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Evaluation of the disintegrin, triflavin, in a rat middle cerebral artery thrombosis model

Seiji Kaku [a,*], Kazuo Umemura [a], Atsuhiro Mizuno [a], Tomihisa Kawasaki [b], Mitsuyoshi Nakashima [a]

[a] *Department of Pharmacology, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-31, Japan*
[b] *Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., Tsukuba City, Ibaraki 305, Japan*

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Abstract

Platelet aggregation plays a important role in the thrombotic cerebral infarction. The final common mechanism in the formation of a platelet aggregate is the linking of adjacent platelets by fibrinogen binding to the platelet integrin $\alpha_{IIb}\beta_3$. In this study, we evaluated the effect of the disintegrin, triflavin, in a rat middle cerebral artery thrombosis model. Thrombus at the left middle cerebral artery in rat was induced by photochemical reaction between rose bengal and green light, which caused endothelial injury at the site of irradiation. We measured the time to occlusive thrombus formation and the size of ischaemic cerebral damage. Triflavin dose dependently prolonged the time to occlusive thrombus formation in this model. Triflavin also reduced the size of ischaemic cerebral damage on examination at 24 h after photochemical reaction. Triflavin dose dependently inhibited ADP- and collagen-induced platelet aggregation and platelet retention in the collagen-coated beads method ex vivo. These effects were thought to result from the blockade of platelet integrin $\alpha_{IIb}\beta_3$. Blockade of platelet integrin $\alpha_{IIb}\beta_3$ may be useful in the prevention of cerebral arterial thrombosis.

Keywords: Platelet aggregation; Cerebral ischemia; Thrombosis; (Rat)

1. Introduction

Platelet aggregation plays an essential role in normal hemostasis and in arterial thrombosis, where it is implicated in the pathogenesis of unstable angina, myocardial infarction and stroke (Davies, 1994). The final common mechanism in the formation of a platelet aggregate is the linking of adjacent platelets by fibrinogen binding to the platelet integrin $\alpha_{IIb}\beta_3$ (Plow and Ginsberg, 1988; Phillips et al., 1988). This step is thought to represent an excellent target for the development of antiplatelet agents.

Recently, a number of antagonists of platelet integrin $\alpha_{IIb}\beta_3$ have been studied in animal models and in clinical trials (Coller et al., 1995). All of these drugs are targeted at the thrombotic complications associated with acute procedures for ischaemic heart disease, such as thrombolysis and angioplasty. Although thrombotic cerebral infarction is also a good target for antiplatelet agents, no animal study or clinical trial of the effect of an antagonist of platelet integrin $\alpha_{IIb}\beta_3$ on this condition has been reported.

Triflavin is an arginine-glycine-aspartic acid-containing snake venom peptide first reported by Huang et al. (1991a). This peptide inhibits human platelet aggregation stimulated by various agonists by interfering with the interaction of fibrinogen with its specific receptor, platelet integrin $\alpha_{IIb}\beta_3$ (Huang et al., 1991a,b; Sheu et al., 1992). Further, it also inhibits platelet thrombus formation in mesenteric venules in mice (Sheu et al., 1994a).

We have developed a thrombosis model whereby the middle cerebral artery in an experimental animal is occluded by photochemical reaction between rose bengal and green light. This reaction causes endothelial injury followed by platelet adhesion, aggregation and formation of a platelet and fibrin-rich thrombus at the site of reaction (Umemura et al., 1993). In this study, we used this model to evaluate the antithrombotic effect of triflavin in the rat middle cerebral artery.

* Corresponding author. Tel.: (81-53) 435-2271; Fax: (81-53) 435-2270.

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2. Materials and methods

2.1. Materials

Rose bengal (Wako Pure Chemicals, Japan), ADP (MC Medical, Japan), Collagen (Moriya, Japan) was purchased commercially.

2.2. Preparation of snake venom peptide

Triflavin was isolated from the venom of *Trimeresurus flavoviridis* obtained from the Japan Snake Institute (Gunma, Japan). Purification was performed by a modification of Huang et al. (1991a) as follows: lyophilized *Trimeresurus flavoviridis* venom (1 g) was dissolved in 80 ml of 0.05 M ammonium acetate (pH 5.0) (buffer A). After centrifugation at $2500 \times g$ for 30 min, the supernatant was applied to a CM Sepharose Fast Flow column (X-K 16/20 column, Pharmacia-LKB, Uppsala, Sweden) equilibrated with buffer A, and eluted with a linear gradient from buffer A to 0.2 M ammonium acetate (pH 6.8) over 400 min at a flow rate of 1 ml/min. Fractions were assayed for their inhibitory activity on ADP-induced platelet aggregation in human platelet-rich plasma, and their purity was judged using sodium dodecyl sulfate/20%-polyacrylamide gel electrophoresis under reducing and non-reducing conditions as described previously (Laemmli, 1970). Homogeneous fractions were pooled, concentrated by ultrafiltration using an Amicon stir cell with a YM3 membrane, and dialyzed against saline. The *S*-pyridylethylated protein was digested with lysil endopeptidase and separated by reversed-phase high performance liquid chromatography. Amino-acid sequencing of separated peptides was performed by a protein sequencer. The protein was confirmed to be triflavin from N- and C-terminal amino-acid sequences and molecular mass. Molecular mass of the purified protein determined by mass spectrometry was 7618. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard, and stored frozen at -70°C until use.

2.3. Agonist-induced platelet aggregation

Male wistar rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). At various times after intravenous administration of triflavin, blood was withdrawn into a plastic syringe containing 3.8% sodium citrate (1:9 citrate/blood, v/v) from the abdominal aorta. Platelet-rich plasma and platelet-poor plasma was prepared by centrifugation of citrate-anticoagulated blood. Platelet counts in platelet-rich plasma were determined with an automatic cell counter (MEK-4150, Nihon Kohden, Tokyo, Japan), and were adjusted to a count of 5×10^8 platelets/ml with platelet-poor plasma. Platelet aggregation in platelet-rich plasma was measured using an aggregometer (NBS Hematracer model 601, Niko Bioscience, Tokyo, Japan), by recording the increase in light transmission through a stirred suspension of platelet-rich plasma maintained at 37°C. Aggregation was induced by ADP at 20 μM or collagen at 20 μg/ml.

2.4. Platelet retention in collagen-coated beads

Platelet retention was measured using a collagen-coated bead column (Pura beads column, ISK, Tokyo, Japan). Male wistar rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). At 5 min after intravenous administration of triflavin, blood was withdrawn into a plastic syringe from the abdominal vein. In order to measure basal platelet counts, 1 ml of blood were poured into sample cup containing EDTA. Then, the syringe was immediately connected to a syringe pump (ISK), and the blood was passed through the collagen-coated bead column at a rate of 2 ml/min for 30 s into sample cup containing EDTA. Platelet counts in both blood samples were measured using an automatic blood cell counter, and the percentage of platelet retention to the beads was calculated.

2.5. Middle cerebral artery thrombosis model in rats

Male Wistar rats weighing 220–270 g were anesthetized with halothane (3% induction/1% maintenance). Body temperature of the animals was maintained at 37.5°C with a heating pad. The middle cerebral artery thrombosis model in the rat has been described previously (Umemura et al., 1993, 1994). Briefly, a catheter for the administration of drug or rose bengal was inserted into the femoral vein. The scalp and temporalis muscle were folded over and a subtemporal craniotomy was performed using a dental drill under an operating microscope to open a 3 mm diameter oval bony window. The window was irradiated with green light (wave length 540 nm) achieved by using a xenon lamp (L4887, Hamamatsu Photonics, Hamamatsu, Japan) with a heat absorbing filter and a green filter. The irradiation was directed by a 3-mm diameter optic fiber mounted on a micromanipulator. The probe (1 mm diameter) of a laser doppler flowmeter (ALF21, Advance, Japan) was placed on the middle cerebral artery to measure middle cerebral artery blood flow. When a steady baseline flow was obtained, rose bengal (7.5 mg/kg) was injected intravenously 1 min after the injection of the drug. Photoirradiation was continued for a further 10 min. Blood flow in the middle cerebral artery was continuously monitored for 15 min after rose bengal injection. The middle cerebral artery was considered to be occluded when blood flow had completely stopped as indicated by the flow monitor. The time taken from the injection of rose bengal to the cessation of blood flow was recorded as the middle cerebral artery occlusion time. When blood flow continued beyond the 15-min observation period, the occlusion time was taken as 15 min (maximum).

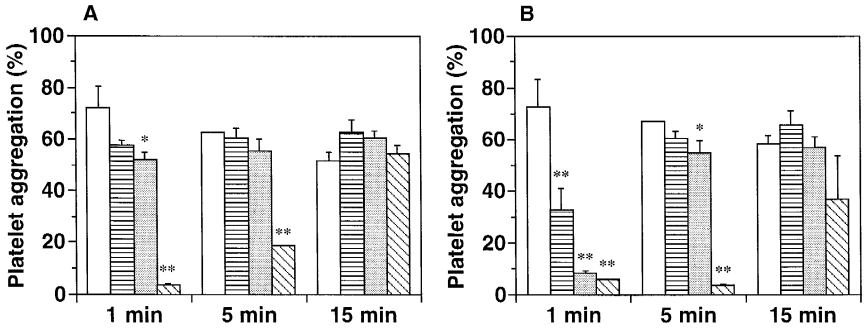

Fig. 1. Inhibitory effect of triflavin on ex vivo ADP (A)- and collagen (B)-induced platelet aggregation in platelet-rich plasma from rats; i.v. bolus of triflavin at a dose of 0.1 (striped), 0.3 (dotted) and 1 (hatched) mg/kg and saline (open). Values are the means ± S.E.M. for each group of 3 animals. * $P < 0.05$, ** $P < 0.01$ versus the control group.

2.6. Preparation of histological specimens

About 24 h after surgery, the brain was quickly removed under pentobarbital anesthesia. The cerebrum was separated and coronally sectioned into 1-mm thick slices from the frontal lobe with a microslicer (D.S.K. DTK-300, Kyoto, Japan). Six consecutive slices were then stained with 1% triphenyltetrazolium chloride (Katayama, Nagoya, Japan) and subsequently photographed. For each animal, the area of infarction was measured using a computerized image analysis system (Videoplane, Germany), and the ratio of infarction area to the whole area of the corresponding cerebrum was calculated.

2.7. Statistical analysis

Data are expressed as means ± S.E.M. Analysis of variance was used for comparison between groups. $P < 0.05$ was considered significant.

3. Results

3.1. Ex vivo platelet aggregation and platelet retention in rats

Isolated triflavin inhibited ADP- and collagen-induced platelet aggregation with IC_{50} values of 75 and 53 nM in human platelet-rich plasma and 540 and 590 nM in rat platelet-rich plasma in vitro. The results of the inhibitory effect of triflavin on ex vivo platelet aggregation are shown in Fig. 1. Triflavin rapidly and dose dependently inhibited ADP- and collagen-induced platelet aggregation in rats, with almost complete inhibition seen at a dose of 1 mg/kg at 1 min after intravenous administration. These inhibitory effects of triflavin decreased rapidly and were not statistically significant at 15 min after administration even at a dose of 1 mg/kg. The result of ex vivo platelet retention in collagen-coated beads are shown in Fig. 2. Triflavin dose dependently inhibited platelet retention, with this effect being significant at a dose of 1 mg/kg ($P <$ 0.01).

3.2. Middle cerebral artery thrombosis

Physiological parameters prior to rose bengal injection were within normal limits (mean arterial blood pressure 107.8 ± 1.7 mmHg; PO_2 121.2 ± 2.1 mmHg; PCO_2 44.1 ± 0.2 mmHg; pH 7.43 ± 0.01). There was no significant difference in middle cerebral artery blood flow before rose bengal injection among the different groups (data not shown). In the control group, the middle cerebral artery was completely occluded within the 15-min observation period in all animals, with the time taken from the injection of rose bengal to the cessation of middle cerebral

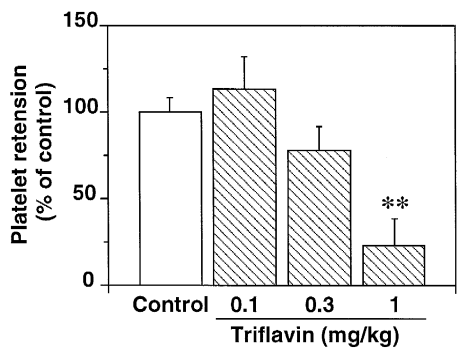

Fig. 2. Effect of triflavin on ex vivo platelet retention in rats. Values are the means ± S.E.M. for each group of 3 animals. ** $P < 0.01$ versus the control group.

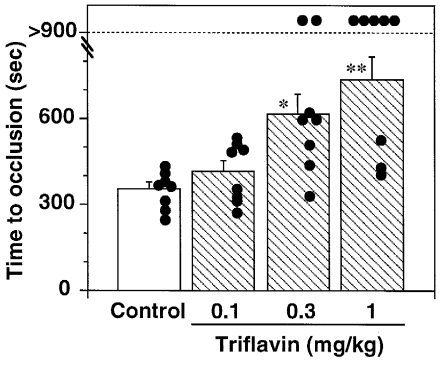

Fig. 3. Effect of triflavin on the formation of occlusive thrombus. Closed circles indicate the time to occlusive thrombus in each animal. Columns indicate the means ± S.E.M. for each group of 8 animals.

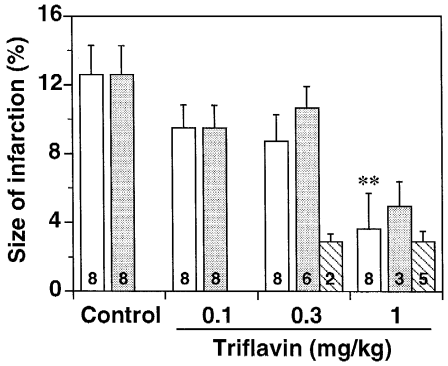

Fig. 4. Effect of triflavin on the percentage of infarcted area 24 h after induction of cerebral ischemia by thrombotic occlusion of the middle cerebral artery. Results are expressed as infarcted ratio, which is infarcted area divided by the whole area. Columns represent the data from total (open), occluded (dotted) and patent (hatched) animals within the observation period. Numbers in the column indicate the number of animals in each column. Values are the means ± S.E.M. ** $P < 0.01$ versus the control group.

artery blood flow being 353.4 ± 2.7 s. Triflavin significantly prolonged the time taken from the injection of rose bengal to the cessation of middle cerebral artery blood flow at doses of 0.3 and 1 mg/kg compared with control group ($P < 0.05$ and $P < 0.01$, respectively, Fig. 3). At the higher dose, triflavin also decreased the occlusion rate; two of eight animals at a dose of 0.3 mg/kg and five of eight animals at a dose of 1 mg/kg did not occlude during the observation period.

The size of ischaemic cerebral damage at 24 h after photochemical reaction is shown in Fig. 4. Infarction size at a dose of 1 mg/kg was significantly smaller than that of the control. Moreover, at a dose of 1 mg/kg, size of cerebral infarction in occluded animals was also decreased.

4. Discussion

In this study, we found that a disintegrin, triflavin, effectively prevented occlusive thrombus formation in rat middle cerebral artery and reduced the size of ischaemic cerebral damage. To our knowledge, this is the first report of the effect of a disintegrin which can block the fibrinogen binding to the platelet integrin $\alpha_{IIb}\beta_3$ on cerebral artery thrombosis and cerebral infarction. The reason these antagonists have not been evaluated in the cerebral artery thrombosis model may be their species specificity (Cox et al., 1992; Cook et al., 1993a,b) and difficulty in preparing the cerebral artery thrombosis model in responsive species. For example, inhibitory activity of which compound in rats was much less potent than that in humans, monkeys, dogs and guinea pigs. The cerebral artery thrombosis model has been reported only in rats (Umemura et al., 1993). In one variation (Umemura et al., 1993; Nakayama et al., 1988), photochemical reaction between rose bengal and green light causes the formation of a single molecular oxygen that damages the endothelium (Vandeplassche et al., 1990). Platelets consequently adhere and aggregate on the damaged vessel, resulting in the formation of an occlusive thrombus at the site of photochemical reaction.

Triflavin dose dependently prolonged the time to occlusive thrombus formation in the rat middle cerebral artery, and improved patency after photochemical reaction. This effect was thought to result from the inhibition of platelet aggregation. The duration of this inhibitory effect of triflavin was very short, and was not significant at 15 min after injection of triflavin even at a dose of 1 mg/kg. Rapid clearance of the snake venom-derived disintegrin from circulation has been reported elsewhere (McLane et al., 1995). These results suggest that the inhibition of platelet aggregation at the early phase of photochemical reaction or endothelial injury may be important to the improvement of the patency of the vessel.

Triflavin dose dependently decreased the size of ischaemic cerebral damage. The effect of triflavin at a dose of 0.3 mg/kg was dependent on the patency of the middle cerebral artery. At a dose of 1 mg/kg, the size of ischaemic cerebral damage was decreased even in the occluded animals. The mechanisms of this decrease are unclear; we speculate that occluded vessels may reperfuse in these animals earlier than in others. A significant correlation between the time of reopening of the middle cerebral artery and area of cerebral infarction has been reported in this model (Umemura et al., 1993). However, the possibility that triflavin acts via mechanisms independent of blockade of the platelet integrin $\alpha_{IIb}\beta_3$ cannot be entirely ruled out because triflavin has broad cross reactivity to other integrins. Triflavin inhibits adhesion of tumor cells to a variety of extracellular matrix components (Sheu et al., 1994b).

In conclusion, triflavin inhibited agonist-induced platelet aggregation and also inhibited platelet retention in the collagen-coated beads method. These effects resulted in the prolongation of time to occlusive thrombus formation and a decrease in size of cerebral infarction. Antagonists of platelet integrin $\alpha_{IIb}\beta_3$ may have therapeutic potential in the prevention of the cerebral thrombosis and cerebral infarction.

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