Inhibitory Effect of Clopidogrel, Vapiprost and Argatroban on the Middle Cerebral Artery Thrombosis in the Rat

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ABSTRACT—This study investigated the roles of thromboxane A_2 (TXA_2), ADP and thrombin in middle cerebral artery (MCA) thrombosis in the rat. The rat MCA was occluded by a thrombus induced by the photochemical reaction of rose bengal by green light that causes endothelial damage followed by platelet adhesion, aggregation and formation of a platelet and fibrin-rich thrombus at the site of the photochemical reaction. Vapiprost, a specific TXA_2-receptor antagonist; clopidogrel, which has the thienopyridine structure of ticlopidine and is a more potent inhibitor of ADP-induced platelet aggregation than ticlopidine; argatroban, a specific thrombin inhibitor; or heparin was administered intravenously before rose bengal injection. The MCA local blood flow was monitored by a laser Doppler flowmeter. The MCA was occluded by thrombus about 5 min after the initiation of the photochemical reaction. Vapiprost, clopidogrel and argatroban all significantly prolonged the time taken for the thrombotic occlusion of the MCA, but in this respect, heparin was ineffective. Our observations suggest that vapiprost and clopidogrel are useful antithrombotic agents against platelet and fibrin-rich thrombi. The effect of argatroban is attributable to inhibition of thrombin-induced platelet activation and fibrin generation. The thrombosis model described in this study is useful for understanding the mechanism(s) of thrombogenesis in the rat MCA and may be applied to other mammalian species.

Keywords: Green light, Rose bengal, Photochemical reaction, Laser Doppler flowmeter, Middle cerebral artery thrombosis (rat)

In patients with transient ischemic attacks, subsequent stroke can be prevented by platelet activation inhibitors (1-6). However, numerous factors such as platelet aggregation, the formation of fibrin, vasoconstriction and interaction between vessel walls and leukocytes (7) may contribute to arterial thrombogenesis. Our understanding of the mechanisms of thrombus formation in the cerebral artery is far from complete. For investigating thrombogenesis in the cerebral artery, suitable animal models are required. In line with this, we have established a model of middle cerebral artery (MCA) thrombosis in the rat (8). In our model, an arterial platelet and fibrin-rich thrombus is induced through endothelial damage by a photochemical reaction (8).

Platelet aggregation in vivo is thought to be induced by at least two main mediators, thromboxane A_2 (TXA_2) and ADP. Platelets have an important role in arterial thrombosis that may result in stroke or myocardial infarction. In this study, the effect of vapiprost (9, 10), a TXA_2-receptor antagonist; clopidogrel, which has the thienopyridine structure of ticlopidine and is a more potent inhibitor of ADP-induced platelet aggregation than ticlopidine (11-13); and argatroban (14, 15), a thrombin inhibitor, were investigated in the MCA thrombosis model in rats. Clopidogrel is an analogue of ticlopidine that has been reported to be effective for the prevention of stroke (5, 6).

Thrombin has two main functions, platelet aggregation and conversion of fibrinogen to fibrin, that are important in thrombosis (16, 17). Accordingly, recently specific thrombin inhibitors including argatroban have been developed and found to inhibit platelet-rich thrombus formation in several animal models (18-20). However, effects of thrombin inhibitors in animal models of cerebrovascular thrombosis have not yet been investigated. Therefore, in the present study, we have also investigated whether or not the thrombin inhibitor argatroban is effective against arterial thrombosis in our rat model.

MATERIALS AND METHODS

Agents

Vapiprost, [1*R*-[1α(*Z*),2β,3β,5α]]-(+)-7-[5-([1,1′-biphenyl]-4-ylmethoxy)-3-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid, hydrochloride was obtained from Glaxo Group Research, Ltd. (Greenford, England); clopidogrel, (*s*)-methyl(2-(2-chlorophenyl)-2-(4,5,6,7-tetrahydrothieno(3,2-C)pyridine-5-yl)acetate, hydrogen sulfate was obtained from Daiichi Pharmaceutical Co., Ltd. (Tokyo); and argatroban, (2*R*,4*R*)-4-methyl-1-[N^2-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginyl]-2-piperidinecarboxylic acid monohydrate, was obtained from Mitsubishi Kasei Co. (Tokyo). Heparin (187 units per 1 mg dry powder) was purchased from Sigma (St. Louis, MO, USA) and rose bengal was purchased from Wako (Osaka).

Animal preparation

Wistar male rats (SLC, Hamamatsu) weighing 240–260 g were used. The body temperature of the animals was maintained at 37.5℃ with a heating-pad (K-module Model K-20; American Pharmaseal Company, Valencia, CA, USA). The MCA thrombosis model in the rat has been described previously (8). In brief, under pentobarbital anesthesia and spontaneous respiration, a catheter for the administration of rose bengal or agents was placed in the femoral vein. The scalp was incised, the temporalis muscle was partialy cut, and a subtemporal craniotomy was performed using a dental drill under an operating microscope to open a 3-mm diameter circular bonny window.

Photo-irradiation

The 3-mm diameter circular window was irradiated with green light and the entire irradiated segment including the proximal end of the lenticulostriate branch became occluded by thrombi. Photo-irradiation with green light (wave length: 540 nm) was achieved by using a xenon lamp (L4887; Hamamatsu Photonics, Hamamatsu) with a heat absorbing filter and a green filter. The irradiation was directed by a 3-mm diameter optic fiber mounted on a micromanipulator. The head of the optic fiber was placed on the window in the skull base at a distance of 2 mm above the vessel, providing an irradiation dose of 0.62 W/cm^2. The probe (1 mm in diameter) of a laser Doppler flowmeter (ALF 2100; Advance, Tokyo), which is an established instrument and suitable for this model, was placed on the proximal end of the MCA observed in the window for measuring the MCA local blood flow (Fig. 1). When a steady local blood flow baseline was obtained during photo-irradiation, rose bengal (20 mg/kg) was injected intravenously. Photo-irradiation was continued for a further 8 min. The local blood flow in the MCA was continuously monitored for 15 min after rose bengal injection. The MCA was considered to be occluded when the local blood flow had completely stopped as indicated by the flow monitor. The time taken from the injection of rose bengal to the cessation of local blood flow was recorded as the MCA occlusion time. This was based on the condition that there was no reflow within one min after occlusion; otherwise, recording was continued until this condition was observed. In untreated animals (controls), no spontaneous reflow was observed. In treated animals, however, intermittent reflow following occlusion was very rare. When the MCA local blood flow continued beyond the 15-min observation period, the occlusion time was taken as 15 min (maximum). In preliminary experiments, blood pressure, heart rate and arterial blood gas were not affected by the combination of rose bengal and green light, the operation procedures or injection of drugs.

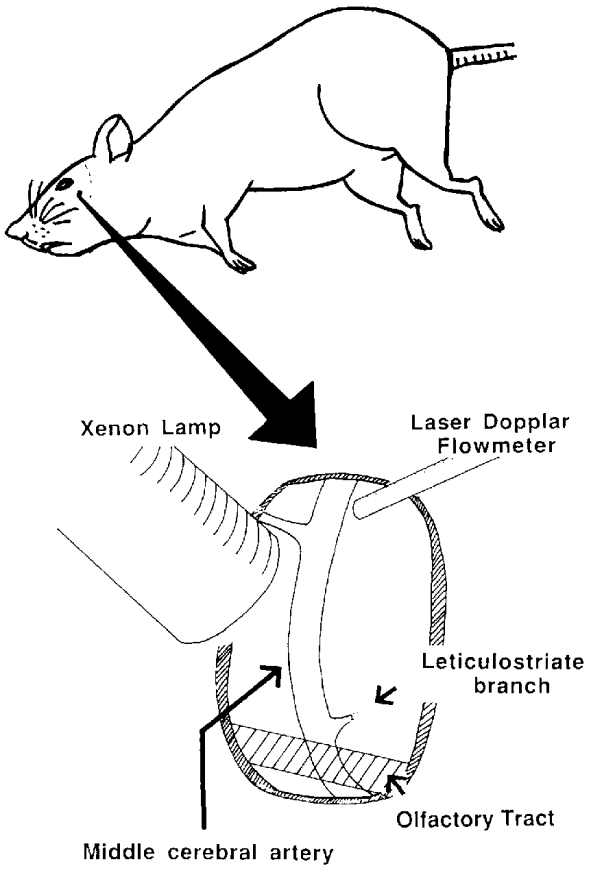

Fig. 1. Illustration of animal preparation.

In collaboration with Hamamatsu Photonics, we designed and developed the green light irradiation system, L4887. The light source is a short-arc type, Super-Quiet xenon lamp. This xenon lamp was chosen to achieve the

high brightness and stable light intensity necessary for irradiating a small area. Light is prefiltered and concentrated by an elliptical reflector that has a special coating for efficient absorption of infrared and ultraviolet radiations. The remaining visible light is spectrally modified by filters to produce green light of band width 80 nm, centered at 540 nm. Elimination of infrared and ultraviolet radiations is essential as these can heat up and damage biological tissues. To verify that the irradiation or photochemical reaction does not unphysiologically heat up the brain, in 4 animals, the temperature in the irradiated area near the MCA was measured with an implanted digital thermometer probe (0.5-mm diameter; Inter Medical, Tokyo).

Drug treatment

Various doses of the TXA_2-receptor antagonist vapiprost (0.1, 0.3 and 1.0 mg/kg) and heparin at the dose of 1.2 mg/kg (187 units/mg dry powder) were intravenously injected 10 min before the injection of rose bengal. Clopidogrel (1.0, 3.0 and 10 mg/kg) was intravenously administered 2 hr before the injection of rose bengal. Argatroban was infused at 10, 30 or 100 μg/kg per minute beginning 15 min before rose bengal injection and was continued for the entire 15-min observation period after rose bengal injection. The activated partial thromboplastin time (APTT) was determined in the blood from rats treated with argatroban or heparin using an APTT test kit (Pathrombin; Behringwerke, Tokyo).

Statistics

All results are expressed as means±S.E. The groups were compared with the analysis of variance. If there was a significant difference, Dunnett's multiple comparison was used. A P value <0.05 was considered as being significant.

RESULTS

Physiological parameters were within the normal range prior to rose bengal injection (mean arterial pressure: 113±2 mmHg, Po_2: 83.4±2.2 mmHg, Pco_2; 40.3±0.6 mmHg and pH: 7.41±0.01). During the irradiation period, the temperature in the irradiated zone increased by only 0.9℃. However, the brain temperature fell by 1℃ within 5 min after the cessation of photo-irradiation.

There was no significant difference in the MCA local blood flow before rose bengal injection among the different groups (Table 1). A typical tracing of the MCA local blood flow is shown in Fig. 2. The MAC local blood flow transiently increased just after the rose bengal injection and did not change up to 4 min. The MCA was occluded completely by thrombus about 5 min after initiation of the photochemical reaction (Table 1). The brief increase in local blood flow just immediately before the precipitous fall may be accounted for as follows: Just before occlusion, the vessel lumen at the irradiation segment becomes critically narrowed by thrombus. This can result in backflow due to a fall in local blood pressure immedi-

Table 1. Effects of vapiprost, clopidogrel, argatroban and heparin on the time for the thrombotic occlusion of the MCA, the MCA local blood flow before rose bengal injection and APTT

Substance	Dose	n	MCA local blood flow (mV)	The time to occlusion (sec)	APTT (sec)
Saline		7	1280±130	287.9±14.6	17.2±0.3
Vapiprost					
	0.1 mg/kg	7	1420±130	352.9±41.0	—
	0.3	7	1200±110	609.3±79.3*	—
	1.0	7	1330±100	742.9±78.0*	—
Clopidogrel					
	1.0 mg/kg	7	1520±120	315.7±53.9	—
	3.0	7	1420±130	421.4±104.5	—
	10	7	1250±110	715.0±102.2*	—
Argatroban					
	10 μg/kg/min	7	1450±210	362.9±50.8	27.1±1.0*
	30	7	1350±110	444.3±20.0*	43.7±3.7*
	100	7	1580±140	774.3±81.3*	86.8±4.8*
Heparin					
	1.2 mg/kg	7	1010±130	298.3±71.0	>200*

Data are expressed as means±S.E. *$P<0.05$ vs control group. The MCA local blood flow was measured before rose bengal injection. The time taken following rose bengal injection to the thrombotic occlusion of the artery was designated the occlusion time.

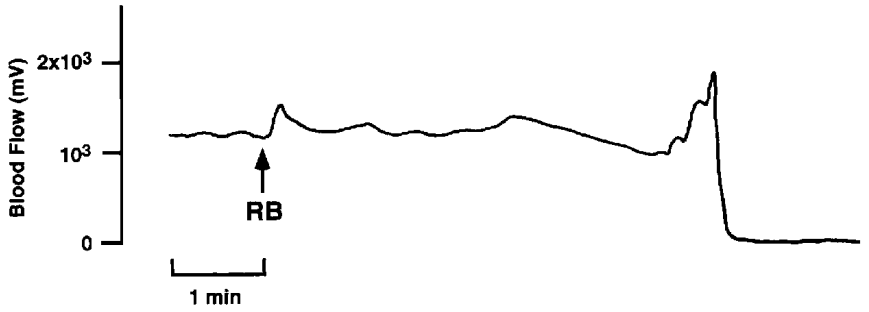

Fig. 2. A typical tracing of the middle cerebral artery local blood flow recorded by the laser Doppler flowmeter. RB: injection of rose bengal.

ately distal to the stenotic site.

In this model, only the segment of the artery that is irradiated with green light in the presence of rose bengal becomes occluded by thrombis (the site of photochemical reaction) (data not presented, K. Umemura et al.). Neither photo-irradiation nor rose bengal alone could induce thrombosis as was verified by monitoring local blood flow for up to 60 min during continuous photo-irradiation and scanning electron microscopic observation of the vessel.

Vapiprost treatment significantly prolonged the time for arterial occlusion in a dose-dependent manner (Table 1). At the doses of 0.3 and 1.0 mg/kg, vapiprost completely inhibited thrombus formation during the 15-min observation period following the photochemical reaction in 2 and 4 out of 7 animals, respectively (2 groups, Fig. 3). Likewise clopidogrel significantly prolonged the occlusion time in a dose-dependent manner (Table 1). At a dose of 10 mg/kg, clopidogrel completely inhibited thrombus formation during the 15-min observation period in 4 out of 7 animals (Fig. 4). When clopidogrel was intravenously administered and 2 hr later, blood was taken for the ex-vivo platelet aggregation test, there was a marked inhibition of ADP-induced platelet aggregation (data not presented, K. Umemura et al.).

At a dose of 30 μg/kg per min, argatroban significantly prolonged the MCA occlusion time; at 100 μg/kg per min, it completely inhibited thrombus formation during the 15-min observation period in 4 out of 7 animals (Fig. 5). The APTT was also significantly prolonged by argatroban in a dose-dependent manner. However, heparin, which prolonged the APTT by a factor of 10, was without effect against the MCA occlusion.

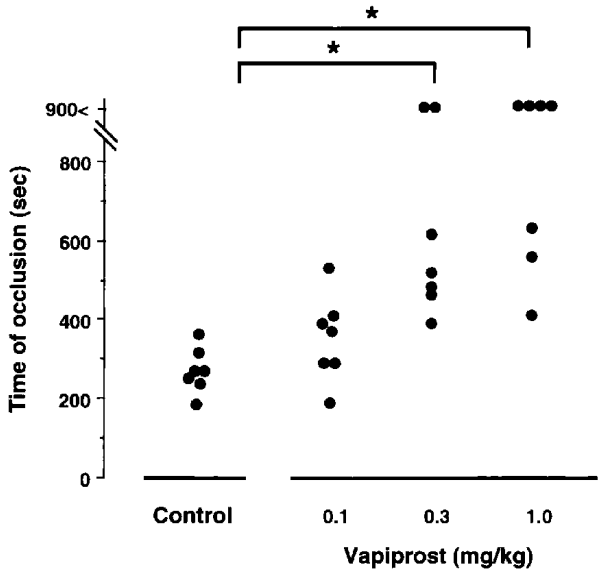

Fig. 3. Effect of vapiprost on the middle cerebral artery thrombosis in rats. *P<0.05 vs control group.

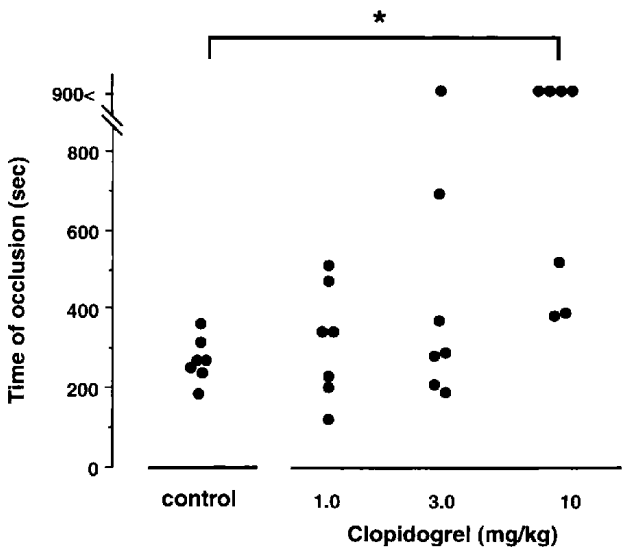

Fig. 4. Effect of clopidogrel on the middle cerebral artery thrombosis in rats. *P<0.05 vs control group.

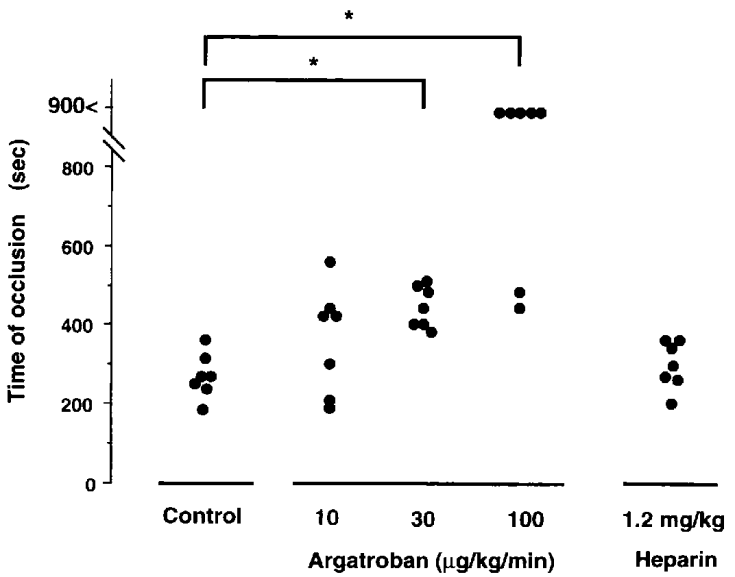

Fig. 5. Effects of argatroban and heparin on the middle cerebral artery thrombosis in rats. *P<0.05 vs control group.

DISCUSSION

In this study, thrombotic occlusion of the rat middle cerebral artery was achieved by photochemical reaction between rose bengal and green light that causes endothelial injury followed by platelet adhesion to the damaged vessel and formation of a thrombus at the site of the photochemical reaction. Irradiation of the vessel in the absence of rose bengal was completely ineffective in inducing thrombosis or altering local blood flow. Against the thrombotic occlusion of MCA, we tested three agents with different mechanisms of actions: vapiprost, a TXA_2-receptor antagonist; clopidogrel, which has the thienopyridine structure of ticlopidine and is a more potent inhibitor of ADP-induced platelet aggregation than ticlopidine; and argatroban, a specific thrombin inhibitor. The prolongation of the time required to achieve thrombotic occlusion of the MCA was used as an index of the antithrombotic effect for each agent. All three preparations significantly slowed the occlusion of MCA, suggesting that platelet activation and thrombin generation contribute to the MCA thrombosis in this model.

It has been reported that platelets of the rat do not strongly aggregate by TXA_2 in vitro (21). However, vapiprost, TXA_2-receptor antagonist, inhibited platelet agrregation induced by collagen in whole blood (22) and was effective in the MCA thrombosis model. This suggests that TXA_2 may be involved in the thrombogenesis of the MCA in this model.

The thrombin inhibitor argatroban that prolonged the MCA occlusion time also significantly increased the APTT, but heparin that showed a striking effect in increasing the APTT was ineffective against the thrombotic occlusion of MCA. In line with our observation, several other investigators have also reported that thrombin has an important role in the formation of platelet-rich thrombi in arteries, but heparin has been ineffective in these models (18–20). The mechanisms of actions of argatroban and heparin on thrombin are, however, different. Heparin is well known to form a complex with plasma antithrombin III, and then this complex neutralizes thrombin. Argatroban, on the other hand, binds directly to the active sites of the thrombin molecule (23). Hanson and Harker (24) have shown that argatroban, which is a relatively small molecule, can diffuse into the thrombus and interact with thrombin that is bound to platelet receptors. Furthermore, the argatroban-thrombin complex binds to the high affinity functional thrombin receptors on platelets without initiating platelet activation (24). In contrast, high molecular weight fractions of heparin are known (25) to activate platelets, but fibrin II monomer (26) and platelet factor 4 (27) are known to inactivate heparin. However, it is not known to us if the concentration of fibrin II monomer and platelet factor 4 at the site of thrombogenesis in our model are high enough to neutralize heparin. Our findings with argatroban suggest that thrombin may play an important role in the cerebral artery thrombosis in this model. Moreover, platelet aggregation may contribute to the thrombogenesis more than blood coagulation in our model.

In conclusion, our observations with vapiprost, clopidogrel and argatroban suggest that thromboxane A_2, ADP and thrombin are involved in the thrombogenesis of the rat middle cerebral artery under the experimental con-

ditions we used.

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REFERENCES

1 Feliste R, Celebassee D, Simon MF, Chap H, Defreyn G, Vallee E, Douste-Blazy L and Maffrand JP: Broad spectrum anti-platelet activity of ticlopidine and PCR-4099 involves the suppression of the effects of released ADP. Thromb Res **48**, 403–415 (1987)

2 Antiplatelet Trialists' Collaboration: Secondary prevention of vascular disease by prolonged antiplatelet treatment. Br Med J **296**, 320–331 (1988)

3 Bousser MG, Eshwege E, Haguenau M, Lefaucconnier JM, Thibult N, Touboul D and Touboul PJ: "AICLA" controlled trial of aspirin and dipyridamole in the secondary prevention of arthero-thrombotic cerebral ischemia. Stroke **14**, 5–14 (1983)

4 Canadian Cooperative Study Group: A randomized trial of aspirin and sulfinpyrazone in threatened stroke. N Engl J Med **229**, 53–59 (1987)

5 Gregory W and Albers MD: Role of ticlopidine for prevention of stroke. Stroke **23**, 912–916 (1992)

6 Grotta JC, Norris JW, Kamm B and the TASS Baseline and Angiographic Data Subgroup: Prevention of stroke with ticlopidine: Who benefits most? Neurology **42**, 111–115 (1992)

7 Matsuno Y, Onodera H, Shiga Y, Nakamura M, Ninomiya M, Kihara T and Kogure K: Correlation between myeloperoxidase-quantified neutrophil accumulation and ischemic brain injury in the rat: Effects of neutrophil depletion. Stroke **25**, 1469–1475 (1994)

8 Umemura K, Wada K, Uematsu T and Nakashima M: Evaluation of the combination of a tissue-type plasminogen activator, SUN9216, and a thromboxane A_2 receptor antagonist, vapiprost, in a rat middle cerebral artery thrombosis model. Stroke **24**, 1077–1081 (1993)

9 Hornby EJ, Foster MR, McCabe PJ and Stratton LE: The inhibitory effect of GR32191, a thromboxane receptor blocking drug, on human platelet aggregation, adhesion and secretion. Thromb Haemost **61**, 429–436 (1989)

10 Humphrey PPA, Hallet P, Hornby EJ, Wallis CJ, Collington EW and Lumley P: Pathophysiological actions of thromboxane A_2 and their pharmacological antagonism by thromboxane receptor blockade with GR32191. Circulation **81**, Supp I, I-42–I-52 (1990)

11 Feliste R, Celebassee D, Simon MF, Chap H, Defreyn G, Vallee E, Douste-Blazy L and Maffrand JP: Broad spectrum anti-platelet activity of ticlopidine and PCR-4099 involves the suppression of the effects of released ADP. Thromb Res **48**, 403–415 (1987)

12 Yao SK, Ober JC, McNatt J, Benedict CR, Rosolowsky M, Anderson HV, Cui K, Maffrand JP, Campbell WB, Buja LM and Wollerson JT: ADP plays an important role in mediating platelet aggregation and cyclic flow variations in vivo in stenosed and endothelium-injured canine coronary arteries. Circ Res **70**, 39–48 (1992)

13 Samama CM, Bonnin P, Bonneau M, Pignaud G, Mazoyer E, Bailliart O, Maffrand JP, Viars P, Caen JP and Drouet LO: Comparative arterial antithrombotic activity of clopidogrel and acetyl salicylic acid in the pig. Thromb Haemostas **68**, 500–505 (1992)

14 Imura Y, Stassen JM, Vreys I, Lesaffre E, Gold HK and Collen D: Synergistic antithrombotic properties of G4120, a RGD-containing synthetic peptide, and argatroban, a synthetic thrombin inhibitor, in a hamster femoral vein platelet-rich thrombosis model. Thromb Haemostas **68**, 336–340 (1992)

15 Imura Y, Stassen JM and Collen D: Comparative antithrombotic effects of heparin, recombinant hirudin and argatroban in a hamster femoral vein platelet-rich mural thrombosis model. J Pharmacol Exp Ther **261**, 895–898 (1992)

16 Neshheim ME and Mann KG: Thrombin-catalyzed activation of a single chain bovine factor V. J Biol Chem **254**, 1426–1434 (1979)

17 Wagner WR, Hubbell JA, McIntyre LV and Mann KG: Importance of thrombin synthesis in mural thrombosis on collagen-coated glass. Circulation Supp **78**, II-312 (1988)

18 Edit JF, Allison P, Norle S, Ashton J, Golino P, McNatt J, Buja LM and Willerson JT: Thrombin is an important mediator of platelet aggregation in stenosed canine coronary arteries with endothelial injury. J Clin Invest **84**, 18–27 (1989)

19 Heras M, Chesebro JH, Webster MW, Mruk JS, Grell DE, Penny WJ, Bowie EJW, Badimon L and Fuster V: Hirudin, heparin, and placebo during deep arterial injury in the pig. The in vivo role of thrombin in platelet-mediated thrombosis. Circulation **82**, 1476–1484 (1990)

20 Jang IK, Gold HK, Ziskind AA, Leinbach RC, Fallon JT and Collen D: Prevention of platelet-rich arterial thrombosis by selective thrombin inhibitor. Circulation **81**, 219–225 (1990)

21 Nishizawa EE, Williams DJ and Connell CL: Arachidonate induced aggregation of rat platelets may not require prostaglandin endoperoxides or thromboxane A_2. Thromb Res **30**, 289–296 (1983)

22 Matsuno H, Uematsu T, Umemura K, Takiguchi Y, Wada K and Nakashima M: Effects of vapiprost, a novel thromboxane receptor antagonist, on thrombus formation and vascular patency after thrombolysis by tissue-type plasminogen activator. Br J Pharmacol **106**, 533–538 (1992)

23 Lindhout T, Braruch D, Schoen P, Franssen J and Hemker HC: Thrombin generation and inactivation in the presence of antithrombin III and heparin. Biochemistry **25**, 269–282 (1986)

24 Hanson SR and Harker LA: Interruption of acute platelet-dependent thrombosis by the synthetic antithrombin D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone. Proc Natl Acad USA **85**, 3184–3188 (1988)

25 Salzman EW, Rosenberg RD, Smith MH, Lindon JN and Favreau L: Effect of heparin and heparin fraction platelet aggregation. J Clin Invest **65**, 64–73 (1980)

26 Hogg PJ and Jackson CM: Fibrin monomer protects thrombin from inactivation by heparin-antithrombin III. Implications for heparin efficacy. Proc Natl Acad Sci USA **86**, 3619–3623 (1989)

27 Rucinski B, Niewiarowski S, James P, Walz DA and Budzynski AZ: Antiheparin proteins secreted by human platelets. Purification, characterization, and radioimmunoassay. Blood **53**, 47–62 (1979)