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Antithrombotic effect of a novel recombinant hirudin analogue, CX-397, in a rat arterial thrombosis model

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1 The antithrombotic effect of a new specific thrombin inhibitor, CX-397, was examined in a photochemically-induced arterial thrombosis model in the rat femoral artery and compared with that of heparin.

2 Pretreatment with CX-397 (10, 20 and 40 $\mu g\ kg^{-1}\ min^{-1}$, i.v.) from 15 min before the experiment prolonged the time required for thrombotic occlusion of the artery in a dose-dependent manner. The antithrombotic efficacy of CX-397 was associated with modest increases in activated partial thromboplastin time (APTT) and template bleeding time.

3 On the other hand, heparin at a dose of 450 $\mu g\ kg^{-1}$ markedly prolonged APTT and the bleeding time, but did not inhibit thrombo-occlusion.

4 CX-397 selectively inhibited platelet aggregation and concurrent secretion of 5-hydroxytryptamine (5-HT) and thromboxane A_2 (TXA_2) production from platelets in response to thrombin, but not to collagen and ADP, in a dose-dependent manner (5-100 ng ml^{-1}).

5 CX-397 at 10 $\mu g\ kg^{-1}$ combined with vapiprost, a TXA_2 receptor antagonist, at 0.1 mg kg^{-1} significantly prevented occlusion, whereas, at these doses, neither drug alone had much effect.

6 These results demonstrate that CX-397 may prove to be more efficient for preventing platelet-rich thrombosis than heparin. Thrombin may play an important role in the rat thrombosis model.

7 The additive antithrombotic effect of the combination of thrombin inhibitor and TXA_2 receptor antagonist at low doses suggests that thrombin and TXA_2 may work in concert to produce thrombosis.

Keywords: CX-397; thrombin inhibitor; heparin; thrombin; TXA_2; arterial thrombosis; platelet aggregation

Introduction

Arterial thrombus is a significant problem in the management of ischaemic disease. Many factors participate in thrombus formation. Thrombin is considered to play a central role in the development of arterial thrombus as the primary mediator of blood coagulation and, perhaps more importantly, as a potent activator of platelets (Fenton *et al.*, 1991; Chesebro *et al.*, 1992). Hirudin, a direct inhibitor of thrombin, has been shown to be effective in preventing platelet-dependent arterial thrombosis in experimental (Heras *et al.*, 1989; Kelly *et al.*, 1991; Imura *et al.*, 1992) and clinical studies (Topol *et al.*, 1994) in which heparin is ineffective. Possible explanations for the greater efficacy of hirudin include its ability to inhibit fibrin-bound thrombin (Weitz *et al.*, 1990) and its insensitivity to heparin-neutralizing proteins released from platelet-rich thrombi, such as a platelet factor 4 (Fareed *et al.*, 1991). Moreover, hirudin is an effective inhibitor of thrombin-induced platelet activation, whereas heparin is not (Meyer *et al.*, 1994).

Recently, a new arterial thrombosis model was developed using the rat femoral artery, in which a photochemical reaction between rose bengal and green light is employed to induce vessel occlusion (Matsuno *et al.*, 1991). The photochemically-induced thrombosis (PIT) model is characterized by endothelial injury with little damage to the media. Previously we demonstrated the important role of thromboxane A_2 (TXA_2) as a platelet activator as well as a vasoconstrictor in platelet-rich thrombus formation in the rat PIT model (Takiguchi *et al.*, 1992). However, the role of thrombin in this model has never been clarified.

CX-397 is a new novel recombinant hirudin analogue having a hybrid amino acid sequence of hirudin variants-1 and -3. It inhibits thrombin in a competitive manner more effectively than its parent hirudins (Komatsu *et al.*, 1993). In the present study we evaluated the antithrombotic effect of this hirudin analogue in the rat PIT model to clarify the role of thrombin. In addition, we examined the hypothesis that the combination of the thrombin inhibitor and a TXA_2 receptor antagonist at low doses would show an additive antithrombotic effect.

Methods

Male Wistar rats weighing from 240 to 270 g were used. These rats were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). The body temperature of the rats was maintained at 37°C with a heating pad (Model K-20, American Pharmaseal Company, U.S.A.).

Induction of arterial thrombus

The experimental procedure to induce a thrombus in the rat femoral artery has been described previously in detail elsewhere (Matsuno *et al.*, 1991). Briefly, a part of the femoral artery was carefully separated and a pulsed Doppler flow probe (PDV-20, Crystal Biotech America, U.S.A.) was placed on the artery. The contralateral femoral artery and vein were cannulated with polyethylene tubes for monitoring blood pressure, pulse rate and drug delivery, respectively. Green light

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(540 nm wavelength) irradiation was achieved with a L4887 irradiation apparatus (Hamamatsu Photonics, Japan). The light was directed by an optic fibre positioned about 5 mm above a segment of the femoral artery proximal to the flow probe. Under the irradiation, the photosensitizer dye, rose bengal (Sigma, U.S.A.) was injected (10 mg kg^{-1}). Light exposure was continued until the blood flow stopped or for 20 min, whichever was the greater. Formation of an occlusive thrombus was indicated by complete cessation of the blood flow. Time to achieve complete occlusion was recorded.

Drug treatment

CX-397 (15,600 antithrombin units mg^{-1}; Japan Energy, Japan) was intravenously infused at 10, 20 and 40 $\mu g\ kg^{-1}\ min^{-1}$ from 15 min before the rose bengal injection until the end of the experiment. Heparin at 450 u kg^{-1} (Sigma, U.S.A.) and a TXA_2 receptor antagonist, vapiprost (Glaxo, U.K.) (Lumley *et al.*, 1989), at 0.1 mg kg^{-1} were administered i.v. 10 min before the experiment. In each control group saline was administered by infusion or injection in an equivalent volume.

Bleeding time

Bleeding time following tail incision was determined with a template blade device (Simplate, Organon Teknika Co. U.S.A.). Uniform incisions were made 3 cm from the tip of the tail, and blood was carefully blotted every 15 s on filter paper until no more blood was absorbed.

Blood coagulation time

The activated partial thromboplastin time (APTT) was determined with an APTT test kit (Wako, Japan). APTT and bleeding time were measured in the same animals treated with CX-397 for 15 min or with heparin for 10 min before the experiment.

Platelet study

Washed platelets were prepared by the method of Tomita *et al.* (1983), and the platelet count was adjusted to 4×10^8 cells ml^{-1}. Aggregation of washed platelets was induced by incubation of them with 0.3 u ml^{-1} thrombin (Mochida, Japan), 20 $\mu g\ ml^{-1}$ collagen (Nycomed Arzneimittel, Germany), or 10 nmol ml^{-1} adenosine 5'-diphosphate (ADP, Sigma, U.S.A.) in the presence of 1.5 mM Ca^{2+}. ADP-induced aggregation was measured in the presence of 0.4 mg ml^{-1} fibrinogen (America Diagnostica, U.S.A.) and 1.5 mM Ca^{2+}. After incubation with CX-397 for 2 min, aggregation in response to each stimulant was measured by the turbidometric method using a NBS hematracer 601 (Niko Bioscience, Japan). Seven minutes after stimulation with agonist, the response was terminated by the addition of a tenth volume of ice-cold stop solution containing 60 mM EDTA and 100 μM indomethacin, and the platelets were centrifuged immediately at 600 *g*, 4°C for 3 min. The supernatant was assayed for 5-hydroxytryptamine (5-HT) released and TXA_2 generated (estimated from the amount of TXB_2) with a 5-HT enzyme immunoassay kit (Dianova Immunotech, Germany) and a TXB_2 enzyme immunoassay system (Amersham, U.S.A.), respectively.

Statistical analysis

Results are expressed as mean ± s.e. In the case that no occlusion was found during the 30 min of observation, time to occlusion was calculated as 30 min. Data were analyzed by nonparametric Williams-Wilcoxon's test. Results were considered to have a significant difference if $P<0.05$.

Results

Antithrombotic effect

Figure 1 shows the inhibitory effect of CX-397 on platelet-rich thrombus formation in response to endothelial injury induced by a photochemical reaction between rose bengal and green light. In the saline control group, the blood flow of the irradiated femoral artery was completely occluded by the formation of a platelet-rich thrombus 9.22 ± 0.79 min ($n=8$) after the injection of rose bengal under green light irradiation. Blood flow variation was rarely found before the establishment of the occlusive thrombus. In the group of rats receiving CX-397 at a dose of 10 $\mu g\ kg^{-1}\ min^{-1}$, the time required to occlude the artery was slightly prolonged (10.78 ± 0.80 min, $n=8$). At the higher doses (20 and 40 $\mu g\ kg^{-1}\ min^{-1}$), the blood flow was gradually diminished with blood flow variation, and the time to achieve occlusion was significantly prolonged (19.31 ± 2.76, 23.65 ± 2.29 min, $n=8$, $P<0.01$, respectively) in a dose-dependent manner. CX-397 even at the highest dose (40 $\mu g\ kg^{-1}\ min^{-1}$) tested did not cause any changes in haemodynamics, i.e., blood pressure, pulse rate and the blood flow of the femoral artery.

The results on bleeding time and APTT are shown in Table 1. CX-397 at doses of 20 and 40 $\mu g\ kg^{-1}\ min^{-1}$ caused moderate prolongation of the APTT (2–3 fold increase).

On the other hand, as also shown in Figure 1, heparin at a dose of 450 u kg^{-1} did not prolong the time to occlusion (9.46 ± 0.95 min, $n=8$), although the bleeding time and APTT were extremely prolonged (Table 1).

Antiplatelet effect

Thrombin (0.3 u ml^{-1}), collagen (20 $\mu g\ ml^{-1}$) and ADP (10 nmol ml^{-1}) induced aggregation of washed platelets, to the same extent (65 ± 5, 60 ± 7 and 58 ± 6%, respectively ($n=7$)). CX-397 inhibited platelet aggregation and concurrent secretion of 5-HT and TXA_2 production by platelets in response to thrombin in a concentration-dependent manner, with mean IC_{50} values (the inhibitory concentration needed to cause 50% of maximal inhibition) of 21, 20 and 15 ng ml^{-1}, respectively (Figure 2). However, CX-397 even at 100 ng ml^{-1} did not inhibit platelet aggregation in response to ADP or collagen.

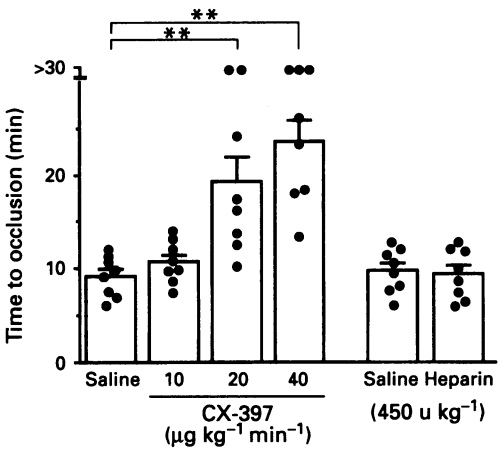

Figure 1 Effects of CX-397 and heparin on the time required to achieve thrombotic occlusion of the rat femoral artery. (●) Indicate time to occlusion of each rat. Each column indicates mean (± s.e.) time to occlusion in each group. In the event that no occlusion was found during the 30 min of observation, the time was taken as 30 min. CX-397 was given by continuous i.v. infusion during the experimental period beginning 15 min before the injection of rose bengal. Heparin was administered i.v. 10 min before the injection of rose bengal. $^{**}P<0.01$.

Table 1 Effects of treatment with CX-397 or heparin on activated partial thromboplastin time (APTT) and bleeding time

	n	*APTT* (s)	*Bleeding time* (min)
Saline	5	28.4±0.2	5.0±0.7
CX-397			
20 μg kg^{-1} min^{-1}	5	51.3±1.7	11.3±3.3
40 μg kg^{-1} min^{-1}	5	67.5±1.6	15.1±5.0
Saline	5	29.0±0.4	4.5±0.4
Heparin			
450 u kg^{-1}	5	>300	>30

Results are shown as mean±s.e.

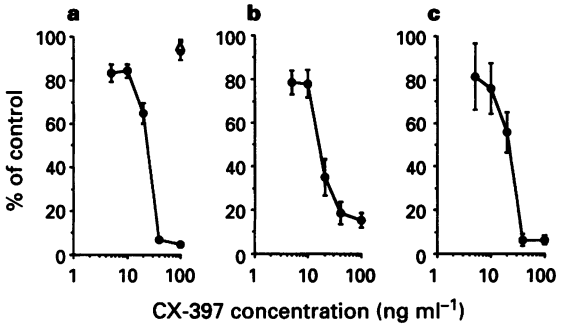

Figure 2 Concentration-response relationship for the inhibitory effects of CX-397 on platelet aggregation induced by thrombin, collagen or ADP (a), and on thromboxane A_2 (TXA_2) production (b) and 5-hydroxytryptamine (5-HT) secretion (c) in response to thrombin. After incubation with CX-397 at a dose range of 5–100 ng ml^{-1} or saline for 2 min, washed platelets were stimulated with 0.3 u ml^{-1} thrombin (●), 20 μg ml^{-1} collagen (○) or 10 nmol ml^{-1} ADP (△). Points show mean±s.e. of 7 rats.

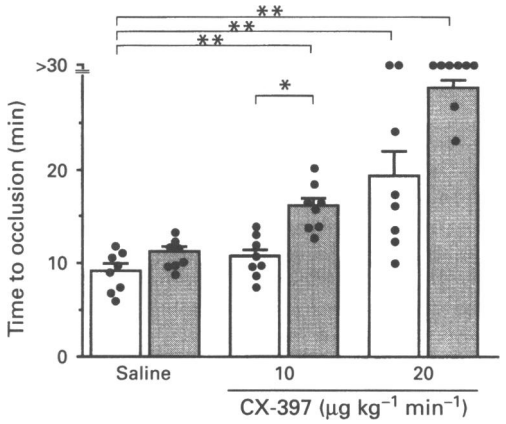

Figure 3 Effects of combination treatment with CX-397 and vapiprost on the time required to achieve thrombotic occlusion of the rat femoral artery. CX-397 (10, 20 μg kg^{-1} min^{-1}) or saline was given with (stippled columns) or without (open columns) vapiprost (0.1 mg kg^{-1}) by continuous i.v. infusion during the experimental period beginning 15 min before the injection of rose bengal. Vapiprost was administered i.v. 10 min before the injection of rose bengal. Each column indicates the mean (±s.e.) time to occlusion in each group. In the event that no occlusion was found during the 30 min of observation, the time was taken as 30 min. (●) Indicate time to occlusion of each rat. *P<0.05, **P<0.01.

Effects of combination

Figure 3 shows the antithrombotic effect of a combination treatment with CX-397 and vapiprost, a TXA_2 receptor antagonist. Vapiprost at a low dose of 0.1 mg kg^{-1} slightly prolonged the time required to occlude the artery (11.29±0.68 min, n=8) as we previously reported (Takiguchi *et al.*, 1992). In animals treated with CX-397 at 10 μg kg^{-1} min^{-1} and vapiprost at 0.1 mg kg^{-1}, the time to occlusion was significantly prolonged (16.11±0.88 min, n=8, P<0.01), whereas neither drug alone at these doses was effective. The time to occlusion for the combination of CX-397 at 10 or 20 μg kg^{-1} min^{-1} and vapiprost was much longer than that for CX-397 alone (P<0.05 at 10 μg kg^{-1} min^{-1}). The combination with CX-397 at 20 μg kg^{-1} min^{-1} did not cause a further prolongation of APTT (48.4±2.2 s, n=5) or the bleeding time (12.0±2.8 min, n=5), as compared with CX-397 alone (Table 1).

Discussion

The arterial thrombotic process begins with platelet adhesion to subendothelial collagen at the injured vessel. The activated platelets release ADP from dense granules and generate TXA_2. At the platelet membrane surface the content of anionic phospholipids increases. The latter allows for efficient assembly of factor Xa and prothrombinase complexes on the negatively charged platelet surface, resulting in generation of thrombin. ADP, TXA_2 and thrombin cause additional circulating platelets to aggregate at the site of injury. Recently, we developed a new platelet-rich arterial thrombosis model in the rat femoral artery and reported that TXA_2 plays a major role in thrombogenesis in this model, whereas ADP is of minor importance (Takiguchi *et al.*, 1992). In the present study, the direct thrombin inhibitor CX-397 was very effective in inhibiting the occlusive thrombus formation, indicating that thrombin is also an important mediator of thrombosis in the rat PIT model.

The dose of CX-397 required for inhibition of platelet-dependent arterial thrombosis (more than 20 μg kg^{-1} min^{-1}) was larger than that necessary to inhibit coagulation-dependent venous thrombosis (0.1–20 μg kg^{-1} min^{-1}) (Hayashi *et al.*, 1993). Prolongation of APTT two fold more was necessary to prevent the arterial thrombus formation, which is consistent with the results of other investigators with hirudin (Heras *et al.*, 1989; Imura *et al.*, 1992). The continuous infusion of the effective dose (20, 40 μg kg^{-1} min^{-1}) of CX-397 resulted in a plasma level of over 10 ng ml^{-1}. CX-397 selectively inhibited platelet aggregation in response to thrombin with a mean IC_{50} value of 21 ng ml^{-1}, but not that to collagen or ADP. Therefore, thrombin as a potent activator of platelets appears to be more important as a producer of platelet-dependent thrombus than as a fibrin generator in the rat model as well as in swine and canine arterial models (Heras *et al.*, 1989; Eidt *et al.*, 1989).

Although both CX-397 and heparin are antithrombin drugs, their interactions with thrombin are different: CX-397 directly inhibits thrombin, whereas heparin accelerates the antithrombin III-mediated inhibition of thrombin. In the present study, heparin was ineffective in the rat platelet-dependent thrombosis model even at a dose which prolongs APTT more than ten times. These findings confirm and extend the observations made in other animal models that platelet-mediated thrombosis is quite resistant to heparin (Eidt *et al.*, 1989; Heras *et al.*, 1989; Kelly *et al.*, 1991; Imura *et al.*, 1992). This is probably because of the release of heparin-neutralizing factors, including platelet factor 4, from activated platelets (Eitzman *et al.*, 1994), and the limited access of the heparin-antithrombin III complex to thrombin sequestered within forming thrombus (Weitz *et al.*, 1990). Moreover, heparin is ineffective in inhibiting platelet-bound meizothrombin during

thrombin generation (Lindhout *et al.*, 1986). Hirudin can displace thrombin from its binding sites on platelets and thus may be the cause of deaggregation (Tam *et al.*, 1979) and a reduction of the mural thrombus (Wysokinski *et al.*, 1993). Hirudin and other low-molecular-weight direct thrombin inhibitors such as argatroban (Jang *et al.*, 1990) and PPACK (Hanson & Harker, 1988) are effective inhibitors of thrombin-induced platelet activation following arterial injury, whereas heparin is not.

Previously we reported the relatively important role of TXA_2 in thrombogenesis in the rat PIT model. The TXA_2 receptor antagonist vapiprost showed a potent inhibitory effect on the occlusive thrombus formation. The antithrombotic effect of vapiprost was believed to arise from not only its antiplatelet action but also its inhibitory effect on vascular contractions induced by platelet-derived TXA_2. In the present study low doses of CX-397 and vapiprost in combination significantly prevented occlusion, whereas neither drug alone was effective. Thrombin directly stimulates platelets to release TXA_2, inducing potent vasoconstriction, especially, if the endothelium has been injured or removed since, under these conditions, the inhibitory effects of endothelium-derived prostacyclin and nitric oxide are lacking (Yang *et al.*, 1994). CX-397 inhibited thrombin-induced TXA_2 production from platelets. Therefore, it is considered that a concomitant inhibition of both thrombin and TXA_2 potently interferes with the platelet-vessel interaction, which may, in part, provide the additive antithrombotic effect of CX-397 and vapiprost in the rat. Such an additive effect of inhibitors of both thrombin and TXA_2 has been demonstrated in coronary thrombosis in canine models (Fitzgerald & FitzGerald, 1989; Yao *et al.*, 1992; White *et al.*, 1994).

In conclusion, CX-397 effectively inhibited the platelet-dependent thrombus formation in the rat femoral artery model. The combination of CX-397 and vapiprost demonstrated an additive antithrombotic effect. The results of this study suggest the importance of thrombin, which may participate in multiple interactions between the coagulation, platelet and vascular systems and produce the platelet-dependent thrombus in concert with TXA_2. Such a combination of thrombin and TXA_2 inhibitors at low doses may be worthy of testing clinically.

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