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Inhibitory activity of kinetin on free radical formation of activated platelets in vitro and on thrombus formation in vivo

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Abstract

Kinetin has been shown to have anti-aging effects on several different systems, including plants and human cells. Recently, we demonstrated that kinetin markedly inhibited platelet aggregation in washed human platelets. In the present study, an electron spin resonance (ESR) method was used to further evaluate the scavenging activity of kinetin on the free radicals formed. Kinetin (70 and 150 μM) concentration dependently reduced the ESR signal intensity of hydroxyl radicals in collagen (1 μg/ml)-activated platelets. Furthermore, kinetin was effective in reducing the mortality of ADP-induced acute pulmonary thromboembolism in mice when administered intravenously at doses of 4 and 6 mg/kg. In addition, intravenous injection of kinetin (4 and 6 mg/kg) significantly prolonged the bleeding time by approximately 1.9- and 2.1-fold as compared with normal saline in severed mesenteric arteries of rats. A continuous infusion of kinetin (0.6 mg/kg/min) for 10 min also significantly increased the bleeding time by about 2.3-fold, and the bleeding time returned to baseline within 120 min after cessation of kinetin infusion. Platelet thrombi formation was induced by irradiation of mesenteric venules with filtered light in mice pretreated intravenously with fluorescein sodium. When kinetin was administered at 13 and 14 mg/kg in mice pretreated with fluorescein sodium (5 mg/kg), the occlusion time was significantly prolonged. In conclusion, these results suggest that kinetin has effective free radical-scavenging activity in vitro and antithrombotic activity in vivo. Treatment with kinetin may lower the risk of thromboembolic-related disorders. Therefore, kinetin may be a potential therapeutic agent for arterial thrombosis, but its toxicity must be further assessed.

Keywords: Kinetin; Hydroxyl radical; Bleeding time; Occlusion time; Arterial thrombosis

1. Introduction

N^6-furfuryladenine (kinetin) belongs to the family of N^6-substituted adenine derivatives known as cytokinins. Cytokinins are plant hormones that promote cell growth, development, and division (Zhang et al., 1996). In addition to these effects, kinetin has also been shown to have anti-aging effects in several different systems including human fibroblasts and the fruit fly, *Zaprionus paravittiger* (Rattan and Clark, 1994; Sharma et al., 1995). Although the detailed mechanisms of action of kinetin have not yet been completely revealed, several lines of evidence indicate that kinetin may act directly as an antioxidant or indirectly as a regulator of antioxidants. For example, kinetin has been shown to (a) have a direct effect on superoxide dismutase activity in plants (Goldstein and Czapski, 1991); (b) prevent oxidation of unsaturated acids in plant membranes (Leshem, 1988); (c) slow down development and aging in insects, by reducing their fecundity and increasing the specific activity of catalase (Sharma et al., 1995); and (d) delay the onset of many age-related characteristics that appear in normal human skin fibroblasts undergoing aging in vitro (Rattan and Clark, 1994).

Intravascular thrombosis is one of the generators of a wide variety of cardiovascular diseases. Initiation of intraluminal thrombosis is believed to involve platelet adherence and aggregation. Thus, platelet aggregation may play a crucial role in atherothrombotic processes. Indeed, antiplatelet agents (e.g., ticlopidine and aspirin) have been shown to reduce the incidence of stroke in high-risk patients (Hass et al., 1989). The activation of platelets leads to the release of free radicals both inside platelets and in the external sur-

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roundings (Salvemini and Botting, 1993). Free radical species modify both the adhesive and aggregatory responses of platelets, and free radical scavengers are therefore important regulators of platelet function. These scavengers can protect platelets against stimulation by free radicals and prevent damage to the vascular endothelium. It should be possible to prevent platelet activation and endothelial damage by drugs that possess both antioxidant and antiplatelet activities.

Recently, we systematically began to study the inhibitory activity of kinetin on platelet aggregation, and found that the antiplatelet activity of kinetin (50–150 μM) may result from inhibition of the activation of phospholipase C and the Na^+/H^+ exchanger. This leads to lower intracellular alkalinization and intracellular Ca^{2+} mobilization, followed by inhibition of thromboxane A_2 formation, and then increased cyclic AMP formation, followed by a further inhibition of the Na^+/H^+ exchanger, ultimately resulting in markedly decreased intracellular Ca^{2+} mobilization and phosphorylation of P47 (Sheu et al., in press).

In this study, we further evaluated the free radical scavenging activity of kinetin in activated platelets by electron spin resonance (ESR) and the antithrombotic activity of kinetin in three in vivo models: ADP-induced acute pulmonary thrombosis in mice, hemostatic bleeding time in rat mesenteric arteries, and irradiation of mesenteric microvessels in fluorescein sodium-pretreated mice. It has been reported that platelet thrombi can be induced by irradiation with filtered light of the microvasculature of mice pretreated with fluorescein sodium (Sato and Ohshima, 1984), and the platelet thrombi thus obtained are localized to the irradiated region in the wall of arterioles or venules. Therefore, we used this model to evaluate the in vivo antiplatelet activity of kinetin.

2. Materials and methods

2.1. Materials

Kinetin, prostaglandin E_1, bovine serum albumin, collagen, heparin, ADP, DMPO (5,5-dimethyl-1-pyrroline *N*-oxide), and fluorescein sodium were purchased from Sigma (St. Louis, MO, USA). Rats (Sprague–Dawley strain) and mice (ICR strain) were anesthetized with sodium pentobarbital (50 mg/kg) by intraperitoneal injection. Kinetin was dissolved in 0.5% dimethyl sulfoxide (DMSO) and stored at −4 °C.

2.2. Preparation of human platelet suspensions

Human platelet suspensions were prepared as previously described (Huang et al., 1991). In this study, human volunteers gave informed consent. In brief, blood was collected from healthy human volunteers who had taken no medicine in the preceding 2 weeks, and was mixed with acid/citrate/glucose (9:1, vol/vol). After centrifugation at 120 × *g* for 10 min at room temperature, the supernatant (platelet-rich plasma) was supplemented with prostaglandin E_1 (0.5 μM) and heparin (6.4 IU/ml), then incubated for 10 min at 37 °C and centrifuged at 500 × *g* for 10 min. The washed platelets were finally suspended in Tyrode's solution containing bovine serum albumin (3.5 mg/ml) and adjusted to about 4.5×10^8 platelets/ml. The final concentration of Ca^{2+} in Tyrode's solution was 1 mM.

2.3. Measurement of free radicals in platelet suspensions by electron spin resonance (ESR) spectrometry

The ESR method used a Bruker EMX ESR spectrometer as described previously (Iuliano et al., 1994). In brief, platelet suspensions (0.4 ml) were prewarmed to 37 °C for 2 min, and then kinetin (70 and 150 μM) or an isovolumetric vehicle solution (0.5% DMSO) was added for 3 min before the addition of collagen (1 μg/ml). The reaction was allowed to proceed for 5 min, followed by the addition of 100 mM DMPO for the ESR study.

ESR spectra were recorded at room temperature on a Bruker EMX ESR spectrometer using a quartz flat cell designed for aqueous solutions. Conditions of ESR spectrometry were as follows: 20 mW power at 9.78 GHz, 1 G modulation, and 100 G scanning in 42 s, with 10 scans accumulated.

2.4. ADP-induced acute pulmonary thrombosis in mice

Acute pulmonary thromboembolism was induced according to our previously described method (Sheu et al., 1995). Various doses of kinetin and vehicle solution (0.5% DMSO) (all in 50 μl) were administered by injection into the tail vein of mice (20–24 g). Four minutes later, ADP (0.9 mg/g) was injected into the contralateral vein. Mortality in each group was determined within 10 min.

2.5. Measurement of bleeding time in mesenteric arteries of rats

The bleeding time of severed mesenteric arteries was measured according to our previous report (Sheu et al., 1995). In brief, rats were anesthetized, and the trachea was cannulated to facilitate spontaneous breathing. Both the femoral artery and vein were cannulated to monitor blood pressure and for drug administration, respectively. The abdomen was opened by a midline incision, and a portion of the small intestine was brought out to display the mesenteric artery. Experimental solutions were infused into the right femoral vein at 0.2 ml/min for a 10-min period or were given as a bolus injection. An arterial vessel (external diameter 125–200 μm) located at the junction of the small intestine wall and the mesentery was incised after the start of the 10-min infusion or immediately after the bolus injection. Blood was flushed away by a superfusion system. Bleeding was observed through a dissecting microscope (× 100), and

bleeding time was recorded from the start of incision until bleeding was arrested by hemostatic plug formation. Each animal was used as its own control with bleeding time determined during the infusion of the control (normal saline), vehicle solution (0.5% DMSO), and various doses of kinetin (2–6 mg/kg). Repeat measurements were made by selecting sequential vessels of the same diameter along the small intestine mesentery. To ensure similar blood flow characteristics for each test, once a vessel had been severed and a plug had formed, it was not used for additional determination of bleeding time. Ten rats were evaluated with a normal saline infusion (0.2 ml/min for 10 min) to ensure that repeated measurements did not influence the subsequent bleeding time response. In other experiments, immediately before and at the end of the infusion, blood (1 ml) was collected from the femoral artery of rats and mixed with heparin (0.2 U/ml) for determination of platelet counts and basic hematological parameters (i.e., red blood cells, white blood cells, platelets, hematocrit, and hemoglobin) with an automatic cell counter (Coulter, A^c T).

2.6. Fluorescein sodium-induced platelet thrombus in mesenteric microvessels of mice

A method from our previous report was used (Sheu et al., 1994). Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After a tracheotomy was performed, an external jugular vein was cannulated with polyethylene tubing (PE-10) for administration of the dye and drug (i.v. bolus), and the femoral artery was cannulated for monitoring blood pressure. A segment of the small intestine with its mesentery attached was loosely exteriorized through a midline incision in the abdominal wall and was placed on a transparent culture dish for microscopic observation. Frequent rinsing of the mesentery with warm saline solution maintained at 37 ± 0.5 °C was performed to prevent the mesentery from drying out. Microvessels in the mesentery were observed under transillumination with a halogen lamp. Venules with diameters of 30–40 μm were selected for irradiation to produce a microthrombus. In the epi-illumination system, light from a 100-W mercury lamp was filtered by a filter (B-2A, Nikon, Tokyo, Japan) with a dichromic mirror (DM 510, Nikon). This filtered light with wavelengths below 520 nm irradiated a microvessel (the area of irradiation was about 100 μm in diameter on the focal plane) through an objective lens ($\times 20$). The doses of fluorescein sodium used were 5 and 7 mg/kg. The injected volume of test solution or normal saline (control) was smaller than 50 μl. Five minutes after administration of the dye, irradiation by filtered light and a timer were started simultaneously, and platelet aggregation was observed on a TV monitor. The time lapse for inducing thrombus formation leading to cessation of blood flow was measured. The elapsed time for inducing platelet plug formation was measured repeatedly every 5 min with irradiation of venules.

2.7. Statistical analysis

Each experiment was repeated several times as indicated (n) using different rats and mice, and a mean and standard error of the mean were thus obtained. Mean blood pressure was expressed as [(systolic pressure − diastolic pressure)/3 + diastolic pressure]. Data were assessed by analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by using the Newman–Keuls method. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Free radical-scavenging activity of kinetin in collagen-activated platelets

The rate of free radical-scavenging activity is defined by the following equation: inhibition rate = 1 − signal height (kinetin)/signal height (control) (Yamaguchi et al., 1999). In this study, a typical ESR signal of the hydroxyl radical was observed, as shown in Fig. 1. Kinetin (70 and 150 μM) markedly suppressed hydroxyl radical formation by about 41% and 76% ($n = 5$), respectively. This observation may provide in vitro evidence of the usefulness of kinetin for its free radical-scavenging activity in activated platelets.

3.2. Effect of kinetin on ADP-induced acute pulmonary thrombosis in mice

In this study, we demonstrated the effect of kinetin in preventing death from acute pulmonary embolism in mice. The results summarized in Table 1 show that kinetin significantly lowered mortality and reversed the changes in platelet numbers in mice challenged with ADP (0.9 mg/g). Kinetin (2, 4, and 6 mg/kg) reduced mortality to 70%, 40% and 35%, respectively. The vehicle solution (0.5% DMSO) did not significantly lower mortality or reverse the change in platelet numbers in mice challenged with ADP (0.9 mg/g). This result indicates that kinetin effectively prevents ADP-induced acute pulmonary thrombosis in mice.

3.3. Effect of kinetin on bleeding time in mesenteric arteries in rats

The reproducibility of the bleeding time was verified in control experiments. In control rats, normal saline was injected into the circulation, and the bleeding time measured in mesenteric arteries was about 3.5 ± 0.4 min. Fig. 2 shows that kinetin administered as a bolus to rats markedly increased the bleeding time in a dose-dependent manner. At 2 mg/kg, kinetin showed no significant effect on the bleeding time. At 4 and 6 mg/kg, kinetin significantly increased the bleeding time by about 1.9- and 2.1-fold as

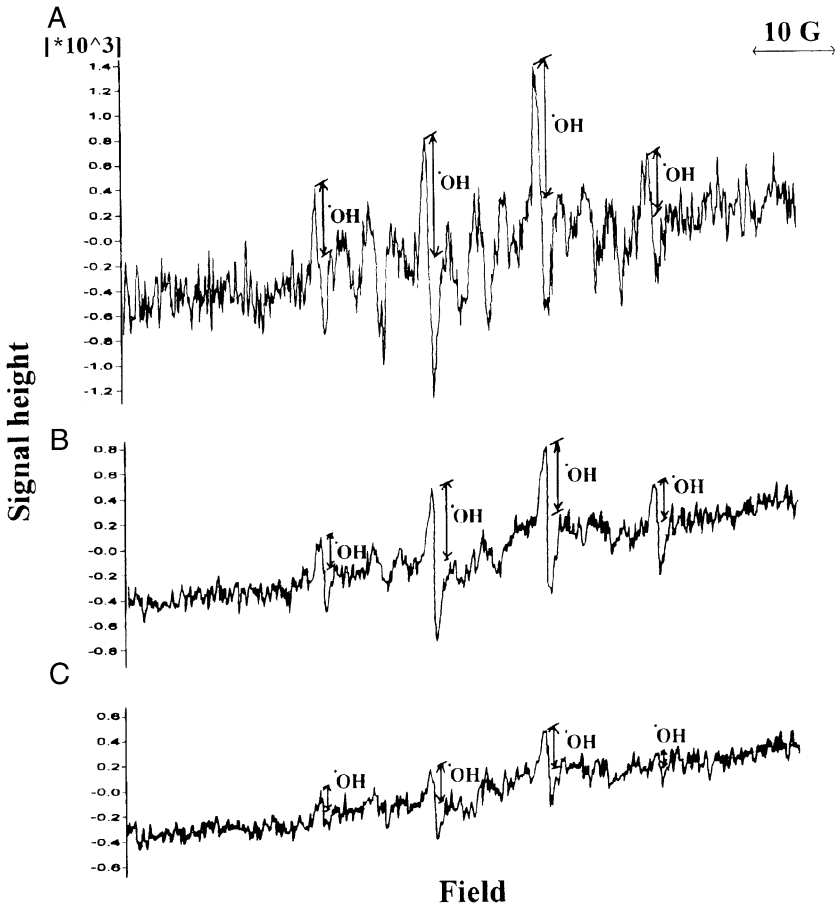

Fig. 1. ESR spectra of kinetin in the inhibition of hydroxyl radical formation in collagen-activated platelets. Platelet suspensions (0.4 ml) were preincubated with (A) vehicle solution (0.5% DMSO) or kinetin at (B) 70 μM and (C) 150 μM for 3 min, and then collagen was added (1 μg/ml) to trigger platelet aggregation as described in Materials and methods. The reaction was allowed to proceed for 5 min, followed by the addition of DMPO (100 mM) for electron spin resonance (ESR) experiments. The spectrum is a representative example of five similar experiments.

compared with normal saline (Fig. 2), respectively. In addition, the vehicle solution (0.5% DMSO) did not significantly increase the bleeding time as compared with normal saline (Fig. 2). The effect of a continuous infusion of kinetin (0.6 mg/kg/min) on the prolongation of the bleeding time is shown in Fig. 3. This result demonstrates that the bleeding time of severed mesenteric arteries was prolonged by about 2.3-fold (4.1 ± 0.6 vs. 7.4 ± 0.5 min) after termination of a 10-min infusion of kinetin. Bleeding

Table 1
Effect of kinetin on mortality and platelet count of acute pulmonary thrombosis caused by intravenous injection on ADP in experimental mice

	Number of deaths	Total number	Mortality (%)	Platelet count (10^3/mm^3)
Control	0	10	0	245 ± 28 (10)
ADP (0.9 mg/g)	14	20	70	148 ± 23* (20)
0.5% DMSO	14	20	75	145 ± 21* (20)
+Kinetin (mg/kg)				
2	15	20	75	141 ± 12* (20)
4	8	20	40	179 ± 21* (20)
6	7	20	35	206 ± 20 (20)

Platelet count is presented as the means ± S.E.M. (n).
* $P < 0.05$ as compared with control group (normal saline).

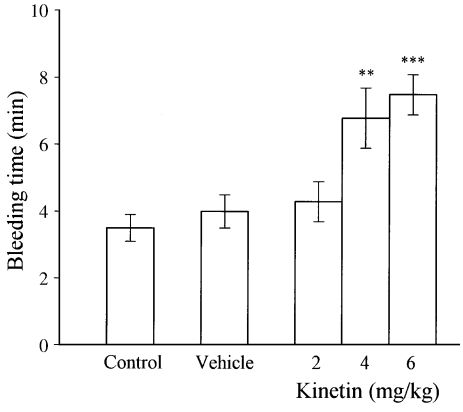

Fig. 2. Effect of kinetin on bleeding time in rat mesenteric arteries. Bleeding time was immediately measured after an intravenous bolus of normal saline (control), vehicle solution (0.5% DMSO), and kinetin (2, 4, and 6 mg/kg), respectively. Data are presented as the means ± S.E.M. ($n = 10$). ** $P < 0.01$ and *** $P < 0.001$ as compared with the control group.

times were also significantly prolonged by about 1.7- and 1.4-fold within 60 and 90 min after termination of kinetin infusion, respectively (Fig. 3). However, the bleeding time returned to the control value within 120 min after termination of the kinetin infusion (Fig. 3).

In addition, the mean blood pressure of rats (with intact mesenteric blood vessels) was continuously monitored during infusion of the same volume of normal saline, vehicle solution (0.5% DMSO), and kinetin. The mean blood pressure of control rats was 98.9 ± 13.6 mm Hg. The baseline blood pressure did not significantly change during the infusion of 0.5% DMSO (91.0 ± 11.2 mm Hg) or kinetin (0.6 mg/kg/min) (82.5 ± 10.4 mm Hg) (data not shown). The steady-state and post-kinetin values of the basic systemic and hematological parameters were also measured in this study. There were no significant differences in the hematological parameters, including hemoglobin, hematocrit, numbers of red blood cells, platelets, and white blood cells, between the control (normal saline), and post-kinetin values (data not shown).

3.4. Effect of kinetin on thrombus formation in microvessels of fluorescein sodium-pretreated mice

The latent period of inducing platelet plug formation was shortened as the administered dose of fluorescein sodium increased. When fluorescein sodium was given at 5 and 7 mg/kg, the occlusion time required was 165 ± 16 and 117 ± 13 s, respectively (Fig. 4). Kinetin is an inhibitor of platelet aggregation (Sheu et al., in press). We therefore examined its inhibitory effect on the formation of platelet-rich thrombi in this model. When kinetin was administered at 13 and 14 mg/kg in mice pretreated with 5 mg/kg of fluorescein sodium, the occlusion time was significantly prolonged (Fig. 4). In contrast, kinetin (13 mg/kg) did not significantly prolong the occlusion time until it was administered at 16 mg/kg in mice with fluorescein sodium (7 mg/kg)-induced platelet plug formation (Fig. 4). Kinetin also exhibited an antithrombotic effect in arterioles (data not shown). However, arterioles sometimes showed slight vasoconstriction with fluorescein sodium irradiation (Sato and Ohshima, 1984), thus venules were chosen for the induction of platelet plug formation in this study.

The effect of the anticoagulant, heparin (1.5 U/g), was also examined at the same time; however, it showed no significant effect on occlusion time (data not shown).

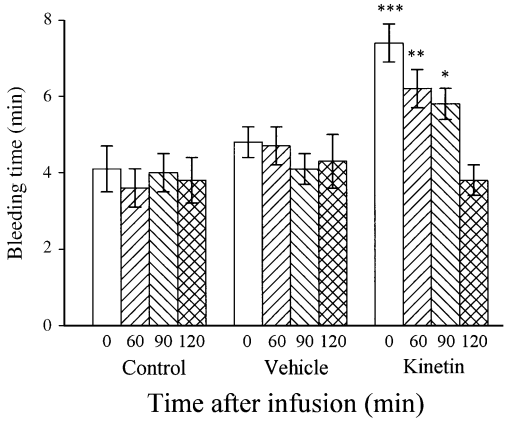

Fig. 3. Effect of a continuous infusion of kinetin (0.6 mg/kg/min) on bleeding time in rat mesenteric arteries. Bleeding time was immediately measured after termination of 10 min of infusion of normal saline (control), vehicle solution (0.5% DMSO), and kinetin; bleeding time was also measured 60, 90, and 120 min after termination of the 10-min drug infusion, respectively. Data are presented as the means ± S.E.M. ($n = 10$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as compared with the control group.

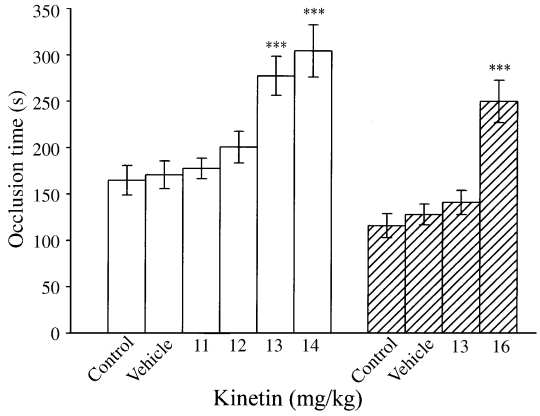

Fig. 4. Effect of kinetin on occlusion time for induction of thrombus formation upon light irradiation of mesenteric venules of mice pretreated with fluorescein sodium (5 mg/kg, open bars; 7 mg/kg, hatched bars). Data are presented as the occlusion time (s) of platelet plug formation (means ± S.E.M.; $n = 10$). ***$P < 0.001$ as compared with the control group.

4. Discussion

The principal objective of this study was to ascertain the inhibitory activity of kinetin on both free radical scavenging in vitro and antithrombosis in vivo. The role of reactive oxygen species in platelet physiology is relevant because tissue damage mediated in this manner can contribute to pathological situations where platelets are involved. These include endothelial damage (Freeman et al., 1986), reperfusion injury of ischemic myocardium (Bolli et al., 1988), and severe vasoconstriction (Katusic and Vanhoutte, 1989). Reactive oxygen species (i.e., hydrogen peroxide, hydroxyl radicals) derived from platelets might affect cells with which they come into intimate contact, such as endothelium, and this could result in an amplification of platelet reactivity during thrombus formation. Therefore, the presence of free radical scavengers at the site of a developing thrombus can alter the pattern of the thrombotic lesion in response to endothelial cell injury. In this study, we found that kinetin (70 and 150 μM) effectively inhibited hydroxyl radical formation in collagen-activated platelets (Fig. 1). It is

known that hydrogen peroxide exerts several biological effects via the Fenton reaction with metal catalysis to produce the very reactive hydroxyl radical (Salvemini and Botting, 1993). Thus, the mechanisms of antithrombotic activity of kinetin may be involved, at least partly, in the inhibition of free radical formation in activated platelets.

Furthermore, platelet activation is intimately involved in acute pulmonary thrombosis (Nordoy and Chandler, 1964), and kinetin was effective in preventing ADP-induced thromboembolic death, as expected. These data are consistent with the fact that platelet aggregation is the crucial event causing thromboembolism in these animal models.

Furthermore, prolongation of the bleeding time was seen in rats receiving kinetin. Animal studies with platelet glycoprotein IIb/IIIa antagonists have shown a prolongation of the bleeding time (Sheu et al., 1995). However, based on a careful analysis of the bleeding time, it has been suggested that prolongation of the bleeding time in humans does not predict the risk of hemorrhage or surgical bleeding, thus this study represents a rational experiment for clinical evaluation of antiplatelet compounds (Rodgers and Levin, 1990).

Electron microscopy has shown that the thrombi formed after irradiation injury are mainly composed of activated platelets, as are those induced by laser irradiation (Sheu et al., 1994). The degree of endothelial cell damage after irradiation seems to depend on the intensity of irradiation and the amount of fluorescein dye given. We have demonstrated that endothelial cell injury induced platelet aggregation and adhesion to the vessel wall in vivo (Sheu et al., 1997, 1999). Kinetin exhibited a marked antithrombotic effect in vivo. It prolonged the occlusion time of thrombus formation induced by irradiation of fluorescein sodium in venules or arterioles. Since the light beam covered the entire microscopic field, arterioles and venules could be observed simultaneously. Our data revealed that platelet aggregation usually occurred first in venules rather than in arterioles. This may be explained by the higher flow velocity in arterioles causing a delayed adhesion of platelets to arteriolar endothelial cells (Callahan et al., 1960). In this system, the occlusion time is related to the blood flow rate, size of the microvessel diameter, and dose of fluorescein dye. Kinetin (13 mg/kg) caused significant prolongation of the occlusion time in mice pretreated with 5 mg/kg of fluorescein sodium, probably mainly through its antiplatelet effects.

The mesenteric venules of mice were continuously irradiated by filtered light during the experimental period; nevertheless, a single incision of the mesenteric arteries of rats was made for the bleeding time measurement. In addition, the animal species used were different in the hemostatic (rat) and thrombotic platelet plug (mouse) studies. These reasons may explain why kinetin prolonged the occlusion time of thrombus formation in mice at a dose higher than that used to measure hemostatic bleeding time in rats. In this study, the mechanisms of kinetin inhibition of thrombotic and hemostatic platelet plug formation in experimental animals are probably related to its antiplatelet effects, including inhibition of platelet aggregation and free-radical formation.

In conclusion, platelet aggregation plays a pathophysiological role in a variety of thromboembolic disorders. Therefore, prevention of platelet aggregation by drugs should provide effective prophylactic and/or therapeutic treatments for such diseases. In this study, our results suggest that kinetin has promising antithrombotic activity, and it may be a potential therapeutic agent for the treatment of arterial thromboembolism. The toxicity of kinetin, however, must be further assessed.

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