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European Journal of Pharmacology 320 (1997) 161–166

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YC-1, a nitric oxide-independent activator of soluble guanylate cyclase, inhibits platelet-rich thrombosis in mice

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Received 4 July 1996; revised 12 November 1996; accepted 15 November 1996

Abstract

YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole), a nitric oxide (NO)-independent activator of soluble guanylate cyclase, has been shown to inhibit platelet activation and aggregation in vitro through the generation of cGMP. In the present study, we assessed the antithrombotic effect of YC-1 in models of experimental thrombosis in mice. YC-1 (10, 30 μg/g, i.p.)-treated mice showed a prolonged tail bleeding time 30 min after injection (from control 91.0 ± 6.4 s to 208.6 ± 22.7 s and 291.8 ± 42.4 s, respectively). In contrast, aspirin at a dose of 30 μg/g (i.p.) prolonged the bleeding time to more than 600 s. Platelet-rich thrombus formation was induced by irradiation of the mesenteric venule with filtered light in mice pretreated intravenously with fluorescein sodium. YC-1 (30 μg/g, i.p.) markedly prolonged the occlusion time of irradiated venules (from control 146.1 ± 19.0 s to 275.6 ± 24.5 s) in heparinized (1 U/g) mice. In the same condition, aspirin (100 μg/g) only slightly prolonged the time required for occlusion (193.2 ± 13.2 s). In a model of fatal pulmonary thromboembolism induced by intravenous injection of ADP (300 μg/g), YC-1 was effective in reducing mortality when administered intraperitoneally at doses of 10–30 μg/g. The antithrombotic effect of YC-1 was correlated with the inhibition of ADP-induced platelet aggregation ex vivo. In contrast, aspirin (30, 100 μg/g) did not inhibit ADP-induced pulmonary thromboembolism in vivo or platelet aggregation ex vivo. YC-1 (3, 10 μg/g) also exhibited profibrinolytic activity ex vivo, as revealed by shortening of the euglobulin clot lysis time. Therefore, YC-1 is an effective antithrombotic agent in preventing thrombosis in animal models, and its antiaggregating and additional profibrinolytic effects may be of potential clinical benefit in the treatment of thromboembolic diseases.

Keywords: YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole) ; Tail bleeding time; Antithrombotic effect; Platelet aggregation; Profibrinolytic effect

1. Introduction

Numerous evidence indicates that platelets contribute significantly to the pathogenesis of thromboembolism, which is one of the major causes of death throughout the world (Dinerman and Mehta, 1990; Schwortz et al., 1990; Ross, 1993). Following endothelial damage or disruption of atherosclerotic plaque in blood vessels, platelets rapidly adhere to newly exposed extracellular matrix and release granular constituents, thereby recruiting more circulating platelets, resulting in the formation of a platelet-rich thrombus. Mural thrombus can restrict the blood flow and lead to myocardial, cerebral or peripheral ischemia. The developing thrombus may embolize with potentially lethal consequences. Therefore, inhibition of platelet function may be a promising approach to prevent and treat thrombotic diseases (Coller, 1992; Antiplatelet Trialists' Collaboration, 1994a).

We reported in our previous studies that YC-1, a chemically synthetic benzylindazole compound (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole), possesses antiplatelet activity in vitro, and demonstrated that YC-1 inhibited platelet aggregation through NO-independent activation of soluble guanylate cyclase (Ko et al., 1994; Wu et al., 1995). YC-1 also inhibited proliferation of rat A10 vascular smooth muscle cells in vitro via cGMP-dependent mechanisms (Yu et al., 1995). In addition, YC-1 was observed to exert a vasorelaxing effect in rat aorta in vitro, and to decrease the mean arterial pressure of spontaneously hypertensive rats (SHR) but not that of normotensive (WKY) rats in vivo (Yu, unpublished data). The objective of the present study is to measure the in vivo antithrombotic effect of YC-1 in several models of experimental thrombosis in order to evaluate the therapeutic

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PII S0014-2999(96)00911-9

potential of this novel guanylate cyclase activator in the treatment of thromboembolism.

2. Materials and methods

2.1. Tail bleeding time

Male ICR (International Cancer Research) mice (18–22 g) were i.p. injected with dimethylslufoxide (DMSO, vehicle, 2.5 μl/g), YC-1 or aspirin. After 30 min, the mouse was placed in a tube holder with its tail protruding and then a cut 2 mm from the tail tip was made. Immediately, the mouse's tail was vertically immersed into normal saline at 37°C. Bleeding time was recorded from the time bleeding started till it had completely stopped for more than 30 s (Dejana et al., 1982).

2.2. Fluorescent dye-induced platelet thrombus formation in mesenteric venules of mice

A modification of the method used in previous reports (Rosenblum and El-Sabban, 1977; Sato and Ohshima, 1984; Chang and Huang, 1994) was used. Male ICR mice (18–22 g) were i.p. injected with YC-1, aspirin or DMSO. After 30 min, fluorescein sodium (12.5 μg/g) with or without heparin (1 U/g) was injected i.v. through a lateral tail vein of the mouse. The mouse was then anesthetized with sodium pentobarbital (50 μg/g, i.p.). A segment of small intestine attached to its mesentery was exteriorized through a midline incision on the abdominal wall and placed onto a transparent plastic plate for microscopic observation. Frequent rinsing of the mesentery with warm saline solution kept at 37°C was performed to prevent the mesentery from drying.

Microvessels in the mesentery were observed under transillumination from a halogen lamp. Venules with diameter of 30–40 μm were selected to be irradiated to produce a microthrombus. In the epi-illumination system, the light from a 100-W mercury lamp was excited by a filter (B-2 A, Nikon) with a dichroic mirror (DM510, Nikon). This filtered light, from which all wave lengths below 520 nm were eliminated, irradiated a microvessel (the area of the irradiation was about 100 μm in diameter on the focal plane) through an objective lens (×20). Five minutes after the administration of fluorescein sodium, the irradiation by filtered light and the video timer were started simultaneously and thrombus formation was observed on a TV-monitor. Occlusion time was recorded from the time when filtered light started to when the blood flow in the irradiated vessel stopped completely.

2.3. ADP-induced acute pulmonary thromboembolism in mice

Acute pulmonary thromboembolism was induced according to the method previously described by Nordoy and Chandler (1964). Male ICR mice (18–24 g) were i.p. injected with YC-1, aspirin or DMSO. After 30 min, ADP (300 μg/g) was injected into the lateral vein of the mouse. The mortality in each group, after injection, was determined within 10 min.

2.4. Measurement of platelet aggregation ex vivo

After i.p. administration of YC-1 or DMSO, male ICR mice (20–25 g) were anesthetized with sodium pentobarbital (50 μg/g) and the chest was opened. A blood sample (1 ml) was withdrawn from the heart with a syringe pre-filled with 3.8% sodium citrate (1:10, v/v). Blood was then centrifuged at $180 \times g$ for 4 min to obtain platelet-rich plasma. The platelet number of platelet-rich plasma was adjusted to about 3×10^8 platelets per ml with platelet-poor plasma. Ex vivo platelet aggregability of platelet-rich plasma was measured at 37°C by using the turbidimetric method (O'Brien, 1962). The aggregation response was measured as the maximal change in light absorbance as compared to that of platelet-poor plasma and expressed as percentage aggregation.

2.5. Assay of fibrinolytic activity

Plasma fibrinolytic activity was assayed ex vivo by the euglobulin clot lysis time, based on the method of Von Kaulla and Schultz (1958). Citrated blood was obtained from mice pretreated i.p. with YC-1 or DMSO, and then incubated with or without ADP (10 μM) at 37°C for 10 min. The blood was centrifuged at $14\,900 \times g$ for 2 min to produce platelet-poor plasma. Platelet-poor plasma (0.25 ml) was diluted with ice-cold distilled water (4.75 ml), and the pH was adjusted to pH 5.9 with acetic acid. This procedure caused precipitation of the euglobulin fraction. After incubation for 10 min at 4°C followed by centrifugation ($3000 \times g$) for 10 min at 4°C, the supernatant was discarded and the euglobulin precipitate was dissolved in 0.5 ml phosphate buffer (13.4 mM KH_2PO_4, 53.6 mM Na_2HPO_4). One unit thrombin (10 U/ml, 0.1 ml) was added to 0.4 ml euglobulin fraction at time 0 to induce clot formation. The clot was then incubated at 37°C and the time taken for complete lysis to occur was recorded.

Plasma fibrinolytic activity was also assayed in vitro. Citrated blood was preincubated with YC-1 (60 μg/ml) or DMSO for 3 min at 37°C, and then ADP (10 μM) was added and the incubations were continued for another 10 min. The preparation of euglobulin fraction and measurement of euglobulin clot lysis time were performed as described above.

2.6. Drugs

YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole] was chemically synthesized as described previously (Yoshina and Kuo, 1978). Aspirin was purchased from Sigma (St. Louis, MO, USA). YC-1 and aspirin were dissolved in dimethylsulfoxide (DMSO). Heparin, fluorescein sodium and ADP were purchased from Sigma and dissolved in normal saline.

2.7. Statistical analysis

Results are expressed as the means ± S.E.M. of n experiments. Unpaired Student's t-test was used to evaluate the data obtained from in vivo and ex vivo experiments. Paired Student's t-test was used to evaluate the differences in euglobulin colt lysis time in vitro. A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of YC-1 on tail bleeding time of mice

The tail bleeding time of control mice (injected with DMSO, 2.5 μl/g, i.p.) was measured to be 91.0 ± 6.4 s (n = 18). Aspirin (3–30 μg/g, i.p.)-treated mice showed a dose-dependent prolongation of the bleeding time 30 min after injection; at a dose of 30 μg/g, aspirin prolonged the bleeding time to more than 600 s. YC-1 (3–30 μg/g, i.p.) also prolonged, but less potently than aspirin, the bleeding time of mice 30 min after injection (Table 1).

After 15, 30, 60 and 120 min of i.p. administration with YC-1 (30 μg/g), the tail bleeding times of mice were recorded as 126.6 ± 24.5, 364.0 ± 92.0, 220.0 ± 89.1 and 162.2 ± 62.3 s (n = 5), respectively. Since YC-1 had its maximal effect on bleeding time 30 min after i.p. injection, this time was chosen for the further experiments reported here.

Platelet counts of mice ($906.8 \pm 40.5 \times 10^3$ platelets/μl for control) were not observed to change 30 min after i.p. administration with YC-1 of 3, 10 and 30 μg/g (data not shown).

3.2. Effect of YC-1 on fluorescein sodium-induced platelet-rich thrombus formation in mesenteric venules of mice

In heparinized (1 U/g, i.v.) mice, fluorescein sodium and filtered light irradiation was observed to cause platelet-rich thrombus formation in mesenteric venules. The occlusion time of the irradiated vessel was 146.1 ± 19.0 s (n = 12). From the data shown in Table 2, YC-1 (10, 30 μg/g, i.p.) dose dependently prolonged the occlusion time to 190.8 ± 18.5 s (n = 8) and 275.6 ± 24.5 s (n = 9), respectively. Aspirin (100 μg/g, i.p.) also increased the occlusion time to 193.2 ± 13.2 s (n = 9) in heparinized mice.

YC-1 (30 μg/g) had no significant effect on the occlusion time in mice which were not pretreated with heparin (188.3 ± 13.8, n = 8 vs. control 165.8 ± 11.5 s, n = 12).

Table 1
Effect of YC-1 on the tail bleeding time of mice

		Tail bleeding time (s)	n
DMSO (control)		91.0 ± 6.4	18
YC-1	3 μg/g	119.0 ± 17.4	10
	10 μg/g	208.6 ± 22.7 [a]	14
	30 μg/g	291.8 ± 42.4 [a]	15
Aspirin	3 μg/g	196.6 ± 17.6 [a]	8
	10 μg/g	538.8 ± 57.3 [a]	8
	30 μg/g	600.0 ± 0 [a]	6

Bleeding times longer than 600 s were recorded as 600 s. Values are presented as means ± S.E.M. of experimental number (n) indicated. [a] $P < 0.001$ as compared with the control.

Table 2
Effect of YC-1 on fluorescent dye-induced platelet-rich thrombus formation in mesenteric venules of heparinized (1 U/g) mice

		Occlusion time (s)	n
DMSO (control)		146.1 ± 19.0	12
YC-1	10 μg/g	190.8 ± 18.5	8
	30 μg/g	275.6 ± 24.5 [a]	9
Aspirin	100 μg/g	193.2 ± 13.2	9

Values are presented as means ± S.E.M. of experimental number (n) indicated. [a] $P < 0.001$ as compared with the control.

3.3. Effect of YC-1 on ADP-induced acute pulmonary thromboembolism

When ADP (300 μg/g) was injected into the tail vein of the mouse to induce acute pulmonary thromboembolism, 95% of treated mice died within 3 min (n = 20). From the results summarized in Table 3, YC-1 (10 and 30 μg/g) dose dependently reduced mortality to 67% and 50%, respectively. In contrast, aspirin (30 and 100 μg/g) was ineffective in protecting the mice from death.

3.4. Effect of YC-1 on platelet aggregation ex vivo

In the platelet-rich plasma obtained from control mice (injected with DMSO), ADP (10 μM) was found to induce 55.9 ± 3.6% platelet aggregation (n = 6). YC-1 (10 and 30 μg/g, i.p., 30 min) inhibited ADP-induced platelet aggregation ex vivo, in a dose-dependent manner (percentages of platelet aggregation were 37.7 ± 9.6 and 26.1 ± 9.3%

Table 3
Effect of YC-1 on mortality due to acute pulmonary thromboembolism induced by intravenous injection of ADP (300 μg/g) in mice

		Number of dead mice	Total number	Mortality (%)
DMSO (control)		19	20	95
YC-1	10 μg/g	8	12	66.7
	30 μg/g	8	16	50
Aspirin	30 μg/g	7	8	87.5
	100 μg/g	8	8	100

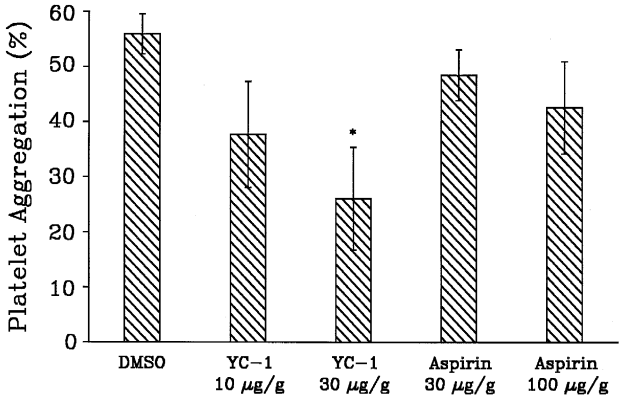

Fig. 1. The antiaggregatory effect of YC-1 and aspirin ex vivo. Platelet aggregation was induced by ADP (10 μM) in platelet-rich plasma (3×10^8 platelets/ml) obtained from mice treated with DMSO (2.5 μl/g, i.p., control), YC-1 (10 and 30 μg/g, i.p.) or aspirin (30 and 100 μg/g, i.p.). Values are presented as means ± S.E.M. ($n=6$). * $P<0.01$ as compared with the control.

respectively, $n=6$) (Fig. 1). Aspirin (30 and 100 μg/g) had no significant effect on ADP-induced platelet aggregation ex vivo.

3.5. Effect of YC-1 on euglobulin clot lysis time

The euglobulin clot lysis time of blood which was very carefully obtained from the control mice was 108.3 ± 1.5 min; however, when the blood was challenged with ADP (10 μM), the euglobulin clot lysis time showed a marked increase to 172.3 ± 14.6 min.

YC-1 (30 μg/g) did not affect the euglobulin clot lysis time of unchallenged blood ex vivo (113.8 ± 15.4 min). However, in ADP-challenged blood, YC-1 (3 and 10 μg/g) ex vivo markedly decreased the euglobulin clot lysis time to 147.2 ± 11.5 and 121.6 ± 13.9 min, respectively (Fig. 2).

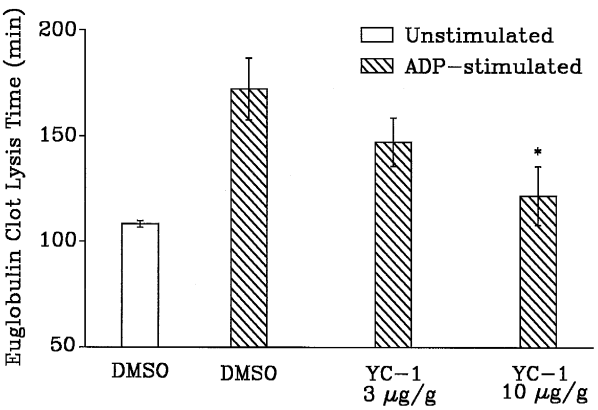

Fig. 2. The profibrinolytic effect of YC-1 ex vivo. Citrated blood was obtained from mice treated with DMSO (2.5 μl/g, i.p., control) or YC-1 (3 and 10 μg/g, i.p.) and then incubated without (blank bar) or with ADP (10 μM, hatched bars) at 37°C for 10 min. The plasma fibrinolytic activity was assayed by the euglobulin clot lysis time as described in Section 2. Values are presented as means ± S.E.M. ($n=6$–8). * $P<0.05$ as compared with the control (hatched bar).

YC-1 (60 μg/ml) also shortened the euglobulin clot lysis time of ADP-challenged blood in vitro (from control 166.7 ± 10.5 to 139.8 ± 15.8 min, $n=6$, $P<0.05$). Furthermore, in the presence of methylene blue (10 μM), an inhibitor of soluble guanylate cyclase, the effect of YC-1 on the euglobulin clot lysis time of ADP-challenged blood in vitro was abolished (141.8 ± 16.1 vs. control 150.8 ± 16.8 min, $n=4$). On the other hand, when YC-1 was directly added to euglobulin fraction, the euglobulin clot lysis time remained unchanged (data not shown).

4. Discussion

YC-1, a novel activator of soluble guanylate cyclase, is known to inhibit platelet activation (e.g., ATP release, thromboxane A_2 formation, phosphoinositide breakdown and intracellular Ca^{2+} mobilization) and platelet aggregation in vitro, through cGMP-dependent mechanisms (Ko et al., 1994; Wu et al., 1995). In the present study, we have shown that YC-1 exhibited antiaggregatory and profibrinolytic activities ex vivo, prolonged the occlusion time in the fluorescent dye-induced thrombosis model and decreased the mortality of ADP-induced pulmonary thromboembolism in mice.

In the fluorescent dye-induced thrombosis model, when intravascular fluorescein sodium absorbed the filtered light, the photoexcited dye generated toxic substances (e.g., reactive oxygen species), leading to endothelial damage and then platelet adherence to the damaged surface and aggregation (Sato and Ohshima, 1984). Electron microscopy revealed that the platelet-rich thrombus was localized at denuded endothelium where the underlying basement membrane was exposed (Rosenblum and El-Sabban, 1977; Sheu et al., 1994). It is possible that during the thrombotic process, the coagulatory pathway is also activated, which further facilitates thrombus formation. This may explain why YC-1 alone showed no significant effect on occlusion of irradiated venules. There are several lines of evidence to suggest that platelets may not be the only factor contributing to the formation of thrombus in this model. First, the antiplatelet agent prostaglandin E_1 had only limited or no effect in this thrombosis model (Sheu et al., 1995). Second, trigramin, a potent antagonist of platelet glycoprotein IIb/IIIa ($k_i=20$ nM, Huang et al., 1987), which inhibited platelet aggregation in vitro ($IC_{50}=0.2$–0.4 μM) and markedly prolonged the bleeding time in hamsters (0.17–2.12 mg/kg, i.v., Cook et al., 1989), had no significant effect on this fluorescent dye-induced thrombosis model, even at a very high dose (20 mg/kg, i.v., Sheu et al., 1995). Third, ancrod, a thrombin-like enzyme, causes defibrinogenation by breaking down plasma fibrinogen to form non-cross-linked fibrin which is rapidly removed from circulation, markedly prolonged the occlusion time in this thrombosis model (Chang and Huang, 1994). Taken together, we suggest that in addition to platelets, the

coagulation cascade also plays a critical role in the fluorescent dye-induced thrombosis model. In fact, by eliminating the effect of coagulatory factors by pretreatment with heparin, YC-1 markedly prolonged the occlusion time of irradiated venules.

In the acute pulmonary thromboembolism model, intravenous injection of ADP causes in the sudden death of mice. Histological examination of the organs of the dead animals reveals massive occlusion of the microvessels of the lungs by platelet aggregates (Nordoy and Chandler, 1964). In contrast to the fluorescent dye-induced thrombosis model, in which the vessel wall is injured and the coagulation pathway may be involved in thrombus formation, in ADP-induced pulmonary thromboembolism model, the vessel wall is not damaged and platelet aggregation is the critical factor. Hence, the antiplatelet agent YC-1 was effective in preventing ADP-induced thromboembolic death as expected.

In the present study, aspirin markedly prolonged the tail bleeding time of mice, but had only little or no effect on fluorescent dye-induced thrombosis and ADP-induced pulmonary thromboembolism. This is consistent with the finding of Sheu et al. (1994), who showed that indomethacin, another inhibitor of cyclooxygenase, is ineffective in these thrombosis models. There is some evidence to imply that different mechanisms may be involved in hemostasis and experimental thrombosis models. Suehiro et al. (1994) have reported that aspirin did not inhibit thrombus formation in rat carotid arteriovenous shunt, while the bleeding time was markedly prolonged. In contrast, ticlopidine inhibited thrombus formation without prolonging the bleeding time. Comparison of the effect of various GP IIb/IIIa antagonists (trigramin, triflavin, eristostatin and albolabrin) on the bleeding time, ADP-induced thromboembolism or photochemical-induced thrombus formation (Beviglia et al., 1993; Sheu et al., 1995), also indicated that antithrombotic activity does not correlate well with the prolongation of bleeding time. However, the precise mechanisms accounting for the discrepancy are still unknown. In contrast to YC-1, which inhibits platelet aggregation caused by various platelet activators through elevation of cGMP levels in platelets, aspirin only inhibits cyclooxygenase, thus blocking platelet thromboxane A_2 production. Thus aspirin may be insufficient to prevent thrombus formation in thrombosis in which multiple factors other than thromboxane A_2 are involved. In addition, blockade of the synthesis of endothelium-derived prostanoids which can prevent platelet deposition and aggregation may also account for the ineffectiveness of aspirin and other inhibitors of cyclooxygenase (Lindberg et al., 1994).

Increased risk of hemorrhage is a major complication in antiplatelet therapy, particularly when it is combined with thrombolytic agents. It has been reported that aspirin and glycoprotein IIb/IIIa receptor blockers dramatically prolong the bleeding time and increase the bleeding episodes in clinical antiplatelet trials (Antiplatelet Trialists' Collaboration, 1994b; The EPIC Investigators, 1994). In the present study, YC-1 moderately prolonged the tail bleeding time in mice, but its potency was much lower than that of aspirin. Hence, it is expected that YC-1 may cause fewer bleeding complications.

There is increasing evidence that platelets play a key role in thrombolysis (Coller, 1990). Platelet-rich thrombi are particularly resistant to thrombolysis in ex vivo and in vivo models. Furthermore, antiplatelet agents enhance the efficacy of thrombolytic therapy and also decrease the incidence of reocclusion (Fareed et al., 1992). Activated platelets are able to release substances which inhibit fibrinolysis, e.g., plasminogen activator inhibitor-1 (PAI-1) and α_2-antiplasmin, thereby mediating thrombolysis resistance (Levi et al., 1992; Stringer et al., 1994; Fay et al., 1994). Studies with NO donors, including SIN-1 and sodium nitroprusside, have indicated that NO donors at doses that do not affect platelet aggregation shorten the euglobulin clot lysis time through increased *t*-PA activity. This suggests that the phenomenon is a result of inhibition of PAI-1 release from platelets (Basista et al., 1985; Lidbury et al., 1990; Drummer et al., 1991). However, it has been reported recently that the activity of *t*-PA could be directly enhanced by NO via *S*-nitrosylation (Stamler et al., 1992; Stamler, 1994). In this study, we used YC-1, a NO-independent activator of soluble guanylate cyclase, to explore the role of cGMP-elevating agents on fibrinolysis. When administered to mice with YC-1, the euglobulin clot lysis time of the ADP-stimulated blood was decreased almost to the unstimulated level. YC-1 did not directly affect the fibrinolytic process in a cell-free system and had no effect on the euglobulin clot lysis time of unstimulated blood ex vivo. Hence, we can surmise that the profibrinolytic activity of YC-1 is mainly due to inhibition of platelet activation, and thus prevention of the release of inhibitors of fibrinolysis. This idea is further supported by the fact that YC-1 shortened the euglobulin clot lysis time of ADP-stimulated blood in vitro. In addition, prevention of the effect of YC-1 on fibrinolysis by methylene blue indicated that the profibrinolytic activity was mediated by soluble guanylate cyclase. Since YC-1 can activate soluble guanylate cyclase in a NO-independent manner, it is suggested that activation of soluble guanylate cyclase is sufficient to account for the profibrinolytic effect of NO donors, although the direct modification of *t*-PA by NO cannot be excluded. Furthermore, YC-1 and NO donors exhibited profibrinolytic activity at doses that have no or only minimal effect on platelet aggregation. Thus, the mechanism that mediates platelet-involved fibrinolytic inhibition is more sensitive to soluble guanylate cyclase activators than that mediating platelet aggregation.

In conclusion, YC-1 is shown to have an antithrombotic effect on platelet-rich thrombus formation in vivo, concomitant with inhibition of platelet aggregation and profibrinolytic activity ex vivo. We suggest that YC-1 may be of

potential clinical benefit in the treatment of thromboembolic diseases.

Acknowledgements

This work was supported by a research grant from the National Science Council of Taiwan (NSC85-2331-B002-002-M25).

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