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Melagatran, a direct thrombin inhibitor, but not edoxaban, a direct factor Xa inhibitor, nor heparin aggravates tissue factor-induced hypercoagulation in rats

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ABSTRACT

There are concerns that some anticoagulants can paradoxically increase thrombogenesis under certain circumstances. We have shown that low-dose administration of a direct thrombin inhibitor, melagatran, significantly worsens the coagulation status induced by tissue factor injection in rats. We compared the effect of inhibition of thrombin and factor Xa for their potential to aggravate tissue factor-induced coagulation in rats. Hypercoagulation was induced by the injection of 2.8 U/kg tissue factor after administration of melagatran, heparin and edoxaban in rats. Blood samples were collected 10 min after tissue factor injection. Platelet numbers, thrombin–antithrombin complex concentrations and plasma compound concentrations were measured. Though a high dose of melagatran (1 mg/kg, i.v.) suppressed platelet consumption and thrombin–antithrombin complex generation induced by tissue factor, lower doses of melagatran (0.01, 0.03 and 0.1 mg/kg, i.v.) significantly enhanced platelet consumption and thrombin–antithrombin complex generation. In addition, although melagatran (3 mg/kg, i.v.) improved coagulation status when tissue factor was given 5 min after the drug administration, and 2, 4 and 8 h after melagatran dosing, it deteriorated coagulation status. These results were well explained by the plasma melagatran concentration. Low concentrations (15–234 ng/ml) of melagatran aggravated coagulation status whereas it was mended by high concentrations (1190 ng/ml or more) of the compound. In contrast, edoxaban and heparin did not show any exacerbation under these examination conditions. These results show that subtherapeutic concentrations of melagatran are associated with coagulation pathway activation, whereas factor Xa inhibition with edoxaban has a low risk of paradoxical hypercoagulation.

1. Introduction

Factor Xa plays an important role in the blood coagulation cascade, serving as the juncture between the intrinsic and extrinsic system leading to the generation of thrombin, thus factor Xa is an attractive target for the prevention and treatment of thromboembolic diseases (Ansell, 2007). Thrombin also has a key role in the blood coagulation cascade (Weitz, 2007). Therefore, several oral direct factor Xa inhibitors and an oral direct thrombin inhibitor have been launched. But, there are some concerns about thrombogenesis by direct thrombin inhibitors. Compared to warfarin/placebo, the elevated risk of arterial cardiovascular events in patients treated with ximelagatran (FDA, 2004), a prodrug of direct thrombin inhibitor melagatran, and dabigatran (Connolly et al., 2009) were reported.

We (Furugohri et al., 2005) and other group (Perzborn et al., 2008a) have previously demonstrated that a direct thrombin inhibitor, melagatran, induces a paradoxical activation of coagulation pathway in a rat model of tissue factor-induced hypercoagulation. Low-dose oral administration of melagatran enhances platelet consumption and thrombin–antithrombin complex generation, suggesting that this paradoxical phenomenon may be implicated in coagulation activation observed with direct thrombin inhibitors in clinical trials. We presented the precise mechanism of this paradoxical activation of coagulation by the direct thrombin inhibitor in our *in vitro* studies (Furugohri et al., 2011; Morishima et al., 2006). In short, direct thrombin inhibitors increase the activation of coagulation by suppression of the thrombin-induced negative-feedback system through the inhibition of protein C activation. The same result was reported by other group (Perzborn and Harwardt, 2008b).

However, the precise mechanism underlying the paradoxical coagulation activation *in vivo* by melagatran remains to be fully elucidated. Thus, in this study, we determined the relationship between the plasma concentrations of melagatran and the coagulation status by changing treatment conditions such as doses of the compound and timing of coagulation induction after the administration of melagatran.

In terms of the paradoxical coagulation activation by other anticoagulants, the effects of edoxaban (the free form of edoxaban tosilate hydrate: Japanese Accepted Name), a recently approved direct factor Xa inhibitor in Japan, on tissue factor-induced hypercoagulation are not being investigated, even though we have shown that a factor Xa

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inhibitor, DX-9065a, does not cause the aggravation of coagulation status induced by tissue factor. Moreover, it is not examined whether a different type of anticoagulant heparin, which exerts an antithrombin-dependent inhibition of thrombin and factor Xa, induces hypercoagulation in rats treated with tissue factor. Therefore, we directly compared the potential of edoxaban and heparin with that of melagatran to aggravate tissue factor-induced coagulation in rats.

2. Materials and methods

2.1. Reagents and drugs

Edoxaban and melagatran were synthesized at Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Heparin sodium was purchased from Novo Nordisk A/S (Copenhagen, Denmark). Tissue factor (Thromboplastin C Plus) and Enzygnost TAT micro were purchased from Siemens AG (Munich, Germany). Halothane was from Takeda Pharmaceutical (Osaka, Japan) and thiopental sodium was from Mitsubishi Tanabe Pharma (Osaka, Japan).

2.2. Animals

Animal facilities, animal care and study programs were in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd. Eight or nine-week-old male Wistar rats were purchased from Japan SLC (Hamamatsu, Japan) and maintained on an 8:00 am/8:00 pm light/dark schedule, temperature (23 ± 2 °C) and humidity ($55 \pm 20\%$). Rats were housed 5–6 per cage and food and water were available ad libitum. They were acclimated for 1 or 2 weeks.

2.3. Tissue factor-induced hypercoagulation model

Rats (232–284 g) were anesthetized with thiopental (100 mg/kg, i.p.). Coagulation was induced by the injection of 2.8 U/kg tissue factor into the femoral vein. Blood samples were collected into plastic syringe containing citrate solution (9 volumes of blood to 1 volume of 3.13% sodium citrate solution) 10 min after tissue factor injection. Immediately after blood collection, platelet number was measured using an automatic hematology analyzer MEK-6358 (Nihon Kohden, Tokyo, Japan). Then, blood samples were centrifuged at 1500 ×*g* for 10 min at 4 °C and plasma samples were prepared. Plasma was stored at −70 °C until the measurement of the following parameters: thrombin–antithrombin complex and plasma concentrations of drugs. Thrombin-antithrombin complex concentrations were assayed according to the previous study with Enzygnost TAT micro (Morishima et al., 1997).

2.4. Drug treatment

2.4.1. Dose-dependent effects of a direct thrombin inhibitor, heparin and a factor Xa inhibitor on hypercoagulation

Under the thiopental (100 mg/kg, i.p.) anesthesia, melagatran at doses of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 mg/kg or heparin at doses of 0.1, 0.3, 1, 10 and 100 U/kg was injected into the jugular vein of rats. Hypercoagulation was induced 5 min after drug administration. Edoxaban (0.1, 0.5 and 2.5 mg/kg) was orally administered by gavage to fasted rats, and then the rats were anesthetized with thiopental. Hypercoagulation was induced 30 min after drug administration.

2.4.2. Time-dependent effects of direct thrombin inhibitor and factor Xa inhibitor after drug treatment on hypercoagulation

The experiment protocol is shown in Fig. 1. Under the halothane (2–3%) anesthesia, melagatran (3 mg/kg) or edoxaban (0.3 mg/kg) was injected into the jugular vein of rats. After their administration, the rats were awoken except 5 min after dosing. Hypercoagulation was induced 5 min, 2, 4, 8 and 16 h after dosing under the thiopental anesthesia.

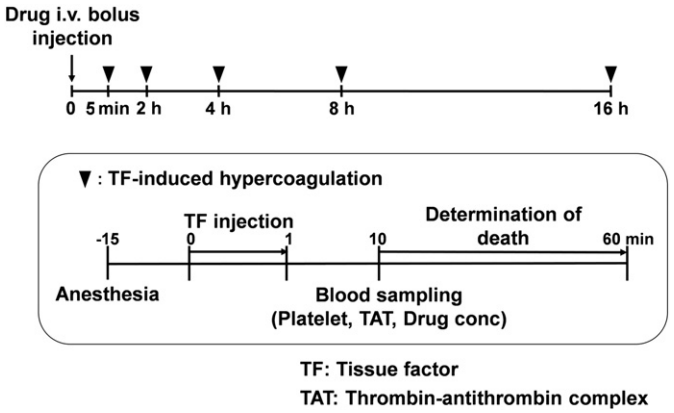

Fig. 1. The experimental protocol of time-dependent experiments.

2.5. Measurement of plasma concentration

The plasma concentrations of drugs were measured according to the previous study (Furugohri et al., 2008; Zafar et al., 2007) by liquid chromatography-tandem mass spectrometry analysis. Lower limit of quantitation is shown Table 1–4.

2.6. Statistical analysis

Analyses were performed using EXSAS ver.7.10 (ARM SYTEX, Osaka, Japan) based on SAS release 8.2 (SAS Institute Japan, Tokyo, Japan). Data are expressed as means ± S.E.M. unless otherwise noted and statistical significance was measured at the level of $P<0.05$. Comparison of the platelet number and thrombin–antithrombin complex concentration between groups were analyzed by *t*-test or Dunnett multiple comparison method. Mortality rate of rats up to 60 min after tissue factor injection was analyzed by Fisher test.

3. Results

3.1. Dose-dependent effects of melagatran, heparin and edoxaban on hypercoagulation

3.1.1. Melagatran

In the sham group, platelet number and thrombin–antithrombin complex concentration were $79.2 \pm 2.1 \times 10^4$ cells/μl and 3.6 ± 0.4 ng/ml, respectively (Fig. 2). Intravenous injection of tissue factor significantly reduced platelet number to $47.5 \pm 2.3 \times 10^4$ cells/μl ($P<0.001$) and increased concentration of thrombin–antithrombin complex to 341.9 ± 37.7 ng/ml ($P<0.001$) (Fig. 2). Compared to the tissue factor-treated control group, a high dose of melagatran (1 mg/kg, i.v.) given 5 min before the hypercoagulation induction suppressed platelet consumption ($68.0 \pm 3.1 \times 10^4$ cells/μl, $P<0.001$) and thrombin–antithrombin complex generation (152.5 ± 6.0 ng/ml, $P<0.01$). The plasma concentration of melagatran was 1190 ± 182 ng/ml (Table 1). Lower doses of melagatran (0.01, 0.03 and 0.1 mg/kg,

Table 1
Dose-dependent effect of melagatran on mortality in tissue factor-induced hypercoagulation rats and plasma melagatran concentrations.

Treatment	Dose (mg/kg, i.v.)	Mortality	Concentration (ng/ml)
Control		0/6	
Melagatran	0.001	0/6	1.3 ± 0.2
	0.003	3/6	4.5 ± 1.2
	0.01	4/6	22 ± 0
	0.03	4/6	81 ± 6
	0.1	3/6	234 ± 31
	0.3	0/6	454 ± 31
	1	0/6	1190 ± 182

Concentrations represent the means ± S.D. Lower limit of quantitation is 1.0 ng/ml.