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Full Length Article

A direct thrombin inhibitor suppresses protein C activation and factor Va degradation in human plasma: Possible mechanisms of paradoxical enhancement of thrombin generation

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ABSTRACT

We have demonstrated that antithrombin (AT)-independent thrombin inhibitors paradoxically increase thrombin generation (TG) in human plasma in a thrombomodulin (TM)- and protein C (PC)-dependent manner. We determined the effects of AT-independent thrombin inhibitors on the negative-feedback system, activation of PC and production and degradation of factor Va (FVa), as possible mechanisms underlying the paradoxical enhancement of TG. TG in human plasma containing 10 nM TM was assayed by means of the calibrated automated thrombography. As an index of PC activation, plasma concentration of activated PC-PC inhibitor complex (aPC-PCI) was measured. The amounts of FVa heavy chain and its degradation product ($FVa^{307–506}$) were examined by western blotting. AT-independent thrombin inhibitors, melagatran and dabigatran (both at 25–600 nM) and 3–30 μg/ml active site-blocked thrombin (IIai), increased peak levels of TG. Melagatran, dabigatran and IIai significantly decreased plasma concentration of aPC-PCI complex at 25 nM or more, 75 nM or more, and 10 and 30 μg/ml, respectively. Melagatran (300 nM) significantly increased FVa and decreased $FVa^{307–506}$. In contrast, a direct factor Xa inhibitor edoxaban preferentially inhibited thrombin generation (≥25 nM), and higher concentrations were required to inhibit PC activation (≥150 nM) and FVa degradation (300 nM). The present study suggests that the inhibitions of protein C activation and subsequent degradation of FVa and increase in FVa by antithrombin-independent thrombin inhibitors may contribute to the paradoxical TG enhancement, and edoxaban may inhibit PC activation and FVa degradation as a result of TG suppression.

1. Introduction

It has been previously shown that antithrombin (AT)-independent thrombin inhibitors, such as melagatran, dabigatran, hirudin, and active site-blocked thrombin (IIai), but not direct factor Xa (FXa) inhibitors (edoxaban and rivaroxaban) or an AT-dependent thrombin inhibitor (heparin), increased thrombin generation (TG) in human plasma containing recombinant human soluble thrombomodulin (TM) in vitro and in a rat model of tissue factor-induced hypercoagulation in vivo [1–5]. This paradoxical increase in TG by AT-independent thrombin inhibitors depends on TM and protein C (PC), because the effect was not observed in the absence of TM or PC [2,4].

PC is an important factor for negative regulation of the coagulation pathway [6,7]. Thrombin-TM complex activates PC, and the activated PC (aPC) suppresses the coagulation response via degradation of coagulation factors Va (FVa) and VIIIa (FVIIIa). Inactivation of FVa and FVIIIa interferes with the functions of the prothrombinase and tenase complexes, and results in inhibition of the coagulation cascade [8,9].

FVa is activated from its zymogen FV by FXa or thrombin [10,11]. On activation, FV is spliced in two chains, a heavy chain (FVa^{HC}, residues 1–709, M_r 105,000) and a light chain (residues 1546–2196, M_r 74,000), which noncovalently bind to each other by a calcium dependent interaction [10,11]. FVa acts as the non-enzymatic cofactor of prothrombinase complex, which comprises FXa, a negatively charged phospholipid membrane, and calcium ions and increases the rate of the activation of prothrombin to thrombin by 5 orders of magnitude compared with the activation of prothrombin by FXa alone [10,11]. Hence, FVa is a critical factor in the coagulation cascade.

aPC cleaves FVa heavy chain at arginine (Arg) 306, Arg506, and Arg679 [6,10,11]. The inactivation cleavages occur in a sequential fashion with cleavage at Arg506 preceding cleavages at Arg306 and Arg679. The cleavage at Arg506 results in an only partial inactivation of FVa activity and the cleavage at Arg306 is necessary for the complete loss of cofactor activity of FVa [11]. Thus, the amount of residues 307–506 of the FVa fragment ($FVa^{307–506}$, M_r 30,000) indicates the full inactivation of FVa by aPC.

Abbreviations: aPC, activated PC; aPC-PCI, activated PC-PC inhibitor complex; AT, antithrombin; $AUC_{0\text{-}TTP}$, area under the curve of integrated intensity from the start of TG to time to peak; FVa, factor Va; FVa^{HC}, FVa heavy chain; $FVa^{307–506}$, FVa degradation product; FVIIIa, factor VIIIa; FXa, factor Xa; IIai, active site-blocked thrombin; PC, protein C; SEM, standard error of the mean; TF, tissue factor; TG, thrombin generation; TM, thrombomodulin.

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We hypothesized that the suppression of the thrombin-induced negative-feedback system, such as the inhibitions of PC activation and subsequent FVa degradation and the increase in FVa, would be involved in the paradoxical enhancement of TG by AT-independent thrombin inhibitors. To test our hypothesis, we evaluated the effects of AT-independent thrombin inhibitors and a direct FXa inhibitor, edoxaban, on TG, PC activation, and production and degradation of FVa in human plasma containing TM.

2. Materials and methods

2.1. Reagents

Edoxaban tosylate (edoxaban), melagatran, and dabigatran were synthesized at Daiichi Sankyo Co., Ltd. (Tokyo, Japan). IIai was purchased from Enzyme Research Laboratories, Inc. (South Bend, IN). A phospholipid reagent (60% phosphatidylcholine, 20% phosphatidylethanolamine, 20% phosphatidylserine) was prepared at Daiichi Sankyo. Normal human plasma and PC-deficient human plasma were purchased from George King Bio-Medical, Inc. (Overland Park, KS) and Affinity Biologicals Inc. (Ancaster, Canada), respectively. Recombinant human soluble TM (Recomodulin) was obtained from Asahi Kasei Pharma Corporation (Tokyo, Japan). PPP reagent (tissue factor + phospholipids), FluCa-kit (2.5 mM *Z*-Gly-Gly-Arg-aminomethylcoumarin and 100 mM $CaCl_2$), and thrombin calibrator were bought from Thrombinoscope BV (Maastricht, Netherlands). APC-PCI ELISA kit was purchased from BioPorto Diagnostics A/S (Hellerup, Denmark). Human FVa and *anti*-human FV mouse monoclonal antibody (AHV-5146, epitope between positions Arg306 and Arg506) were obtained from Haematologic Technologies, Inc. (Essex Junction, VT).

2.2. Thrombin generation assay

TG in human platelet-poor plasma was assayed by calibrated automated thrombogram method as previously reported [2] using a fluorometer Fluoroskan Ascent (Thermo Fisher Scientific, Waltham, MA) and the thrombinoscope software (Thrombinoscope BV). Briefly, the assay was performed as follows: 75 μl plasma containing 10 nM TM and 5 μl of anticoagulant solutions were pipetted into the well of a microtiter plate together with 20 μl of a mixture of 15 pM tissue factor (TF) and 24 μM phospholipids. After 5–10 min of preincubation at 37 °C, the reaction was started by adding 20 μl of FluCa-kit. The final concentrations in plasma of melagatran, dabigatran and edoxaban were 1–1200 nM, and IIai was 0.1–30 μg/ml. Peak plasma concentrations of melagatran, dabigatran, and edoxaban at the therapeutic doses in clinical settings are 380 nM, 230 nM, and 550 nM, respectively [12–14]. Final concentrations of TF, phospholipids, the fluorogenic substrate, and $CaCl_2$ were 2.5 pM, 4 μM, 417 μM, and 16.7 mM, respectively. The fluorescence was measured for 90 min at 37 °C (ex. 390 nm, em. 460 nm). Data were obtained from six independent experiments of duplicate measurements.

2.3. Evaluation of PC activation

PC activation was assessed by measuring the concentration of aPC-PCI complex [15,16]. TG was induced in human plasma as mentioned above without the fluorogenic substrate. The reactions were stopped by adding 13 μl of 0.5 M sodium citrate 15 min after the time to peak in the TG assay. APC-PCI complex was measured using ELISA according to the manufacturer's protocol.

2.4. Measurement of FVa^{HC} and $FVa^{307–506}$

The amounts of FVa^{HC} and $FVa^{307–506}$ were measured as indices of FVa generation and degradation, respectively [17]. TG was induced in human plasma as mentioned above without the fluorogenic substrate. The concentrations of anticoagulants were 300 nM for melagatran and 75 and 300 nM for edoxaban. The reactions were stopped by adding 13 μl of 0.5 M sodium citrate buffer (pH 4.3) every 2 min from 0 min to the time to peak in the TG assay. The plasma was diluted with Hepes buffer (20 mM Hepes, 140 mM NaCl, pH 7.4) to prepare 4 mg protein/ml solutions and mixed with the same volume of 5% mercaptoethanol in Laemmli sample buffer. The samples were boiled for 5 min.

Equal amounts of proteins (20 μg) were loaded in 10% SDS-PAGE slab gels (XV PANTERA Gel, DRC Co., Ltd., Tokyo, Japan) under the reducing condition and transferred to PVDF membranes using iBlot Transfer Stack system (Thermo Fisher Scientific Inc., Waltham, MA). FVa^{HC} and its degradation product $FVa^{307–506}$ were detected with *anti*-human FV mouse monoclonal antibody (2.5 μg/ml) and visualized using a Alexa Fluor 680 goat *anti*-mouse IgG (dilution 1:10,000, Thermo Fisher Scientific Inc.). Fluorescent signals were detected using an ODYSSEY system (LI-COR, Inc., Lincoln, NE) and quantified with Image Studio software (LI-COR, Inc.) as the integrated intensity. Area under the curves of integrated intensity from the start of TG to time to peak ($AUC_{0\text{-}TTP}$) were calculated by the trapezoidal method. Control and drug-treated samples were loaded on the same gel and $AUC_{0\text{-}TTP}$ of FVa^{HC} and $FVa^{307–506}$ were compared between the samples to calculate ratio to control. As a loading control, *anti*-human IgG antibody (3E8, dilution 1:2000, Santa Cruz Biotechnology, Inc., Dallas, TX) was used.

2.5. Statistical analysis

Analyses were performed using SAS System Release 8.2 or 9.2 (SAS Institute Inc., Cary, NC). All data represent the mean ± standard error of the mean (SEM). The statistical significance between the control and treatment groups was analyzed by F test for the homoscedasticity followed by Student *t*-test or Welch's test. A P value of <0.05 (two-tailed) was considered as a significant difference.

3. Results

3.1. Effects of anticoagulants on TF-induced TG in TM-containing human plasma

To confirm the paradoxical enhancement of TG by AT-independent thrombin inhibitors and check the time to peak of the response, TG was measured in human plasma in the presence of 10 nM TM. Melagatran and dabigatran, direct thrombin inhibitors, and IIai significantly increased peak height of TG at 25–600 nM and 3–30 μg/ml, respectively (Fig. 1A–C and Fig. 2A–C). Peak height of TG reached a maximum at 300 nM of melagatran, 300 nM dabigatran, and 30 μg/ml of IIai with 2.3-fold, 2.6-fold, and 2.3-fold increase, respectively (Fig. 2A–C). Melagatran and dabigatran at a higher concentration (1200 nM) suppressed TG. Edoxaban did not enhance TG and demonstrated only a significant inhibitory effect at concentrations of 25 nM or more (Fig. 2D). Time to peak of TG in the presence of these anticoagulants at each concentration are shown in Table 1. Melagatran, dabigatran, and edoxaban, but not IIai, prolonged time to peak.

3.2. Effects of anticoagulants on PC activation

As an index of PC activation, we measured the plasma concentration of aPC-PCI complex [15,16]. Since melagatran, dabigatran, and IIai increased peak height of TG, inhibition of PC activation may occur by the time to peak. In addition, it is reported that 15–30 min is required for binding of aPC and PCI in plasma [18]. Therefore we measured aPC-PCI complex 15 min after the time to peak in the TG assay.

Melagatran, dabigatran, and IIai significantly inhibited the generation of aPC-PCI complex at concentrations of 25 nM or more, 75 nM or more, and 10 and 30 μg/ml, respectively (Fig. 2AC). The concentrations of these thrombin inhibitors for the inhibition of PC activation were