Effects of vapiprost, a novel thromboxane receptor antagonist, on thrombus formation and vascular patency after thrombolysis by tissue-type plasminogen activator

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1 A thrombus was induced in the rat femoral artery by endothelial damage due to the photochemical reaction between systemically-injected Rose Bengal and transillumination with green light (wavelength: 540 nm). The artery of the control rat was completely occluded in 302.8 ± 27.0 s after the initiation of the reaction.

2 Pretreatment with vapiprost (0.1, 0.3 and 1.0 mg kg⁻¹, i.v., 5 min before the reaction) prolonged the time required to occlude the femoral artery in a dose-dependent manner. The efficacy of vapiprost on the time required for occlusion was over 10 times higher than that of aspirin which was administered 30 min before the reaction.

3 The thrombolytic effects of tissue-type plasminogen activator (tPA) on the established arterial thrombus in the presence and absence of vapiprost were also studied in the same model. When vapiprost (0.3 mg kg⁻¹, i.v.) was administered just before tPA infusion (100 µg kg⁻¹ min⁻¹ for 30 min), the time required to reperfuse the occluded artery was reduced, the incidence of the reperfusion was increased and the arterial blood flow after reperfusion was improved.

4 When vapiprost (1.0 mg kg⁻¹ daily p.o.) was administered for 1 week after the establishment of reperfusion by tPA combined with vapiprost, the patency of the reperfused artery was improved and the femoral arterial blood flow was better preserved than after treatment with only tPA.

5 These findings suggest that this thromboxane receptor antagonist may be a useful adjunct to anti-thrombotic therapy. The combination therapy with tPA may be more effective than treatment with tPA alone and provides greater protection against reocclusion after reperfusion.

Keywords: Vapiprost; thromboxane receptor antagonist; antithrombotic effect; tissue-type plasminogen activator; reperfusion; patency of reperfused vessel

Introduction

Platelets play an important role in thrombus formation associated with various ischaemic vascular events. Recently, the so-called 'arachidonic cascade', which liberates various prostaglandins from the vascular wall and platelets, was fully elucidated. It has been suggested that deterioration of the balance between thromboxane A₂ (TXA₂) production by platelets and prostaglandin I₂ (PGI₂) production by endothelial cells may be a trigger to ischaemic disease because TXA₂ subsequently causes powerful platelet aggregation and contraction of blood vessels, while PGI₂ counteracts TXA₂ (Aiken *et al.*, 1980). Anti-platelet drugs which inhibit platelet aggregation and possess a relatively low risk of general bleeding, are frequently prescribed for the treatment of ischaemic disease and thrombosis. On the other hand, thrombolytic therapy with tissue-type plasminogen activator (tPA) is highly effective for recanalization of the coronary artery obstructed by intraluminal thrombi (Collen *et al.*, 1984; Williams *et al.*, 1986). However, the thrombolytic therapy is not necessarily free from spontaneous reocclusion of the reperfused vessel, especially in the vessel with a residual stenosis of 80% or more (Gold *et al.*, 1986). Recent data suggest that TXA₂ may play a role in reocclusion following tPA-induced thrombolysis (Shebski *et al.*, 1988). We have established a model of arterial thrombosis in the rat femoral artery (Matsuno *et al.*, 1991). This thrombus is platelet-rich and quite representative of that observed clinically (Friedman *et al.*, 1966). It has been suggested that platelet-rich regions are

more resistant to antithrombotic or thrombolytic interventions than erythrocyte-rich ones (Ik-Kyung *et al.*, 1989). In the present study, we have therefore examined the efficacy of the novel thromboxane receptor antagonist, vapiprost, on the prevention of platelet-rich thrombus formation in the rat femoral artery induced by a photochemical reaction, in comparison with a well-known cyclo-oxygenase inhibitor, aspirin and inhibition of reocclusion of the femoral artery reperfused with tPA.

Methods

Male Wistar rats (weighing from 240 to 260 g) were used. These rats were anaesthetized by intraperitoneal injection of 50 mg kg⁻¹ of sodium pentobarbitone. Animals were allowed to breathe spontaneously. Body temperature of the rats was maintained at 37.5°C with a heating-pad (American Pharmaceutical Company, K-module Model K-20).

Induction of thrombus in the femoral artery

The experimental procedure to induce a thrombus in the rat femoral artery has been described elsewhere (Matsuno *et al.*, 1991) in detail. In brief, a short incision was made in the skin and subcutaneous tissue on the right groin region and the femoral neurovascular sheath was gently exposed. A 5 mm-long portion of the right femoral artery distal to the inguinal ligament was isolated by rubbing it against the blade of a forceps, and a pulsed doppler flow probe (Model PDV-20 Crystal Biotech, U.S.A.) positioned for monitoring blood

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flow. The contralateral femoral artery and vein were cannulated for monitoring blood pressure and pulse rate and for drug delivery by intravenous injection, respectively. Transillumination with green light was achieved by use of a xenon lamp with a heat absorbing filter and a green filter (a band width of 54 nm centered at 540 nm; Hamamatsu Photonics Inc., Japan). The light was directed by an optic fibre mounted on a micromanipulator so that the head would be approximately 5 mm away from the intact right femoral artery, proximal to the flow probe. About 10 min after establishing the baseline blood flow, the irradiation with green light was started, and after 10 min, Rose Bengal (10 mg kg^{-1}) was injected. The arterial blood flow, blood pressure and pulse rate were continuously monitored on a multi-channel thermo-pen writing oscillograph (WS-681G, Nihon Kohden, Japan). The femoral artery was judged to be occluded when the blood flow fell almost to zero.

Experimental protocol

Aspirin (3 and 10 mg kg^{-1}) and vapirost (0.1, 0.3 and 1.0 mg kg^{-1}) were administered intravenously 30 min and 5 min before the injection of Rose Bengal, respectively. The transillumination with green light was continued until the artery was occluded. The observation was continued for 1 h after the injection of Rose Bengal. Because both agents were dissolved in different solvents, i.e. aspirin was dissolved in saline and vapirost was dissolved in 50 mM phosphate buffer (pH 7.4), control experiments were performed separately for each of the agents using the appropriate solvent.

In the thrombolytic study with tPA, the i.v. infusion of which with an infusion pump (Model 22, Harvard, U.S.A.) was started immediately after the establishment of occlusion of the femoral artery and continued for 30 min at a rate of $100 \mu\text{g kg}^{-1} \text{ min}^{-1}$, observation was continued for 1.5 h after the cessation of tPA infusion. Just before starting the infusion with tPA, care was taken to flush out any heparin solution (about 100 u kg^{-1}) remaining in the catheter. The patency of the reperfused artery was assessed at 24 h and at 1 week after the above procedures. Control values were obtained by infusion of saline instead of tPA. The effects of co-administration of vapirost were assessed when vapirost (0.3 mg kg^{-1} , i.v.) was injected once just before the infusion of tPA and when oral administration of vapirost (1.0 mg kg^{-1}) was subsequently continued for 1 week following the acute i.v. bolus injection. The artery was defined as reperfused when the blood flow was restored to more than 25% of the baseline value.

Ex vivo whole blood aggregation

In a separate experiment using 10 rats, the time-profiles of plasma concentration and suppression of whole blood aggregation *ex vivo* were investigated after i.v. injection of vapirost (1.0 mg kg^{-1}). Blood samples (5 ml each) were collected into plastic tubes containing $300 \mu\text{l}$ of 50 u ml^{-1} heparin from the descending aorta under anaesthesia with sodium pentobarbitone (50 mg kg^{-1} , i.p.) after either 5, 30 or 60 min after the injection and, then, the rat was killed. An impedance aggregometer (C550S, Chrono-log Corp., U.S.A.) was used. Collagen was used and the increase in electrical impedance was observed until the tracing reached a plateau.

Histopathological observation

At the end of some experiments using tPA only or in combination with vapirost, whether the occluded artery ($n = 4$) was reperfused or not, the rats were killed with an overdose of pentobarbitone and the irradiated segments of femoral arteries were excised. These segments were immediately fixed

with 2.0% glutaraldehyde in 0.1 M sodium phosphate buffer for 1 h. Each segment was cut open longitudinally to allow visual inspection, and these sections were processed for routine scanning with an electron microscope (SEM).

Agents

Aspirin and heparin were purchased from Sigma and Novo-industry, Denmark, respectively; vapirost and tPA (TD-2061) were a gift from Glaxo Group Research Ltd, England and Daiichi C.L., Japan, respectively.

Statistical analysis

All data are expressed as the mean $\pm$ standard deviation (s.d.). Comparisons of femoral blood flow and the time to reperfusion were performed with unpaired Student's *t* test. Comparison among groups was carried out by use of Wilcoxon's test for the time to occlusion and χ^2 test for the reperfusion rate and the reocclusion rate.

Results

Antithrombotic effects of vapirost and aspirin in the *in vivo* study

Animals from all groups studied had similar body weights and haemodynamic conditions before induction of thrombosis. Typical changes in arterial blood pressure, pulse rate and femoral arterial blood flow from a control animal are illustrated in Figure 1a. Vapirost-treated groups are also shown in Figure 2. The lowest dose of vapirost had only a slight effect on the occlusion time ($681.28 \pm 58.61 \text{ s}$). However, 0.3 mg kg^{-1} of vapirost was significantly effective in preventing a femoral arterial thrombus ($P < 0.01$ vs control). In six of the nine rats, occlusive thrombi did not develop within 60 min. The times required for occlusion in the three rats in this group in which the femoral arteries had become occluded by the formation of thrombi (11, 18 and 21 min, respectively), were much more prolonged than those with aspirin. At the highest dose of vapirost studied (1.0 mg kg^{-1}), complete cessation of femoral arterial blood flow could not be obtained in the first 60 min of observation, although the blood flow itself was diminished as compared with the control value. Figure 1b and c shows the effects of vapirost, i.e., the dose-dependent prolongation of time required to occlude the artery and cyclic flow variations (CFVs) in the femoral arterial blood flow. The result of aspirin treatment is illustrated in Figure 2. Occlusive femoral arterial thrombi occurred in all 7 rats of the aspirin vehicle-treated groups in a mean time of $326.6 \pm 26.9 \text{ s}$. In the group of rats receiving aspirin at a dose of 3.0 mg kg^{-1} , the time required to occlude the artery was only slightly prolonged ($341.5 \pm 32.6 \text{ s}$). At 10.0 mg kg^{-1} the time to occlusion was further prolonged ($417.0 \pm 103.4 \text{ s}$), but with either dose level the thrombus was finally established within 12 min in all animals.

Ex vivo study

When platelet aggregation was induced with $1.0 \mu\text{M}$ collagen, extensive inhibition by vapirost was observed as compared with that of non-treated rats. Vapirost was very effective in blocking collagen-induced platelet aggregation *ex vivo* in this experiment, inhibition rates of aggregation at 5, 30 and 60 min after administration of vapirost being $90.6 \pm 8.2\%$, $75.7 \pm 13.3\%$ and $81.9 \pm 8.9\%$, respectively. In this case, plasma concentrations of vapirost at 5, 30 and 60 min were 1675.5 ± 69.3 , 869.7 ± 43.3 and $560.5 \pm 17.9 \text{ ng ml}^{-1}$, respectively.

Figure 1 (a) Simultaneous records of typical changes in blood velocity of the irradiated femoral artery and, pulse rate and blood pressure from the contralateral femoral artery. (1) Baseline measurements; (2) change before and after the i.v. injection of Rose Bengal (RB, 10 mg kg⁻¹); (3) 30 min after thrombus formation. Continuous records of femoral blood flow from two animals receiving vapirost at a dose of 0.3 mg kg⁻¹ (b) and 1.0 mg kg⁻¹ (c). Thrombosis was induced by the photochemical reaction between xenon light and Rose Bengal injection.

Thrombolytic effects of tPA alone and vapirost on tPA

The control resting flows before the formation of thrombus did not differ significantly between these groups of rats or any groups tested. The incidence of reperfusion within the 1.5 h observation period was 38.5% for tPA (100 µg kg⁻¹ min⁻¹) and the time to reperfusion was 70.8 ± 11.6 min (Table 1). Following reperfusion the femoral arterial blood flow was much decreased as compared with that before thrombosis. Animals given either saline or vapirost (1.0 mg kg⁻¹) alone failed to reperfuse in all cases without tPA (Table 1). tPA was much more effective in restoring femoral arterial blood flow in the presence of vapirost (0.3 mg kg⁻¹) than in the absence of thromboxane inhibition. tPA at 100 µg kg⁻¹ min⁻¹ alone produced reperfusion in 5 of 12 rats

Figure 2 Time to occlusive femoral arterial thrombosis in the individual experiments. The solvent group was divided into 2 groups of animals receiving the vehicle of either vapirost (●) or aspirin (▲). ***P* < 0.01 versus control group.

whereas in the presence of vapirost, 11 of 13 rats reperfused. Likewise, femoral arterial blood flow of reperfusion (Figure 3) and the time to reperfusion (Table 1) also indicate the greater effectiveness of tPA in the presence of vapirost.

Inhibition of reocclusion with vapirost

The incidence of reocclusion with animals reperfused (*n* = 5) was 60% during the 1 week of observation and the femoral arterial blood flow of the group treated with tPA alone was much decreased. In rats receiving i.v. injection of vapirost just before the infusion of tPA, reocclusion incidence after 1 week was unchanged; however, reocclusion incidence after 24 h was improved as well as the blood flow (Figure 3). Additionally, after receiving vapirost for 1 week (1.0 mg kg⁻¹ daily, p.o.), reocclusion incidence and femoral arterial blood flow were improved in comparison with the other groups (Table 1, Figure 3).

Histopathological observations

Scanning electron microscopy of femoral arteries collected after the interruption of blood flow showed that mural thrombi were present in the lumen of the arteries. The luminal thrombi were composed of aggregates of platelets which had entrapped red blood cells within them, and the central portions of the mural thrombi were rich in fibrin (Figure 4). Following reperfusion with tPA, platelets remained anchored to the disrupted arterial wall in spite of restoration of femoral arterial blood flow. However, red blood cells were lost (Figure 5a). Figure 5b shows a femoral artery after reperfusion with vapirost and tPA. When vapirost (0.3 mg kg⁻¹) was injected before infusion of tPA, platelet aggregates

Table 1 Frequency of femoral artery reperfusion and reocclusion in the photochemical induced thrombosis model in the rat

Groups	n	Reperfusion incidence	Reperfusion time (min)	Reocclusion incidence		
				90 min	24 h	1 week
tPA alone	12	5/12	70.8 ± 11.6	2/5	1/3	0/2
Group I	13	11/13*	55.7 ± 8.7*	1/11	2/10	4/8
Group II	6	5/6	52.8 ± 11.2*	0/5	1/5	0/4

Data are expressed in means ± s.d. **P* < 0.05 versus tPA-only treated group. Group I was treated with tPA in combination with i.v. injection of vapirost just before the start of tPA infusion. Group II: vapirost (p.o.) was administered for 7 days in addition to single i.v. administration. Denominators of reocclusion incidence express the number of animals maintaining the reperfusion state at that time.

were also observed but in general, aggregates were less prevalent in the vapiprost-treated rats than in the tPA-only treated group, and reocclusion state was similar to the control without tPA treatment. The arterial endothelia were repaired after 1 week, however, examination over a longer period was not carried out. In the case of the arterial segments in which blood flow decreased after 1 week, many platelets adhered to the repaired endothelial surface and the reocclusion state was observed (Figure 6a). These adherent platelets were also decreased by administration of vapiprost (0.3 mg kg^{-1} daily) for 1 week (Figure 6b).

Figure 3 Femoral blood flow during the continuous administration of vapiprost after reperfusion was more increased than that after tPA alone. The effect of chronic treatment with vapiprost upon tPA-induced reperfusion and maintenance of blood flow. Infusion of tPA ($100 \mu\text{g kg}^{-1}$) was started 30 min after the complete cessation of blood flow and continued for 30 min (indicated by horizontal bar). Group I was treated with tPA in combination with i.v. injection of vapiprost just before the start of tPA infusion. Group II was treated with vapiprost (1 mg kg^{-1} daily, p.o.) which was administered for 7 days in addition to a single i.v. administration. * $P < 0.05$ versus tPA-only treated group. Symbols: tPA only (○); Group I (●); Group II (▲). Values are mean with s.d. shown by vertical bars.

Discussion

The present study has shown vapiprost to be an effective and potent antithrombotic drug *in vivo* in a photochemically induced thrombosis (PIT) model in the rat. The thrombus produced subsequent to the injury to the vascular endothelium, is platelet-rich and resembles clinically observed thrombi (Matsuno *et al.*, 1991). TXA_2 , although relatively labile, is synthesized in large quantities by platelets in response to a variety of stimuli (Best *et al.*, 1980; Ambler *et al.*, 1985). Thromboxane receptor antagonists (TRAs) such as vapiprost (Lumley *et al.*, 1989), sulotoroban (Shebuski *et al.*, 1988; Fujita *et al.*, 1988) and Ah-23848 (Brezinski *et al.*, 1988) have been shown to interfere with platelet function through specific competitive inhibition of the platelet membrane receptor that binds either TXA_2 or the cyclic endoperoxides intermediate, PGH_2 . By preventing the recruitment of platelets and the secondary release reaction, TRAs may effect a moderate reduction in TXA_2 levels (Ashton *et al.*, 1986), but their mechanism of action is almost entirely through their antagonism of the TXA_2 - PGH_2 receptor (Ogletree *et al.*, 1985; Hanasaki *et al.*, 1988). Vapiprost (GR32191) prevents stimulation of platelet aggregation both by TXA_2 and its less potent but longer-lived precursor, PGH_2 , in a dose-dependent manner and has been shown to inhibit partially platelet deposition on damaged arteries and artificial surfaces (McCabe *et al.*, 1987). In our study of its antithrombotic effect, vapip-

Figure 4 Electronmicrograph of femoral artery showing complete interruption of blood flow. The lumen of the irradiated arterial segment is packed with red blood cells; many platelets with pseudopods and fibrin nets on the swollen endothelial cells are shown. The bar in the photograph denotes $20 \mu\text{m}$.

rost (1.0 mg kg^{-1}) proved to be effective in preventing the formation of occlusive femoral thrombi. Indeed, occlusion did not occur in any animals during the 60 min observation period. However cyclic flow variations (CFVs) were observed after administration of a single bolus injection of vapiprost as was observed in dogs (Shebski *et al.*, 1988). The animals which received the lower dose of vapiprost or the vehicle all had occlusive thrombi at times similar to those in the control group (Matsuno *et al.*, 1991), despite the platelet aggregation response to collagen being inhibited *ex vivo*. These results are supported by the findings of Hornby *et al.* (1989). The above investigations suggest that TXA_2 is an important element in the thrombus formation on the vascular wall. Schmitz *et al.* (1985) have reported that the increased production of thromboxane at the site of the damaged arterial wall is a very potent trigger for irreversible platelet aggregation and thrombus formation. Therefore, TRAs, like vapiprost, may be very effective antithrombotic agents in clinical conditions of arterial damage and platelet deposition. The doses of vapiprost used in this experiment did not influence systemic haemodynamic parameters. Aspirin, acetylsalicylic acid (ASA), reduces platelet aggregation by irreversibly inhibiting cyclo-oxygenase and thereby preventing platelet PGH_2 and TXA_2 synthesis (Folts *et al.*, 1988). Although concurrent inhibition of endothelial cell PGI_2 production is potentially detrimental, this does not appear to have been a problem in models in which high-dose ASA has resulted in a striking improvement (Kelton *et al.*, 1978; Wu *et al.*, 1981; Zammit *et al.*, 1984).

The results of the thrombolytic study demonstrated that the administration of vapiprost increased the incidence of reperfusion, shortened the time to tPA-induced arterial thrombolysis and increased the femoral arterial blood flow after reperfusion. These results suggest that inhibition of

Figure 5 The luminal surface of a femoral artery after successful thrombolysis. Note the presence of platelets with pseudopods adherent to the surface of the vessel in spite of the restoration of blood flow (a). After combination thrombolytic therapy with tPA and vapirost ($0.3 \text{ mg kg}^{-1} \text{ i.v.}$), platelets on the surface of the vessel decreased as compared with the tPA only treatment (b). The bars in both photographs denote $5 \mu\text{m}$.

Figure 6 The luminal surface of a femoral artery fixed 1 week after successful thrombolysis with tPA alone. There are few blood cells, however, more platelets remain on the repaired endothelial cells (a). Following vapirost treatment for 1 week, blood elements are scarcely present on the luminal surface. Arrows indicate the border line between the repaired surface of endothelial cells after injury by photochemical reaction and non-damaged surface (b). The bars in both photographs denote $5 \mu\text{m}$.

TXA₂-mediated platelet aggregation may be beneficial in the therapeutic effectiveness of a fibrinolytic agent such as tPA. The effect may be related to platelet activation and an increase in TXA₂ that occurs as a consequence of fibrinolytic administration in experimental animals (Fitzgerald & Fitzgerald, 1989). Platelet activation may slow the rate at which thrombolysis proceeds and may play an important role in acute rethrombosis. Furthermore, scanning electron microscopy has confirmed that platelet aggregation is involved in spontaneous reocclusion in this study.

Reocclusion after reperfusion has been recognized as one of the major limitations of thrombolytic therapy. For example, the benefit of establishing coronary reperfusion is significantly offset by an acute spontaneous reocclusion rate of 20.0% to 45.0%, despite full heparinization (Collen *et al.*, 1984; Williams *et al.*, 1986; Sheehan *et al.*, 1987). In patients with myocardial infarction, coronary reocclusion can be prevented by a maintenance infusion of low doses of rTPA for up to 4 h (Gold *et al.*, 1986). However, these prolonged infusions caused a significant depletion of circulating fibrinogen of up to 50% of the initial values. In contrast, our findings have demonstrated that prevention of reocclusion by the use of combination therapy of tPA and low-dose vapirost has been successful without prolonged tPA infusion. This supports the concept that residual platelets are one of the causes of the reocclusion. In the present study, the combination of vapirost and tPA produced a depression of platelet reactivity and increase of blood flow after reper-

fusion. However, in the case of once only treatment with vapirost, arterial blood flow was not maintained after 1 week. Scanning electron microscopy of arteries collected in animals with decreased blood flow after 1 week showed that mural thrombi were present in the lumen of the arteries. However, the depression of blood flow was prevented by continuous therapy of vapirost ($1.0 \text{ mg kg}^{-1} \text{ daily, p.o.}$) for 1 week. These examinations strongly suggest that platelet aggregation plays an important role in spontaneous and cyclical reocclusion after successful thrombolysis. The results also suggest that TXA₂ generation may be activated limiting the response to fibrinolysis as TXA₂-mediated platelet activation occurs simultaneously with clot dissolution. The inhibition of TXA₂-mediated platelet activation, as with a combination therapy between tPA and vapirost, may provide benefit to thrombolytic therapy.

In conclusion, the results of the present study indicate that vapirost is capable of preventing experimentally induced artery thrombosis. A combination of vapirost and tPA may be more effective than tPA alone in enhancing the outcome of thrombolytic therapy. Continuous therapy with vapirost after reperfusion would prevent the acute reocclusion after reperfusion.

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