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Short communication

Inhibitory effect of hericenone B from *Hericium erinaceus* on collagen-induced platelet aggregation

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ABSTRACT

Platelet aggregation in the blood vessel causes thrombosis. Therefore, inhibitors of platelet aggregation promise to be preventive or therapeutic agents of various vascular diseases, including myocardial infarction and stroke. In the present study, we found that hericenone B had a strong anti-platelet activity and it might be a novel compound for antithrombotic therapy possessing a novel mechanism. Prior to this study, we examined anti-platelet aggregation activity of ethanol extracts of several species of mushrooms, and found that extract of *Hericium erinaceus* potently inhibited platelet aggregation induced by collagen. Therefore, we first fractionated the ethanol extract of *H. erinaceus* to identify the active substances. The anti-platelet activity of each fraction was determined using washed rabbit platelets. As a result, an active component was isolated and identified as hericenone B. Hericenone B selectively inhibited collagen-induced platelet aggregation, but it did not suppress the aggregation induced by U46619 (TXA_2 analogue), ADP, thrombin, or adrenaline. Furthermore, hericenone B did not inhibit arachidonic acid- or convulxin (GPVI agonist)-induced platelet aggregation. Therefore, hericenone B was considered to block collagen signaling from integrin α2/β1 to arachidonic acid release. Moreover, we found that collagen-induced aggregation was inhibited by hericenone B in human platelets, similar to in rabbit platelets.

Introduction

Platelets play an important role in thrombus formation in injured blood vessels. However, platelet aggregation is also cause for various clinical disorders, including vascular diseases. One of the major clinical problems in developed countries is arterial thrombosis caused by unnecessary platelet aggregation and subsequent thrombus formation at atherosclerotic lesions causing myocardial infarction and stroke (Jennings 2009; McNicol and Israels 2003; Varga-Szabo et al. 2008). Therefore, inhibitors of platelet aggregation promise to be preventive or therapeutic agents of thrombosis.

Prior to this study, we examined anti-platelet aggregation activity of ethanol extracts of several species of mushrooms using washed rabbit platelets, and found that extract of *Hericium erinaceus* potently inhibited platelet aggregation induced by collagen (data not shown). *H. erinaceus* is a mushroom that grows on old or dead broadleaf trees. *H. erinaceus* has been used as a food and herbal medicine in East Asia since ancient times. Hericenones C–H and erinacines A–I have been isolated from the fruit body and mycelium of *H. erinaceus*, respectively. All of these promote nerve growth factor (NGF) synthesis in cultured rodent astrocytes (Kawagishi et al. 1991, 1993, 1994, 1996). These results spotlighted the usefulness of *H. erinaceus* for the treatment and prevention of dementia. Moreover, we have previously reported that *H. erinaceus* extract induced NGF mRNA expression in 1321N1 human astrocytoma cells (Mori et al. 2008), and that oral administration of *H. erinaceus* increased NGF mRNA expression in mouse hippocampus (Mori et al. 2008). Furthermore, scores on a cognitive function scale were improved by ingestion of *H. erinaceus* in patients with mild cognitive impairments (Mori et al. 2009). However, the anti platelet effect of *H. erinaceus* has not been investigated until now. We fractionated ethanol extracts of *H. erinaceus* repetitively to isolate and identify an anti platelet aggregation compound. Furthermore, the characteristics of the active substance were investigated in detail for its effects on platelet aggregation.

Materials and methods

Materials

Fruit bodies of *H. erinaceus* were cultivated by the Hokuto Corporation (Nagano, Japan). Collagen (Collagenreagent Horm)

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was obtained from Nycomed Pharma GmbH (Zurich, Switzerland). 9,11-Dideoxy-9α,11α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) was obtained from Cayman Chemical Company (Ann Arbor, MI, United States). Thrombin was obtained from Wako Pure Chemichals (Osaka, Japan). Convulxyn was obtained from Alexis Biochemicals (San Diego, CA, United States). All other chemicals used were of reagent grade or highest quality available.

Isolation of hericenone B from H. erinaceus

The Fresh fruit bodies (8.0 kg) were freeze-dried and extracted three times with ethanol (15 l) at room temperature for 2 days to produce the extract (52.0 g). The extract was concentrated and fractionated by solvent partition between ethyl acetate and water to yield an ethyl acetate soluble fraction. The ethyl acetate solubles (20.5 g) were chromatographed over SiO_2 using *n*-hexane–ethyl acetate solutions with increasing polarity as the eluent. The *n*-hexane–ethyl acetate (1:3) eluent (845.2 mg) was further chromatographed over SiO_2 with chloroform–methanol with increasing polarity. Then the chloroform–methanol (99:1) eluent (69.3 mg) was chromatographed over ODS with water–methanol to give an anti platelet aggregation compound (9.0 mg). The structure of the active compound was deduced by its ^1H and ^{13}C NMR spectra.

Determination of platelet aggregation

Fresh blood was obtained from male rabbits (Japanese white rabbits weighing about 2.5–3.5 kg), or healthy Japanese male volunteers who had not taken any medication for 2 weeks prior to the study. The experimental procedures using rabbits were performed in accordance with the guidelines of the Institution for Animal Care and the Use Committee of Tohoku University. All human participants provided informed consent and this experiment was conducted with approval of the Institutional Review Board of Tohoku University Graduate School of Pharmaceutical Sciences. Washed platelets were prepared and suspended in the Tyrode/HEPES solution (pH 7.35) with a final density of 3×10^8 platelets/ml as previously described (Iwashita et al. 2007a,b). Platelet aggregation was determined by a standard turbidimetric method using an aggregometer (PAM-6C, Merbanix, Tokyo, Japan), and expressed as the level of light transmission. The level of light transmission was calibrated as 0% for a platelet suspension and 100% for the Tyrode/HEPES solution (pH 7.35). 300 μl of platelet suspension was preincubated in a glass cuvette at 37 °C for 2 min with stirring (1000 rpm). Platelets were then incubated with 1 mM $CaCl_2$ for 2 min. After preincubation with hericenones, or 5 μM aspirin (positive control) for 5 min, aggregation stimulants were added and platelet aggregation was monitored for 10 min. The value of maximal light transmission was used for calculation of the aggregation percentage.

After stimulation by collagen, platelets were fixed by addition of 15 μl of the Tyrode-HEPES solution containing 20% glutaraldehyde for the observation by scanning electron microscopy. The platelets were incubated on cover glasses coated with poly-L-lysine at 4 °C over night. The samples adhering to the cover glasses were washed with PBS and dehydrated by increasing concentrations of ethanol (50, 70, 80, 90, and 100%) and t-butyl alcohol. The samples were then lyophilized using a freeze-dryer (ES-2030, Hitachi, Tokyo, Japan) and sputter-coated with gold and palladium using an ion sputter (E-1010, Hitachi, Tokyo, Japan). These specimens were then observed under a scanning electron microscope (S-3200N, Hitachi, Tokyo, Japan).

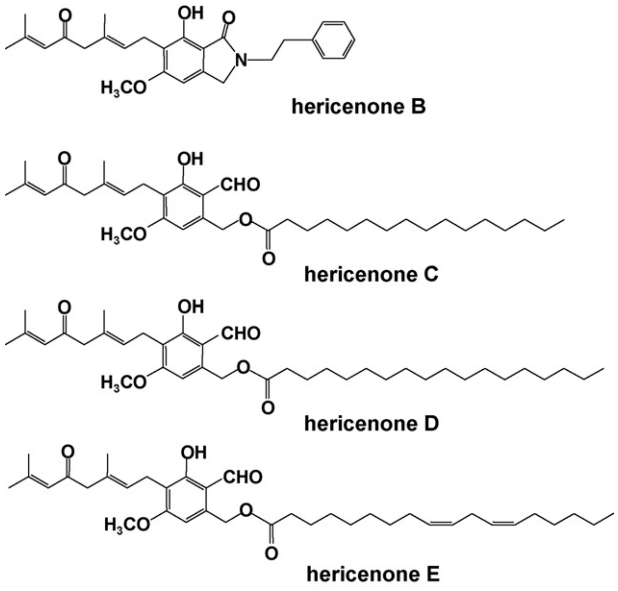

Fig. 1. Chemical structures of hericenone B, C, D and E.

Statistical methods

Data were expressed as mean ± S.E.M. Significant differences ($p < 0.01$) were determined by unpaired *t*-test (Fig. 3(B)), or one-way ANOVA followed by a Dunnett's test (Fig. 2(C)).

Results and discussion

In order to identify the anti-platelet substances of *H. erinaceus*, we fractionated the extract by solvent partition and repeated column chromatography using SiO_2 or ODS, and an active substance was isolated. It was identified as hericenone B (Fig. 1) by spectra data of ^1H NMR and ^{13}C NMR, completely agreeing with previously reported data (Kawagishi et al. 1990). During the purification of the extract, we also obtained hericenone C, D and E (Fig. 1) (Kawagishi et al. 1991). This is the first study reporting that hericenone B has anti-platelet aggregation activity.

Hericenone B inhibited collagen-induced (3 μg/ml) platelet shape change and aggregation in a concentration-dependent manner. Complete inhibition was obtained at 30 μM (Fig. 2(A) and (B)). However, hericenone C, D, and E (100 μM) did not show any inhibitory activity on the aggregation (Fig. 2(C)). Therefore, we assume that hericenones containing a fatty acid chain may not inhibit platelet aggregation, and that the inhibitory activity of platelet aggregation is distinctive to hericenone B. The anti-platelet activity of hericenone B may be due to its characteristic γ-lactam and N-substituent.

Platelet aggregation is initiated by several agonists, such as adrenaline, adenosine 5′-diphosphate (ADP), 5-hydroxytryptamine (5-HT), thromboxane A_2 (TXA_2) and thrombin besides collagen (Jackson et al. 2003; Varga-Szabo et al. 2008). In order to examine the selectivity of the inhibitory activity, we investigated the effect of hericenone B on platelet aggregation induced by U46619 (TXA_2 analogue) (3 μM), ADP (100 μM), thrombin (0.01 U/ml), and adrenaline (10 μM) + ADP (10 μM) in addition to collagen (3 μg/ml) (Fig. 3(A)). Prior to this experiment, we examined concentration-dependency of these agonists on platelet aggregation, and in the present study we used the minimal concentrations of these agonists that elicited a full aggregatory response. Adrenaline was used with ADP because by itself it did not induce strong aggregation of rabbit platelets. We found that hericenone B specifically suppressed

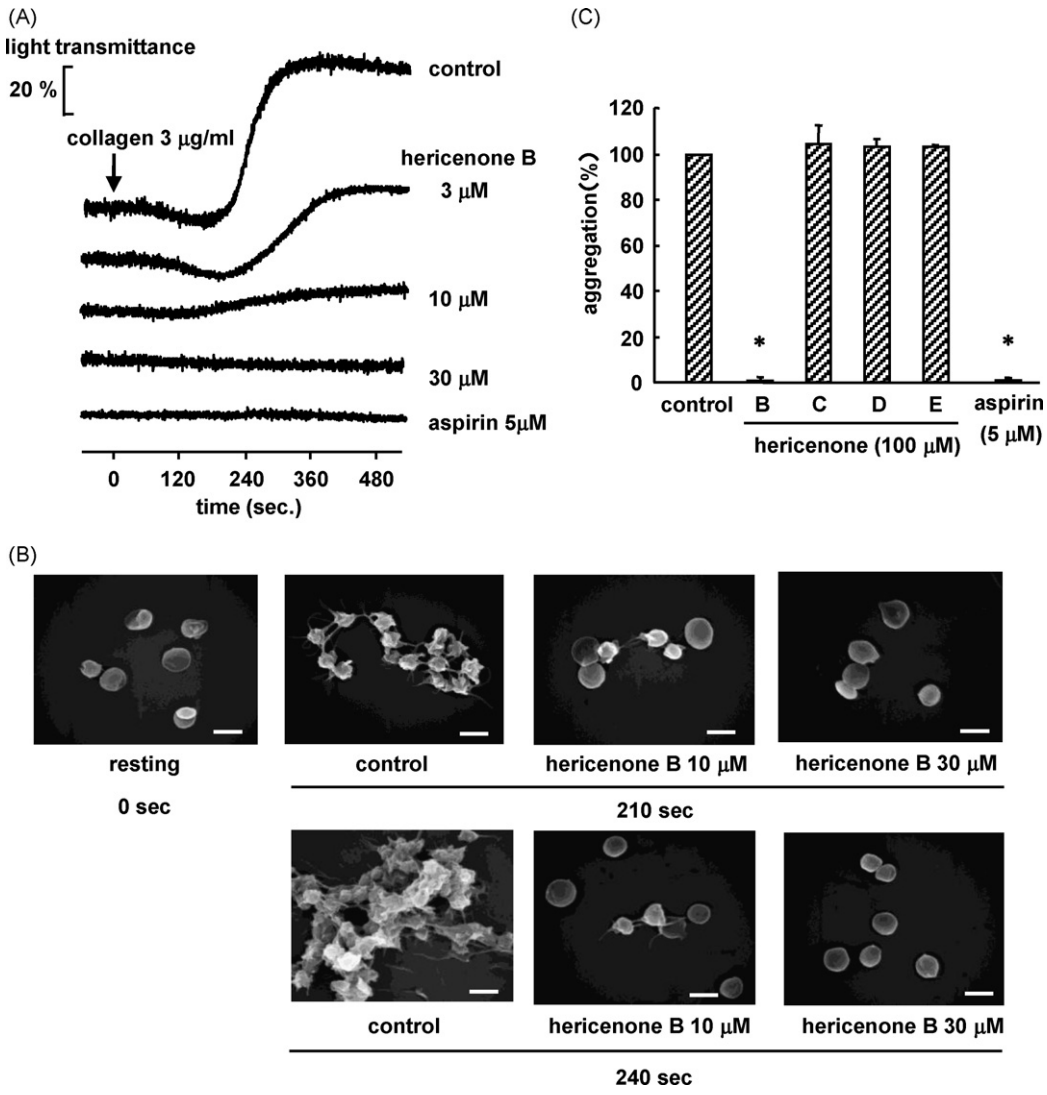

Fig. 2. The inhibitory effect of hericenone B, C, D and E on collagen-induced platelet aggregation. (A) The inhibitory effect of hericenone B on collagen-induced platelet aggregation. Representative traces of collagen-induced platelet aggregation. (B) Scanning electron micrographs of platelets at 0, 210 or 240 s after stimulation with collagen (3 μg/ml). (C) Comparison of the effects among hericenone B, C, D and E. Washed rabbit platelets were preincubated with or without hericenones in the presence of 1 mM $CaCl_2$ for 5 min at 37 °C. Aspirin (5 μM) was used as a positive control. The platelets were stimulated with collagen (3 μg/ml). Each bar indicates 2 μm. Values represent the mean ± S.E.M. of three individual experiments. $^{*}p < 0.01$ vs. control.

collagen-induced platelet aggregation at low concentrations with an IC_{50} value of about 4 μM. The IC_{50} value on U46619-induced aggregation was about 23 μM. Moreover, the inhibitory activity on ADP, adrenaline + ADP, and thrombin-induced aggregation was very weak ($IC_{50} > 100$ μM).

Several collagen receptors have been identified on the platelet surface. The immunoglobulin superfamily member GPVI and integrin α2/β1 are most notable and essential for collagen-induced platelet activation and aggregation (Farndale et al. 2004; Surin et al. 2008). Both the selective agonist of GPVI (convulxin) (Francischetti et al. 1997; Polgar et al. 1997) and integrin α2β1 (aggretin) (Huang et al. 1995) initiate platelet aggregation by different signaling pathways. It is also known that several antibodies against GPVI and integrin α2/β1 inhibit platelet aggregation (McNicol and Israels 2003; Takayama et al. 2008). Platelet activation via these two prominent receptors results in the liberation of arachidonic acid from the plasma membrane. Arachidonic acid is subsequently converted to prostaglandin H_2 (PGH_2) by cyclooxygenase-1 and then to TXA_2 by thromboxane synthase. TXA_2, released from platelets, is a strong mediator of platelet aggregation (Clemetson and Clemetson 2007; Surin et al. 2008). Therefore, we examined the inhibitory activity of hericenone B on platelet aggregation induced by arachidonic acid and convulxin, a specific agonist of GPVI, in order to investigate the mechanism of action of hericenone B in collagen signaling. Hericenone B did not inhibit platelet aggregation induced by arachidonic acid and convulxin (Fig. 3(B)). In addition, hericenone B did not show the inhibitory activity of cyclooxygenase-1 *in vitro* (Mori et al., unpublished observation). These results suggest that the site of hericenone B may be upstream of arachidonic acid liberation and GPVI is not involved in the inhibitory effect of hericenone B on collagen-induced aggregation.

Moreover, hericenone B inhibited collagen-induced aggregation in human platelets in a concentration-dependent manner with an IC_{50} value of about 3 μM. The effective concentration was approximately equal to that observed in rabbit platelet aggregation (Fig. 4). Therefore, hericenone B may be applicable to prevent thrombosis in human.

Anti-platelet therapy is currently applied to treat and prevent cardiovascular disease and stroke, and aspirin and $P2Y_{12}$ ADP receptor antagonists such as clopidogrel and ticlopidine are widely used clinically (Jennings 2009; Kiernan et al. 2009). Aspirin irreversibly inhibits cyclooxygenases, suppressing the synthesis of

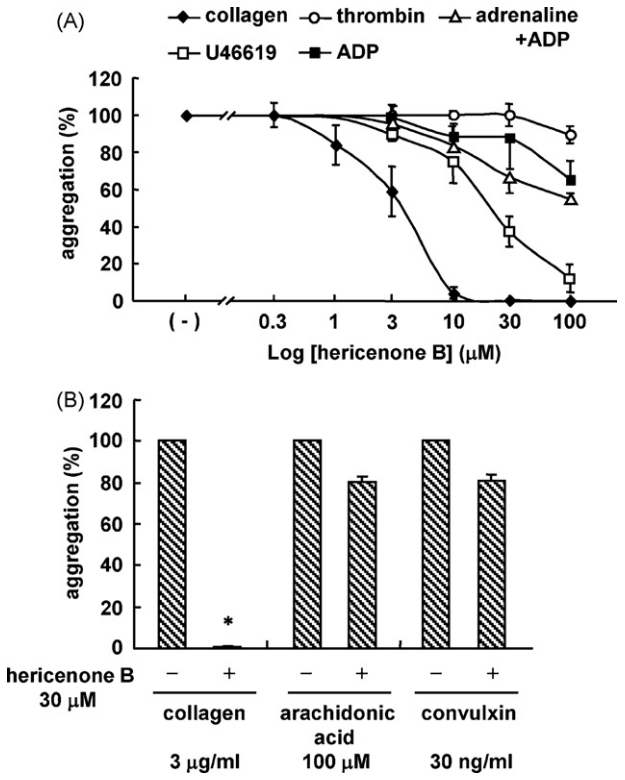

Fig. 3. The effect of hericenone B on platelet aggregation induced by various agonists. (A) The effect of hericenone B on platelet aggregation induced by collagen, U46619, thrombin, ADP or adrenaline. (B) The effect of hericenone B on platelet aggregation induced by arachidonic acid or convulxin. Washed rabbit platelets were preincubated with hericenone B (0.3–100 μM) for 5 min at 37 °C in the presence of 1 mM of $CaCl_2$. Platelets were then stimulated by collagen (3 μg/ml), U46619 (3 μM), ADP (100 μM), thrombin (0.01 U/ml), adrenaline (10 μM) + ADP (10 μM), arachidonic acid (100 μM) or convulxin (30 ng/ml). The aggregation was expressed as the percentage of the aggregation in the absence of hericenone B. Values represent the means ± S.E.M. of three individual experiments. $^{*}p < 0.01$ vs. absence of hericenone B (−).

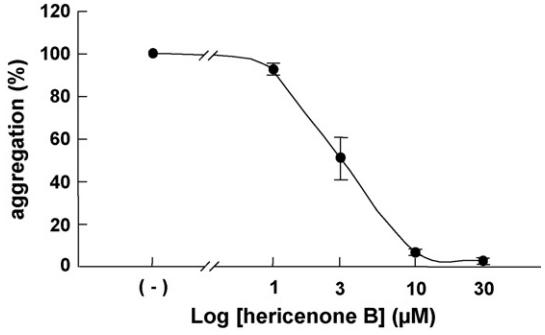

Fig. 4. The effect of hericenone B on collagen-induced aggregation in human platelets. Washed human platelets were preincubated with hericenone B (1–30 μM) for 5 min at 37 °C in the presence of 1 mM of $CaCl_2$. Platelets were then stimulated by collagen (3 μg/ml). The aggregation was expressed as the percentage of the aggregation in the absence of hericenone B. Values represent the mean ± S.E.M. of three individual experiments.

TXA_2 from arachidonic acid and TXA_2-mediated platelet activation. However, hericenone B inhibits upstream of the arachidonic acid release in collagen signaling. Therefore, hericenone B may be a novel compound for anti platelet therapy possessing a novel antiplatelet mechanism.

In conclusion, hericenone B from *H. erinaceus* specifically inhibited collagen-induced platelet aggregation through the inhibition of upstream of arachidonic acid liberation in integrin α2/β1 signaling. It is necessary to investigate the effects *in vivo* and the mechanism of action of hericenone B more precisely in the future.

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