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A comparative study of the antithrombotic effect of aurintricarboxylic acid on arterial thrombosis in rats and guinea-pigs

[1]Yoshiharu Takiguchi, Masamitsu Shimazawa & Mitsuyoshi Nakashima

Department of Pharmacology, Hamamatsu University School of Medicine, Hamamatsu 431-31, Japan

1 The antithrombotic effect of aurintricarboxylic acid (ATA) which inhibits binding of von Willebrand factor (vWF) to platelet glycoprotein Ib (GPIb) receptor was evaluated in photochemically-induced thrombosis models in the femoral artery of rats and guinea-pigs.

2 ATA at a dose of 10 mg kg^{-1} significantly prolonged the time required for thrombotic occlusion of the artery in rats. The antithrombotic efficacy was associated with a significant inhibition of platelet retention and *ex vivo* botrocetin-induced platelet aggregation.

3 On the other hand, in guinea-pigs, ATA at the same dose inhibited the platelet retention and the platelet aggregation, but did not prevent thromboocclusion.

4 ATA inhibited aggregation of washed platelets from rats or guinea-pigs in response to botrocetin and thrombin in a dose-dependent manner (1-30 μM), and to the same extent.

5 ATA moderately increased activated partial thromboplastin time and bleeding time in both species.

6 These results indicate that vWF may play a role in the development of occlusive arterial thrombosis in the rat, but not in the guinea-pig.

7 The antithrombotic activity of ATA may partly arise from its inhibitory effect on thrombin, in addition to that on the vWF-GPIb pathway.

Keywords: von Willebrand factor; aurintricarboxylic acid; thrombin; arterial thrombosis; platelet aggregation

Introduction

Platelet-dependent thrombus formation is preceded by anchoring of platelets to the site of vascular injury. The initial event, platelet adhesion to subendothelium is mediated by von Willebrand factor (vWF) through its binding to platelet glycoprotein Ib (GPIb) receptor (Sakariassen *et al.*, 1986). The important role of the vWF-GPIb pathway in the development of arterial thrombosis has been shown in an *in vivo* study in pigs with von Willebrand disease caused by a defect in platelet GPIb receptors (Nichols *et al.*, 1986). This result was also supported by the findings that a monoclonal antibody against vWF and an antagonist against GPIb prevented arterial thrombosis in coronary stenosis models in pigs (Bellinger *et al.*, 1987), baboons (McGhie *et al.*, 1994) and dogs (Yao *et al.*, 1994).

We have established new arterial thrombosis models in the femoral artery of rats and guinea-pigs, in which a photochemical reaction between rose bengal and green light is employed to produce endothelial injury (Matsuno *et al.*, 1991; Takiguchi *et al.*, 1992a). Rose bengal is known to be one of the most efficient photodynamic generators of molecular oxygen singlet. This highly reactive form of oxygen may react with structural proteins and lipids in cellular membranes to initiate a sequence of direct peroxydation reactions leading to endothelial damage (Saniabadi *et al.*, 1995). Previously we have demonstrated different roles of some thrombogenic mediators between rats and guinea-pigs with these models (Takiguchi *et al.*, 1992b; 1995a; Hirata *et al.*, 1993). Thrombin was found to be responsible for occlusive thrombus formation in the rat, but not in the guinea-pig, whereas ADP is the main mediator in the guinea-pig, but is of minor importance in the rat. Thromboxane A_2 has a major role in both animals. However, the role of vWF has never been clarified.

Aurintricarboxylic acid (ATA) has been shown to be a potent inhibitor of both ristocetin-induced, vWF-mediated platelet agglutination and shear-induced, vWF-mediated platelet aggregation (Phillips *et al.*, 1988). This inhibition results from the binding of ATA to vWF, not to platelets, which blocks the interaction between vWF and GPIb (Girma *et al.*, 1992). The effectiveness of ATA in preventing platelet-dependent thrombus formation has been demonstrated in canine and rat models (Strony *et al.*, 1990; Kawasaki *et al.*, 1994). In the present study we evaluated the antithrombotic effect of ATA in the arterial thrombosis models of the femoral artery of rats and guinea-pigs to clarify the possible role of vWF.

Methods

Male Wistar rats weighing between 250 and 290 g and Hartley guinea-pigs weighing between 390 and 500 g were anaesthetized with sodium pentobarbitone (50 mg kg^{-1} for rats and 40 mg kg^{-1} for guinea-pigs, i.p.). The body temperature of the animals 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 a femoral artery has been described previously in detail (Matsuno *et al.*, 1991; Takiguchi *et al.*, 1992a). Briefly, a part of the femoral artery was carefully separated and a pulsed Doppler flow probe (PVD-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

[1]Author for correspondence at present address: Department of Clinical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokushima, 1-78 Sho-machi, Tokushima 770, Japan.

(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. At the end of some experiments, the animals were killed with an overdose of pentobarbitone and the irradiated segments of femoral arteries were excised for histopathological observation. These segments were immediately fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer for 1 h. Each segment was cut open longitudinally and processed for routine scanning with an electron microscope.

Drug treatment

ATA (Sigma, U.S.A.) was dissolved in 0.1 M phosphate buffer, pH 7.4, and administered intravenously 5 min before the injection of rose bengal or blood collection. The maximal dosage of ATA was determined so as not to affect haemodynamics, i.e., blood pressure, pulse rate and blood flow of the femoral artery. In the control group vehicle was administered in an equivalent volume.

Platelet retention to collagen-coated bead

Blood (2.5 ml) was drawn without anticoagulant from the abdominal aorta of animals treated with ATA or vehicle. Immediately, 1 ml of the blood was collected into a sample cup with EDTA-2K. The remaining fresh blood was pulled through a microadhesion column with type I collagen coated-plastic beads (Igaku Shoin, Japan) by a constant output pump (Igaku Shoin, Japan) at a flow rate of 2 ml min^{-1}, and collected into a sample cup with EDTA-2K. Platelets were counted in the pre and post column samples by a cell counter (MEK-4150, Nihon Kohden, Japan). Platelet retention was expressed as percentage decrease in platelet count from the precolumn sample.

Bleeding time

Bleeding time was determined by the methods of tail incision for rats and ear incision for guinea-pigs as we previously described (Takiguchi *et al.*, 1995a,b). Uniform incisions were made with a template blade device (Simplate, Organon Teknika Co., U.S.A.), 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 times were measured in the same animals treated with ATA or vehicle for 5 min before the experiment.

Platelet aggregation

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 with botrocetin (Sigma, U.S.A.), thrombin (Mochida, Japan) or collagen (Nicomed Arzneimittel, Germany) in the presence of 1.5 mM Ca^{2+}. After incubation with ATA for 2 min, aggregation in response to each stimulant was measured for 5 min by the turbidimetric method with a NBS hematracer 601 (Niko Bioscience, Japan).

In another *ex vivo* platelet study, platelet-rich plasma (PRP) was prepared and aggregation was induced by botrocetin.

Statistical analysis

Results are expressed as mean ± s.e. The occlusion time data were analyzed by nonparametric Williams-Wilcoxon's test. Other data were statistically analyzed by Student's *t* test when only two groups were involved. Comparison of more than two groups was performed by analysis of variance followed by Dunnett's multiple range test. Results were considered to have a significant difference if $P < 0.05$.

Results

Effect on thrombus formation

Figure 1 shows the antithrombotic effect of ATA on photochemically-induced arterial thrombosis in the femoral artery of rats and guinea-pigs. Typical changes in blood flow of the irradiated femoral artery of rats are shown in Figure 2. In the vehicle group of rats, the blood flow of the irradiated femoral artery was completely occluded by the formation of a platelet-rich thrombus 5.5 ± 1.0 min ($n = 6$) after the injection of rose bengal under green light irradiation, and occlusion persisted throughout the observation period (Figure 2a). ATA prolonged the time required to achieve occlusion in rats in a dose-dependent manner. In all rats receiving 10 mg kg^{-1} ATA, blood flow variation was scarcely observed and complete ces-

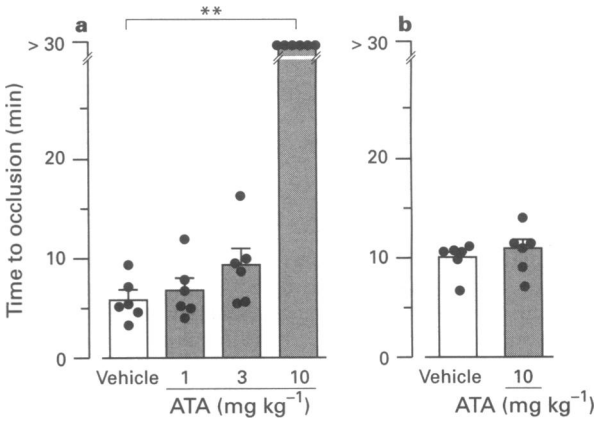

Figure 1 Effects of aurintricarboxylic acid (ATA) on the time required to achieve thrombotic occlusion of the femoral artery of rats (a) and guinea-pigs (b). (●) Indicate time to occlusion of each animal. Columns indicate mean (± s.e.) time to occlusion in each group. ATA was administered i.v. 5 min before the injection of rose bengal. **P < 0.01 versus vehicle.

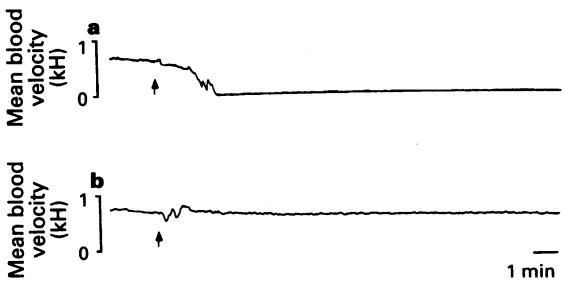

Figure 2 Recordings of typical changes in mean blood flow velocity of the irradiated femoral artery of two rats receiving vehicle (a) and aurintricarboxylic acid (ATA) at a dose of 10 mg kg^{-1} (b) after the i.v. injection of rose bengal (arrow) under green light. Thrombosis was induced by the photochemical reaction between green light and rose bengal injection. ATA was administered 5 min before the experiment.

sation of the blood flow could not be found during the 30 min of observation (Figure 2b). Scanning electron microscopy of the irradiated nonocclusive femoral arteries from rats treated with 10 mg kg^{-1} ATA showed adherent platelets on the luminal surface without thrombus formation (Figure 3).

In guinea-pigs, occlusive thrombus was formed with a mean time of 10.0 ± 0.7 min ($n=6$) in the vehicle-treated group. ATA did not affect the time to occlusion (10.8 ± 0.9 min, $n=6$) at a dose of 10 mg kg^{-1} (Figure 1).

Effect on platelet retention

The inhibitory effect of ATA on *ex vivo* platelet retention to collagen-coated bead column in rats and guinea-pigs is shown in Table 1. ATA significantly inhibited the platelet retention in rats at 10 mg kg^{-1}, but not at 1 and 3 mg kg^{-1}. In guinea-pigs ATA at 10 mg kg^{-1} inhibited the platelet retention, to the same extent as in rats.

Effect on platelet aggregation

Figure 4 shows the effects of ATA on aggregation of washed platelets from rats and guinea-pigs in response to botrocetin, thrombin and collagen. The concentration of each stimulant was determined to induce about 60% aggregation in rats and guinea-pigs, respectively. ATA inhibited rat platelet aggregation in response to botrocetin (0.75 $\mu g\ ml^{-1}$) and thrombin (0.2 u ml^{-1}) in a concentration-dependent manner, with mean IC_{50} values (the inhibitory concentration needed to cause 50% of inhibition) of 4.1 and 6.0 μM, respectively. ATA inhibited botrocetin (2.4 $\mu g\ ml^{-1}$)- and thrombin (0.1 u ml^{-1})-induced

Table 1 Effect of aurintricarboxylic acid (ATA) on platelet retention in rats and guinea-pigs

	n	*Platelet retention (%)* *Rat*	*Guinea-pig*
Vehicle	5	28.7 ± 1.8	38.8 ± 9.0
ATA 1 mg kg^{-1}	5	26.8 ± 2.1	
3 mg kg^{-1}	5	26.4 ± 1.9	
10 mg kg^{-1}	5	4.5 ± 2.6**	6.3 ± 4.3**

Results are shown as mean $\pm$ s.e. **$P<0.01$ versus vehicle.

Figure 3 The luminal surface of a nonocclusive femoral artery of rat receiving aurintricarboxylic acid (10 mg kg^{-1}). The artery was fixed 30 min after injection of rose bengal. Note the presence of adherent platelets on the injured luminal surface. Bar = 5 μm.

aggregation of washed platelets from guinea-pigs with similar IC_{50} values (4.2 and 5.1 μM, respectively) as those in rats. However, ATA even at 30 μM did not inhibit platelet aggregation in response to collagen (20 μg ml^{-1} for rats; 10 μg ml^{-1} for guinea-pigs) in both species.

ATA at a dose of 10 mg kg^{-1} almost completely inhibited platelet aggregation *ex vivo* in response to botrocetin (3 μg ml^{-1} for rats; 10 μg ml^{-1} for guinea-pigs) in both species (Table 2).

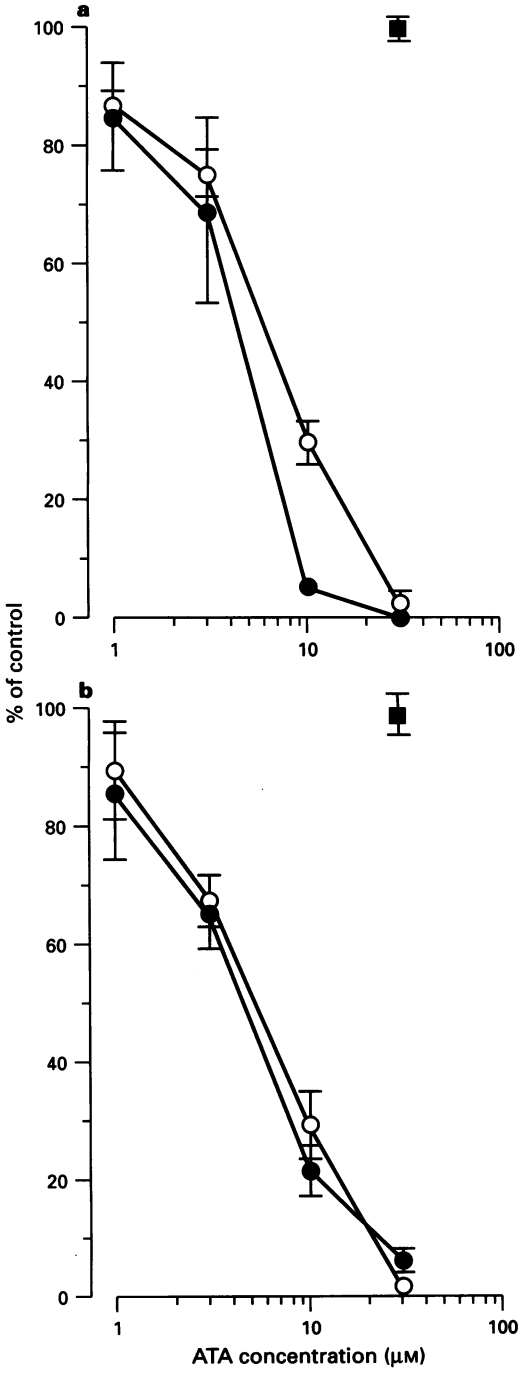

Figure 4 Concentration-response relationships for the inhibitory effects of aurintricarboxylic acid (ATA) on aggregation of washed platelets from rats (a) and guinea-pigs (b). After incubation with ATA at a dose range of 1–30 μM or vehicle for 2 min, platelets were stimulated with botrocetin (●, 0.75 μg ml^{-1} for rats and 2.4 μg ml^{-1} for guinea-pigs), thrombin (○, 0.2 u ml^{-1} for rats and 0.1 u ml^{-1} for guinea-pigs) or collagen (■, 20 μg ml^{-1} for rats and 10 μg ml^{-1} for guinea-pigs). Points show mean and vertical lines ± s.e. of 6 animals.

Effects on coagulation and bleeding time

The results on APTT and bleeding time in rats and guinea-pigs are shown in Table 3. ATA at 10 mg kg^{-1} caused moderate prolongation of APTT and the bleeding time (1.8 to 2.7 fold increase) in both species.

Discussion

Von Willebrand factor plays an important role in normal haemostasis (Ruggeri & Zimmerman, 1987) and, perhaps, in the development of thrombotic vascular disease (Nichols *et al.*, 1991). Binding of vWF to GPIb receptor on the platelet surface initiates platelet adhesion (Sakariassen *et al.*, 1986), which results in platelet activation, secretion and aggregation (Kroll *et al.*, 1991). Thus, inhibition of the initial contact between vWF and the platelet GPIb may be effective in preventing platelet-dependent thrombus formation. In the present study, with photochemically-induced thrombosis models in the femoral artery of rats and guinea-pigs, we demonstrated that ATA which blocks the platelet GPIb recognition site on vWF (Phillips *et al.*, 1988) potently inhibited thrombus formation in the rat, but not in the guinea-pig. It is likely that the relative role of the vWF-GPIb pathway in the development of occlusive thrombosis is different between rats and guinea-pigs.

Intravenous injection of ATA at 10 mg kg^{-1} significantly inhibited the thrombotic occlusion and the *ex vivo* platelet retention and botrocetin-induced aggregation in rats. The effective dosage was the same as that found by Strony *et al.* (1990) to prevent thrombosis in a canine model of constricted coronary artery. Recently Kawasaki *et al.* (1994) also found that ATA at 10 mg kg^{-1} exhibited antithrombotic activity with an inhibition of platelet retention in an electrically-in-

Table 2 Effects of aurintricarboxylic acid (ATA) on *ex vivo* platelet aggregation in response to botrocetin in rats and guinea-pigs

	n	*Platelet aggregation* (%)
Rat		
Vehicle	3	63.0±5.5
ATA (10 mg kg^{-1})	3	2.0±0.6**
Guinea-pig		
Vehicle	3	81.3±6.4
ATA (10 mg kg^{-1})	3	6.0±2.5**

Platelet aggregation was induced by botrocetin at 3 μg ml^{-1} for rats and 10 μg ml^{-1} for guinea-pigs. Results are shown as mean ± s.e. **$P<0.01$ versus vehicle.

Table 3 Effects of aurintricarboxylic acid (ATA) on activated partial thromboplastin time (APTT) and bleeding time in rats and guinea-pigs

	n	*APTT* (s)	*Bleeding time* (min)
Rat			
Vehicle	5	29.4±1.2	4.9±0.2
ATA (10 mg kg^{-1})	5	79.1±4.3**	9.7±0.6**
Guinea-pig			
Vehicle	5	28.7±0.9	5.0±0.4
ATA (10 mg kg^{-1})	5	69.1±2.4**	9.3±0.8**

Results are shown as mean ± s.e. **$P<0.01$ versus vehicle.

duced thrombosis model of rat carotid artery. From these results vWF is considered to play an important role in occlusive thrombus formation in the rat femoral artery model.

However, in guinea-pigs, ATA at a dose of 10 mg kg^{-1} inhibited the platelet retention and the platelet aggregation induced by botrocetin, and increased bleeding time, to the same extent as in rats, but did not prolong the time to occlusion. The failure of ATA to prevent thrombotic occlusion may be due to factors other than vWF and GPIb. This hypothesis is supported by the clinical observation that some patients with von Willebrand disease still develop acute myocardial infarction (Humphries *et al.*, 1992). The data suggest that a blockade of the vWF-GPIb pathway cannot necessarily prevent arterial thrombosis.

The interaction of vWF with GPIb induces platelet aggregation and thrombus formation under high shear conditions (Moake *et al.*, 1988; Ikeda *et al.*, 1991). Vasospasm affects the shear stress in an artery so that platelet activation would be influenced by vWF. Our previous studies suggested the involvement of platelet-mediated local vasoconstriction at the site of arterial injury in the thrombotic occlusion in the rat, but not in the guinea-pig (Takiguchi *et al.*, 1992b; 1995b). A thromboxane A_2 receptor antagonist, vapiprost and a 5-HT_2 receptor antagonist, ketanserin showed powerful antithrombotic effects compared to their antiplatelet effects in the rat model, as compared with other different antiplatelet drugs. The potent effects of these drugs were considered to reflect their abilities to inhibit vascular contraction induced by the platelet-derived factors in addition to their anti-platelet actions. However, such beneficial effects of these drugs could not be found in the guinea-pig model. From these findings, the thrombus formation in the rat could be considered to depend on the high shear stress induced by vascular contraction, which is a possible reason for the efficacy of ATA.

ATA inhibited platelet aggregation in response to botrocetin and thrombin in both animals, which was consistent with the results of a study by Kinlough-Rothbone & Packham (1992). Interestingly, the IC_{50} values against thrombin and botrocetin were almost the same. Therefore, ATA possibly acts as a thrombin inhibitor *in vivo*. APTT was increased in ATA treated-animals. The inhibitory effect of ATA on coagulation is probably due to inhibition of the activity of thrombin. Our previous study demonstrated that a direct thrombin inhibitor, CX-397 prevented thrombotic occlusion in the rat thrombosis model and suggested that thrombin plays an important role in thrombogenesis as a potent activator of platelets, perhaps rather than as the primary mediator of coagulation (Takiguchi *et al.*, 1995a). Therefore, it is possible that the efficacy of ATA in the rat model may partly arise from its inhibitory effect on thrombin, in addition to that on vWF-GPIb binding. Thrombin is believed not to participate in thrombotic occlusion in the guinea-pig model (Hirata *et al.*, 1993); therefore, this may partly account for the ineffectiveness of ATA in the guinea-pig. Morphological observation showed injured endothelium covered with adherent platelets in ATA-treated rats without thrombus formation. This is consistent with platelet adhesion at the injured site in pigs with von Willebrand disease (Nichols *et al.*, 1986). Platelet aggregation, rather than adhesion, may be important for thrombus formation and growth.

In conclusion, ATA exhibited potent antithrombotic activity in the photochemically induced thrombosis model in the rat femoral artery. This finding suggests a role of vWF in thrombogenesis in the rat. ATA inhibited platelet aggregation in response to thrombin as well as that to botrocetin. Therefore, the antithrombotic activity of ATA may be, in part, due to its inhibitory effect on thrombin. On the other hand, ATA had no effect on thrombotic occlusion in the guinea-pig model. Therefore, the role of the vWF-GPIb pathway in the development of arterial thrombosis seems to be different between rats and guinea-pigs.

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