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Thromboxane A_2 synthase inhibitor enhanced antithrombotic efficacy of GPIIb–IIIa receptor antagonist without increasing bleeding

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Abstract

The advantage of platelet integrin GPIIb–IIIa receptor antagonists in the prevention of thrombotic occlusion was clearly proven in patients who underwent interventional treatment of the coronary artery, but its value in cerebral ischemia is still under investigation. The expectation of intracranial hemorrhage on strong inhibition of platelet function restricts its application in cerebral ischemia. To minimize bleeding while keeping antithrombotic activity, we have tried to find an appropriate approach using a combination of platelet integrin GPIIb–IIIa receptor antagonist and some other antithrombotic agents. The time to thrombotic occlusion was measured using a photothrombotic occlusion model of guinea pig middle cerebral artery. A platelet integrin GPIIb–IIIa receptor antagonist, ME3277 (sodium hydrogen [4-[(4,5,6,7-tetrahydrothieno [3,2-*c*] pyridin-2-yl) carbonylamino] acetyl-*o*-phenylene] dioxydiacetate), delayed occlusion time from 7.3 min in vehicle to 15.0, 20.6 and 25.9 min ($P < 0.05$) at 0.1, 0.3 and 1 mg/kg, respectively. ME3277 profoundly inhibited ex vivo platelet aggregation and the highest dose of ME3277 prolonged (3.5 folds, $P < 0.01$) the bleeding time measured in the hind paw. A thromboxane A_2 synthase inhibitor, sodium ozagrel, significantly delayed occlusion time to 19.5 min at 30 mg/kg ($P < 0.05$) while it did not affect bleeding time or platelet aggregation. ME3277 (0.1 mg/kg) in combination with 10 mg/kg sodium ozagrel synergistically delayed occlusion time (sodium ozagrel alone; 7.9 min, combination; 26.1 min, $P < 0.05$ vs. ME3277 alone). Sodium ozagrel did not affect ex vivo platelet aggregation or bleeding time when combined with 0.1 mg/kg of ME3277. This synergy was cancelled by combination with 30 mg/kg aspirin (14.7 min). A thromboxane A_2 receptor antagonist, vapiprost (0.1 mg/kg), did not enhance the antithrombotic efficacy of ME3277. These results imply that local prostacyclin production enhances the in vivo antithrombotic effect of the platelet integrin GPIIb–IIIa receptor antagonist. Therefore, the thromboxane A_2 synthase inhibitor allowed a reduction in the dose level of the platelet integrin GPIIb–IIIa receptor antagonist for cerebral thrombosis, which resulted in a reduced risk of bleeding.

Keywords: Platelet integrin GPIIb–IIIa receptor; Thromboxane A_2 synthase; Vessel occlusion; Bleeding time

1. Introduction

Recently, the common final pathway of platelet aggregation for all platelet stimulators was found to involve fibrinogen and integrin GPIIb–IIIa (Phillips et al., 1991). Therefore, antagonists of the platelet integrin GPIIb–IIIa receptor are expected to be useful for the treatment of cerebral thrombosis. While clinical studies did not show any crucial adverse effect of platelet integrin GPIIb–IIIa receptor antagonists, a possibility of intracranial hemorrhage has not been denied (The Abciximab in Ischemic Stroke Investigators, 2000). Therefore, investigators have attempted to develop agents that have potent antiplatelet activity as do platelet integrin GPIIb–IIIa receptor antagonists but with minimal bleeding.

We have investigated the mechanisms of in vivo thrombus formation in the guinea pig middle cerebral artery using a photothrombotic occlusion model. A thrombotic occlusion was induced by a photochemical reaction between Rose Bengal injection and transluminal photoirradiation. This reaction damaged the endothelium at the site of photoirradiation (Matsuno et al., 1991; Watson et al., 1985), which was followed by platelet adhesion and aggregation, and resulted in an occlusive thrombus at the artery. In a previous study, we found that the platelet integrin

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GPIIb–IIIa receptor antagonist did not sufficiently prevent vessel occlusion at the dose that profoundly inhibited platelet aggregation (Kawano et al., 1999). Several reports demonstrated that platelet integrin GPIIb–IIIa receptor antagonists did not prevent thromboxane A_2 generation in activated platelets at the dose that effectively inhibited platelet aggregation (Byrne et al., 1997; Carroll et al., 1997). Thrombus formation was accompanied by the release of thromboxane A_2 and several substances from activated platelets. These substances, in turn, facilitate platelet activation and constrict the vessel wall around the damaged region (Ellis et al., 1977; Hamberg et al., 1975; McGoon and Vanhoutte, 1984). Therefore, we speculated that vascular constriction would be mostly attributable to the photothrombotic occlusion of the guinea pig middle cerebral artery. Since the thromboxane A_2 synthase inhibitor did not prolong bleeding time (Patrono, 1990), the combination of GPII–IIIa receptor antagonist with the thromboxane A_2 synthase inhibitor could synergistically inhibit thrombus formation without affecting bleeding time.

2. Materials and methods

2.1. Animal preparation

The protocol was approved by the local Committee on ethics of animal experimentation and extra care was taken to avoid animal suffering. The experimental protocol to induce thrombotic occlusion in the middle cerebral artery was taken from our previous report and slightly modified (Kawano et al., 1999). Briefly, male Hartley guinea pigs weighing 300–450 g were anesthetized with 1% isoflurane in 30% O_2 and 70% N_2O mixture using a face mask. Animal body temperature was maintained at 38°C with a heating pad (K-module K-30, Baxter). The middle cerebral artery was observed under an operation microscope connected to a 3-CCD video camera. Photoirradiation with green light (wavelength, 520–620 nm) was achieved using a xenon lamp (model L-4887, Hamamatsu Photonics, Hamamatsu, Japan) with a heat absorbing filter and a green filter. The tip of a 3-mm-diameter optic fiber was placed on the middle cerebral artery that contains the proximal end of the lenticulostriate branch, providing an irradiation dose of 0.636 W/cm^2. A photochemical reaction was initiated 10 min after the intravenous administration of antithrombotic agents. The antithrombotic agents used in the present study were as follows; ME3277 (a platelet integrin GPIIb–IIIa receptor antagonist, sodium hydrogen [4-[(4,5,6,7-tetrahydrothieno [3,2-*c*] pyridin-2-yl) carbonylamino] acetyl-*o*-phenylene] dioxydiacetate, 0.1–1 mg/kg), sodium ozagrel (a thromboxane A_2 synthase inhibitor, 10 and 30 mg/kg), vapiprost (a thromboxane A_2 receptor antagonist, 0.1–3 mg/kg), aspirin (30 mg/kg) and heparin (200 IU/kg). Rose Bengal (10 mg/kg) was

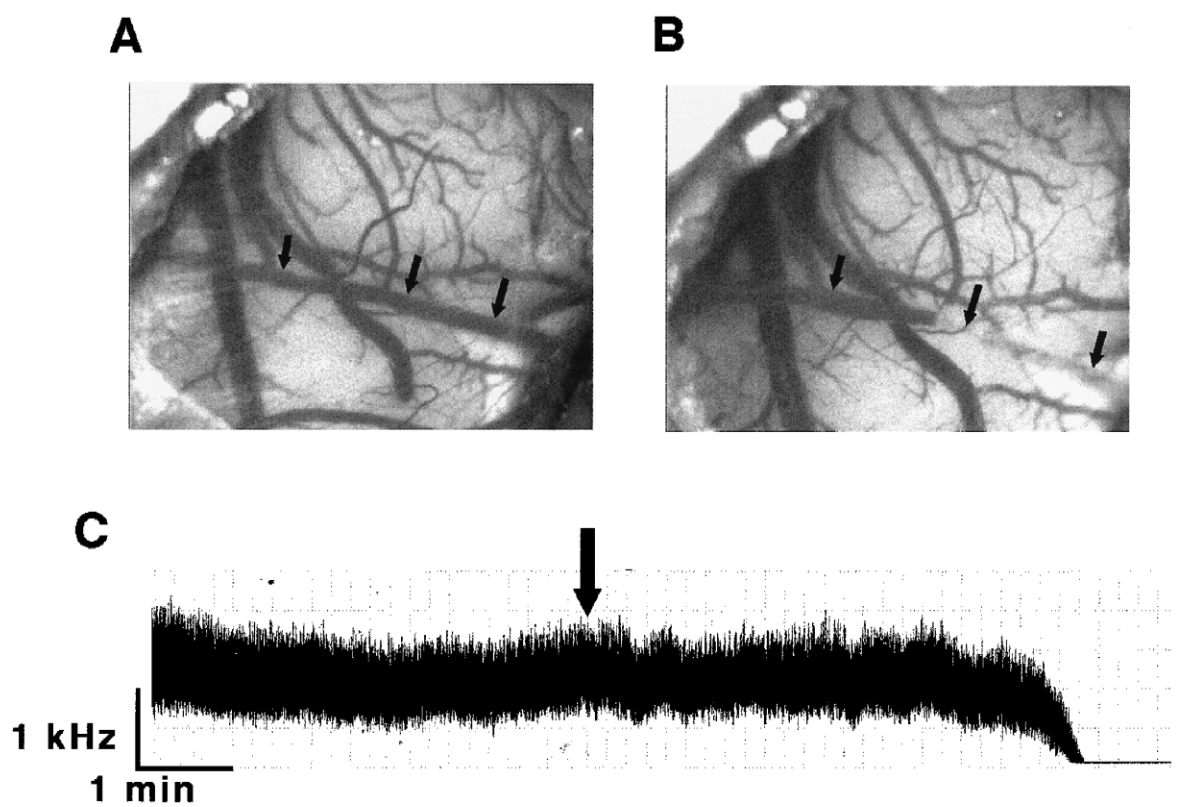

Fig. 1. Surgical microscopic view of the middle cerebral artery (A) before and (B) 10 min after the photochemical reaction in a vehicle-treated animal. The arrow indicates the middle cerebral artery. A typical recording of blood flow of guinea pig middle cerebral artery during thrombotic occlusion is shown in (C). The arrow indicates the start of the photochemical reaction.

injected via the jugular vein and subsequently photoirradiation was continued for 10 min. Blood flow in the middle cerebral artery was monitored for 30 min after Rose Bengal injection with a pulse Doppler flow probe (PVD-20, Crystal Biotech). The occlusion time was taken as the time to achieve zero flow of the middle cerebral artery from the start of Rose Bengal injection. When the middle cerebral artery did not occlude for 30 min, the occlusion time was taken as 30 min.

2.2. Ex vivo platelet aggregation in platelet-rich plasma

Each drug was intravenously administered to a series of animals under pentobarbital (30 mg/kg i.p.) anesthesia ($n = 4$). Twenty minutes after drug administration, blood samples were collected from the abdominal aorta using a syringe containing 1:9 sodium citrate (final 0.38%). Platelet-rich plasma was obtained by centrifugation of the blood samples at $150 \times g$ for 10 min at room temperature. Platelet-poor plasma was obtained by recentrifugation at $2000 \times g$ for 10 min. Platelets were counted with a cell counter (MEK-6158, Nihon Kohden, Japan) and adjusted to 300,000/μl. Platelet aggregation was measured according to the method of Born (1962) using an aggregometer (NBS Hematracer model 601, Niko Bioscience, Tokyo). Inducers of platelet aggregation and final concentrations were as follows; ADP, 5 μM; collagen, 2 μg/ml; arachidonic acid, 100 μM. The light transmission for the various inducers was recorded for 7 min after a 1-min preincubation period and the maximal platelet aggregation amplitude was measured.

2.3. Measurement of activated partial thromboplastin time

Heparin was administered via the right jugular vein under pentobarbital (30 mg/kg i.p.) anesthesia ($n = 4$). Twenty minutes after drug administration, a blood sample, 45 μl, was collected from a branch of the left jugular vein.

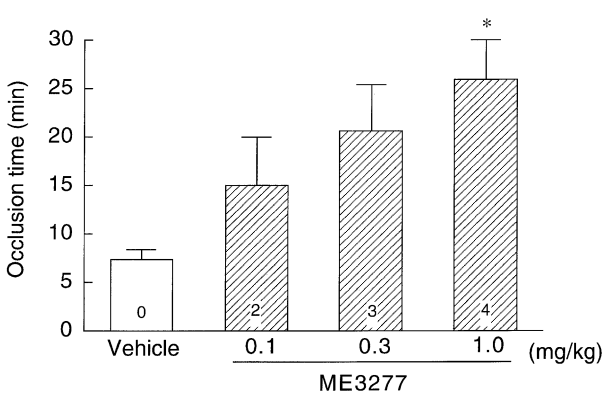

Fig. 2. Effect of ME3277 (0.1–1 mg/kg) alone on thrombotic occlusion of the middle cerebral artery. Occlusion time is presented as the mean ± S.E.M. ($n = 6$). Numbers inside each column indicate the number of animals in which occlusion time is > 30 min. $^{*}P < 0.05$ vs. vehicle-treated animals.

Table 1
Effects of antithrombotic agents on ex vivo platelet aggregation
Values are presented as the means ± S.E.M. ($n = 4$). Blood was collected 20 min after drug administration under pentobarbital anesthesia. Values are significantly different from those for the vehicle group at $^{a}P < 0.05$, $^{b}P < 0.01$ (Dunnett's test), $^{c}P < 0.05$ and $^{d}P < 0.01$ (Student's *t*-test). AA, arachidonic acid; ND, not determined.

Treatment	Aggregation (%)		
	ADP (5 μM)	Collagen (2 μg/ml)	AA (100 μM)
Vehicle	90.5 ± 3.5	86.3 ± 2.8	89.0 ± 1.9
ME3277 0.1 mg/kg	71.3 ± 7.3	74.5 ± 4.2	58.5 ± 19.5
0.3 mg/kg	20.3 ± 6.4^{b}	14.0 ± 8.1^{b}	15.8 ± 6.2^{b}
1.0 mg/kg	3.3 ± 1.0^{b}	4.8 ± 0.5^{b}	3.0 ± 0.6^{b}
Ozagrel Na 10 mg/kg	94.3 ± 5.4	91.8 ± 5.7	71.8 ± 24.2
30 mg/kg	86.8 ± 4.0	66.0 ± 18.9	87.3 ± 2.5
Aspirin 30 mg/kg	86.2 ± 5.6	45.3 ± 17.5^{c}	7.3 ± 1.3^{d}
Vapiprost 0.1 mg/kg	77.3 ± 4.3	71.0 ± 2.4	5.3 ± 0.5^{b}
0.3 mg/kg	84.3 ± 4.9	44.8 ± 23.9	8.8 ± 1.3^{b}
3.0 mg/kg	90.8 ± 3.2	30.0 ± 9.7^{a}	8.0 ± 2.4^{b}
ME3277 0.1 mg/kg + Ozagrel Na 10 mg/kg	70.8 ± 6.8^{c}	76.0 ± 4.2	33.8 ± 18.1^{c}

Activated partial thromboplastin time was measured with the CoaguCheck Plus system® (Boehringer Mannheim). An activated partial thromboplastin time of less than 18 s was taken as 18 s and more than 150 s was 150 s.

2.4. Effect of antithrombotic agents on bleeding time

The bleeding time was determined, using Ivy method (Ivy et al., 1941) under pentobarbital (30 mg/kg i.p.) anesthesia ($n = 4$). Twenty minutes after drug administration, the pad of the hind paw was cut with a Simplate® (Organon Teknika, USA). Blood from the incision was blotted at 30-s intervals until bleeding ceased (maximal

Table 2
Effects of antithrombotic agents on the bleeding time evaluated in guinea pig hind paw
Values are presented as the means ± S.E.M. ($n = 4$). Each drug was administered alone or in combination with 0.1 mg/kg ME3277. The hind paw was cut with a retractable blade 20 min after drug administration under pentobarbital anesthesia. Values are significantly different from those for the vehicle group at $^{a}P < 0.01$ (Dunnett's test), $^{b}P < 0.05$ and $^{c}P < 0.01$ (Student's *t*-test). ND, not determined.

Treatment	Bleeding time (min)	
	alone	+ ME3277
Vehicle	4.1 ± 0.6	–
ME3277 0.1 mg/kg	5.8 ± 1.3	–
0.3 mg/kg	8.1 ± 0.9	–
1.0 mg/kg	14.5 ± 1.5^{a}	–
Ozagrel Na 10 mg/kg	5.3 ± 0.8	5.9 ± 0.4
30 mg/kg	7.0 ± 0.5	ND
Aspirin 30 mg/kg	6.3 ± 1.0	5.6 ± 0.9
Vapiprost 0.1 mg/kg	5.6 ± 0.5	7.1 ± 0.9
3 mg/kg	7.5 ± 0.9	ND
Heparin 200 IU/kg	13.3 ± 3.4^{c}	11.6 ± 2.6^{b}

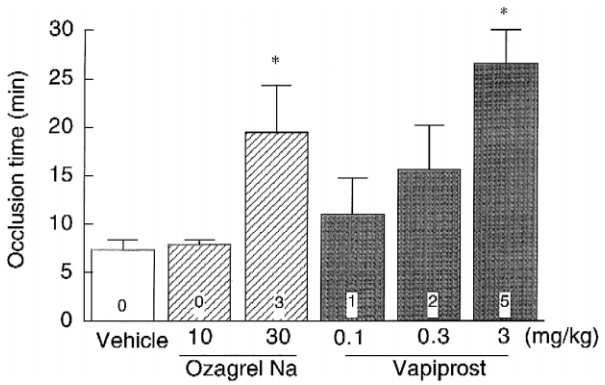

Fig. 3. Effect of vapiprost (0.1, 0.3 and 3 mg/kg) alone and sodium ozagrel (10 and 30 mg/kg) alone on thrombotic occlusion of the middle cerebral artery. Occlusion time is presented as the mean ± S.E.M. ($n = 6$). Numbers inside each column indicate the number of animals in which occlusion time is > 30 min. * $P < 0.05$ vs. vehicle-treated animals.

duration: 30 min). The bleeding time was counted to the nearest half minute.

2.5. Drugs

ME3277 was obtained from Meiji Seika Kaisha, Tokyo. Vapiprost was obtained from Glaxo, UK. Sodium ozagrel was purchased from Ono Pharmaceutical, Osaka, Japan. Aspirin and Rose Bengal were purchased from Wako, Osaka. ADP and collagen were donated by MC Medical, Tokyo. Sodium arachidonic acid was obtained from Sigma.

2.6. Statistical analysis

Data are expressed as the means ± S.E.M. Student's unpaired *t*-test or Wilcoxon's test was used for comparison of differences between two groups. Dunnett's test or Steel's test was used for comparisons of three groups or more. A P value less than 0.05 was considered significant.

3. Results

Blood pressure, heart rate, basal middle cerebral arterial blood flow and arterial blood gas did not differ among groups during the experimental procedure (data not shown). Approximately 10 min after the photochemical reaction, a platelet-rich thrombus filled the lumen of the middle cerebral artery. A slight decrease in vessel diameter was observed at the irradiated region in vehicle-treated animals (Fig. 1A,B). Simultaneously, the middle cerebral artery blood flow was reduced to zero (Fig. 1C). ME3277 at 0.1, 0.3 and 1 mg/kg dose dependently delayed occlusion time from 7.3 ± 1.1 min in vehicle to 15.0 ± 5.0, 20.6 ± 4.8 and 25.9 ± 4.1 min ($P < 0.05$), respectively (Fig. 2). The prolongation of occlusion time corresponded to the inhibitory rate of ex vivo platelet aggregation (Table 1). The bleeding time was significantly ($P < 0.01$) prolonged only at the highest dose, 1 mg/kg ME3277 (Table 2). Administration of vapiprost at 0.1, 0.3 and 3 mg/kg dose dependently prolonged occlusion time to 11.0 ± 3.8, 15.6 ± 4.6 and 26.5 ± 3.5 min ($P < 0.05$), respectively (Fig. 3).

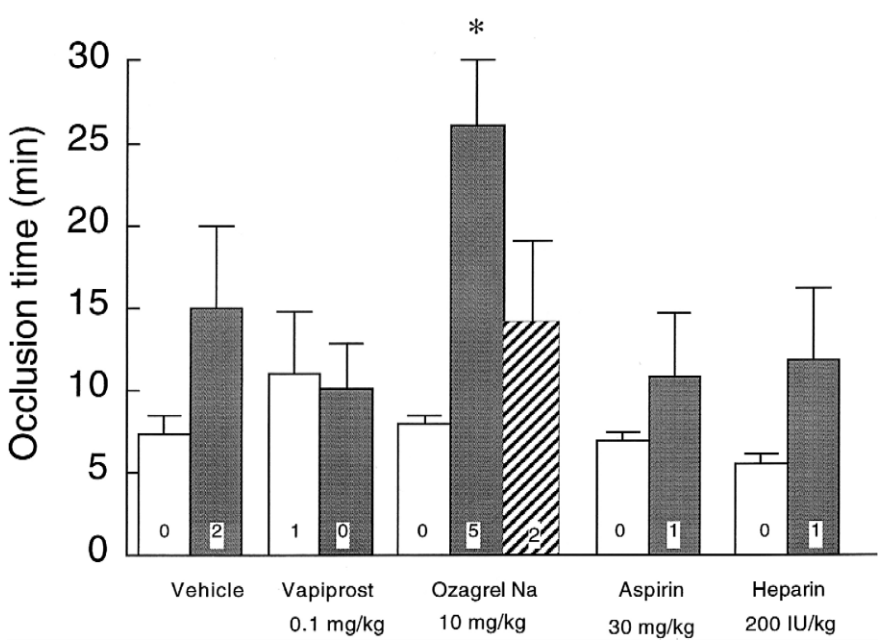

Fig. 4. The combined effect of ME3277 and various antithrombotic agents. Occlusion time of the middle cerebral artery was measured in the presence (striped column) or absence (open column) of 0.1 mg/kg ME3277. Each column presents the mean ± S.E.M. ($n = 6$). Hatched columns indicate the combination of 10 mg/kg sodium ozagrel, 0.1 mg/kg ME3277 and 30 mg/kg aspirin. Numbers inside each column indicate the number of animals in which occlusion time is > 30 min. * $P < 0.01$ vs. animals treated with sodium ozagrel alone.

Sodium ozagrel also delayed occlusion time to 7.9 ± 0.5 min at 10 mg/kg and 19.5 ± 4.8 min at 30 mg/kg ($P < 0.05$), while it did not inhibit ex vivo platelet aggregation (Fig. 3, Table 1). In contrast, aspirin did not delay occlusion time at a dose of 30 mg/kg (6.9 ± 0.5 min, Fig. 4), although it inhibited collagen- and arachidonic acid-induced platelet aggregation (Table 1). Heparin did not affect occlusion time (5.6 ± 0.5 min, Fig. 4) at the dose that significantly prolonged activated partial thromboplastin time (18 s in vehicle to 146 ± 4 s with heparin treatment, $P < 0.01$).

ME3277 (0.1 mg/kg), in combination with a threshold dose of sodium ozagrel (10 mg/kg), significantly delayed occlusion time (26.1 ± 3.9 min, $P < 0.05$, Fig. 4) without affecting ex vivo platelet aggregation (Table 1) or the bleeding time (Table 2). The synergy between ME3277 and sodium ozagrel was blocked by 30 mg/kg aspirin (14.7 ± 4.8 min). In contrast, ME3277 in combination with vapiprost (0.1 mg/kg, 10.1 ± 2.7 min), aspirin (30 mg/kg, 10.8 ± 3.9 min) or heparin (200 IU/kg, 11.9 ± 4.4 min) did not synergistically delay occlusion time (Fig. 4).

4. Discussion

We evaluated the combined effects of platelet GPIIb–IIIa receptor antagonists on antithrombotic action, platelet aggregation and bleeding time in the photothrombotic occlusion model of guinea pig middle cerebral artery. Use of several combinations showed that the threshold dose of sodium ozagrel enhanced the in vivo antithrombotic effect of ME3277 without affecting ex vivo platelet aggregation.

Although sodium ozagrel or vapiprost produced a weaker platelet inhibition than ME3277, all showed a similar in vivo efficacy. We thought that vasoconstriction could explain this phenomenon. In fact, microscopic observation indicated that vascular constriction occurred when the vessel was occluded by endothelial injury in vehicle-treated animals (Fig. 1b). Previous reports suggested that the platelet integrin GPIIb–IIIa receptor antagonist did not inhibit thromboxane A_2 production even if platelet aggregation was suppressed (Byrne et al., 1997; Carroll et al., 1997). Because thromboxane A_2 is known to constrict vascular smooth muscle, we thought it possible that the combination of sodium ozagrel enhanced in vivo efficacy of ME3277 by reducing the production of thromboxane A_2. However, the thromboxane A_2 receptor antagonist, vapiprost, did not enhance the antithrombotic efficacy when used in combination with ME3277. This indicated that inhibition of thromboxane A_2 production did not predominately contribute to vessel occlusion in the present model. It is also known that the thromboxane A_2 synthase inhibitor increased prostacyclin, a vasodilating substance, as "prostaglandin H_2 steal" (Kuzuya et al., 1986). When thromboxane A_2 synthase is blocked, its substrate, prostaglandin H_2, accumulates in the platelets and is converted to prostacyclin in the endothelial cells. Thus, we examined the combined effect of aspirin, a cyclooxygenase inhibitor, which inhibits prostacyclin formation in the endothelium as well as thromboxane A_2 formation in the platelets. Abolishment of the synergy of ME3277 and sodium ozagrel by aspirin suggested that prostacyclin formation rather than thromboxane A_2 inhibition could be important in preventing the thrombus formation by the thromboxane A_2 synthase inhibitor. This suggestion is based on the fact that thrombotic occlusion involves several factors such as thromboxane A_2, thrombin, serotonin, endothelin and platelet activating factor. These substances facilitate thrombus formation as well as cause vasoconstriction (Adner et al., 1993; Nakamura et al., 1985; Reid et al., 1995; Shirahase et al., 1987; Uski and Reinstrup, 1990). Therefore, each antagonist of these vasoconstrictors no longer effectively inhibits vasoconstriction on single use. Alternatively, sodium ozagrel, by converting thromboxane A_2 to prostacyclin, could directly dilate the vessel and effectively prolong occlusion time when combined with ME3277. We were interested in evaluating vasodilators including prostacyclin analogues and a Ca^{2+} channel blocker on thrombotic occlusion. However, these vasodilators did not influence occlusion time at a dose range which preserves a normal hemodynamic condition (data not shown). Thus, so far, the thromboxane A_2 synthase inhibitor is useful in combination with the platelet integrin GPIIb–IIIa receptor antagonist for prevention of vascular thrombosis.

Although the combination of sodium ozagrel with the lowest dose of ME3277 delayed thrombus formation as much as did 1 mg/kg ME3277, bleeding time was not synergistically prolonged. In general, the smooth muscle layer is rich in resistance arteries and conductance arteries, while poor in capillaries and venules. In the middle cerebral artery, therefore, thrombus formation after endothelial injury depends on both vasoconstriction and thrombus formation. The dose at which ME3277 prevents thrombotic occlusion in the middle cerebral artery is decreased when vasoconstriction is inhibited with sodium ozagrel. In the skin, however, thrombotic occlusion is mostly dependent on thrombus formation rather than on vasoconstriction, because large parts of the skin vessels consist of capillaries and venules. Therefore, bleeding depended directly on the extent of platelet inhibition. Since the combination of ME3277 with sodium ozagrel did not enhance ex vivo platelet aggregation, the combination did not affect bleeding time. Also, in the present study, bleeding time was prolonged by heparin without an effect on thrombotic occlusion in the middle cerebral artery. This indicates that the coagulation system is dominant in hemostasis in the skin but not in thrombotic occlusion in the middle cerebral artery. This possibility is supported by the hypothesis that brain hemostasis is more likely to be dependent on an intrinsic procoagulant than platelet aggregation (MacDonald et al., 1994).

In summary, the thromboxane A_2 synthase inhibitor enhanced the in vivo antithrombotic effect of platelet integrin GPIIb–IIIa receptor antagonist in guinea pig middle cerebral artery. This combination had a more potent antithrombotic activity in vivo than did the high dose of ME3277. The combination did not prolong the bleeding time more than did the highest dose of ME3277 alone. The local formation of prostacyclin by sodium ozagrel may contribute to the phenomenon, possibly by inhibiting vessel constriction. Therefore, the combination of thromboxane A_2 synthase inhibitor and platelet integrin GPIIb–IIIa receptor antagonist could be useful for the treatment of cerebral thrombosis.

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