The Potent Inhibition of Vapiprost, a Novel Thromboxane A₂ Receptor Antagonist, on the Secondary Aggregation and ATP Release of Human Platelets

Shuichi HORIE,^{*,a} Mayumi YAMADA,^b Megumi SATOH,^b Shinobu NORITAKE,^b Sayuri HIRAISHI,^a Keiichiro KIZAKI,^a Osamu KURUSU,^b Tamio NAKAHARA,^b Hidemi ISHII,^a and Mutsuyoshi KAZAMA^a

Department of Clinical Biochemistry, Faculty of Pharmaceutical Sciences, Teikyo University,^a 1091-1 Suarashi, Sagamiko, Tsukui, Kanagawa 199-01, Japan and Pharmacology Group, Preclinical Research Center, Tsukuba Research Laboratories, Nippon Glaxo Ltd.,^b 43 Wadai, Tsukuba, Ibaraki 300-42, Japan.

Received September 20, 1996; accepted February 12, 1997

The inhibitory effects of vapiprost hydrochloride (vapiprost), a novel thromboxane A₂ receptor antagonist, on platelet aggregation and ATP release were studied using platelet rich plasma (PRP) of humans, guinea pigs, rabbits and rats. In *in vitro* experiments with human platelet, vapiprost inhibited the aggregation and ATP release stimulated with U-46619, collagen or arachidonic acid (AA) at an IC₅₀ of less than 2.1×10^{-8} M. Vapiprost did not inhibit the primary aggregation or ATP release of human platelets stimulated with adenosine 5'-diphosphate (ADP), epinephrine (Epi) or platelet activating factor (PAF), but inhibited the secondary aggregation stimulated with those agonists at an IC₅₀ of less than 1.3×10^{-7} M. The sensitivity of platelets in various species of animals to vapiprost was in the following order: human $\geq$ guinea pigs $>$ rats $>$ rabbits. In *ex vivo* experiments with guinea pigs which received a single oral dose of vapiprost, the agent demonstrated strong inhibition of ATP release from platelets stimulated with U-46619, collagen or AA at an ID₅₀ of less than 25.8 μ g/kg. These inhibitory effects were observed within 30 min and sustained for 24 h at a single dosage of 5 mg/kg of vapiprost. In AA-induced pulmonary infarction models of mice, the sudden death rates decreased significantly with the oral administration of 10 mg/kg or more of vapiprost.

These results indicate that vapiprost effectively inhibits the secondary aggregation and ATP release of human platelets stimulated with various agonists, and that guinea pig and human platelets are similar in response to vapiprost. Furthermore, it was demonstrated in *ex vivo* experiments with guinea pigs that the inhibitory action of vapiprost appears rapidly and lasts for long periods.

Key words vapiprost; thromboxane A₂ receptor antagonist; platelet aggregation; platelet ATP-release

Antiplatelet therapy has been widely applied for the prevention and treatment of coronary diseases and ischemic cerebrovascular diseases.^{1–3)} Recent studies have revealed that thromboxane A₂ (TXA₂) plays a key role in the amplification of platelet activation.^{4,5)} TXA₂ and prostaglandin H₂ (PGH₂) produced in platelets stimulated with agonists are released into extraplatelet. The released TXA₂ and PGH₂ amplify platelet activation through binding to a TXA₂ receptor on the platelet membrane and thereby induce the secondary aggregation. Thus, the compounds that inhibit platelet release and secondary aggregation reactions, accompanied by TXA₂ production, are perceived as attractive drugs in the prevention of thrombus formation. Specific antagonists to TXA₂ receptors possibly inhibit secondary platelet aggregation by inhibiting the binding of both PGH₂ and TXA₂ produced in platelets to TXA₂ receptors, as compared with inhibitors for TXA₂ synthetase, which cannot prevent PGH₂-dependent platelet aggregation because the PGH₂ produced in platelet activation binds to TXA₂ receptors.⁶⁾

Vapiprost has been developed as a TXA₂ receptor antagonist.^{7–9)} Its structure is shown in Fig. 1. The inhibitory effect of the agent on platelet aggregation has been studied by Hornby *et al.*¹⁰⁾ using human platelet rich plasma (PRP), by Thomas *et al.*¹¹⁾ using human whole blood and by Lumley *et al.*⁹⁾ using human PRP and whole blood. However, the effects of vapiprost alone, that is, without aspirin addition, on the secondary aggregation of platelets have not been reported. Furthermore, it is known

that stimulant induced platelet aggregation differs among species and that platelet sensitivity differs among human species.

In the present study, the inhibitory effects of vapiprost on platelet aggregation and ATP release were examined in PRP of humans, guinea pigs, rabbits and rats using various agonists, and were compared with those of aspirin, which is widely used clinically. In addition, the inhibitory effect of the agent by oral administration was examined *ex vivo* in guinea pigs.

MATERIALS AND METHODS

Test Agents and Reagents Vapiprost hydrochloride ([1R-[1 α (Z),2 β ,3 β ,5 α]]-(+)-7-[5-[[[(1,1'-biphenyl)-4-yl]methoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid hydrochloride) was used throughout this study and was provided by Nippon Glaxo, Ltd. Ara-

Fig. 1. Chemical Structure of Vapiprost

* To whom correspondence should be addressed.

chidonic acid (sodium salt, AA), platelet activating factor (3-*O*-hexadecyl-2-acetyl-sn-glycerol-1-phosphocholine, PAF), epinephrine (Epi), luciferase-luciferin, thrombin, and ATP (sodium salt) were obtained from Sigma Chemical Co., U.S.A. Aspirin (Yoshitomi Pharmaceutical Co., Japan), U-46619 (Cayman Chemical Co., U.S.A.), collagen (Horm-Chemie, Germany), adenosine 5'-diphosphate (potassium salt, ADP, Boehringer-Mannheim-Yamanouchi Co., Japan), and pentobarbital (sodium salt, Nenbtal, Dainippon Pharmaceutical Co., Japan) were used. Other reagents were purchased from Wako Pure Chemical Industries, Ltd., Japan.

Human Human platelets were obtained from healthy volunteers (20 to 43 years of age) who did not take any drug within one month before being tested.

Animals Animals used in this study were guinea pigs (male Hartley strain, body weight 260 to 680 g), rabbits (male Nippon white species, body weight 2.5 to 3.0 kg), rats (male Wistar strain, body weight 320 to 350 g), and mice (male ddy strain, body weight 30 to 35 g). Test animals were kept at room temperature ($23 \pm 3^\circ\text{C}$) and allowed to eat solid feed and drink water freely throughout preliminary and feeding periods.

Preparation of Platelet Blood samples were collected from brachial cubital veins of humans, auricular veins of unanesthetized rabbits, and ventral aortae of etherized guinea pigs and rats. The anticoagulants used were 1/10 volume of 3.8 or 3.15% sodium citrate for the blood of human or guinea pigs and rabbits, and 1000 units/ml heparin for rats. PRP was prepared by the centrifugation of citrated or heparinized whole blood (human: $190 \times g$ for 15 min, rabbit: $270 \times g$ for 15 min, guinea pig: $190 \times g$ for 25 min, rat: $190 \times g$ for 10 min, at room temperature). Platelet counts in PRP were measured with a hemocytometer (Sysmex F-800, Toa Medical Electronics Co., Ltd., Japan) and PRP was diluted with platelet poor plasma (PPP) to acquire appropriate platelet counts (mentioned later). Washed platelets were used for thrombin stimulation.

Platelet Aggregation and ATP Release Vapiprost, aspirin or solvent Tyrode-Hepes buffer solution with a 1/20 final volume was added to a measuring cuvette containing PRP. The mixture was stirred with a magnetic stirrer (1000 rpm, 37°C) for 2 min. After luciferase-luciferin of 1/10 final volume (the final concentration was 4 mg/ml) was added to the mixture and stirred in the same manner for 1 min, a 1/20 volume of agonists was added. Platelet aggregation and ATP release were measured over time using a lumi-aggregometer (Baxter, C-560). Platelet counts were set at, in the final volume of 350 or 500 μl , 2.0×10^5 cells/ μl (human), 3.0×10^5 cells/ μl (guinea pigs), 2.4×10^5 cells/ μl (rabbits) and 3.6×10^5 cells/ μl (rats). The platelet aggregation was measured as an increase in light transmission and according to minimum and maximum light transmission set on a chart recorder using PRP and PPP. The difference between the minimum and maximum light transmissions (before and after the addition of agonists, respectively) was regarded as maximum aggregation. Maximum aggregation obtained by an agonist on the addition of Tyrode-Hepes buffer solution, the control, was regarded as 100%, and the maximum ag-

gregation obtained by an agonist on the addition of each test agent was expressed as a % relative to the control. A secondary aggregation reaction was observed in human platelets after treatment with ADP, Epi or PAF, and the maximum transmittance of the second peak after the addition of each agonist was measured. The ATP release in the presence of each test agent was expressed as the % relative to the maximum ATP release of each agonist alone.

In *ex vivo* experiments, animals were orally given 500 μl /kg of various concentrations of vapiprost or aspirin, and blood samples were collected 1 h later. The same amount of distilled water was administered to animals as a control. To determine the time profile of the inhibitory effect of agents on platelet aggregation and ATP release, guinea pigs received 5 mg/kg of vapiprost or distilled water, and the blood samples were collected at 0.5, 1, 3, 6, 12, 18 and 24 h after the administration. PRP was prepared in the same manner as described above, and the platelet aggregation and ATP release at 7 min after the addition of 5 μM of U-46619, 1 $\mu\text{g}/\text{ml}$ collagen and 0.5 mM AA were measured. For the platelet release reaction, the results in Fig.3 are expressed as the concentration of ATP released.

Setting of Concentrations of Agonists In order to differentiate secondary aggregation from primary aggregation, when various agonists were added to the reaction system, the minimum effective concentrations of each agonist for maximum aggregation and ATP release were examined. The concentrations for the subsequent tests were determined as follows: U-46619: 5 μM (human, guinea pigs, rats), 10 μM (rabbits); collagen: 1 $\mu\text{g}/\text{ml}$ (human, guinea pigs, rats), 10 $\mu\text{g}/\text{ml}$ (rabbits); AA: 1 mM (human), 0.5 mM (guinea pigs), 0.25 mM (rabbits), 4 mM (rats); ADP: 10 μM (human, rabbits), 2 μM (guinea pigs, rats); Epi: 5 μM (human); PAF: 0.5 μM (human); thrombin: 0.1 U/ml (human).

AA-Induced Pulmonary Infarction Model One hour after the oral administration of distilled water or vapiprost to mice, 100 mg/kg of AA was administered into the caudal veins. The numbers of mice which succumbed to sudden death within 3 min were counted.

Statistical Processing Results are presented as the means $\pm$ S.E. For significance tests, Student's *t*-test and Fisher's exact probability test were applied in an *ex vivo* study on the time profile of vapiprost-effect and in the AA-induced pulmonary infarction models, respectively.

RESULTS

Inhibitory Effect of Vapiprost and Aspirin on Aggregation and ATP Release of Human Platelets *in Vitro* In this experiment, the minimum concentrations of various agonists to induce maximum aggregation and ATP release were used. The IC_{50} of vapiprost in platelet aggregation and ATP release induced by various agonists was determined using human PRP (Table 1). Vapiprost inhibited the aggregation of platelets stimulated with U-46619, collagen and AA; their IC_{50} were $2.1 \pm 0.5 \times 10^{-9}$ to $2.1 \pm 0.6 \times 10^{-8}$ M. Vapiprost did not inhibit primary platelet aggregation stimulated with ADP, Epi or PAF,

Table 1. Inhibitory Effect of Vapiprost on the Aggregation and ATP Release of Human Platelets Stimulated by Various Inducers *in Vitro*

Inducers (Concentration)	Vapiprost (nm)				Aspirin (nm)			
	Inhibition of aggregation (IC ₅₀)		Inhibition of ATP release (IC ₅₀)		Inhibition of aggregation (IC ₅₀)		Inhibition of ATP release (IC ₅₀)	
U-46619 (5 μ M)	2.1 $\pm$ 0.5	(5)	0.88 $\pm$ 0.3	(5)	> 10000	(7)	> 10000	(7)
Collagen (1 μ g/ml)	5.1 $\pm$ 1.1	(3)	6.4 $\pm$ 1.1	(3)	> 10000	(3)	> 10000	(3)
Arachidnate (1 mM)	21 $\pm$ 6	(5)	18 $\pm$ 5	(5)	> 10000	(4)	> 10000	(4)
ADP (10 μ M) 1st	> 10000	(4)			> 10000	(3)		
ADP (10 μ M) 2nd	130 $\pm$ 60	(3)	2.6 $\pm$ 1.3	(3)	> 10000	(3)	2100 $\pm$ 1600	(3)
Epi (5 μ M) 1st	> 10000	(4)			> 10000	(5)		
Epi (5 μ M) 2nd	6.3 $\pm$ 2.2	(3)	2.2 $\pm$ 1.5	(3)	> 10000	(5)	> 10000	(5)
PAF (0.5 μ M) 1st	> 10000	(4)			> 10000	(3)		
PAF (0.5 μ M) 2nd	4.4 $\pm$ 1.9	(3)	3.4 $\pm$ 1.7	(3)	> 10000	(3)	> 10000	(3)
Thrombin (0.1 U/ml)	> 10000	(4)	> 10000	(4)	> 10000	(4)	> 10000	(4)

Human PRP was prepared, and the effects of vapiprost and aspirin on the aggregation and ATP release reaction were measured by lumi-aggregometer after stimulation with various inducers. Results are expressed as the means $\pm$ S.E. of each IC₅₀ value, and the number of data is shown in parenthesis.

Table 2. Inhibitory Effect of Vapiprost on the Aggregation and ATP Release of Guinea Pig, Rabbit and Rat Platelets Stimulated by Various Inducers *in Vitro*

Inducers (Concentration)		Vapiprost (nm)				Aspirin (nm)			
		Inhibition of aggregation (IC ₅₀)		Inhibition of ATP release (IC ₅₀)		Inhibition of aggregation (IC ₅₀)		Inhibition of ATP release (IC ₅₀)	
Guinea pig	U-46619 (5 μM)	140 ± 80	(6)	2.3 ± 0.8	(6)	> 10000	(4)	> 10000	(4)
	Collagen (1 μg/ml)	8.1 ± 5.5	(4)	0.24 ± 0.16	(4)	> 10000	(4)	> 10000	(4)
	Arachidonate (0.5 mM)	20 ± 5	(4)	4.3 ± 0.5	(4)	> 10000	(3)	> 10000	(3)
	ADP (2 μM)	> 10000	(4)	0.24 ± 0.17	(4)	> 10000	(3)	> 10000	(3)
Rabbit	U-46619 (10 μM)	4600 ± 2100	(4)	3300 ± 900	(3)	> 10000	(4)	> 10000	(4)
	Collagen (10 μg/ml)	> 10000	(4)	> 10000	(4)	> 10000	(3)	> 10000	(3)
	Arachidonate (0.25 mM)	3900 ± 1700	(5)	150 ± 60	(4)	> 10000	(4)	> 10000	(4)
	ADP (10 μM)	> 10000	(5)	> 10000	(4)	> 10000	(4)	> 10000	(4)
Rat	U-46619 (5 μM)	130 ± 60	(3)	62 ± 10	(3)	> 10000	(3)	> 10000	(3)
	Collagen (1 μg/ml)	> 10000	(3)	370 ± 180	(3)	> 10000	(3)	> 10000	(3)
	Arachidonate (4 mM)	> 10000	(3)	6.3 ± 6.4	(3)	> 10000	(3)	> 10000	(3)
	ADP (2 μM)	> 10000	(4)	> 10000	(4)	> 10000	(3)	> 10000	(3)

PRP from guinea pig, rabbit and rat were prepared, and the effects of vapiprost and aspirin on the aggregation and ATP release reaction were measured by lumi-aggregometer after stimulation with various inducers. Results are expressed as the means $\pm$ S.E. of each IC₅₀ value, and the number of data is shown in parenthesis.

but it inhibited secondary aggregation; the IC₅₀ were $1.3 \pm 0.6 \times 10^{-7}$, $6.3 \pm 2.2 \times 10^{-9}$ and $4.4 \pm 1.9 \times 10^{-9}$ M, respectively. Vapiprost inhibited ATP release from platelets induced by agonists at approximately the same or lower IC₅₀ values as required to inhibit platelet aggregation. On the other hand, vapiprost did not inhibit platelet aggregation or ATP release stimulated by thrombin in washed platelets.

Although aspirin inhibited the ATP release stimulated by ADP at an IC₅₀ of $2.1 \pm 1.6 \times 10^{-6}$ M, it did not inhibit platelet aggregation or ATP release induced by other agonists, even at the concentration of 10^{-5} M, indicating that the inhibitory effect of aspirin on platelet aggregation and ATP release was approximately 1/100 less than that of vapiprost, except in thrombin and ADP stimulation.

Inhibitory Effect of Vapiprost and Aspirin on Platelet Aggregation and ATP Release in Guinea Pigs, Rabbits and Rats *in Vitro* The IC₅₀ of vapiprost and aspirin in platelet aggregation and ATP release was measured when various agonists were added to PRP of guinea pigs, rabbits and rats (Table 2). Because primary and secondary aggregations of PRP by ADP in animals were difficult to differentiate, unlike those in humans, both aggregations

were evaluated as a single aggregation.

In guinea pigs platelets, vapiprost inhibited the aggregation stimulated by U-46619, collagen and AA at IC₅₀ values of $1.4 \pm 0.8 \times 10^{-7}$, $8.1 \pm 5.5 \times 10^{-9}$ and $2.0 \pm 0.5 \times 10^{-8}$ M, respectively, and inhibited ATP release at approximately 1/4 to 1/60 of the IC₅₀ in the corresponding aggregation inhibition. In ADP stimulation, vapiprost inhibited ATP release at the lower IC₅₀ of $2.4 \pm 1.7 \times 10^{-10}$ M, whereas it did not affect platelet aggregation.

In rabbit platelets, vapiprost showed weaker inhibition on aggregation and ATP release stimulated with various agonists than in human or guinea pig platelets (Table 2). The IC₅₀ values of vapiprost in aggregation and ATP release stimulated by U-46619 were $4.6 \pm 2.1 \times 10^{-6}$ and $3.3 \pm 0.9 \times 10^{-6}$ M, respectively. Vapiprost inhibited platelet aggregation and ATP release stimulated by AA at IC₅₀ values of $3.9 \pm 1.7 \times 10^{-6}$ and $1.5 \pm 0.6 \times 10^{-7}$ M, respectively. The inhibitory effects of vapiprost in rabbit platelets were mild compared with those in human platelets. Vapiprost did not inhibit aggregation or ATP release stimulated by collagen or ADP, even at concentrations of 10^{-5} M.

Fig. 2. Effect of Administration of Vapiprost on Aggregation and ATP Release of Guinea Pig Platelets Stimulated by U-46619, Collagen and AA

Vapiprost was administered orally and PRP was prepared 1 h later. The platelet aggregation and ATP release reactions were measured after stimulation by $5 \mu\text{M}$ of U-46619, $1 \mu\text{g/ml}$ of collagen and 0.5 mM of AA, as described in Materials and Methods. Results are expressed as means $\pm$ S.E. of four data. A: aggregation; B: ATP release. $\bullet$, U-46619; $\blacktriangle$, collagen; $\blacksquare$, AA.

Table 3. Fifty Percent Inhibitory Doses of Vapiprost and Aspirin on the Aggregation and ATP Release of Guinea Pig Platelets Stimulated by Various Inducers *ex Vivo*

Inducers (Concentration)	Vapiprost ($\mu\text{g/kg}$)				Aspirin (mg/kg)			
	Inhibition of aggregation (ID_{50})		Inhibition of ATP release (ID_{50})		Inhibition of aggregation (ID_{50})		Inhibition of ATP release (ID_{50})	
U-46619 ($5 \mu\text{M}$)	359.0	(4)	25.8	(4)	> 54	(4)	> 54	(4)
Collagen ($1 \mu\text{g/ml}$)	12.2	(4)	15.6	(4)	20.2	(3)	24.5	(3)
Arachidonate (0.5 mM)	46.6	(4)	6.3	(4)	48.7	(3)	45.1	(3)
ADP ($2 \mu\text{M}$)	> 50000	(3)	> 50000	(3)	> 54	(6)	> 54	(6)

Vapiprost and aspirin were administered 1 h prior to preparations of PRP. Results are expressed as the means of each ID_{50} value from 3–6 samples, as shown in parentheses.

In rat platelets, vapiprost inhibited 50% of the aggregation and ATP release stimulated by U-46619 at concentrations of $1.3 \pm 0.6 \times 10^{-7}$ and $6.2 \pm 1.0 \times 10^{-8} \text{ M}$, respectively. Vapiprost inhibited 50% of the ATP release stimulated by collagen and AA at the concentrations of $3.7 \pm 1.8 \times 10^{-7}$ and $6.3 \pm 6.4 \times 10^{-9} \text{ M}$, respectively, while it did not inhibit aggregation stimulated by the above agonists or the aggregation and ATP release stimulated by ADP, even at the concentration of 10^{-5} M (Table 2).

Aspirin, on the other hand, did not inhibit 50% of the aggregation or ATP release stimulated by the four agonists in any of the animals, even at the high concentration of 10^{-5} M .

Inhibitory Effects of Vapiprost on Aggregation and ATP Release in Guinea Pig Platelets *ex Vivo* The ID_{50} of vapiprost in aggregation and ATP release of platelets stimulated by various agonists was evaluated using guinea pig PRP after the oral administration of vapiprost (Fig. 2). Blood samples were collected 1 h after the oral administration of vapiprost at various concentrations, since the half-life of vapiprost in plasma is reported to be 0.99 to 1.1 h.¹²⁾ The 50% inhibition of platelet aggregation stimulated by U-46619 ($5 \mu\text{M}$), collagen ($1 \mu\text{g/ml}$) or AA (0.5 mM) by the oral administration of vapiprost occurred

at doses of 359, 12.2 and $46.6 \mu\text{g/kg}$, respectively (Fig. 2, Table 3). Fifty percent of the ATP release was inhibited by vapiprost doses of 25.8, 15.6 and $6.3 \mu\text{g/kg}$, respectively (Fig. 2, Table 3). However, in ADP-stimulated platelets, the oral administration of vapiprost did not inhibit the aggregation or release, even at the dose of 50 mg/kg (Table 3).

Oral administration of aspirin, even at the dose of 54 mg/kg , did not inhibit platelet aggregation or ATP release stimulated by U-46619 or ADP (Table 3). Aspirin inhibited 50% of the platelet aggregation and ATP release induced by collagen at doses of 20.2 and 24.5 mg/kg , respectively, and those stimulated by AA at dose of 48.7 and 45.1 mg/kg , respectively. In the case of aspirin, a 1000 times higher dose than vapiprost was required.

Time profiles of the effect of vapiprost on platelet aggregation and ATP release induced by U-46619, collagen and AA were investigated after a single administration of vapiprost (5 mg/kg) to guinea pig (Fig. 3). At 30 min after the administration, vapiprost had already significantly inhibited all the platelet aggregation and ATP release reactions, except in the case of U-46619-induced ATP release. These inhibitory effects of vapiprost on U-46619- and collagen-induced ATP release were retained, even at

Fig. 3. Duration of Inhibitory Action of Vapiprost on Aggregation and ATP Release of Guinea Pig Platelets Stimulated by U-46619, Collagen and AA

Guinea pigs were orally administered 5 mg vapiprost/0.5 ml H₂O/kg or 0.5 ml H₂O/kg at 0 time, and the platelet aggregation and ATP release were measured using PRP samples prepared at each time, as described in Materials and Methods. Each point represents the mean $\pm$ S.E. of five data, except as follows: three data (control of AA-stimulated at 0.5 h), four data (control of U-46619-stimulated at 12 h, control of collagen-stimulated at 0.5 and 18 h, vapiprost of collagen-stimulated at 18 h), and six data (control and vapiprost of collagen-stimulated at 3 h). An asterisk represents significance ($p < 0.05$) against the control of the same time. $\bullet$, $\blacktriangle$, $\blacksquare$, vapiprost-administered; $\circ$, $\triangle$, $\square$, control.

24 h after the administration of vapiprost, and both the platelet aggregation and ATP release reactions stimulated by AA were completely blocked for 24 h after the administration. Significant inhibitory effects of vapiprost on the aggregation of platelets stimulated by U-46619 and collagen were also retained until 18 h after vapiprost administration (Fig. 3).

Effects of Vapiprost on AA-Induced Pulmonary Infarction Models of Mice

Seventeen out of 22 mice that were given distilled water alone 1 h prior to the intravenous administration of AA died within 3 min, resulting in a survival rate of 22.7% (Table 4). The survival rates of mice that were pre-administered with vapiprost dissolved in distilled water increased dose-dependently: 70.0% or higher survived at 10 and 20 mg/kg of vapiprost, demonstrating the significant inhibitory effects of vapiprost on the AA-induced pulmonary infarction model. On the other hand, aspirin has traditionally been administered to man at a

Table 4. Inhibitory Effect of Vapiprost on Mice Death Induced by Intravenous Injection of AA *in Vivo*

Vapiprost (mg/kg)	No. of animal	No. of death	% of survival	Significance
0	22	17	22.7%	—
0.5	10	6	40.0%	N.S.
1.0	9	5	44.4%	N.S.
5.0	9	4	55.6%	N.S.
10.0	14	4	71.4%	$p > 0.05$
20.0	10	3	70.0%	$p < 0.05$

Male mice received distilled water or 0.5–20 mg/kg of vapiprost orally before 1 h i.v. injection of AA (100 mg/kg), and the number of dead mice within 3 min after administration of AA was measured. Statistical evaluations against the control were performed by Fishers exact probability test. N.S. = not significant.

dose of 80 mg, two times a day, which corresponds to approximately 1.5 mg/kg/12 h. However, a faint protective effect of aspirin (10 mg/kg) on the AA-induced mouse

death has been reported.¹³⁾

DISCUSSION

Vapiprost has been developed as an agent which competes with TXA₂ by binding specifically to TXA₂ receptors and inhibits the TXA₂ which facilitates platelet aggregation, vasoconstriction and the constriction of bronchial smooth muscles.^{9,14,15)} In the present study, the inhibitory effect of vapiprost on platelet aggregation and ATP release, as well as differences in its effect among animal species and various agonists, were evaluated using PRP of human, guinea pigs, rabbits and rats, and, as a control, aspirin which is widely used clinically^{1,16,17)} was employed.

U-46619, a stable derivative of TXA₂, is a compound that induces platelet activation by binding to TXA₂ receptors.¹⁸⁾ U-46619-induced aggregation and the ATP release of platelets for four animal species were examined in the study. Platelets of human, guinea pigs and rats aggregated and released ATP to approximately the same degree with the same concentrations of U-46619, while rabbit platelets required a double concentration of U-46619 for the maximum reactions, suggesting that the sensitivity of rabbit platelets to U-46619 was lower than that of human, guinea pigs and rats. Vapiprost inhibited platelet aggregation and ATP release induced by U-46619 in all animal species in this study. The IC₅₀ of vapiprost in the above aggregation and release was lowest in human platelets among those species of animal platelets tested. The results indicated that the sensitivity to U-46619 was only slightly different among human, guinea pigs and rats, and the sensitivity to vapiprost was strongest in human platelets.

In human platelets, the IC₅₀ value of vapiprost in aggregation stimulated by collagen and AA was 0.5 and 2.1×10^{-8} M, which was almost consistent with the IC₅₀ in the aggregation results reported in studies by Hornby *et al.*¹⁰⁾ Collagen and AA are known to produce TXA₂ in platelets and to amplify platelet activation.^{4,5)} The results in the present study indicated that platelet aggregation and ATP release were suppressed when vapiprost inhibited the binding between TXA₂, which was newly produced in the platelets by collagen and AA, and its receptors. Platelet aggregation in humans is biphasic regarding the stimulation of ADP, Epi or PAF. Vapiprost did not inhibit the primary aggregation by the agonists, but inhibited the secondary aggregation at IC₅₀ values of 10^{-9} to 10^{-7} M, suggesting that the agent did not affect aggregation induced by exogenous coagulants, but inhibited the release reaction and aggregation, accompanied by a release reaction that followed the former reaction. Vapiprost had no effect on the platelet aggregation stimulated by thrombin. Thrombin-stimulated platelet aggregation and release are mediated by the platelet activation pathways of a wide range of agents other than that of TXA₂,¹⁹⁾ suggesting that vapiprost has high specificity to TXA₂ receptors.

The inhibitory action of aspirin on platelet aggregation and ATP release was not effective for any of the agonists except in the ATP release of human platelets induced by

ADP, and this inhibition was considerably weaker than that of vapiprost. Probably, the maximal concentration of aspirin (1×10^{-5} M) was too low to exert any inhibitory action. On the other hand, a specific TXA₂ synthetase inhibitor, such as OKY-1581 or OKY-046, has been shown to be an effective inhibitor of human platelet aggregation induced by various agonists. OKY-1581, which is a more potent inhibitor of platelet thromboxane synthetase than OKY-046,²⁰⁾ inhibited 50% of human platelet aggregation stimulated by AA *in vitro* at the concentration of approximately 100 nM, though the ADP-induced aggregation was only slightly inhibited by the same concentration of the agent.²¹⁾ Compared with the concentration of OKY-1581, the IC₅₀ of vapiprost in AA-induced human platelet aggregation was one fifth in this study.

The present study demonstrated that there were differences in platelet responses to vapiprost among species, and the sensitivity to vapiprost was in the following order; human $\geq$ guinea pigs $>$ rats $>$ rabbits. The differences may be due to different characteristics of the TXA₂ receptor in species,^{22,23)} or possibly due to differences in how TXA₂ influences the platelet stimulus conducting system after platelets are stimulated by various agonists. In guinea pigs, while the inhibitory effect of vapiprost on U-46619-induced platelet aggregation was slightly weaker than in human, vapiprost inhibited collagen-induced ATP release and AA-induced platelet aggregation and ATP release at low concentrations, as it did in human. The reactions of guinea pig platelets were most similar to those of human platelets, suggesting that this animal was most suitable for evaluation of the efficacy of vapiprost *ex vivo*.

In *ex vivo* experiments with guinea pigs, vapiprost, even at the dose of 50 mg/kg, did not inhibit platelet aggregation or ATP release stimulated by ADP, but the ID₅₀ values for inhibiting aggregation stimulated by U-46619, collagen and AA were 359, 12.2 and 46.6 μ g/kg, respectively. It was reported that ONO-3708 and SQ 29548,²⁴⁾ synthetic TXA₂ receptor antagonists, were required at doses of more than 3 and 1 mg/kg, respectively, for 50% inhibition of 1 μ g/ml collagen-induced aggregation of guinea pig platelets. It was further suggested that vapiprost effectively inhibited platelet aggregation and release reactions by oral administration, as compared with the TXA₂ receptor antagonists. Vapiprost had a tendency to inhibit platelet ATP release stimulated by U-46619 and AA at lower doses than were required for platelet aggregation, confirming that vapiprost strongly inhibited platelet release reaction in guinea pigs induced by U-46619 and AA *ex vivo* as well as *in vitro*. At the present time, it is unclear why the ID₅₀ of vapiprost *ex vivo* against platelet aggregation stimulated by collagen was lower than that of the release reaction. The present paper indicates that the inhibitory action of vapiprost on platelet aggregation and ATP release reactions by U-46619, collagen or AA was rapid, as seen within 30 min after administration, and that the duration of the inhibitory action was much longer than that of other TXA₂ receptor antagonists, such as BM 13177, SQ 29548, ONO-3708 and S-145.²⁴⁻²⁸⁾ The inhibitory action of these antagonists, other than vapiprost, on platelet aggregation are lost within 6 h after administration to guinea pig.²⁴⁻²⁸⁾ No active metabolite of vapiprost has

been identified; consequently, the progressive and sustained inhibition of platelet aggregation and release reactions are possibly achieved through a slow dissociation of the agent from the platelet TXA₂ receptor, which has been shown by an *in vitro* radio-ligand binding study.²⁹⁾ Armstrong *et al.*³⁰⁾ also reported that the exposure of human platelet to vapiprost, followed by its removal, reduced the number of TXA₂ receptors upon the platelets.

Rabbits and mice die within a few minutes of the intravenous administration of AA.^{31,32)} The sudden death is thought to be due to intravascular platelet aggregation, especially, in pulmonary circulation, to pulmonary and general vasoconstriction and constriction of bronchial smooth muscles caused by increases in blood concentrations of TXA₂.³³⁻³⁵⁾ The oral dose of 10 or 20 mg/kg of vapiprost significantly decreased the death rates in AA-induced pulmonary infarction fatal models of mice. Vapiprost is reported to inhibit U-46619-induced vasoconstriction and bronchial constriction,^{9,14,15)} suggesting that the agent inhibits these constrictions by competitive inhibition with TXA₂ receptors on smooth muscles, in addition to its anti-platelet functions.

In recent years, percutaneous transluminal coronary angioplasty (PTCA) has become popular as an important treatment for ischemic heart disease, particularly coronary arteriosclerosis. However, re-stenosis occurs in 30 to 50% of cases within 6 months, becoming a new issue.³⁶⁾ It will be important, in further studies, to examine whether vapiprost could prevent the formation of coronary thrombosis and re-stenosis through its strong inhibitory action upon the platelet releasing response.

REFERENCES

- 1) Antiplatelet Trialists' Collaboration, *Br. Med. J.*, **296**, 320—331 (1988).
- 2) Sandercock P., *Platelet*, **3**, 107 (1992).
- 3) Collier B. S., *N. Engl. J. Med.*, **322**, 33—42 (1990).
- 4) Kume S., *et al.*, "Kohza Prostaglandin (in Japanese)," Vol. II, ed. by Miyoshi Y., Uchida Y., Murota S., Yamamoto S., Tokyo Kagaku Dojin, Tokyo, 1989, pp. 107—158.
- 5) Okuma M., "Kohza Prostaglandin (in Japanese)," Vol. II, ed. by Miyoshi Y., Uchida Y., Murota S., Yamamoto S., Tokyo Kagaku Dojin, Tokyo, 1989, pp. 265—279.
- 6) Armstrong R. A., Jones R. L., Wilson N. H., *Br. J. Pharmacol.*, **79**, 953—964 (1983).
- 7) Thomas M., Lumley P., Ballard P., O'Brien J. R., *Thromb. Haemost.*, **58**, 181 (1987).
- 8) Lumley P., Collington E. W., Hallett P., Hornby E. J., Humphrey P. P. A., Wallis C. J., Jack D., Brittain R. T., *Thromb. Haemost.*, **58**, 261 (1987).
- 9) Lumley P., White B. P., Humphrey P. P. A., *Br. J. Pharmacol.*, **97**, 783—794 (1989).
- 10) Hornby E. J., Foster M. R., McCabe P. J., Stratton L. E., *Thromb. Haemost.*, **61**, 429—436 (1989).
- 11) Thomas M., Lumley P., *Circulation* (Suppl. I), **81**, I-53—I-58 (1990).
- 12) Uematsu T., Nagashima S., Mizuno A., Hirano K., Nakashima M., *J. Clin. Pharmacol.*, **31**, 815—822 (1991).
- 13) Yokota K., Yamashita A., Oda M., *Jpn. J. Pharmacol.*, **68**, 201—206 (1995).
- 14) Noguchi K., Ojiri Y., Chibana T., Matsuzaki T., Sakanashi M., *Jpn. J. Pharmacol.*, **60**, 341—348 (1992).
- 15) Matsuzaki T., Noguchi K., Nakasone J., Uezu M., Higuchi M., Sakanashi M., *Arzneim.-Forsch./Drug Res.*, **42(II)**, 1318—1322 (1992).
- 16) Hennekens C. H., Buring J. E., Sandercock P., Collins R., Peto R., *Circulation*, **80**, 749—756 (1989).
- 17) Willard J. E., Lange R. A., Hillis L. D., *N. Engl. J. Med.*, **327**, 175—181 (1992).
- 18) Coleman R. A., Humphrey P. P. A., Kennedy I., Levy G. P., Lumley P., *Br. J. Pharmacol.*, **73**, 773—778 (1981).
- 19) Ganguly P., Fossett N. G., *Blood*, **57**, 343—352 (1981).
- 20) Oshima T., McCluskey E. R., Honda A., Needleman P., *J. Pharmacol. Exp. Ther.*, **229**, 598—602 (1984).
- 21) Uotila P., Matintalo M., Dahl M.-L., *Prostaglandins, Leukotrienes and Medicine*, **12**, 299—303 (1983).
- 22) Burke S. E., Lefer A. M., Nicolaou K. C., Smith G. M., Smith J. B., *Br. J. Pharmacol.*, **78**, 287—292 (1983).
- 23) Narumiya S., Okuma M., Ushikubi F., *Br. J. Pharmacol.*, **88**, 323—331 (1986).
- 24) Kaku S., Shikata T., Uchida K., *Folia Pharmacol. Jpn.*, **93**, 171—178 (1993).
- 25) Patscheke H., Staiger C., Neugebauer G., Kaufmann B., Strein K., Ende R., Stegmeier K., *Clin. Pharmacol. Ther.*, **39**, 145—150 (1986).
- 26) Staiger C., Patscheke H., Neugebauer G., Kaufmann B., Strein K., Ende R., Stegmeier K., *Clin. Pharmacol. Ther.*, **29**, 573—579 (1986).
- 27) Friedhoff L. T., Manning J., Funke P. T., Ivashkiv E., Tu J., Cooper W., Willard D., *Clin. Pharmacol. Ther.*, **40**, 634—642 (1986).
- 28) Suga H., Hamanaka N., Kondo K., Kiyake H., Ohuchida S., Arai Y., Kawasaki A., Abstract of Papers, 6th International Conference on Prostaglandin and Related Compounds, Florence, 1986, p. 331.
- 29) Armstrong R. A., Humphrey P. P. A., Lumley P., *Br. J. Pharmacol.*, **110**, 539—547 (1993).
- 30) Armstrong R. A., Humphrey P. P. A., Lumley P., *Br. J. Pharmacol.*, **110**, 548—552 (1993).
- 31) Silver M. J., Hoch W., Kocsis J. J., Ingberman C. M., Smith J. B., *Science*, **183**, 1085—1087 (1974).
- 32) Penhos J. C., Rabbani F., Myers A., Ramsey E., Ramwell P., *Proc. Soc. Exp. Biol. Med.*, **167**, 98—100 (1981).
- 33) Lefer A. M., Okamatsu S., Smith E. F., III, Smith J. B., *Thromb. Res.*, **23**, 265—272 (1981).
- 34) Lefer A. M., Burke S. E., Smith J. B., *Thromb. Res.*, **32**, 311—320 (1983).
- 35) Ma X. L., Karasawa A., Lefer A. M., *Meth. Find. Exp. Clin. Pharmacol.*, **13**, 105—110 (1991).
- 36) Popma J. J., Califf R. M., Topol E. J., *Circulation*, **84**, 1426—1436 (1991).