Full Paper

Effects of DX-9065a, an Inhibitor of Factor Xa, on Ellagic Acid-Induced Plantar Skin Thrombosis Assessed in Tetrodotoxin- and *N*^ω-Nitro-L-Arginine-Treated Rats

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Abstract. Effects of DX-9065a ((+)-2*S*-2-[4-[[*(3S)*-1-acetimidoyl-3-pyrrolidinyl]oxy]phenyl]-3-[7-amidino-2-naphthyl]propanoic acid hydrochloride pentahydrate), a dibasic propanoic acid and an inhibitor of factor Xa, were compared with those of argatroban, a low molecular weight thrombin inhibitor, on the ellagic acid-induced plantar skin thrombosis in the rats treated with tetrodotoxin and *N*^ω-nitro-L-arginine. Plantar skin blood flow was measured with laser Doppler flow meters, and skin temperature of the hindlimb was monitored simultaneously. In order to induce thrombus in plantar skin vasculature, ellagic acid (300 μg, i.a.) was injected into a branch of femoral artery. The formation of thrombus in femoral and plantar vessels was assessed by light microscopy. Ellagic acid decreased plantar skin blood flow and skin temperature. Intravenous injections of DX-9065a (3 mg/kg) and argatroban (1–3 mg/kg) significantly inhibited the ellagic acid-induced disturbance of plantar skin blood flow and lowering skin temperature without affecting bleeding time. The oral administration of DX-9065a (30–100 mg/kg) significantly prevented the decrease in skin temperature induced by ellagic acid, but it partially inhibited the disturbance of plantar skin blood flow. DX-9065a and argatroban also prolonged prothrombin time in a dose-dependent manner. These results suggest that DX-9065a effectively prevented thrombosis produced by ellagic acid in the skin circulation without a risk of bleeding.

Keywords: DX-9065a, ellagic acid, skin blood flow, thrombosis, tetrodotoxin- and *N*^ω-nitro-L-arginine-treated rat

Introduction

DX-9065a ((+)-2*S*-2-[4-[[*(3S)*-1-acetimidoyl-3-pyrrolidinyl]oxy]phenyl]-3-[7-amidino-2-naphthyl]propanoic acid hydrochloride pentahydrate), dibasic propanoic acid, is a selective inhibitor of factor Xa (1, 2) and has been reported to be an orally active anticoagulant (3–5). DX-9065a exhibited anticoagulant activity in various animal models of thrombosis, such as thromboplastin-induced disseminated intravascular coagulation in the

rat (4, 6, 7), mouse, and rabbit (5), or arterial-venous (A-V) shunt in the rat (4, 8, 9). The clinical trial of this drug in humans at phase I was recently conducted (10).

In the present study, in order to further elucidate the potential action of DX-9065a to improve disturbed skin circulation, we established ellagic acid-induced thrombosis in the plantar skin area of the rat hindlimb.

We previously reported the preparation of acutely-denervated rats (11) by the combined treatments with tetrodotoxin (TTX), a Na⁺ channel inhibitor, and *N*^ω-nitro-L-arginine (L-NNA), a NO synthetase inhibitor. TTX is known to acutely denervate nervous elements involved in efferent and afferent reflex mechanisms (12). L-NNA (13), administered via the i.v. route, could induce a pressor response (14), and thus it can counteract the lowered systemic blood pressure and peripheral

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vascular tone following elimination of sympathetic nerve activity.

Ellagic acid is known to be one of the most efficient initiators of blood coagulation by conversion of Hageman factor to an active serine protease (15). Consequently, intraarterial injection of ellagic acid into a branch of a femoral artery can produce locally an ischemic change in the plantar skin of one hindlimb, while the other hindlimb can serve as the control. This experimental model enabled us to properly compare the effects of DX-9065a with those of argatroban on the disturbed skin blood flow in the rats. Our results suggest that DX-9065a effectively prevented ellagic acid-induced disturbance of plantar skin blood flow (PSBF) and the decrease in skin temperature (ST) without affecting the bleeding time.

Materials and Methods

The present study was reviewed by the ethics committee on animal experiments at the University of Shizuoka and was carried out in accordance with the institutional guidelines for animal experiments, the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society, and the law (No. 105) and notification (No. 6) of care and protection of animals established by the Japanese Government.

Preparation of TTX- and L-NNA-treated rat

Male Wistar rats, 9–13-week-old and weighing 250–320 g (Japan SLC, Hamamatsu), were lightly anesthetized with diethyl ether. After disappearance of the corneal reflex, each animal was placed on a heating mat. After tracheotomy, the trachea was intubated and the animal was ventilated with room air (1 ml/100 g body weight) with an artificial respirator (SN-480-7; Shinano Seisakusho, Tokyo) operating at 85 strokes/min. The left jugular vein was cannulated for the intravenous injection of drugs and sampling of blood serum. The right carotid artery was cannulated for recording arterial blood pressure (TDN-R; Gould, Oxnard, CA, USA) and heart rate (HR) triggered by the pulse of blood pressure signal. These outputs were fed into a Macintosh computer set at an input of 10 V via an analog-digital signal converter (LAB STAC; Keisokugiken, Tokyo). A side branch of a femoral artery was cannulated for the intra-arterial injection of ellagic acid. In order to eliminate nervous activity totally, TTX (50 μ g/kg, i.v.) was injected. Afterwards, L-NNA (30 mg/kg, i.v.) was injected to counteract (for about 2 h) the low blood pressure due to the TTX-induced loss of vascular tone. We previously reported that TTX (50 μ g/kg, i.v.) could totally

inhibit the pressor response to electrical stimulation of the spinal cord, and the reflex tachycardia due to the depressor response to acetylcholine (1 μ g/kg, i.v.) (11). Room temperature was controlled at $24 \pm 1^\circ\text{C}$ with a humidity of $55 \pm 1\%$, and the body temperature of the animal was maintained at $36 \pm 0.5^\circ\text{C}$ by a rectal thermistor probe linked to a temperature controller of the heating mat.

Measurements of plantar skin blood flow and skin temperature

The PSBFs of both hindlimbs were continuously measured with laser Doppler flow meters (FLO-N1; Omega Wave Co., Ltd., Tokyo) with a time constant of 1 s. Each flow probe was held at a 2.5-mm distance from the skin surface of the plantar area of each hindlimb. The two blood flow signals were fed into a Macintosh computer via an analog-digital signal converter. The blood flow was expressed as milliliter per minute per 100 g tissue weight or percent change of the value before drug administration. The vascular resistance ($\text{mmHg } 100 \text{ g} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$) was calculated as the mean blood pressure (MBP) (mmHg) divided by the PSBF ($\text{ml} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$). The skin temperature (ST) at each dorsum pedis was measured with a sticker type temperature indicator (ProChecker; Kyowa Medics Co., Ltd., Tokyo).

To disturb PSBF by causing thrombosis, we intra-arterially injected ellagic acid (300 μ g), which had been suspended in the rat serum prepared according to the method previously reported by Hara et al. (16), into a side branch of one of the femoral arteries.

In order to evaluate the effectiveness of drug action via two different routes of drug administration on the ellagic acid-induced disturbance of PSBF, we adopted the following experimental protocols, as depicted in Fig. 1.

In the intravenous administration study, the drug (DX-9065a or argatroban) or vehicle was injected into a jugular vein 20 min after the injection of TTX (50 μ g/kg, i.v.) and L-NNA (30 mg/kg, i.v.). DX-9065a (0.3–3.0 mg/kg) was injected over a 15-s period, whereas argatroban (0.3–3.0 mg/kg) was injected in 15 to 60 s, depending on its injection volume. The effects of drugs per se on HR, MBP, PSBF, and ST were observed for 15 min. Afterwards, ellagic acid, suspended in rat serum (3.0 mg/ml) in a volume of 100 μ l, was injected into a side branch of a femoral artery, and the effects of drugs on the PSBF as well as other parameters (ST, HR, and MBP) were observed for 60 min.

In the oral administration study, the rats were fasted overnight. DX-9065a (30–100 mg/kg) or vehicle was administered via a gastric tube 30 min prior to admin-

A. Intravenous injection study

B. Oral administration study

Fig. 1. Schematic diagrams of experimental protocols. Perpendicular arrows indicate the treatment with drugs or vehicle. PSBF, ST, HR, and MBP represent plantar skin blood flow, skin temperature, heart rate, and mean blood pressure, respectively.

istration of TTX ($50 \mu\text{g}/\text{kg}$, i.v.) and L-NNA ($30 \text{ mg}/\text{kg}$, i.v.). Ellagic acid was injected into a side branch of a femoral artery 60 min after administration of the drug, and the effect of the drug on the PSBF was observed for about 1 h.

Since the PSBF reduced less than $2.0 \text{ ml} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$ when the femoral artery was completely occluded with a clip, we considered that the blood flow of the plantar skin area was completely occluded when the PSBF decreased less than $2.0 \text{ ml} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$. Thus, the total duration of complete occlusion time was estimated.

Measurements of prothrombin time and bleeding time

In the intravenous injection study, the blood sample ($270 \mu\text{l}$) was collected 15 min after injection of DX-9065a or argatroban from the jugular vein of each rat in a 1-ml syringe containing $30 \mu\text{l}$ of 3.1% trisodium citrate dihydrate. In the oral administration study, the blood sample ($270 \mu\text{l}$) was collected from the jugular vein 60 min after the administration of DX-9065a. Blood samples were centrifuged at $1,500 \times g$ for 10 min at 4°C , and platelet-poor plasma was stored at -20°C until assayed. Frozen plasma was thawed at 37°C and placed in a crushed-ice bath. Plasma ($20 \mu\text{l}$) was placed in the process tube, and the coagulation was started by addition of Simplastin (Sankyo, Tokyo) ($40 \mu\text{l}$). After that, prothrombin time (PT) was measured with a Micro-coagulometer (Greiner Electronics AG, Langenthal, Switzerland).

Bleeding time was determined by transection of the rat tail at the end of experiments, as shown in Fig. 1. After the intravenous injection and oral administration of drugs for 75 and 120 min, respectively, bleeding was induced by transection of the extremity of the tail 3 mm from the tip. A drop of blood from the tail was gently blotted with tissue paper every 15 s, and the time to cessation of bleeding was noted. To avoid bleeding again, we took care not to touch the cut area of the tail. If the bleeding time was prolonged over 15 min, the value was taken as 15 min.

Histological study

In order to ascertain whether thrombus formation *in vivo* was induced by ellagic acid or not, we did a histological study. Male Wistar rats, 9–10-week-old and weighing 250–300 g, were prepared as described above; 30 min after the injection of ellagic acid ($300 \mu\text{g}$, i.a.), which actually disturbed PSBF, the rats were killed by exsanguination and perfused transcardially for 1 min with saline (total volume of about 20 ml). Both hindlimbs were immediately removed and fixed overnight in 10% formaldehyde. Plantar and femoral sections were cut into $5\text{-}\mu\text{m}$ -thick sections by use of a microtome, and then each section was stained with hematoxylin and eosin (HE), elastica-van Gieson stain, or phosphotungstic acid hematoxylin (PTAH) for evaluation by light microscopy.

Materials

The following drugs were used: DX-905a, Slonnon injection (argatroban) and ellagic acid dihydrate were gifts from Daiichi (Tokyo). TTX was purchased from Wako (Osaka), and L-NNA from Aldrich (Milwaukee, WI, USA). Other drugs of reagent grade were commercially available. Slonnon injection (20 ml) contained argatroban (10 mg), and D-sorbitol (1.08 g) as a additive. In the case of intravenous injection of argatroban (3.0 ml/kg), the drug was dissolved in distilled water to a concentration of 1.2 mg/ml, because the Slonnon injection volume was too much for a bolus injection. L-NNA was dissolved in 0.1 N HCl to a concentration of 10 mg/ml. Other drugs were dissolved in physiologic salt solution. The injection volume of each drug was 50 μ l/100 g body weight except for that of argatroban and L-NNA.

Statistical analyses

The data were expressed as the mean $\pm$ S.E.M. Evaluation of the statistical significance among groups was carried out by Dunnett's multiple comparison test after analysis of variance (ANOVA). A *P* value of less than 0.05 was considered significant.

Results

Ellagic acid-induced thrombosis of plantar skin area in TTX- and L-NNA-treated rats

To determine the appropriate dose of ellagic acid to disturb the PSBF in TTX- and L-NNA-treated rats, we performed preliminary experiments in which the drug was injected up to 300 μ g into a branch of the left femoral artery. Ellagic acid decreased PSBF dose-dependently, and ID_{50%PSBF}, i.e., the dose required to decrease PSBF up to 50% of the basal value, was 99.1 $\pm$ 5.3 μ g, i.a. (*n* = 4).

Hemodynamic variables before and after intravenous injection of TTX (50 μ g/kg) and subsequent injection of L-NNA (30 mg/kg) in rats (*n* = 4) are given in Table 1. TTX significantly decreased the HR, MBP, and vascular

resistance to PSBF. However, the lowered MBP and vascular resistance to PSBF were restored by the subsequent injection of L-NNA to almost the same level as those before the injection of TTX. Thus, the PSBF was not largely affected. The intravenous injection of L-NNA was effective in counteracting the lowered MBP by TTX for more than 2 h (data not shown).

Figure 2A shows the typical responses of PSBF to intraarterial injection of ellagic acid (300 μ g). Apparently, ellagic acid interrupted PSBF in a variety of ways; i.e., PSBF fluctuated from one minute to the next, as shown in panel A of Fig. 2. However, the summarized data indicated that a bolus injection of ellagic acid (300 μ g, i.a.) inhibited PSBF of the injected side very severely to about 20% of the the basal blood flow for an observation period of 60 min, whereas the contralateral PSBF remained constant (Fig. 2B). Intraarterial injection of ellagic acid had no apparent effect on HR and MBP (data not shown).

Histological surveys confirmed the formation of thrombi. Figure 3 shows light microscopic observations of femoral blood vessels and those in the plantar skin area excised from the injected hindlimb 30 min after the injection of ellagic acid (300 μ g/kg, i.a.) and stained with HE or elastica-van Gieson stain. The contralateral hindlimb served as the control.

The vessel lumens of the ellagic acid-injected side were packed with thrombi. The fibrin deposits in the thrombi were confirmed when the vessels were stained with phosphotungstic acid hematoxylin. To the contrary, there was no thrombus formation in the contralateral vessels. These results suggest that the disturbance of PSBF by ellagic acid can be attributed to the formation of thrombus in femoral blood vessels and those in the plantar skin area.

Antithrombotic effects of DX-9065a and argatroban administered intravenously on the ellagic acid-induced plantar skin thrombosis

The PSBF and ST of the left hindlimb and the HR and MBP before the intraarterial injection of ellagic acid

Table 1. Hemodynamic variables before and after intravenous injection of TTX and subsequent injection of L-NNA in rats anesthetized with diethyl ether

	Before	After TTX (50 μ g/kg, i.v.)	After TTX (50 μ g/kg, i.v.) and L-NNA (30 mg/kg, i.v.)
HR (beats/min)	433 $\pm$ 14	345 $\pm$ 9**	349 $\pm$ 9**
MBP (mmHg)	145 $\pm$ 4	69 $\pm$ 3**	147 $\pm$ 4
PSBF (ml $\cdot$ min ⁻¹ $\cdot$ 100 g ⁻¹)	10.5 $\pm$ 0.6	10.7 $\pm$ 0.2	10.8 $\pm$ 0.40
Resistance to PSBF (mmHg 100 g $\cdot$ min ⁻¹ $\cdot$ ml ⁻¹)	13.9 $\pm$ 0.4	6.5 $\pm$ 0.2**	13.6 $\pm$ 0.1

Each value represents the mean $\pm$ S.E.M. of the same 4 animals. L-NNA was administered 15 min after injection of TTX. ***P* < 0.01 vs corresponding values before injection of TTX.

Fig. 2. Effects of ellagic acid on the plantar skin blood flow (PSBF) in tetrodotoxin (TTX)- and N^G -nitro-L-arginine (L-NNA)-treated rats. Panel A shows typical responses of PSBF to ellagic acid ($300 \mu\text{g}$, i.a.) taken from the control group. Ellagic acid ($300 \mu\text{g}$) was injected into a branch of the left femoral artery in each animal. Panel B: Time-courses of changes in PSBF. PSBF of ellagic acid-injected side (closed circles) and that of the contralateral side (open circles) were simultaneously measured. Ellagic acid ($300 \mu\text{g}$) was injected intra-arterially at time zero. Each point with a vertical bar represents the mean $\pm$ S.E.M. of 14 experiments. Each value before injection of ellagic acid was taken as 100% on the ordinate.

in the total number of rats used in the present series of experiments ($n = 60$) were $11.0 \pm 0.3 \text{ ml} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$, $32.5 \pm 0.2^\circ\text{C}$, $340 \pm 4 \text{ beats/min}$, and $128 \pm 3 \text{ mmHg}$, respectively. These control values among the groups were not significantly different (each $n = 7 - 14$).

The preventive effects of either DX-9065a or argatroban pretreatment for 60 min on the maximum ellagic acid ($300 \mu\text{g}$, i.a.)-induced changes in PSBF and ST of the ipsilateral hindlimb were shown in Fig. 4. In the vehicle-treated rats, ellagic acid ($300 \mu\text{g}$, i.a.) decreased PSBF to about 20% of the basal value in about 2 min (Fig. 2B). Consistent with the disturbed PSBF, the ST of the hindlimb on the ellagic acid-injected side was lowered to $-4.9 \pm 0.4^\circ\text{C}$ from the control ($32.6 \pm 0.2^\circ\text{C}$) (each $n = 14$). The intravenous administration of DX-9065a or argatroban significantly and dose-dependently

inhibited the disturbance of PSBF and the decrease in ST induced by ellagic acid. In the control group, the total duration of complete occlusion time was $35.1 \pm 7.0 \text{ min}$ ($n = 14$). DX-9065a at 1 and 3 mg/kg reduced it to $13.7 \pm 9.2 \text{ min}$ ($n = 7$) and $6.4 \pm 5.8 \text{ min}$ ($n = 9$) ($P < 0.05$ vs corresponding vehicle), respectively. Argatroban at 1 mg/kg also reduced the total duration of complete occlusion time to $15 \pm 9.8 \text{ min}$ ($n = 8$), and the drug at 3 mg/kg abolished the occurrence of complete occlusion, ($n = 8$) ($P < 0.01$ vs corresponding vehicle).

Effects of the oral administration of DX-9065a on the ellagic acid-induced plantar skin thrombosis

The PSBF and ST of the left hindlimb and the HR and MBP before the intraarterial injection of ellagic acid were $10.0 \pm 0.4 \text{ ml} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$, $31.8 \pm 0.3^\circ\text{C}$,

Fig. 3. Light microscopic views of transected plantar (panels A and C) and femoral (panels B and D) vessels which had been excised 30 min after injection of ellagic acid (300 μ g, i.a.) into a branch of femoral artery in tetrodotoxin (TTX)- and *N*^o-nitro-L-arginine (L-NNA)-treated rats. Panels A and B show the preparations of ellagic acid-injected side. Panels C and D show the preparations of the contralateral side. Each upper panel shows the preparation stained with hematoxylin and eosin (HE). Each lower panel shows the preparation stained with elastica-van Gieson stain. Note that the venous lumens are evidently occluded by thrombi. (A) and (V) represent artery and vein, respectively.

353 $\pm$ 7 beats/min, and 107 $\pm$ 5 mmHg, respectively (total animals, $n = 22$). These basal values were not significantly different among the vehicle- and drug-treated groups (each $n = 6 - 9$).

In the vehicle-treated group, ellagic acid (300 μ g, i.a.) induced the disturbance of PSBF and the decrease of ST. The oral administration of DX-9065a (30 and 100 mg/kg) tended to inhibit the disturbance of PSBF,

and significantly prevented the decrease in ST induced by ellagic acid ($P < 0.01$, Fig. 4D), but it partially inhibited the disturbance of PSBF. In the control group, the total duration of complete occlusion time was 36.9 $\pm$ 10 min ($n = 7$). DX-9065a at 30 mg/kg and 100 mg/kg tended to reduce it to 11.9 $\pm$ 8.4 min ($n = 6$) and 11.8 $\pm$ 6.7 min ($n = 9$), respectively.

Fig. 4. Effects of DX-9065a and argatroban on the maximum ellagic acid (300 μ g, i.a.)-induced changes in plantar skin blood flow (PSBF) (panels A and B) and skin temperature (ST) (panels C and D) in tetrodotoxin (TTX)- and *N*^o-nitro-L-arginine (L-NNA)-treated rats. Panels A and C show the results of the intravenous injection study and panels B and D, those of the oral administration study. Each PSBF before injection of ellagic acid was taken as 100% on the ordinate. Each ST was expressed as Δ decrease from the control. Data represent the mean $\pm$ S.E.M. of 7–14 experiments. * P <0.05, ** P <0.01 vs corresponding value of vehicle.

Cardiovascular effects of DX-9065a and argatroban administered intravenously to TTX- and L-NNA-treated rats

The cardiovascular effects of the intravenous injection of the drugs (DX-9065a or argatroban) were evaluated before the injection of ellagic acid and are summarized in Table 2.

The HR, MBP, and PSBF before the intravenous injection of the drugs were 347 ± 3 beats/min, 139 ± 3 mmHg, and 11.0 ± 0.2 ml $\cdot$ min⁻¹ $\cdot$ 100 g⁻¹, respectively (total animals, $n = 60$). These values were not significantly different among the groups (each $n = 7 - 14$).

Intravenous injection of DX-9065a did not affect the HR, MBP, and PSBF at any dose used. On the other hand, the intravenous injection of argatroban at a dose range between 1 and 3 mg/kg significantly increased MBP.

Effects of DX-9065a and argatroban on prothrombin time and bleeding time

The value of prothrombin time for the vehicle-treated group in the intravenous injection study was 18.6 ± 1.1 s ($n = 5$). Intravenous injection of DX-9065a and argatroban prolonged prothrombin time in a dose-dependent manner. There was a significant prolongation of prothrombin time when DX-9065a (3 mg/kg) or argatroban (3 mg/kg) was administered (each P <0.01, vs corresponding vehicle) (Fig. 5A).

Prothrombin time in the vehicle-treated group of the oral administration study was 16.5 ± 1.1 s ($n = 8$). Oral administration of DX-9065a also dose-dependently prolonged prothrombin time. A significant prolongation of prothrombin time was induced by DX-9065a at 100 mg/kg, p.o. (Fig. 5B).

The effects of DX-9065a and argatroban on the bleeding time in TTX- and L-NNA-treated rats were also evaluated by the tail transection method (Fig. 5: C, D). Neither of these drugs had apparent effect on the bleeding time.

Table 2. Effects of intravenous injection of DX-9065a and argatroban on heart rate (HR), mean blood pressure (MBP) and plantar skin blood flow (PSBF) in TTX-L-NNA-treated rats

Drug	n	Control values (as 100%)			After drug treatment		
		HR (beats/min)	MBP (mmHg)	PSBF (ml·min ⁻¹ ·100 g ⁻¹)	HR (%)	MBP (%)	PSBF (%)
Vehicle (saline)	14	356 ± 9	141 ± 5	10.1 ± 0.5	100.7 ± 0.3	102.8 ± 0.5	107.4 ± 0.9
DX-9065a							
0.3 mg/kg	7	355 ± 7	153 ± 7	11.9 ± 0.9	100.8 ± 0.3	102.2 ± 0.6	106.8 ± 1.4
1.0 mg/kg	7	342 ± 8	139 ± 7	10.1 ± 0.4	100.4 ± 0.3	103.8 ± 0.4	104.1 ± 1.2
3.0 mg/kg	9	348 ± 6	139 ± 9	11.9 ± 0.5	100.2 ± 0.1	104.4 ± 1.4	105.0 ± 1.3
Argatroban							
0.3 mg/kg	7	338 ± 10	133 ± 8	11.8 ± 0.6	100.3 ± 0.2	105.2 ± 1.0	103.5 ± 0.2
1.0 mg/kg	8	345 ± 8	138 ± 7	11.2 ± 0.9	100.3 ± 0.2	107.8 ± 0.9*	109.0 ± 2.1
3.0 mg/kg	8	334 ± 11	130 ± 4	10.7 ± 0.5	100.6 ± 0.4	111.7 ± 2.3**	108.8 ± 1.4

Control values were measured before drug treatments. The maximal change in response is expressed as % of the control value (= 100%). Each value represents the mean ± S.E.M. of number of experiments (n). **P*<0.05, ***P*<0.01 vs corresponding value of vehicle.

Fig. 5. Effects of DX-9065a and argatroban on prothrombin time (PT) (panels A and B) and bleeding time (panels C and D) in tetrodotoxin (TTX)- and *N*^o-nitro-L-arginine (L-NNA)-treated rats. Panels A and C show the results of the intravenous injection study and panels B and D, those of the oral administration study. Each PT was normalized to the mean value of the vehicle-treated group. Bleeding time was determined at the end of each experiment; i.e., 75 min after the intravenous injection of each drug and 120 min after oral administration of DX-9065a. Data represent the mean ± S.E.M. of 5–14 experiments. **P*<0.05, ***P*<0.01 vs corresponding value of vehicle.

Discussion

The present study demonstrated that the intravenous and oral administrations of DX-9065a, an inhibitor of factor Xa, effectively attenuated the disturbance in PSBF by ellagic acid in TTX- and L-NNA-treated rats, which was possibly due to thrombus formation and erythrocyte aggregation in the plantar and/or femoral vasculature, whereas the drug did not affect the bleeding time.

Ellagic acid is known to be one of the most efficient initiators of blood coagulation by converting Hageman factor to an active serine protease (15). There are several reports on ellagic acid-induced thrombosis in various animal models for evaluation of antithrombotic effects of drugs (16–19). However, in these experimental models, prior to local injection of ellagic acid into the femoral artery in rats (17) and rabbits (19) or into the cerebral arteries via the carotid artery in rats (16), a ligation or clipping of the arteries was necessary to locally induce thrombus and the subsequent disturbance of blood flow.

In the present study, the intraarterial injection of ellagic acid (300 μ g) into a branch of a femoral artery could induce the disturbance of PSBF without any additional procedures, such as the ligation of a femoral artery. It was previously reported that a NO synthase inhibitor, such as *N*^ω-nitro-L-arginine methyl ester (L-NAME), increased the volume of the thrombus induced by iontophoretic application of adenosine diphosphate (ADP) into the lumen of mesenteric venules of rats (20). Since endothelium-derived relaxing factor (EDRF)/NO inhibits thrombus growth (20), it could be highly probable that the L-NNA-treated rats were more susceptible to induction of thrombosis due to the reduced production of EDRF/NO by L-NNA.

Histologically, platelet-rich thrombi were observed in veins, and the aggregates of erythrocytes were recognized in arteries of the ellagic acid-injected hindlimb. These findings suggest that the thrombus formation and erythrocyte aggregation that occurred in the vascular regions of the femoral blood vessels and plantar skin area probably led to the disturbance in PSBF.

As neuronal reflex mechanisms are well developed in the cutaneous circulation (21), sympathetic efferents exert a strong influence on the skin blood flow (22). Here, we prepared acutely denervated rats by the combined use of TTX and L-NNA (11). TTX (50 μ g/kg, i.v.) could eliminate basal sympathetic nerve activity and inhibit excitation of sympathetic nerve through the reflex mechanisms including depressor responses. Although multiple pressor mechanisms of L-NNA, including inhibition of NO synthetase, have been reported

(14), a bolus injection of L-NNA (30 mg/kg, i.v.) apparently counteracted the lowered blood pressure and peripheral vascular tone that resulted from elimination of sympathetic nerve activity as well as inhibition of endothelium-derived relaxing factor (EDRF)/NO-induced vasodilation. This experimental model thus enabled us to observe direct actions of drugs on skin blood flow without neuronal interference (11).

Here, we chose argatroban as a control drug, because the drug showed a more potent antithrombotic action in comparison with a conventional coagulant such as heparin (16, 23–25). Argatroban has been used clinically in Japan since the early 1980's, and it was also recently approved for clinical use in the United States to treat with patients suffering from heparin-induced thrombocytopenia (26).

It has been reported that DX-9065a has a high selectivity as an inhibitor of factor Xa ($K_i = 41$ nM) and that its inhibitory activities toward thrombin and other serine proteases are very low ($K_i = 200$ μ M) (3). On the other hand, argatroban, a low-molecular-weight arginine derivative, selectively and directly inhibits thrombin (27). Our present study showed that the dosage of DX-9065a (3 mg/kg, i.v.) required to inhibit significantly the disturbance in PSBF by ellagic acid was comparable to that of argatroban (1–3 mg/kg, i.v.; Fig. 4). In addition, DX-9065a (3 mg/kg, i.v.) significantly prolonged prothrombin time to an extent similar to that observed with argatroban (1 mg/kg, i.v.).

It has been reported that the plasma thrombin-antithrombin III complex level was suppressed after the administration of argatroban in patients with peripheral arterial obstructive disease, along with the improvement of their clinical symptoms and a decrease in the size of ischemic skin ulcers (28). Taken together, the inhibition of factor Xa by DX-9065a seems to be an alternative therapeutic approach to treat peripheral thrombosis often encountered in peripheral arterial obstructive disease.

We showed that DX-9065a prolonged prothrombin time after intravenous or oral administration in a dose-dependent manner, as previously reported by Hara et al. (3). The oral administration of DX-9065a inhibited significantly the decrease in ST. The drug attenuated the disturbance of PSBF, although not to a statistically significant extent (Fig. 4B). However, we noted that the ST decreased, depending on the time of disturbed PSBF. In the present study, the oral administration of DX-9065a tended to reduce the total duration of complete occlusion time. Therefore, the oral administration of DX-9065a inhibited significantly the decrease in ST without significant effect on the maximum inhibition of PSBF.

In our study, DX-9065a and argatroban did not pro-

long the bleeding time. A selective factor Xa inhibitor, such as recombinant tick anticoagulant peptide and hirudin, hardly prolonged bleeding time in a canine thrombosis model (29). DX-9065a was also reported not to prolong bleeding time in rats (4, 7) or rabbits (5). On the other hand, it was reported that the intravenous infusion of argatroban prolonged the bleeding time at the dose showing antithrombotic effects in guinea pigs (30). This discrepancy can be explained as follows: The plasma concentration of argatroban declines exponentially with an elimination half-life of about 20 min in humans (31), in whom it also has a short duration of action (32), as in rats (33). In present study, the measurements of bleeding time started at 75 min after the injection of the drugs (see Fig. 1), so that most of the argatroban in plasma would have been degraded. That would be a reason why no significant prolongation of bleeding time was observed in the argatroban-treated group. On the other hand, the half-life of DX-9065a after oral administration was 2.3 h in humans (34). It was reported that the activated partial thromboplastin time and prothrombin time were significantly prolonged at least for 2 h after intravenous injection and oral administration of DX-9065a in rats (3). Therefore, our data show that DX-9065a did not affect bleeding time in spite of sufficient plasma concentration to prolong both activated partial thromboplastin time and prothrombin time at 75 and 120 min after intravenous injection and oral administration, respectively.

In conclusion, both the intravenous injection and oral administration of DX-9065a showed inhibitory effects on the plantar skin thrombosis induced by the ellagic acid without the prolongation of bleeding time. Intravenous injection of argatroban presented almost the similar inhibitory effect on the disturbance of PSBF and decrease in ST in response to ellagic acid. Therefore, our study suggests that the inhibition of factor Xa by DX-9065a would be an alternative therapeutic approach to treat peripheral thrombosis occurring in the skin circulation without a risk of bleeding.

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References

- 1 Nagahara T, Yokoyama Y, Inamura K, Katakura S, Komoriya S, Yamaguchi H, Hara T and Iwamoto M: Dibasic (amidinoaryl) propanoic acid derivatives as novel blood coagulation factor Xa inhibitors. *J Med Chem* **37**, 1200 – 1207 (1994)
- 2 Hara T, Yokoyama A, Morishima Y and Kunitada S: Species differences in anticoagulant and anti-Xa activity of DX-9065a, a highly selective factor Xa inhibitor. *Thromb Res* **80**, 99 – 104 (1995)
- 3 Hara T, Yokoyama A, Ishihara H, Yokoyama Y, Nagahara T and Iwamoto M: DX-9065a, a new synthetic, potent anticoagulant and selective inhibitor for factor Xa. *Thromb Haemost* **71**, 314 – 319 (1994)
- 4 Hara T, Yokoyama A, Tanabe K, Ishihara H and Iwamoto M: DX-9065a, an orally active, specific inhibitor of factor Xa, inhibits thrombosis without affecting bleeding time in rats. *Thromb Haemost* **74**, 635 – 639 (1995)
- 5 Herbert JM, Bernat A, Dol F, Herault JP, Crepon B and Lormeau JC: DX 9065A a novel, synthetic, selective and orally active inhibitor of factor Xa: in vitro and in vivo studies. *J Pharmacol Exp Ther* **276**, 1030 – 1038 (1996)
- 6 Yamazaki M, Asakura H, Aoshima K, Saito M, Jokaji H, Uotani C, Kumabashiri I, Morishita E, Ikeda T and Matsuda T: Effects of DX-9065a, an orally active, newly synthesized and specific inhibitor of factor Xa, against experimental disseminated intravascular coagulation in rats. *Thromb Haemost* **72**, 392 – 396 (1994)
- 7 Yamazaki M, Asakura H, Aoshima K, Saito M, Jokaji H, Uotani C, Kumabashiri I, Morishita E, Ikeda T and Matsuda T: Protective effects of DX-9065a, an orally active, novel synthesized and selective inhibitor of factor Xa, against thromboplastin-induced experimental disseminated intravascular coagulation in rats. *Semin Thromb Haemost* **22**, 255 – 259 (1996)
- 8 Wong PC, Crain EJ Jr, Ngan O, Watson CA and Racanelli A: Antithrombotic actions of selective inhibitors of blood coagulation factor Xa in rat models of thrombosis. *Thromb Res* **83**, 117 – 126 (1996)
- 9 Morishima Y, Tanabe K, Terada Y, Hara T and Kunitada S: Antithrombotic and hemorrhagic effects of DX-9065a, a direct and selective factor Xa inhibitor: comparison with a direct thrombin inhibitor and antithrombin III-dependent anticoagulants. *Thromb Haemost* **78**, 1366 – 1371 (1997)
- 10 Porcari AR, Chi L and Leadley R: Recent advances in clinical trials of the direct and indirect selective Factor Xa inhibitors. *Expert Opinion on Investigational Drugs* **9**, 1595 – 1600 (2000)
- 11 Chino D, Akimaru S, Kataha K, Ishii K and Nakayama K: Specific augmentation of plantar skin blood flow by lipo-PGE₁ assessed in tetrodotoxin- and N^G-nitro-L-arginine-treated rats. *J Cardiovasc Pharmacol* **35**, 630 – 637 (2000)
- 12 Nunoki K and Iijima T: Modification of the baroreceptor reflex by superfusion of the canine posterior hypothalamus with tetrodotoxin. *Naunyn Schmiedeberg's Arch Pharmacol* **321**, 185 – 189 (1982)
- 13 Ishii K, Chang B, Kerwin JF Jr, Huang ZJ and Murad F: N^ω-Nitro-L-arginine: a potent inhibitor of endothelium-derived relaxing factor formation. *Eur J Pharmacol* **176**, 219 – 223 (1990)
- 14 Nakahara T, Ishii K, Tanaka Y and Nakayama K: Involvement of neurohumoral factors in the pressor mechanism of N^G-nitro-L-arginine. *Eur J Pharmacol* **287**, 49 – 56 (1995)

- 15 Bock PE, Srinivasan KR and Shore JD: Activation of intrinsic blood coagulation by ellagic acid: insoluble ellagic acid-metal ion complexes are the activating species. *Biochemistry* **20**, 7258–7266 (1981)
- 16 Hara T, Iwamoto M, Ishihara M and Tomikawa M: Preventive effect of argatroban on ellagic acid-induced cerebral thromboembolism in rats. *Haemostasis* **24**, 351–357 (1994)
- 17 De Clerck F, Goossens J, Vermeylen J, Hornstra G and Reneman RS: Modification of venous stasis thrombosis in the rat by platelet-active drugs and by heparin. *Arch Int Pharmacodyn Ther* **222**, 233–242 (1976)
- 18 Suehiro A, Kakishita E and Nagai K: The role of platelet hyperfunction in thrombus formation in hyperlipidemia. *Thromb Res* **25**, 331–339 (1982)
- 19 Uzunova AD, Ramey ER and Ramwell PW: Gonadal hormones and pathogenesis of occlusive arterial thrombosis. *Am J Physiol* **234**, H454–H459 (1987)
- 20 Peire MA and Puig-Parellada P: Oxygen-free radicals and nitric oxide are involved in the thrombus growth produced by iontophoresis of ADP. *Pharmacol Res* **38**, 353–356 (1998)
- 21 Johnson JM, Brengelmann GL, Hales JR, Vanhoutte PM and Wenger CB: Regulation of the cutaneous circulation. *Fed Proc* **45**, 2841–2850 (1986)
- 22 Cooke JP, Creager MA, Osmundson PJ and Shepherd JT: Sex differences in control of cutaneous blood flow. *Circulation* **82**, 1607–1615 (1990)
- 23 Imura Y, Stassen JM and Collen D: Comparative antithrombotic effects of heparin, recombinant hirudin and argatroban in a hamster femoral vein platelet-rich mural thrombosis model. *J Pharmacol Exp Ther* **261**, 895–898 (1992)
- 24 Kawai H, Yamamoto T, Hara H and Tamao Y: Inhibition of factor Xa-induced platelet aggregation by a selective thrombin inhibitor, argatroban. *Thromb Res* **74**, 185–191 (1994)
- 25 Umemura K, Kawai H, Ishihara H and Nakashima M: Inhibitory effect of clopidogrel, vapirost and argatroban on the middle cerebral artery thrombosis in the rat. *Jpn J Pharmacol* **67**, 253–258 (1995)
- 26 Moledina M, Chakir M and Gandhi PJ: A synopsis of the clinical uses of argatroban. *J Thromb Thrombolys* **12**, 141–149 (2001)
- 27 Okamoto S, Hijikata A, Kikumoto R, Tonomura S, Hara H, Ninomiya K, Maruyama A, Sugano M and Tamao Y: Potent inhibition of thrombin by the newly synthesized arginine derivative No. 805. The importance of stereo-structure of its hydrophobic carboxamide portion. *Biochem Biophys Res Commun* **101**, 440–446 (1981)
- 28 Matsuo T, Kario K, Matsuda S, Yamaguchi N and Kakishita E: Effect of thrombin inhibition on patients with peripheral arterial obstructive disease: A Multicenter Clinical Trial of Argatroban. *J Thromb Thrombolys* **2**, 131–136 (1995)
- 29 Sitko GR, Ramjit DR, Stabilito II, Lehman D, Lynch JJ and Vlasuk GP: Conjunctive enhancement of enzymatic thrombolysis and prevention of thrombotic reocclusion with the selective factor Xa inhibitor, tick anticoagulant peptide. Comparison to hirudin and heparin in a canine model of acute coronary artery thrombosis. *Circulation* **85**, 805–815 (1992)
- 30 Kawamura M, Imura Y, Moriya N, Kita S, Fukushi H, Sugihara H, Nishikawa K and Terashita Z: Antithrombotic effects of TAK-029, a novel GPIIb/IIIa antagonist, in guinea pigs: comparative studies with ticlopidine, clopidogrel, aspirin, prostaglandin E₁ and argatroban. *J Pharmacol Exp Ther* **277**, 502–510 (1996)
- 31 Clarke RJ, Mayo G, Fitzgerald GA and Fitzgerald DJ: Combined administration of aspirin and a specific thrombin inhibitor in man [see comments]. *Circulation* **83**, 1510–1518 (1991)
- 32 Verstraete M: Pharmacotherapeutic aspects of unfractionated and low molecular weight heparins. *Drugs* **40**, 498–530 (1990)
- 33 Sasaki Y, Morii S, Yamashita T and Yamamoto J: Antithrombotic effect of argatroban on the pial vessels of the rat: a study with He-Ne laser-induced thrombus formation. *Haemostasis* **23**, 104–111 (1993)
- 34 Murayama N, Tanaka S, Kikuchi T, Nakaoka M and Sudo K: Radioimmunoassay method for DX-9065a, an anticoagulant agent. Development, evaluation and application to human plasma. *J Pharm Biomed Anal* **14**, 1435–1445 (1996)