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Brief Communication

Ginkgo biloba extract enhances antiplatelet and antithrombotic effects of cilostazol without prolongation of bleeding time

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ABSTRACT

Thrombosis and thromboembolic occlusions of major and minor blood vessels are a major complication in various peripheral vascular diseases. Antiplatelet agents (APA), key tools in the treatment of atherothrombosis, therefore became a mainstay medication for a wide range of vascular diseases. Cilostazol and *Ginkgo biloba* extract (GB), commonly used remedies for peripheral arterial disease, inhibit platelet aggregation with distinct therapeutic mechanisms. In this study, we have investigated if GB can potentiate the antiplatelet effects of cilostazol to explore the utility of combination therapy of cilostazol and GB against peripheral occlusive vascular diseases. GB or cilostazol was evaluated alone or in combination for the antiplatelet activity using *in vitro* and *in vivo* models. In addition, potential bleeding side effect of the combinative therapy was assessed by measuring bleeding time, prothrombin time (PT) and activated partial thromboplastin time (aPTT) *in vivo* after oral administration. In *in vitro* assays using freshly isolated human platelets, the combination of cilostazol and GB showed superior inhibition of both the shear and the collagen-induced platelet aggregation to those of each drug alone. In accordance with these enhanced *in vitro* antiplatelet activities, the combinative therapy showed enhanced anti-thrombotic effects in *in vivo* pulmonary embolism model and arterial thrombosis model. In particular, the increase of survival rate in pulmonary embolism model by combination treatment of cilostazol (25 mg/kg) and GB (20 mg/kg) was higher more than two-fold of those of the respective drugs. Notably, the combination of cilostazol and GB did not show a significant effect on the bleeding time, PT and aPTT increase, suggesting that GB may potentiate the antiplatelet effect of cilostazol without the prolongation of bleeding time or coagulation time. With these studies, we suggest that combinative therapy of GB and cilostazol might offer enhanced anti-thrombotic efficacies without increasing side-effects.

Introduction

Platelets are essential in the maintenance of vascular integrity and the control of bleeding through forming blood clot, but they are also implicated in the pathological progression of atherosclerotic lesions and arterial vascular thrombosis [1]. Antiplatelet agents (APA) are therefore, considered as a key tool in the treatment and/or prevention of vascular thrombotic diseases [2]. However, commonly used APA such as cilostazol, clopidogrel, diprydamole and aspirin has various side effects including headache [3], internal bleeding, prolonged bleeding time, gastrointestinal bleeding and palpitation [4,5], impeding the wide and active use of APA for the various types of cardiovascular protection. Recently, several trials have explored the combinative therapy of multiple APA, to pursue the synergy in efficacy and at the same time, improvement of the safety [6].

Cilostazol, a potent inhibitor of cAMP phosphodiesterase 3 (PDE3), is known to attenuate platelet aggregation through intracellular cAMP increase [7]. It has been routinely used as an antithrombotic agent for the treatment of peripheral arterial occlusive disease. Furthermore, stroke prevention has been recently approved as a new indication of cilostazol in Japan [8,9]. However, similar to the other APA, it has serious side effects such as headache and palpitation [10], raising an urgent need for novel therapeutic approach to reduce its adverse effects without impairing the efficacy.

Ginkgo biloba extract (GB) is one of the most popular herbal products, available in various countries including Germany, US and Asian countries. It has been commonly used as a herbal medicine or health food for its beneficial effects in cerebral and peripheral arterial diseases like dementia and claudication [11,12]. Its efficacies in

Abbreviations: APA, antiplatelet agents; aPTT,, activated partial thromboplastin time; CS, cilostazol; GB, *Ginkgo biloba* extract; MAR, maximum aggregation rate; PAF, platelet activating factor; PDE3, phosphodiesterase 3; PPP, platelet poor plasma; PRP, platelet rich plasma; PT, prothrombin time.

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circulation have been proved by several clinical studies where GB has shown beneficial effects on pain-free walking in the intermittent claudication patients. GB is known to inhibit ADP and collagen-induced platelet aggregation *in vitro* and *in vivo* through cAMP increase and inhibition of thromboxane A2 synthesis [13–15]. In addition, ginkgolides, major active ingredients of GB were reported to have inhibitory effects on platelet aggregation through cAMP and cGMP dependent pathways and MMP9 activation [16,17].

Kim et al. [18] reported that the antithrombotic effects of ticlopidine could be enhanced by the combination with GB, suggesting the applicability of GB as an adjunctive therapy with the conventional APA. However, it is important to assess the adverse effects of APA thoroughly because the side-effects such as prolongation of bleeding time or coagulation can be elevated with increased pharmacological activity of APA. In this study, we investigated whether GB can potentiate the antiplatelet effect of cilostazol, using *in vivo* and *in vitro* models. More importantly, we also evaluated the possibility of the prolongation of bleeding time and blood coagulation time, critical side effect of APA to assure the therapeutic utility of the combination therapy of GB and cilostazol.

Materials and methods

Materials and animals

Cilostazol (99.0%) was obtained from Chemagis Ltd (Israel). GB is the extract from *Ginkgo biloba* leaves provided by SK Chemical Co. (Korea), which contains ginkgoflavone-glycosides 24.74%, and terpene lactone 7.55%. Trisodium citrate, dimethylsulfoxide (DMSO), β-nicotiniamide adenine dinucleotide reduced form (β-NADH), ethylenediamine-tetra-acetic acid (EDTA), Trizma base (tris-hydroxymethyl- aminomethane), pyruvic acid , triton X-100, digitonin, urethane, ferric chloride, carboxyl methyl cellulose and glutaraldehyde were purchased from Sigma Chemical Co. (St. Louis, USA). Collagen and epinephrine were obtained from Chrono-log Corp. (Havertown, USA). Prothrombin reagent and activated partial thromboplastin time reagent were purchased from Dade Behring Inc. (Deerfield, USA).

Animals were purchased from Charles River Japan. Animals were housed in a conventional animal facility with free access to food and water where the environmental temperature and relative humidity were monitored and controlled. The lighting schedule in the animal facility gave 12 hr light and 12 hr dark. Animals were allowed to acclimatize for at least 7 days before experimentation. All the protocols for the animal study were approved by the internal animal Ethics Committee of SK chemical and Ethics Committee of Animal Service Center at Seoul National University.

Sample preparation

For *in vitro* tests, each compound was dissolved in dimethyl sulfoxide. For *in vivo* experiment, each single drug was suspended in 0.5% carboxyl methyl cellulose and mixed to proper ratio for combinatory treatment. Each drug was administered by oral gavage in a volume of 10 ml/kg (mouse and rat). The control animals received vehicle only. All agents were prepared just before use.

Shear-induced platelet aggregation

Blood was collected from human volunteers by venipuncture and it was drawn into a plastic syringe containing 3.8% trisodium citrate solution (1:9 citrate/blood, v/v). The healthy, male volunteers had not taken any drugs for at least 7 days. Platelet rich plasma (PRP) was prepared by centrifugation at room temperature for 15 min at 150 g. Platelet poor plasma (PPP) was obtained from the precipitated fraction of PRP by centrifugation at room temperature for 20 min at 2000 g. The platelet count in PRP was adjusted to 3×10^8 platelets/ml using PPP. After incubation with test drugs at 37 °C for 3 min, PRP was loaded on the cone-plate viscometer (HAAKE, Germany) and shear stress was applied at 10,800 s^{-1} for 3 min. The resultant platelet suspension was obtained and diluted with 0.5% glutaraldehyde. The platelet aggregation was determined by counting single cells. Aggregation and inhibition rate was calculated as follows. Aggregation (%) = $(1\text{-}A/A_0)\times100$, Inhibition (%) = (Aggregation % of sample/Aggregation % of control) × 100. A is number of single platelets in samples, and A_0 is number of single platelets in unsheared control.

In vitro collagen-induced platelet aggregation

PRP was prepared as described above. The platelet aggregation was determined by turbidometric method using an aggregometer (Chrono-log, Havertown, USA). PRP was incubated at 37 °C for 4 min in the aggregometer with stirring at 1000 rpm and then stimulated with collagen (Chrono-Log, USA). The light transmission on the aggregation response curve was measured for 4 min. Test drugs were added to the PRP 3 min before adding the collagen. The maximum aggregation rate (MAR) was estimated by the percent of maximum increase in light transmission with the PPP representing 100% transmission and inhibition rate was calculated as follows. Inhibition rate = [(Av – At) / Av] × 100. Av is MAR of vehicle control and At is MAR of test compound. Collagen concentration (2~3 μg/ml) was adjusted to induce 65~80% MAR of vehicle.

Determination of cytotoxicity

Platelet cytotoxicity was determined by the leakage of lactate dehydrogenase (LDH) from platelets. After incubation with test drugs at 37 °C for 3 min, aliquots were collected and centrifuged at room temperature for 2 min at 12,000 g. A 50 μl aliquot of the resulting supernatant was added to 1 ml of Tris-EDTA-NADH buffer (56 mM Tris (hydroxymethyl)aminomethane, 5.6 mM EDTA, 0.17 mM β-NADH, pH 7.4) and then incubated for 10 min at 37 °C. After incubation, 100 μl of 14 mM pyruvate solution that had been preincubated at 37 °C was added. The reduction in absorbance at 340 nm by the conversion of NADH to NAD^+ was measured for the evaluation of LDH activity in the aliquots. The extent of LDH leakage was expressed as % of total enzyme activity measured in platelets completely lysed with 0.3% Triton X-100.

Arterial thrombosis animal model

Male SD rat weighing 200~300 g were used in this experiment. Rats were fasted overnight and each drug was administered orally to rats. Two hr after administration of test drugs, rats were anesthetized with urethane (1.25 g/kg, i.p.) and approximately 15 mm of the right carotid artery was exposed and dissected free of nerve and connective tissue. Thrombosis was induced by the $FeCl_3$ application method. Briefly, a filter paper (1 × 2 mm, Whatman No. 1) was soaked in $FeCl_3$ (35% w/v in saline) and applied to the external surface of the carotid artery segment for 10 min. An ultrasonic flow-probe was placed around the arterial segment proximal to the injured site. The flow-probe was connected to a Doppler flowmeter (Transonic Systems, Ithaca, USA) to monitor blood flow. The time needed for occlusion to occur was measured for up to 60 min.

Acute thrombosis induced by a combination of collagen and epinephrine in mice

Male ICR mice weighing 20~30 g were used in this experiment. Mice were fasted overnight and divided into groups of 12 animals. Each drug suspended in 0.5% carboxyl methyl cellulose was administered orally to mice. A mixture of collagen (500 μg/kg) plus epinephrine (50 μg/kg) was injected to the tail vein of mice to induce