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Short communication

Synergistic effect of aurintricarboxylic acid and triflavin in a photochemically induced thrombosis model in rats

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Abstract

We report here the synergistic antithrombotic effect of aurintricarboxylic acid in combination with a snake venom-derived disintegrin, triflavin, in a photochemically induced thrombosis model in rats. The time to initiation of thrombus was prolonged by i.v. bolus injection of aurintricarboxylic acid at 10 mg/kg. In contrast, time to occlusion was dose-dependently prolonged by both agents, this prolongation being significant with aurintricarboxylic acid at 10 mg/kg i.v. and with triflavin at more than 3 mg/kg i.v. Interestingly, the combination of aurintricarboxylic acid at 3 mg/kg i.v. and triflavin at 1 mg/kg i.v. prolonged not only the initiation of thrombus, but also the time to occlusion.

Keywords: Aurintricarboxylic acid; Triflavin; Von Willebrand factor; Glycoprotein IIb/IIIa; Photochemically induced thrombosis

1. Introduction

Platelet thrombus formation is an important factor in thromboembolic diseases (Davies et al., 1986). The interaction between von Willebrand factor and platelet glycoprotein (GP) Ib plays a key role in the initial contact adhesion of platelets to exposed subendothelium (Sakariassen et al., 1987), and is followed by the formation of platelet aggregates via the linking of adjacent platelets by fibrinogen binding to the platelet GPIIb/IIIa receptor (Plow and Ginsberg, 1988). On this basis, substances which inhibit the binding of von Willebrand factor to GPIb or the binding of fibrinogen to GPIIb/IIIa are thought to possess promise as antithrombotic agents.

Among these, several specific inhibitors of the interaction of von Willebrand factor with GPIb have been reported (Peng et al., 1993; Yao et al., 1994; Fujimura et al., 1995). Aurintricarboxylic acid binds to large von Willebrand factor multimers but not to GPIb in vitro (Phillips et al., 1988), and effectively prevents thrombus formation in vivo (Strony et al., 1990; Bernat et al., 1994; Kawasaki et al., 1994a). Further, many GPIIb/IIIa receptor antagonists, including anti-GPIIb/IIIa monoclonal antibodies (Pidard et al., 1983; Coller et al., 1986), disintegrins (Yasuda et al., 1991) and disintegrin-like peptides (Scarborough et al., 1991) derived from snake venoms and non-peptide low molecular mass GPIIb/IIIa receptor antagonists (Peerlinck et al., 1993; Carteaux et al., 1993; Nicholson et al., 1995) have been reported so far. However, the antithrombotic effect of an inhibitor of von Willebrand factor-GPIb interaction and a GPIIb/IIIa receptor antagonist given in combination in vivo has not yet been reported.

Using a previously reported model of photochemically induced thrombus in which platelet-rich thrombi are induced in the microvasculature by the irradiation of filtered light in combination with intravascular administration of fluorescent sodium (Rosenblum and El-Sabban, 1977; Sato and Ohshima, 1984), we first evaluated the antithrombotic effect of aurintricarboxylic acid and a snake venom derived Arg-Gly-Asp-containing peptide, triflavin (Huang et al., 1991a). We next investigated the antithrombotic effect of aurintricarboxylic acid used together with triflavin in this model.

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2. Materials and methods

2.1. Materials

Aurintricarboxylic acid ammonium salt (lot CTE1737) was purchased from Wako Chemical Co. (Osaka, Japan) and dissolved in saline before use. Triflavin was isolated from the venom of *Trimeresurus flavoviridis*, obtained from the Japan Snake Institute (Gunma, Japan). Purification was performed by the method of Huang et al. (1991a). Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. The protein was stored frozen at −70°C until use.

2.2. Photochemically induced thrombosis model

Male Wistar rats weighing 230–320 g were anesthetized with sodium pentobarbital injected into the femoral muscle (60 mg/kg). The right jugular vein and artery were cannulated for the injection of dye and the monitoring of arterial blood pressure and heart rate, respectively. The small intestine was exteriorized via a midline incision in the abdominal wall into a bath and perfused with saline kept at 37°C. The mesentery around the ileum was spread out carefully on a small circular glass stage, and the other parts of the intestine were covered with moistened gauze. Microvessels in the mesentery were observed under transillumination with a halogen lamp for selection of venules of 41–62 μm diameter in which to produce microthrombi. Thrombus formation was induced in microvessels as previously described by Sato and Ohshima (1984) and by our group (Kaku et al., 1995; Kawasaki et al., 1994b). Briefly, filtered light at a wavelength of 420–490 nm was passed through an objective lens. Using a field stop, the area of irradiation around the microvessels was adjusted on the focal plane to a diameter of about 130 μm. The light intensity was controlled at 13.8 mW/mm^2.

The experimental protocol was as follows: the test agent was administered by i.v. bolus injection into the jugular vein. One minute after injection, irradiation with filtered light was started. At 1 min after the start of irradiation, a solution of sodium fluorescein (2.5% w/v, Nihon Alcon Co., Tokyo, Japan) was injected through the jugular vein (1 ml/kg body weight). Irradiation with the filtered light was continuously monitored with a TV camera and recorded on videotape for 30 min after the injection of sodium fluorescein. The time when the thrombus began to form (time to initiation) and the time when blood flow completely stopped (time to occlusion) were used as indexes of antithrombotic activity. Time was measured from playback of the video tape. If blood flow did not stop within 30 min, the result was calculated as 30 min. At the end of the experiments the rats were killed with an overdose of potassium chloride. These experiments were approved by the Animal Ethical Committee of Yamanouchi Pharmaceutical Co.

2.3. Statistical analysis

The experiments were performed on groups of 5–15 rats each. Data are expressed as the mean ± S.E.M. Statistical analyses were performed among groups using Dunnett multiple range test, with a P value less than 0.05 considered significant.

3. Results

3.1. Antithrombotic effect of aurintricarboxylic acid and triflavin in a photochemically induced thrombosis model

No significant differences in body weight or vessel diameter of rats were observed among the groups (data not shown), nor was any significant change in blood pressure or heart rate observed in the groups (data not shown). Platelet thrombus formation was clearly evident in all animals injected with saline.

Fig. 1 shows the time to initiation of thrombus after i.v. injection of sodium fluorescein. In the saline group, the time to initiation ranged from 0 min 09 s to 2 min 09 s (mean ± S.E.M.: 1 min 00 s ± 10 s). Aurintricarboxylic acid dose-dependently prolonged the time to initiation, this effect being significant at 10 mg/kg ($P < 0.01$). In contrast, triflavin had no effect on time to initiation of thrombus.

Fig. 2 shows the time to occlusive thrombus after i.v. injection of sodium fluorescein. In the saline group, the time to occlusion ranged from 2 min 44 s to 10 min 36 s (mean ± S.E.M.: 5 min 40 s ± 44 s). Both agents dose-dependently prolonged the time to occlusive thrombus formation. Significant prolongation of time to occlusion was

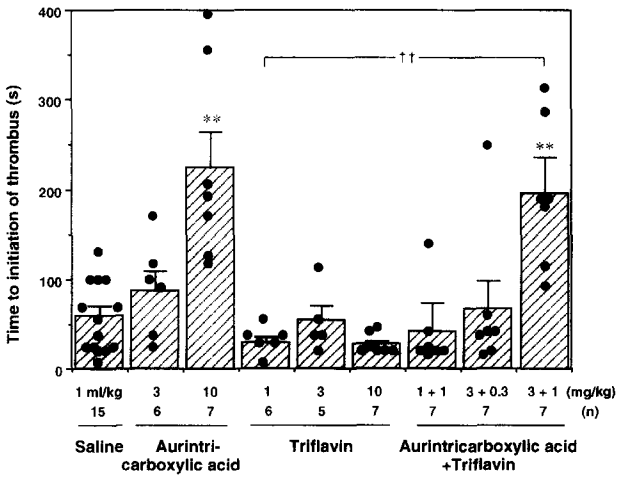

Fig. 1. Effects of aurintricarboxylic acid and triflavin on the time to initiation of thrombus in a photochemically induced thrombosis model in rats. Hatched columns indicate the means ± S.E.M. for 5–15 animals. Closed circles represent the time to initiation of thrombus in each rat. ** $P < 0.01$ vs. the saline group by Dunnett multiple comparison test. †† $P < 0.01$ vs. triflavin 1 mg/kg by Dunnett multiple comparison test.

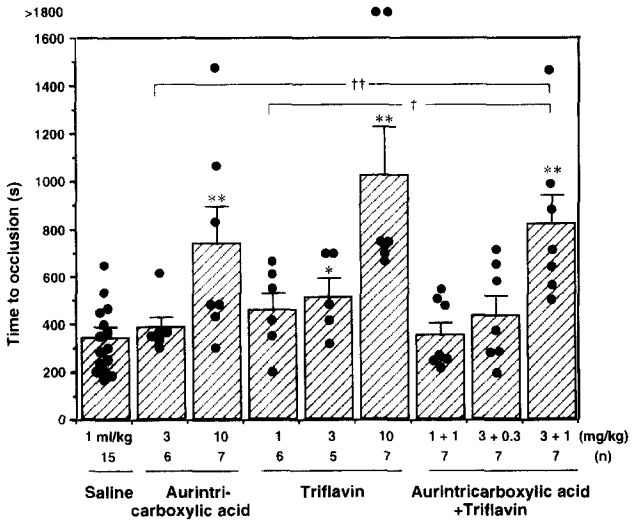

Fig. 2. Effects of aurintricarboxylic acid and triflavin on the time to occlusive thrombus in a photochemically induced thrombosis model in rats. Hatched columns indicate the means ± S.E.M. for 5–15 animals. Closed circles represent the time to occlusion in each rat. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. the saline group by Dunnett multiple comparison test. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. aurintricarboxylic acid 3 mg/kg or triflavin 1 mg/kg by Dunnett multiple comparison test.

observed with aurintricarboxylic acid at 10 mg/kg ($P < 0.01$) and with triflavin at 3 ($P < 0.05$) and 10 mg/kg ($P < 0.01$) compared with the saline group.

We next investigated the combination effect of aurintricarboxylic acid and triflavin in this model. The agents were consecutively administered (aurintricarboxylic acid first) by i.v. bolus injection into the jugular vein. The combination of aurintricarboxylic acid at 1 mg/kg with triflavin at 1 mg/kg or aurintricarboxylic acid at 3 mg/kg with triflavin 0.3 mg/kg had no significant effect on either time to initiation or time to occlusion. However, the combination of aurintricarboxylic acid at 3 mg/kg with triflavin 1 mg/kg significantly prolonged not only the time to initiation of thrombus ($P < 0.01$) but also the time to occlusion ($P < 0.01$). Significant differences were also seen in the time to initiation of thrombus between the combination of aurintricarboxylic acid at 3 mg/kg with triflavin at 1 mg/kg and that with triflavin at 1 mg/kg alone ($P < 0.01$), and in the time to occlusion between the combination of aurintricarboxylic acid at 3 mg/kg with triflavin at 1 mg/kg and that with aurintricarboxylic acid at 3 mg/kg ($P < 0.01$) or triflavin at 1 mg/kg ($P < 0.05$).

4. Discussion

In this study, we found that aurintricarboxylic acid and triflavin effectively prevented occlusive thrombus formation in a photochemically induced platelet-rich thrombosis model, and that the combination of both agents produced a synergistic antithrombotic effect. Although the single injection of these agents dose-dependently prolonged the time to occlusion, their pharmacological efficacy in this action was clearly distinct. The time to initiation of thrombus was prolonged only by aurintricarboxylic acid at 10 mg/kg. This indicates that aurintricarboxylic acid effectively inhibited platelet adhesion to damaged endothelium, whereas triflavin had no effect on platelet adhesion to endothelium but rather effectively inhibited the growth of the platelet thrombus after adhesion. Effective doses of aurintricarboxylic acid and triflavin in the present study were consistent with those of previous reports (Kawasaki et al., 1994a; Sheu et al., 1994). A surprising finding was that the combination of aurintricarboxylic acid at 3 mg/kg i.v. and triflavin at 1 mg/kg i.v. significantly prolonged not only the initiation of thrombus, but also the time to occlusion. As a significant effect on either variable was not produced by the single injection of aurintricarboxylic acid at 3 mg/kg or triflavin at 1 mg/kg, we consider that the combination of these agents produced a synergistic effect, probably resulting from the difference in the pharmacological action of the agents. To our knowledge, this is the first report to describe a synergistic antithrombotic effect of aurintricarboxylic acid and a GPIIb/IIIa antagonist in vivo.

For the present, several problems remain to be solved before the clinical use of inhibitors of von Willebrand factor-GPIb interaction and GPIIb/IIIa receptor antagonists. Several specific inhibitors of the interaction of von Willebrand factor with GPIb have now been suspended because of their induction of thrombocytopenia (Fujimura et al., 1995; Taniuchi et al., 1995; Kawasaki et al., 1995) or their instability in vivo. Further developmental study of aurintricarboxylic acid has also been suspended because of its unexpected in vivo toxicity. Furthermore, most GPIIb/IIIa receptor antagonists are reported to prolong bleeding time at the same dose at which they produce their antithrombotic action (Coller et al., 1989; Ramjit et al., 1993), a phenomenon which limits the clinical use of these compounds. Our results suggest that the usage of an inhibitor of the von Willebrand factor-GPIb interaction together with a GPIIb/IIIa receptor antagonist enables a decrease in the effective dose, and can produce a promising antithrombotic effect without a marked prolongation of bleeding time.

High-shear stress-induced platelet aggregation (h-SIPA) has recently come to be considered a clinically important phenomenon (Ikeda et al., 1991). Studies using a device to produce SIPA indicate that under high shear stress von Willebrand factor interacts with GPIb and subsequently binds to GPIIb/IIIa via its RGD region. Disintegrins including triflavin can block the binding of von Willebrand factor to GPIIb/IIIa (Plow et al., 1985) as well as that of fibrinogen to GPIIb/IIIa (Huang et al., 1991b), and inhibit h-SIPA in human platelet-rich plasma (Kawasaki et al., unpublished study). The synergistic effect of aurintricarboxylic acid and triflavin in the present study may have resulted from effective inhibition of both the interaction of

von Willebrand factor-GPIb by aurintricarboxylic acid and of the interaction of von Willebrand factor-GPIIb/IIIa as well as fibrinogen-GPIIb/IIIa by triflavin. Further studies are needed to confirm this.

In the present study, the antithrombotic effects of aurintricarboxylic acid and triflavin were evaluated in a photochemically induced thrombosis model. The method used (Rosenblum and El-Sabban, 1977; Sato and Ohshima, 1984) causes the formation of active oxygens that damage the endothelium (Sato et al., 1987). Consequently, platelets adhere to and aggregate on the damaged vessel, resulting in formation of an occlusive platelet thrombus. To date, we have used this model to evaluate the pharmacological efficacy of a mutant tissue-type plasminogen activator (Kawasaki et al., 1994b) and a humanized anti-GPIIb/IIIa monoclonal antibody (Kaku et al., 1995). This model permits continuous observation of the process of platelet adhesion to injured subendothelium and subsequent platelet aggregation in real time, and provides a useful tool for investigating the pharmacological action of antithrombotic agents.

In summary, aurintricarboxylic acid and triflavin effectively prevented thrombus formation in a photochemicaly induced thrombosis model, and the combination of both agents could produce a synergistic antithrombotic effect in vivo. The combination of a von Willebrand factor-GPIb inhibitor and a GPIIb/IIIa receptor antagonist may represent a safe and effective therapeutic regimen.

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