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Full Length Research Paper

The anti-platelet activity of *Hypsizygus marmoreus* extract is involved in the suppression of intracellular calcium mobilization and integrin $\alpha_{IIb}\beta_3$ activation

Ji Young Park[1], Won-Jun Oh[1], Dong-Mi Kwak[1], Min-Gyeong Kim[2], Geon-Sik Seo[2], Seung-Bok Hong[3] and Man Hee Rhee[1]*

[1]College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Republic of Korea.
[2]Department of Industrial Crops, Korea National College of Agriculture and Fisheries, Hwasung 445-760, Republic of Korea.
[3]Department of Clinical Pathology, Juseong College, Chungbuk 363-794, Republic of Korea

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Hypsizygus marmoreus **are wild and edible mushrooms found in East Asia that are included in the *Shimeji* family. *H. marmoreus* have emerged as a pivotal entity and therapeutic target in cardiovascular diseases, but there is little information of their effects on platelet function. Therefore, our study was designed to investigate the effect of this extract on platelet aggregation induced by various agonists, $[Ca^{2+}]_i$ mobilization, extracellular signal-regulated kinase (ERK) phosphorylations, ATP secretion, and integrin $\alpha_{IIb}\beta_3$ activation. We found that *H. marmoreus* methanol extract dose-dependently inhibited platelet aggregation that was induced by collagen, but not by thrombin or ADP. Collagen-induced intracellular calcium concentration $[Ca^{2+}]_i$ was also dose-dependently suppressed in *H. marmoreus* extract treated platelets. In addition, collagen-activated ATP secretion was lowered by the *H. marmoreus* extract treatment. Moreover, *H. marmoreus* extract was revealed to attenuate fibrinogen binding initiated by collagen. However, ERK phosphorylation was not affected. In conclusion, *H. marmoreus* extract inhibit platelet aggregation induced by collagen, intracellular calcium mobilization, and dense granule secretion while suppressing integrin $\alpha_{IIb}\beta_3$ activation. Finally, this suggests that *H. marmoreus* could be developed as a functional food or phytomedicine against platelet related cardiovascular disease, including thrombosis, stroke and atherosclerosis.**

Key words: *Hypsizygus marmoreus*, mushroom, platelet aggregation, collagen, calcium, integrin $\alpha_{IIb}\beta_3$.

INTRODUCTION

Not only do platelets play a key role in hemostasis, they are also involved in some pathophysiological conditions such as thrombosis, atherosclerosis, and inflammation (Tucker et al., 2008). Upon vascular injury, platelets become exposed to subendothelial matrix of the blood vessels, particularly collagen, to which they initially adhere through the glycoprotein (GP) Ib-V-IX complex and the plasma von Willebrand factor (vWF). This interaction with collagen is stabilized through direct interactions between the platelet collagen receptor glycoprotein VI (GPVI) (Tucker et al., 2008). This, in turn, triggers the activation of the integrin $\alpha_{IIb}\beta_3$, leading to integrin-dependent stable platelet adhesion, spreading, and aggregation (Li et al., 2003a).

The binding of collagen to receptors triggers a signaling pathway that leads to phospholipase $C\gamma_2$ ($PLC\gamma_2$) phosphorylation, whereas thrombin, one of most abundant

*Corresponding author. E-mail: rheemh@knu.ac.kr. Tel: +82-53-950-5967. Fax: +82-53-950-5955.

Abbreviations: GP, Glycoprotein; **vWF,** von Willebrand factor; **$PLC\gamma_2$,** phospholipase $C\gamma_2$; **PIP2,** Phosphatidylinositol (4,5)-bisphosphate; **IP3,** inositol (1,4,5)-trisphosphate; **DG,** diacylglycerol; **PKC,** protein kinase C; **TXA2,** thromboxane A2; **PAR,** Protease-activated receptor.

and potent soluble agonists, activates PLCβ (Grenegård et al., 2008; Mazharian et al., 2007; Voss et al., 2007). By activating members of the PLC family, Phosphatidylinositol (4,5)-bisphosphate (PIP2) is cleaved into inositol (1,4,5)-trisphosphate [IP3] and diacylglycerol (DG). IP3 and DG are responsible for the mobilization of calcium from intracellular stores and the activation of the protein kinase C (PKC), respectively, both of which lead to secretion and aggregation (Gibbins, 2004).

In addition, the exposure of platelets to various soluble agonists, such as thrombin and ADP, elevates the cytosolic free Ca^{2+} and, thereby contributes to the platelet's shape change, secretion, and aggregation in a highly orchestrated manner (Geiger et al., 1992; Sarratt et al., 2005). Although different agonists activate divergent signaling pathways, diverse platelet agonists lead to stimulation of the PLC family followed by calcium mobilization and eventually activation of the ligand binding function of the integrin $\alpha_{IIb}\beta_3$ that mediates platelet aggregation (Akbar et al., 2007; Stojanovic et al., 2006). In normal circulating platelets, the integrin $\alpha_{IIb}\beta_3$ is in a resting state with a low affinity for its ligands, such as fibrinogen and vWF. At sites of vascular injury, integrin $\alpha_{IIb}\beta_3$ is activated by intracellular signaling (inside-out signaling), initiated by exposure of platelets to the subendothelial matrix collagen or soluble agonists such as thrombin and adenosine diphosphate (ADP). Activated integrin $\alpha_{IIb}\beta_3$ can bind ligands such as fibrinogen, vWF, and fibronectin. This, in turn, induces platelet signaling and activation, which leads to stable aggregation through $\alpha_{IIb}\beta_3$ binding to fibrinogen and, thus, mediates platelet spreading on vascular surfaces during hemostasis and thrombosis through reorganization of the actin cyto-skeletal network and the release of bioactive substances stored in the granules (Ablooglu et al., 2009; Tabuchi et al., 2003; Yacoub et al., 2006). Subsequently, a second level of $\alpha_{IIb}\beta_3$ activation, induced by secondarily released molecules like ADP or thromboxane A2 (TXA2), is required to complete irreversible and firm aggregation of platelets, especially in events occurred by low concentration of the agonist, such as thrombin and collagen (Li et al., 2004; Moers et al., 2004; Van de Walle et al., 2007).

The functions of the ERK pathway - a member of the mitogen-activated protein kinase (MAPK) family - in platelets have not been fully understood. That is, it has been reported that inhibition of the MAPK cascade by PD 98059, which has been described as a potent and highly selective inhibitor of MEK, did not affect platelet responses to the physiological stimuli collagen and thrombin. In particular, pharmacological inhibition of the ERK pathway did not interfere with collagen-stimulated phospholipase A2 (PLA2) phosphorylation (Börsch-Haubold et al., 1996; Borsch-Haubold et al., 1995). The secretion of granule contents is an important cellular function shared by platelets, leukocytes, neurons, endocrine glands, and many other cell types (Li et al., 2004). In platelets, the secretion of granules provides an important mechanism, thus enhancing activation and the stabilization of platelet aggregation at the site of vascular injury. Alpha granules contain polypeptides, such as coagulation proteins (e.g., notably fibrinogen) and membrane adhesion molecules (e.g., integrin $\alpha_{IIb}\beta_3$). Dense granules of platelets contain small molecules, such as ADP and ATP, and calcium that initiate self-amplification of platelet activation (Reed et al., 2000; Stojanovic et al., 2006; Toth-Zsamboki et al., 2003).

Hypsizygus marmoreus (Agaricales, *H. marmoreus*) also known as Bunashimeji, is a highly praised cultivated culinary and medicinal mushroom in East Asia. Previous studies have reported that *H. marmoreus* has an antioxidant effect (Matsuzawa et al., 1998) and an anti-atherosclerotic effect that lowers total serum cholesterol (Mori et al., 2008). Moreover, when polysaccharide, s-(1-3)-D-glucan is isolated from *H. marmoreus*, it has shown a very high anti-tumor activity (Akavia et al., 2009). However, the effect of *H. marmoreus* on the platelet function has not been reported yet. These beneficial effects may correlate with the platelet function, thus the aim of our study was focused on the effect of *H. marmoreus* methanol extract (HMME) on platelet physiology and intracellular pathways.

MATERIALS AND METHODS

Thrombin, ADP, and Fura-2/AM were obtained from Sigma (St. Louis, MO, USA). Collagen was procured from Chrono-log (Havertown, PA, USA). The antibodies that were used against total-ERK and phospho-ERK were from Cell Signaling (Beverly, MA, USA). The ATP assay kit was purchased from Biomedical Research Service Center (Buffalo, NY, USA). The Fibrinogen Alexa fluor® 488 conjugate was obtained from Invitrogen molecular probes (Eugene, OR, USA). All other chemicals were of reagent grade.

Platelet preparation

The preparation of platelets has been described previously (Kim et al., 2006). Male Sprague Dawely rats of 60 days old and weighing from 240 to 250 g were obtained from Orient Co. (Seoul, Korea). The rats were maintained in a standard laboratory animal facility with free access to feed and water, and acclimated for at least two weeks before use. Whole blood from the rats was collected using a 23G needle, which was inserted into the abdominal aorta and transferred to a 15 ml test tube containing 1 ml of ACD (85 mM trisodium citrate, 83 mM dextrose and 21 mM citric acid) as an anticoagulant. Blood was centrifuged at 170 x g for 7 min to obtain platelet-rich plasma.

In order to remove any residual erythrocytes, the platelet-rich plasma samples were again centrifuged at 120 x g for 7 min. In order to isolate the platelets and remove the ACD solution, platelet-rich plasma was centrifuged twice at 350 x g with a washing buffer for 10 min. The platelets of the precipitate were adjusted to the proper number [10^8/ml for the aggregation assay in Tyrode buffer (137 mM of NaCl, 12 mM of $NaHCO_3$, 5.5 mM of glucose, 2 mM of KCl, 1mM of $MgCl_2$, and 0.3 mM of $NaHPO_4$ pH 7.4)]. All platelet preparations were conducted at room temperature, and all experimental procedures and protocols used in this investigation were reviewed and approved by the Kyungpook National University Ethics Committee.

Platelet aggregation

Platelet aggregation was performed as previously described (Kamruzzaman et al., 2010). Aggregation was monitored by measuring light transmission in an aggregometer (Chrono-Log, Havertown, PA, USA). The washed platelets were pre-incubated at 37°C for 2 min with either HMME or a vehicle, and stimulated with agonists. The reaction mixture was further incubated for 5 min by stirring at 170 x g, after which the aggregation rate was determined. The concentration of the vehicle was kept at less than 0.1% so as to exclude an artificial effect.

Determining the $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ was determined with Fura-2/AM as described previously (Kim et al., 2006). Briefly, the platelets were incubated with 5 μM of Fura-2/AM for 30 min at 37°C and then washed. The Fura-2-loaded washed platelets (10^8/ml) were then pre-incubated with HMME for 3 min at 37°C in the presence of 1 mM $CaCl_2$. Next, the platelets were stimulated with collagen for 5 min. Fluorescence signals from platelet suspensions were recorded using a Hitachi F-2500 fluorescence spectrofluorometer (F-2500, Hitachi, Japan). Fluorescence emission was determined at 510 nm, with simultaneous excitation at 340 and 380 nm, changing every 0.5 s. Fura-2 fluorescence was measured in a spectrofluorometer by the method of Schaeffer (Schaeffer and Blaustein 1989): $[Ca^{2+}]_i$ in cytosol=224 nM x (F–Fmin)/(Fmax–F), where 224 nM is the dissociation constant of the Fura-2-Ca^{2+} complex, and Fmin and Fmax represent the fluorescence intensity levels at very low and very high Ca^{2+} concentrations, respectively. In our experiment, Fmax is the fluorescence intensity of the Fura-2- Ca^{2+} complex at 510 nm after the platelet suspension containing 1 mM of $CaCl_2$ had been solubilized by Triton X-100 (0.1%). Fmin is the fluorescence intensity of the Fura-2- Ca^{2+} complex at 510 nm, after the platelet suspension containing 20 mM Tris/3 mM of EGTA had been solubilized by Triton X-100 (0.1%). F represents the fluorescence intensity of the Fura-2-complex at 510 nm after the platelet suspension was stimulated by collagen, with and without HMME, in the presence of 1 mM $CaCl_2$.

Immunoblotting

Platelets treated with HMME or a vehicle were stimulated with collagen and incubated for 5 min in an aggregometer. Lysates were then prepared by solubilizing and centrifuging platelets in a sample buffer (0.125 M Tris–HCl at pH 6.8, 2% SDS, 2% β-mercapto-ethanol, 20% glycerol, 0.02% bromophenol blue in the presence of these protease inhibitors: μg/ml:1 phenylmethylsulfonylfluoride (PMSF), 2 aprotinin, 1 leupeptin, and 1 pepstatin A). The protein concentration was determined using the BCA Assay (PRO-MEASURE, iNtRON Biotechnology, Korea). Equal volumes of platelet proteins were resolved in a 10% SDS-PAGE and transferred to a nitrocellulose membrane in a transfer buffer (25 mM Tris [pH 8.5), 0.2 M glycine, and 20% methanol). Immunoblots were blocked with TBS-T containing 5% nonfat dry milk and incubated with primary antibodies diluted in a blocking solution. The immunoblots were again incubated with the horseradish peroxidase secondary antibody and the membranes were visualized using en-hanced chemiluminescence, ECL (iNtRON Biotechnology, Korea).

ATP release assay

Washed platelets were pre-incubated for 3 min at 37°C with various concentrations of HMME and then stimulated with collagen. The reaction was terminated, and samples were centrifuged. The supernatants were used for the assay. ATP release was measured in a luminometer (GloMax 20/20, Promega, Madison, USA) using the ATP assay kit (Biomedical Research Service Center, Buffalo, USA).

Flow cytometry

Alexa Fluor 488-fibrinogen binding to PRP was quantified by flow cytometry. Briefly, the washed platelets were preincubated with various concentrations of HMME at room temperature in the presence of 0.1 mM $CaCl_2$, and then stimulated with collagen for 5 min, added to Alexa Fluor 488-human fibrinogen (20 μg/ml) for 5 min, and fixed with 0.5% paraformaldehyde at 4°C for 30 min. Platelets were pelleted by centrifugation at 5,500 rpm at 4°C and resuspended in 500 μl PBS. Since activation of integrin $\alpha_{IIb}\beta_3$ is largely dependent on the generation of Ca^{2+}, nonspecific binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ was measured by measuring fibrinogen binding in the presence of calcium chelator EGTA 1 mM. The fluorescent intensity of each platelet sample was analyzed using a FACS Caliber cytometer (BD Biosciences, San Jose, USA), and data were analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, USA).

RESULTS

HMME inhibited platelet aggregation induced by collagen

We previously determined that collagen (1.0 μg/ml) completed platelet aggregation and the present study employed this agonist concentration for platelet activation.

As shown in (Figure 1A), platelet aggregation induced by collagen was markedly inhibited by HMME. To analyze whether the inhibition mediated by this extract was also observed in other agonists or whether it was specific for collagen, platelets were stimulated with other agonists that act through different mechanisms. (Figure 1B) shows that thrombin (0.05 U/ml)-induced aggregation was not changed at all in HMME-treated platelet. In addition, HMME treatment could marginally attenuate ADP (5 μM)-induced aggregation (Figure 1C). Thus, these results in various agonists-induced platelet aggregations suggest that the extract has specificity on the signaling pathways triggered by collagen.

HMME inhibited ATP release in collagen-treated platelet

In order to investigate the suppressive effect on platelet function, we examined the influence of HMME on ATP release. As such, (Figure 2) displays that the ATP release triggered by collagen was significantly inhibited by treatment of HMME in a dose-dependent manner.

HMME suppressed collagen-induced intracellular calcium elevation

Since intracellular calcium mobilization is known to be a

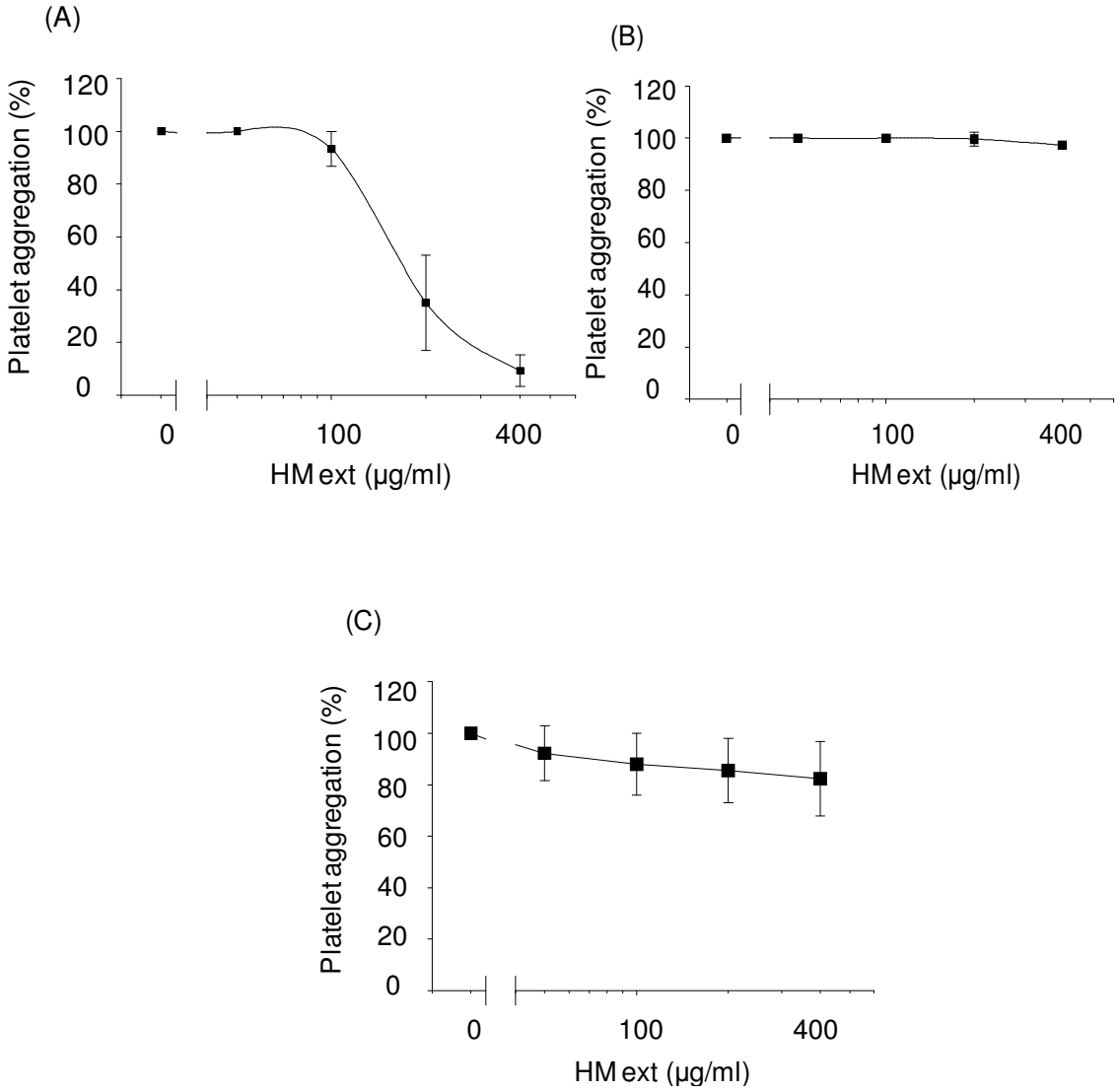

Figure 1. The effects of HMME on collagen (1.0 μg/ml), thrombin (0.05 U/ml), and ADP (5 μM) induced platelet aggregation. Platelets (10^8/ml) were preincubated with or without extracts in the presence of 1 mM $CaCl_2$ for 2 min at 37°C. The platelets were stimulated with collagen (A) 1.0 μg/ml, thrombin (B) 0.05 U/ml or ADP (C) 5 μM for 5 min. Agonists-induced platelet aggregation was recorded using a Chrono-Log aggregometer. Bar graphs show the means ± SEM of at least 4 independent experiments, which were performed in duplicate.

key step of platelet activation triggered by collagen (Harper and Poole, 2010), we examined the effect of HMME on collagen-induced intracellular calcium concentration. As shown in (Figure 3), we found that preincubation with HMME dose-dependently suppressed $[Ca^{2+}]_i$ elevation that was induced by collagen.

HMME did not affect collagen-activated platelet ERK2 phosphorylation

Since it has been established that ERK2 is involved in cytosolic calcium arise and ATP release in platelets (Lee et al., 2010; Rosado et al., 2001), we examined ERK2 phosphorylation in HMME-treated platelet under collagen stimulation. As shown in (Figure 4), pretreatment of HMME did marginally suppress ERK2 phosphorylation at the maximal dose (200 μg/ml). HMME at concentrations that inhibited platelet aggregation significantly could not affect ERK2 phosphorylation, and this result suggests the possibility of a pathway independent of the ERK pathway.

HMME attenuated collagen-activated fibrinogen binding to integrin $\alpha_{IIb}\beta_3$

Since activation of integrin $\alpha_{IIb}\beta_3$ is required for stable clot formation and further activation of platelet after initial

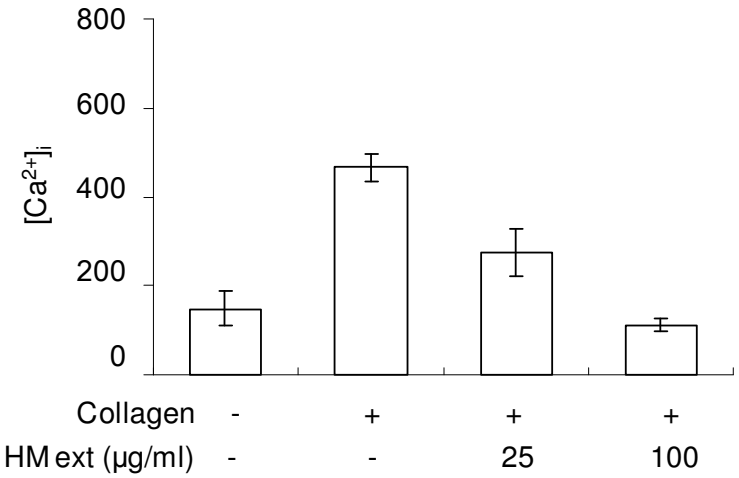

Figure 3. The effect of HMME on collagen-induced $[Ca^{2+}]_i$ elevation. Washed platelets were loaded with Fura-2/AM at 37°C for 1 h, and the concentration of $[Ca^{2+}]_i$ was determined as described in "Materials and Methods." Bar graphs show the means ± SEM of 3 independent experiments, which were performed in duplicate.

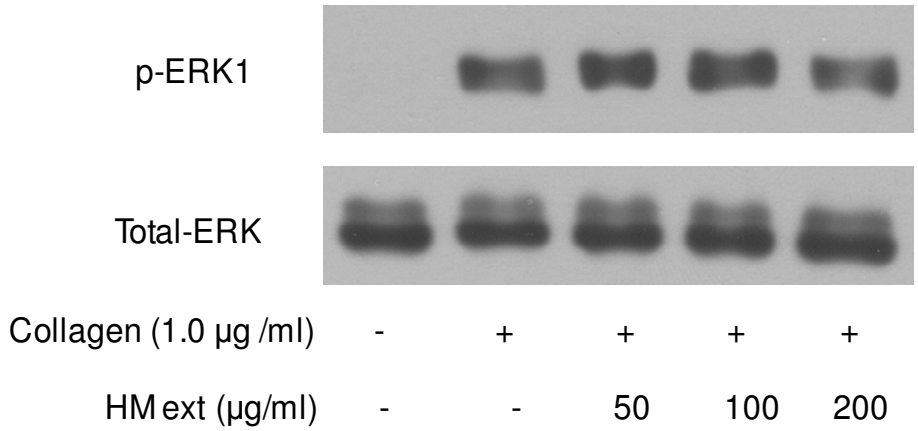

Figure 4. The effect of HMME on collagen-induced ERK phosphorylations. Washed platelets were pre-incubated with a vehicle or HMME at the concentration indicated for 2 min. The platelets were added by collagen (1.0 μg/ml) and incubated for 5 min. Protein extraction and immunoblotting were carried out as described in "Materials and Methods". Blots were visualized by ECL, and the immunoblots are representatives of 3 to 4 independent experiments.

agglutination (Ablooglu et al., 2009), we investigated the role of the extract on functional response of integrin $\alpha_{IIb}\beta_3$ activation. As shown in (Figure 5), a strong inhibition of fibrinogen binding was observed in high concentration of HMME (200 μg/ml)-treated platelets, when compared with the control group, indicating that HMME influences the binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$.

DISCUSSION

As part of our ongoing search for bioactive substances from the wild mushrooms extract library, we decided to examine the anti-platelet activity of *H. marmoreus* extract and its mechanism of action on platelet function. To our knowledge, this study, for the first time, revealed that HMME inhibited platelet aggregation induced by collagen dose-dependently. An increase in $[Ca^{2+}]_i$ is a key signal to activate the downstream event used by a wide range of platelet agonists. The central role of $[Ca^{2+}]_i$ increase within the physiological range is to activate most functional responses in the platelets, including shape change, secretion, aggregation, and pro-coagulant activity. In this study, we found that HMME inhibited

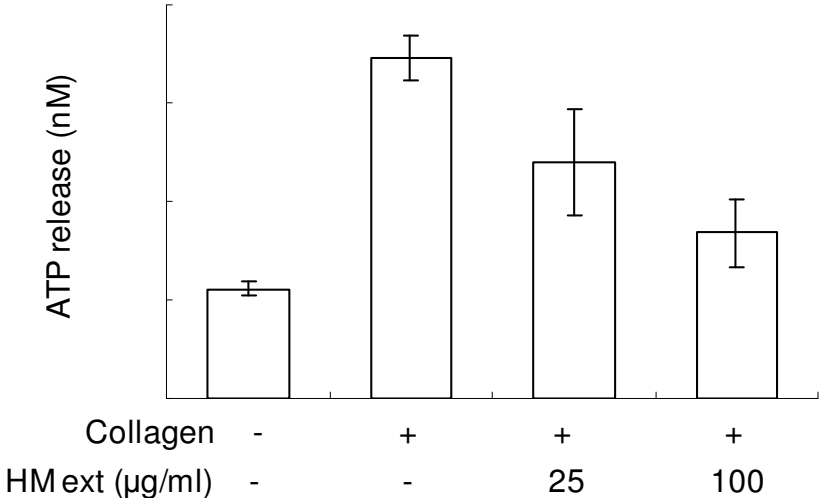

Figure 2. The effects of HMME on dense granule secretion in collagen-activated platelets. Washed platelets were pre-incubated with HMME at the concentrations indicated, and stirred in an aggregometer for 3 min prior to collagen stimulation for 5 min, and then the reactions were terminated followed by ATP release assay. ATP release in response to agonist stimulation was performed as described in "Material and Methods." Bar graphs show the mean ± SEM of at least 3 independent experiments, which were performed in duplicate.

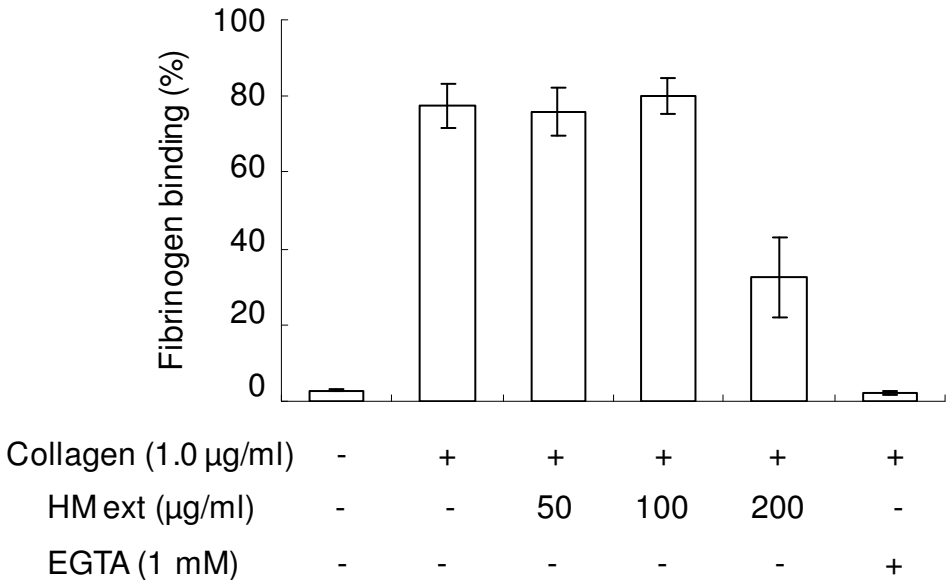

Figure 5. The effect of HMME on fibrinogen binding to integrins in collagen-activated platelets. The inhibitory effect of HMME on fibrinogen binding to the integrin $\alpha_{IIb}\beta_3$ in collagen-stimulated platelets was measured by flow cytometric analysis. Washed platelets were pre-treated with PBME, and then collagen (1.0 μg/ml) was mixed with Alexa Fluor 488-human fibrinogen (20 μg/ml) for 5 min and fixed with 0.5% paraformaldehyde at 4°C for 30 min. Nonspecific binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ was measured by measuring fibrinogen binding of the washed platelets in the presence of EGTA 1 mM. Bar graphs show the means of ± SEM of at least 4 independent experiments, which were performed in duplicate.

$[Ca^{2+}]_i$ increase triggered by collagen, suggesting that the anti-aggregatory ability of the extract may be mediated by inhibition of $[Ca^{2+}]_i$. Platelets are anuclear and secretory cells that can release effector molecules such as calcium, ADP, ATP, and serotonin at the site of injury, thus providing an important role in hemostasis (Reed et al., 2000). Secretion of dense granules is essential for platelet activation and those contents are indispensable

for clot stabilization and enhancing secretion of platelet (Konopatskaya et al., 2009). Our result shows that HMME suppressed the ATP release triggered by collagen, suggesting that anti-platelet activity of the extract is carried out by inhibiting dense granule release. The event of collagen activating its downstream molecule PLCγ2 is a crucial step in the early dense granule release (Toth-Zsamboki et al., 2003). It is important to note that there is a significant difference in platelet activation properties between the high-dose agonist induced and the low-dose agonist induced platelet activation. The latter activation should induce a "second wave" of platelet secretion as a series of rapid positive feedback loop to accomplish successful aggregation that is not dissipated under flowing conditions. However, the high-dose agonist induced does not allow this to occur (Li et al., 2003b). With the consideration of a low dosage of collagen (1.0 µg/ml) in the present study, the contents of a dense granule release, especially ADP, is required for full platelet activation by pushing the platelets above the threshold stimuli required for subsequent downstream, integrin $\alpha_{IIb}\beta_3$ activation (Cho et al., 2002; Li et al., 2003b; Mazzucato et al., 2002; Ren et al., 2010). Nevertheless, it is possible that platelet aggregate in the presence of an ADP scavenger or cyclooxygenase can be inhibited with higher doses of collagen (Cho et al., 2002; Grenegård et al., 2008; Li et al., 2003b; Pleines et al., 2009; Zhang and Colman, 2007).The binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ is well known to stimulate platelets further, and thereby establishing a positive feedback loop for platelet activation. Knowing that integrin-dependent aggregationis essential for thrombus formation (Kasirer-Friede et al., 2004; Li et al., 2006; Tucker et al., 2008) under stimulation of the collagen receptor GPIV, we therefore examined whether treatment of HMME attenuated fibrinogen binding to integrin $\alpha_{IIb}\beta_3$. Although platelet initial adhesion is not affected by the blockade of fibrinogen binding to integrin (Sarratt et al., 2005; Tucker et al., 2008), in order to achieve irreversible aggregation the platelet has to undergo signal amplification assisted by granule contents molecules like ADP (Li et al., 2003b; Mazzucato et al., 2002) and activation of integrin αIIbβ3 (Tucker et al., 2008). Several studies demonstrate that fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ under collagen stimulation is indispensable in activating another collagen receptor integrin $\alpha_2\beta_1$, and thus results in stable clot formation (Tucker et al., 2008; Van de Walle et al., 2007). Our results suggest that the final step of platelet aggregation, accomplished by the adhesive interaction of platelet surface receptors (integrins) through fibrinogen to another adjacent platelet receptors, was inhibited by the extract treatment. In addition, ERK2 is known to be phosphorylated in collagen-activated platelets, depending on TXA2 formation and ADP release (Konopatskaya et al., 2009a; Lee et al., 2010). Moreover, U0126, a selective inhibitor of MAPK/ERK kinase, is reported to preferentially inhibit low-doses of collagen-induced platelet aggregation rather than low-doses of thrombin induced aggregation (Adam et al., 2010). We, therefore, determined whether our extract affect ERK phosphorylation was induced by collagen. Unexpectedly, our result revealed that HMME did not affect collagen-induced ERK activation at all, which was contrary to previous studies (Adam et al., 2010; Konopatskaya et al., 2009; Lee et al., 2010). Debate has surrounded whether the role of ERK is involved in low-dose collagen induced platelet activation. In the contrast to the studies above, Börsch-Haubold et al. (1996) have reported that pharmacological inhibition of ERK cascade (PD98059) could not interfere with collagen and thrombin stimulated PLA2 phosphorylation, which promotes arachidonic acid cleavage and, thus, activates pro-aggregatory eicosanoids like TXA2. Moreover, they reported that platelet functional responses, such as an increase in $[Ca^{2+}]_i$ concentration, rearrangement of cytoskeleton, and dense granule release, were not altered significantly by the inhibitor, suggesting a distinct pathway of ERK (Börsch-Haubold et al., 1996; Borsch-Haubold et al., 1995). Consistent with this, another study has reported that U0126 at concentration that inhibits MEK enzyme, which prevents ERK activation, had no effect on the aggregation of human platelets in response to collagen (1.0 µg/ml), although aggregation was blocked at higher doses of U0126 (McNicol and Jackson, 2003). Mazharian et al. (2005) reported that inhibition of ERK by treatment of PD 98059 did not reduce platelet spreading over a collagen matrix (Mazharian et al., 2005). There is a vast amount of evidence showing that activation of the ERK pathway leads to integrin-ligand interaction or *vice versa* (Chen et al., 1994; Li et al., 2006; Nadal et al., 1997). This suggests that the ERK pathway is strongly activated with integrin engagement. Conversely, many reports declare contradictions to previous studies, indicating that ERK is down-regulated by integrin active-tion, thus suppressing ERK phosphorylation (Flevaris et al., 2009; Nadal et al., 1997). However, it has been also documented that a pharmacological blockade of integrin $\alpha_{IIb}\beta_3$ leads to neither the activation of ERK pathway (Yacoub et al., 2006) nor its spreading on the collagen-coated surface (Sarratt et al., 2005). Taken together, these findings are supporting the strong evidence of the biphasic role of the ERK pathway, possibly due to the effects of the downstream of ERK, thereby providing the complexity of the ERK signaling network. This is in agreement with previous observations suggesting that a complicated ERK pathway is multifactorial as much as multifarious (Adam et al., 2010; Börsch-Haubold et al., 1996; Nadal et al., 1997; Oury et al., 2006; Schwarz et al., 2000). Many of the unidentified signaling molecules in the ERK pathway, therefore, mediate platelet activation induced by low-dose collagen, and further studies are required to determine the signaling molecules to resolve these discrepancies.

In conclusion, *H. marmoreus* extract inhibited collagen-

induced platelet aggregation in a dose-dependent manner. It also significantly suppressed dense granule secretion, which was determined by ATP release assay. In addition, the extract significantly and dose-dependently inhibited collagen-induced $[Ca^{2+}]_i$ elevation. *H. marmoreus* extract significantly inhibited fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ at the maximal dose (200 µg/ml). Taken together, these results suggest that *H. marmoreus* extracts inhibit platelet activation with generalities through the well-known classical pathway of platelet inhibition, and that it could be applied to dietary food with beneficial medical effects.

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