by both the extrinsic and intrinsic pathway activation.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

It has been previously shown [1–4] that low doses of a direct thrombin inhibitor, melagatran, aggravated coagulation status initiated by the injection of tissue factor (TF) in rats, and that antithrombin (AT)-independent thrombin inhibitors such as melagatran, dabigatran, hirudin, and active site-blocked thrombin increased TF-induced thrombin generation (TG) in thrombomodulin (TM)-containing human plasma in vitro. In contrast, direct factor Xa (FXa) inhibitors such as edoxaban and rivaroxaban, and AT-dependent anticoagulants such as unfractionated heparin (UFH) and low molecular weight heparin solely inhibited TG, but not enhance it. This paradoxical enhancement of TG by AT-independent thrombin inhibitors was not observed in the absence of TM or protein C (PC), suggesting mediation by the suppression of the thrombin-TM-mediated negative-feedback system through the inhibition of PC activation.

The blood coagulation cascade is a stepwise sequence of proteolytic reactions of the coagulation factors, and consists of two pathways, the TF-dependent “extrinsic pathway” and the contact factor-dependent “intrinsic pathway”. Until now, most TG measurements evaluated the coagulation reaction of the extrinsic pathway stimulated by TF.

In terms of the intrinsic pathway, factor XII (FXII), an initiator of the intrinsic pathway, —deficient patients do not exhibit an abnormal bleeding tendency despite having markedly prolonged activated partial thromboplastin time (aPTT) [5]. On the other hand, thrombus formation is suppressed in FXII-deficient mice without excessive bleeding [6]. Moreover, elevated levels of activated FXII (FXIIa) have been reported to be associated with increased risk for coronary heart disease [7] and to be a prognostic risk factor for recurrent coronary events [8]. Factor XI (FXI)—deficient patients do not always have a bleeding diathesis, whereas FXI-deficient mice are protected from carotid artery thrombus formation triggered by FeCl3 [9]. These data suggest that the intrinsic pathway plays an important role in the formation of pathologic thrombus but not in hemostasis. However, the effects of direct thrombin inhibitors and other anticoagulants on the intrinsic pathway-induced TG remain to be investigated.

The aim of the present study was to investigate the effects of a direct thrombin inhibitor, melagatran, a FXa inhibitor, edoxaban, and UFH on TG induced by the intrinsic pathway activation using kaolin as a stimulant in human plasma, whether melagatran enhances the intrinsic pathway-induced TG, and if this enhancement is mediated by inhibition of the thrombin-TM and PC system.
2. Materials and Methods

2.1. Reagents

Edoxaban tosylate and melagatran was synthesized at Daiichi Sankyo Co., Ltd. (Tokyo, Japan). A phospholipid reagent (60% phosphatidylcholine, 20% phosphatidylethanolamine, 20% phosphatidylserine) was prepared at Daiichi Sankyo. UFH was purchased from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). Normal human plasma was purchased from George King Bio-Medical, Inc. (Overland Park, KS, U.S.A.) and PC-deficient human plasma was obtained from Affinity Biologicals Inc. (Ancaster, Canada). The thrombin calibrator and FluCa-kit (2.5 mM Z-Gly-Gly-Arg-aminomethylcoumarin and 100 mM CaCl₂) were bought from Thrombinoscope BV (Maastricht, The Netherlands). Recombinant human soluble TM (Recomodulin) was from Asahi Kasei Pharma Corp. (Tokyo, Japan) and kaolin was from Sigma-Aldrich Co. LLC (St. Louis, MO, U.S.A.).

2.2. TG Assay

TG in platelet-poor plasma was assayed by calibrated automated thrombogram method according to the previous method[10] using a fluorometer Fluoroskan Ascent (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and the thrombinoscope software (Thrombinoscope BV). TM-containing plasma was prepared by adding 16 μL of 500 nM TM solution to 984 μL of plasma. The TG assay was performed as follows: 75 μL of plasma in the presence and absence of TM and 5 μL of anticoagulant solutions (final concentrations in plasma: melagatran 9.38—1500 nM, edoxaban 9.38—900 nM, or UFH 1.56—50 mU/mL) was pipetted into the well of a microtiter plate together with 20 μL of a mixture of 600 ng/mL kaolin[11,12] and 24 μM phospholipids. After 10 min of preincubation at 37 °C, the reaction was started by adding 20 μL of FluCa-kit. Final concentrations were 5 nM TM, 100 ng/mL kaolin, 4 μM phospholipids, 417 μM fluorogenic substrate, and 16.7 mM CaCl₂.

The fluorescence was measured for 120 min at 37 °C (ex. 390 nm, em. 460 nm). TG curves were described in terms of peak height, lag time, and endogenous thrombin potential (ETP). Data were obtained from three or four independent experiments of a single measurement.

2.3. Statistical Analysis

Analyses were performed using SAS release 9.2 (SAS Institute Japan, Tokyo, Japan). All data represent the mean ± standard error of the mean (SEM). The statistical significance between the control and treatment groups was analyzed by Dunnett test. The concentration-response relationship was evaluated by a regression analysis. A P value of less than 0.05 (two-tailed) was considered as a significant difference.

3. Results

3.1. Effects of Anticoagulants on Kaolin-induced TG in TM-containing Normal Human Plasma

TG was determined using normal human plasma in the presence of 5 nM TM to evaluate the coagulation response under conditions where the negative feedback system is active as in an in vivo situation. Melagatran, a direct thrombin inhibitor, significantly increased peak height and ETP of TG at 150 and 300 nM compared with the control (Figs. 1A, 3A,B). Peak height of TG reached a maximum at 300 nM of melagatran with 2.4-fold increase (Figs. 1A, 3A). At a higher concentration (600 nM), melagatran did not enhance TG. In terms of the time parameter, melagatran prolonged lag time with increasing concentrations (Fig. 3C).

Edoxaban, a direct FXa inhibitor, and UFH, an AT-dependent anticoagulant, concentration-dependently decreased TG (Fig. 1B,C). Edoxaban (Fig. 4A,B) and UFH (data not shown) suppressed the peak height and...
ETP. Edoxaban (Fig. 4C) prolonged lag time, whereas UFH (data not shown) did not affect it except at 12.5 mU/mL.


In normal human plasma without TM, the enhancement of peak height and ETP of TG by melagatran disappeared (Figs. 2A, 3A,B). Melagatran prolonged lag time with increasing concentrations (Figs. 2A, 3C).

Next, the effect of melagatran on TG was examined in PC-deficient human plasma. Melagatran inhibited TG concentration dependently and failed to enhance TG in PC-deficient plasma even in the presence of 5 nM TM (Fig. 3A,B).

Edoxaban (Fig. 4A,B) and UFH (data not shown) decreased TG in normal human plasma without TM and in PC-deficient plasma in a concentration dependent manner.

4. Discussion

We previously demonstrated that AT-independent thrombin inhibitors paradoxically increased TG induced by TF in TM-containing normal human plasma in vitro [1,2]. In contrast, edoxaban, a direct FXa inhibitor, and UFH, an AT-dependent anticoagulant, did not show the paradoxical enhancement of TG [2]. In the present study, we investigated whether a direct thrombin inhibitor, melagatran, enhances the intrinsic pathway-induced TG in human plasma, and the role of the negative feedback system mediated by thrombin-TM-PC in the enhancement of TG by the direct thrombin inhibitor.

The blood coagulation cascade is described as two pathways that are initiated either by exposure of blood to a damaged vessel wall (the extrinsic pathway) or by contact with negative charge (the intrinsic pathway). The extrinsic pathway, which is considered to be the physiologic trigger for hemostasis or thrombus formation, is initiated when factor VIIa (FVIIa) forms a complex with TF. Deficiency of FVII in humans and mice, or low TF levels in mice, is associated with impaired coagulation and severe hemorrhage [13,14]. In addition, TF contained in atherosclerotic plaques or microparticles induces hypercoagulation and pathological thrombus formation in coronary heart diseases [15,16] or venous thromboembolism [17]. These facts indicate that TF/FVIIa contributes to both hemostasis and pathologic thrombus formation. Therefore, a lot of studies of coagulation have been focused on the extrinsic pathway.

Until recently, there was less focus on thrombosis research in the intrinsic pathway, because it was considered not highly relevant to thrombotic diseases or hemostasis. Observations of FXII and FXI-deficient patients indicate that these coagulation factors in the intrinsic pathway play relatively minor roles in normal hemostasis [18–21]. However, recent animal studies revealed that thrombus formation is suppressed in FXII- and FXI-deficient mice without excessive bleeding [6,9]. Moreover, it has been reported that elevated levels of FXII(a) positively associate with arterial thrombosis, ischemic stroke, and coronary heart disease [7,22,23] and FXI(a) is also associated with deep vein thrombosis [24] and ischemic stroke [25]. At present, the intrinsic pathway proteases, FXIIa and FXIa, are considered to have important roles in the pathologic thromboembolism but not in the normal hemostasis.

Until now, we studied the effects of anticoagulants on the TG induced by TF, but not by the intrinsic pathway activation. One of the most potent contact pathway activators is kaolin, which is used in aPTT and TG assays [11,12,26]. So, we investigated the effects of several types of anticoagulants on TG induced by kaolin.

In the present study, melagatran showed similar effects on TG induced by the intrinsic pathway activator to that on TF-induced TG, namely melagatran significantly increased peak height and ETP of TG at 150 and 300 nM compared with the control in the presence of TM, whereas melagatran did not enhance TG in the absence of TM in normal human plasma or in PC-deficient plasma. These results suggest that the enhancement of TG by melagatran
is mediated by the suppression of thrombin-induced negative-feedback system through inhibiting PC activation as well as in the extrinsic pathway-induced TG assay.

Our study clearly demonstrates that melagatran enhanced TG at lower concentrations but not at a high concentration. Therefore, we speculate that when the plasma concentration of the direct thrombin inhibitor declines below the therapeutic range, the paradoxical enhancement of coagulation activity might occur.
Melagatran, a direct thrombin inhibitor, elevated the risks of arterial cardiovascular events [27]. Dabigatran, another direct thrombin inhibitor, also increased the rate of myocardial infarction (MI) than warfarin in Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) trial [28]. Although more detailed analysis of the RE-LY study of dabigatran showed that the increase in the occurrence of MI seen with dabigatran did not reach statistical significance [29], meta-analysis including recent studies (RE-COVER II, RE-ALIGN etc.) demonstrated that dabigatran is associated with a significant increase in the risk of MI [30]. Dabigatran increased rates of thromboembolic events compared with warfarin (5% vs. 0%) in patients with mechanical heart valves (RE-ALIGN study) [31]. Eikelboom et al. explained three reasons for this finding. First, inadequate plasma levels of the drug; second, very few events in warfarin group, and finally, the different mechanism of action between dabigatran and warfarin. They described that in patients with mechanical heart valves, coagulation activation and TG were induced by TF released from damaged tissues during the surgery, which activates the extrinsic pathway of coagulation, and by the exposure of blood to the artificial surface of the valve leaflets and sewing ring, which activates the intrinsic pathway. In the RE-ALIGN study, dose adjustment or discontinuation of dabigatran to lower its plasma concentration was required in 52 of 162 patients (32%). Thus, the paradoxical enhancement of TG by dabigatran at insufficient plasma concentrations might have occurred and resulted in the failure of the study. Edoxaban solely inhibited but did not enhance TG in the absence and presence of TM and PC-depletion in mice lacking these factors [16]. Warfarin, dabigatran, and rivaroxaban induced TG in the absence of TF in mice lacking tissue factor, the cell-associated inhibitor of blood coagulation, in hemostasis and thrombosis, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 1687–1693. If a prothrombotic clot against lysis—a role for the intrinsic pathway of coagulation in fibrinolysis, Thrombosis. Haemost. 80 (1998) 24–27.

