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The mechanism of anti-platelet activity of davallialactone: Involvement of intracellular calcium ions, extracellular signal-regulated kinase 2 and p38 mitogen-activated protein kinase

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Abstract

This study was designed to investigate the effect of davallialactone, which was isolated from the mushroom *Inonotus xeranticus*, on platelet aggregation induced by collagen, thrombin and ADP. We found that davallialactone dose-dependently inhibited platelet aggregation that was stimulated either by collagen (2.5 μg/ml), a potent ligand of integrin α2β1 and glycoprotein VI, or by thrombin (0.1U/ml), a potent agonist of the protease-activated receptors (PARs) PAR1 and PAR3. In addition, davallialactone inhibited platelet aggregation induced by ADP, an agonist of P2Y receptor. To understand the mechanism of anti-platelet activity, we determined whether davallialactone affected the downstream signaling in collagen-activated platelets. Using the fura-2/AM fluorometric assay, we found that davallialactone dose-dependently inhibited intracellular calcium concentration levels ($[Ca^{2+}]_i$). Moreover, davallialactone inhibited the phosphorylation of extracellular signal-regulated protein kinase (ERK)-2 and p38 mitogen-activated protein kinase (MAPK), in a dose-dependent manner. The tyrosine phosphorylation of 60 and 85kDa proteins, which were activated by collagen, were differentially inhibited by davallialactone. Taken together, these data suggest that davallialactone may have potential anti-platelet aggregation activity via suppression of intracellular downstream signaling pathways.

Keywords: Davallialactone; *Inonotus xeranticus*; Platelet aggregation; Thrombin; Collagen; ADP; ERK2; p38 MAPK; $[Ca^{2+}]_i$

1. Introduction

It is well known that platelet aggregation plays an essential role in the physiology of primary haemostasis and is related to the pathogenesis of cardiovascular diseases such as thrombosis (Shattil et al., 1998; Stouffer and Smyth, 2003). Aberrant intravascular thrombosis is the main cause of a wide variety of cardiovascular diseases (Grenache et al., 2003; Huo and Ley, 2004). Upon the activation of a platelet receptor towards a plasma membrane, the bioactive substances (e.g., calcium, growth factor, and aggregation-related materials) in the granules are released in an energy-dependent process that requires ionized calcium (Leclerc, 2002; Savage et al., 2001). In particular, intracellular calcium ($[Ca^{2+}]_i$) is the main integrator of platelet aggregation, which is induced by the activation of a G protein-coupled receptor (e.g., the protease-activated receptors [PARs] PAR1 and PAR3) and an integrin-type receptor (e.g., α2β1). The signaling of these

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endogenous and exogenous molecules is the main stimulus for platelet aggregation, which is involved in serious cardiovascular diseases, including atherosclerosis, stroke, and diabetes (Huo and Ley, 2004). Therefore, searching for natural products or compounds that have anti-platelet activity is of great importance in the prevention of such cardiovascular diseases.

Mushrooms are a nutritionally functional food and a source of physiologically beneficial medicines, including antimicrobial, antitumor, and antiviral activities (Lull et al., 2005; Seo et al., 2003; Sullivan et al., 2006). Although it has been reported that the *Inonotus* species has therapeutic effects, such as anti-inflammatory and hepatoprotective (Wasser and Weis, 1999), anti-nociceptive (Park et al., 2005), anti-cancer (Burczyk et al., 1996; Jarosz et al., 1990; Kim et al., 2006b), and antimicrobial activities (Al-Fatimi et al., 2005; Awadh Ali et al., 2003), the biological activities of *Inonotus xeranticus* (*I. xeranticus*) have been studied only by us (Lee et al., 2006; Lee and Yun, 2006). We found that hispidin analogs (i.e., davallialactone and inoscavin) were isolated from *I. xeranticus* and that they showed potent free radical scavenging activity; however, the biological activities of the principal ingredients of *I. xeranticus*, such as davallialactone and inoscavin A, remain to be studied. Therefore, we were curious to determine whether davallialactone modulates platelet aggregation. In order to investigate the anti-platelet properties of davallialactone, platelet aggregation assays that were induced by collagen, thrombin and ADP, were carried out. In addition, we sought to determine the mechanism of davallialactone-induced anti-platelet activity by using a downstream signaling assay. In conclusion, we found that davallialactone showed potent anti-platelet activity, which could have a beneficial therapeutic effect on aberrant platelet aggregation-mediated cardiovascular diseases.

2. Materials and methods

2.1. Materials

Davallialactone was isolated from the medicinal fungus *I. xeranticus* (Lee et al., 2006). The purity of this compound was more than 95% by HPLC analysis. Thrombin and fura-2/AM were obtained from Sigma (St. Louis, MO). Collagen and ADP were obtained from Chrono-log Corp. (Havertown, PA). Antibodies to ERK and phospho-ERK were obtained from Promega. The antibodies to p38 MAPK and phospho-p38 MAPK were from Cell Signaling. The antibody to phosphotyrosine was from Upstate. All other chemicals were of reagent grade.

2.2. Platelet preparation

The preparation of the platelets has been described previously with minor modifications (Kim et al., 2006a). Rat blood (8 ml) was obtained via a venipuncture using a 23G needle which was inserted into the abdominal aorta. The blood was transferred to a 15 ml test tube containing 1 ml of a citrate phosphate dextrose solution (90 mM $Na_3C_6H_5O_7{\cdot}2H_2O$, 14 mM $C_6H_8O_7{\cdot}H_2O$, 128.7 mM $NaH_2PO_4{\cdot}H_2O$, 2.55 g/100 ml dextrose). The blood was centrifuged at 120 g for 7 min in order to achieve platelet-rich plasma. In order to remove residual erythrocytes, the platelet-rich plasma samples were again centrifuged at 40 g for 7 min. In order to isolate the platelets and remove the citrate phosphate dextrose solution, the platelet-rich plasma was centrifuged twice at 300 g for 10 min. The platelets in the precipitate were adjusted to the proper number [10^8/ml for an aggregation assay with a Tyrode buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 2 mM KCl, 1 mM $MgCl_2$, 0.3 mM $NaHPO_4$, pH 7.4)]. All of the steps for platelet preparation were conducted at room temperature, and all experimental procedures and protocols were reviewed and approved by the Ethics Committee of the College of Veterinary Medicine, Kyungpook National University.

2.3. Platelet aggregation assay

Platelet aggregation was performed as previously described (Kim et al., 2006a). Aggregation was monitored by measuring light transmission via an aggregometer (Chrono-log Corp., Havertown, PA). The washed platelets were preincubated at 37 °C for 2 min with either davallialactone or vehicle. The reaction mixture was further incubated for 5 min, with stirring, at 1200 rpm. The concentration of the vehicle was kept at less than 0.1% so as to exclude an artificial effect.

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

$[Ca^{2+}]_i$ was determined with fura-2/AM as described previously (Cho et al., 2006). Briefly, the platelet-rich plasma was incubated with 5 μM of fura-2/AM for 60 min at 37 °C. The fura-2-loaded washed platelets (10^8/ml) were preincubated with davallialactone for 2 min at 37 °C in the presence of 1 mM $CaCl_2$. Next, the platelets were stimulated with collagen for 5 min. Fura-2 fluorescence was measured in a spectrofluorometer (F-2500, Hitachi, Japan) with an excitation wavelength that ranged from 340 nm and 380 nm, changing every 0.5 s; the emission wavelength was 510 nm. The $[Ca^{2+}]_i$ was calculated by the method of Schaeffer (Schaeffer and Blaustein, 1989): $[Ca^{2+}]_i$ in cytosol = 224 nM × $(F-F_{min})/(F_{max}-F)$, where 224 nM is the dissociation constant of the fura-2-Ca^{2+} complex, and F_{min} and F_{max} represent the fluorescence intensity levels at very low and very high Ca^{2+} concentrations, respectively. In our experiment, F_{max} 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%). F_{min} 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 davallialactone, in the presence of 1 mM $CaCl_2$.

2.5. Western blot analysis

Platelet extracts were prepared by the standard Laemmli method (Laemmli, 1970). After the platelet aggregation assay, the sample buffer (0.125 M Tris–HCl, 2% SDS, 2% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue, pH 6.8, 2×) was added and the protein concentration level was

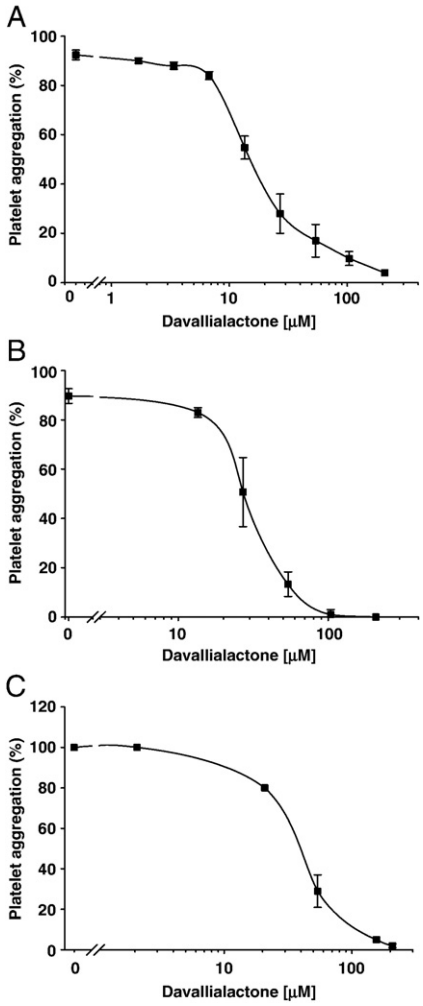

Fig. 1. The inhibitory effect of davallialactone on collagen (2.5 μg/ml)-, thrombin (0.1 U/ml)-, and ADP (10 μM)-induced platelet aggregation. Platelets (10^8/ml) were preincubated with or without davallialactone in the presence of 1 mM $CaCl_2$ for 2 min at 37 °C. The platelets were stimulated with (A) collagen (2.5 μg/ml), (B) thrombin (0.1 U/ml) or (C) ADP (10 μM). Platelet reaction was terminated at 5 min and the percentage of aggregation rate was determined. Data are given as means±S.E.M. (n=3 experiments).

determined with PRO-MEASURE (iNtRON Biotechnology, Korea). Following centrifugation at 15,000×g for 5 min, 50 μg of aliquot proteins were resolved by 10% SDS-PAGE. The resolved proteins were electrotransferred to nitrocellulose membranes in 25 mM Tris (pH 8.5), 0.2M glycerin, and 20% methanol at 100 V for 2 h. Blots were blocked for at least 2h with PBS-tween-20 (PBST) containing 5% nonfat dry milk, and then incubated with an appropriate antibody (~ 1:1000 dilution ratio in a blocking solution). After washing in PBST three times, the blot was incubated with a secondary antibody (1:5000 dilution ratio in a blocking solution) for 1h and the antibody-specific proteins were visualized by an ECL detection system (Supex Co. Pohang, Korea) according to the manufacturer's instructions.

2.6. Statistical analysis

Data were analyzed by a one-way analysis of variance followed by Dunnett's test post-hoc in order to determine the statistical significance of the differences. All data are presented as means±S.E.M. P values of 0.05 or less were considered to be statistically significant.

3. Results

3.1. The inhibitory effect of davallialactone on rat platelet aggregation

To examine the inhibitory effect of davallialactone on rat platelet aggregation, washed rat platelets were preincubated with various concentrations of davallialactone and exposed to collagen (2.5 μg/ml). Using 2.5 μg/ml of collagen treatment, we obtained an appropriate amount of platelet aggregation (up to 98.0±4.5%). As shown in Fig. 1A, davallialactone inhibited the aggregation in a concentration-dependent manner with an IC_{50} value of 11.4±1.9 μM. Since collagen is known to be a potent ligand against integrin-type receptors such as α2β1 and glycoprotein VI, we were interested to determine whether davallialactone still had inhibitory activity towards other types of plasma membrane receptors, such as PAR and ADP receptors. Similar to collagen-induced platelet aggregation, Fig. 1B shows the inhibitory activity of davallialactone in thrombin-induced platelet aggregation, with an IC_{50} value of 13.0±2.6 μM. In addition, davallialactone dose-

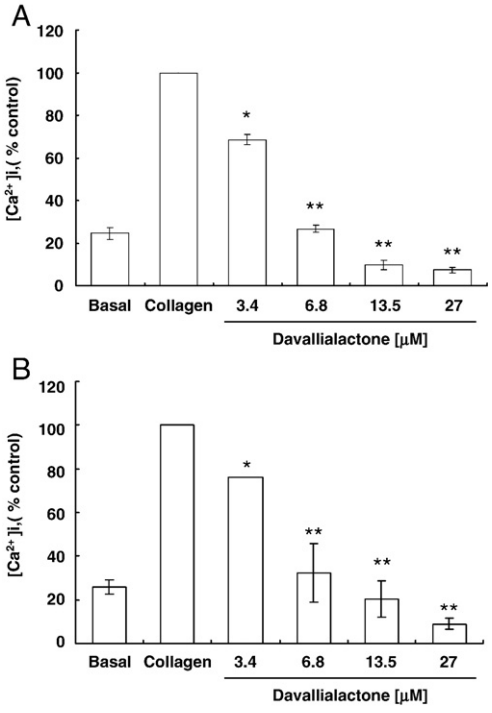

Fig. 2. The effect of davallialactone on collagen (2.5 μg/ml)-induced intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ either in the absence of 1 mM EGTA (A) or in the absence of 1 mM EGTA (B). Platelets were loaded with fura-2/AM as described in Materials and methods. The platelets (10^8/ml) were preincubated with or without davallialactone in the presence of 1 mM $CaCl_2$ for 2 min at 37 °C. The platelets were stimulated with collagen (2.5 μg/ml) for 3 min at 37 °C. $[Ca^{2+}]_i$ levels were determined as described in Materials and methods. Data are given as means±S.E.M. (n=4 experiments). *P<0.05 versus collagen-activated platelets, **P<0.01 versus collagen-activated platelets.

dependently inhibited ADP (10 μM)-induced platelet aggregation with an IC_{50} value of 41.7±11.7 μM (Fig. 1C).

3.2. The effect of davallialactone on calcium mobilization

Next, we determined whether davallialactone affected the $[Ca^{2+}]_i$ in collagen-induced platelet aggregation. The basal level of ($[Ca^{2+}]_i$ was 52.7±5.3 nM in resting platelets, which was increased by 2.5 μg/ml collagen to 227.8±11.0 nM in the presence of 1 mM $CaCl_2$. As shown in Fig. 2A, davallialactone reduced the intracellular level of Ca^{2+} that was stimulated by collagen (2.5 μg/ml) in a dose-dependent manner. In order to show the inhibitory effect of davallialactone on calcium mobilization from internal stores, measurement of the intracellular level of Ca^{2+} was performed in the presence of 1 mM EGTA. Fig. 2B shows that davallialactone dose-dependently inhibited the calcium mobilization in collagen (2.5 μg/ml)-activated platelets.

3.3. The effect of davallialactone on the phosphorylation of extracellular signal-regulated protein kinase 2 (ERK2) and p38 mitogen-activated protein kinase (MAPK)

Since $[Ca^{2+}]_i$ is known to be the most important integrator in the signaling pathways of various aggregating agents, we analyzed downstream signaling using an immunoblotting technique with specific antibodies. In order to determine whether davallialactone affected the phosphorylation of ERK2 and p38 MAPK,

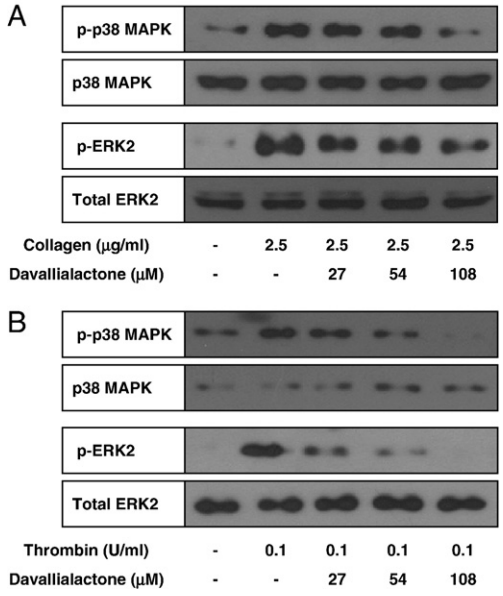

Fig. 3. The effect of davallialactone on the phosphorylation of extracellular signal-regulated kinase 2 (ERK2) and p38 mitogen-activated protein kinase (MAPK) in collagen-activated rat platelets. The preincubation with davallialactone and the stimulation by collagen in platelets are described in Fig. 1. After platelet aggregation was conducted for 5 min, the reaction was terminated and the sample buffer (2×) was added. The protein extraction and the protein concentration of platelets are described in Materials and methods. The platelet protein (50 μg) was separated with 10% SDS-PAGE and was electrotransferred onto a nitrocellulose membrane. Primary antibody and secondary antibody treatment of the membrane is described in Materials and methods. Specific bands were visualized by an ECL kit (Supex Co).

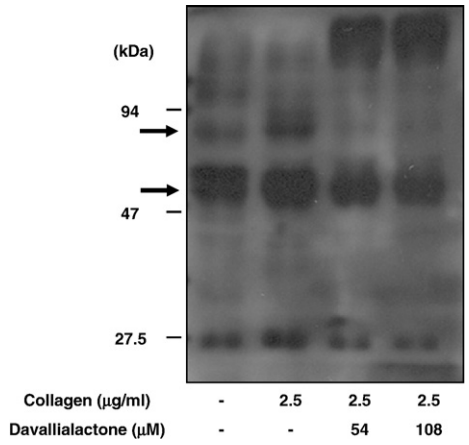

Fig. 4. The effect of davallialactone on tyrosine phosphorylation in collagen-activated rat platelets. The preincubation with davallialactone and the stimulation by collagen in platelets are described in Fig. 1. After platelet aggregation was conducted for 5 min, the reaction was terminated and sample buffer (2×) was added. The protein extraction and the protein concentration of platelets are described in Materials and methods. The platelet protein (50 μg) was separated with 10% SDS-PAGE and was electrotransferred onto a nitrocellulose membrane. Primary antibody and secondary antibody treatment of the membrane is described in Materials and methods. Specific bands were visualized by an ECL kit (Supex Co).

representative positive signals in platelet aggregation using collagen-activated rat platelets were determined. Platelet MAPKs include ERK, p38 MAPK and c-Jun NH_2-terminal kinase (JNK) (Borsch-Haubold et al., 1995; Bugaud et al., 1999; Kau et al., 2005; Nadal et al., 1997). It is well documented that collagen induces ERK and p38 MAPK activation (Borsch-Haubold et al., 1995; Mazharian et al., 2005; Roger et al., 2004). As shown in Fig. 3A, davallialactone inhibited the phosphorylation of ERK2 and p38 MAPK in collagen-stimulated platelets, without altering their total levels, assessed by total ERK2 and p38 MAPK antibodies. In addition, we carried out immunoblotting to determine the phosphorylation of ERK2 and p38 MAPKs in thrombin-activated platelets (Fig. 3B). As expected, davallialactone dose-dependently abrogated the phosphorylation of ERK2 and p38 MAPK proteins that was stimulated by 0.1U/ml thrombin (Fig. 3B).

3.4. The effect of davallialactone on tyrosine phosphorylation

In order to elucidate the involvement of upstream signaling components of MAPKs, we set out to find the activation of phosphotyrosine (with immunoblotting) by using a polyclonal antibody specific for tyrosine phosphorylation. Fig. 4 displays the phosphorylation of 60 and 85 kDa proteins that was induced by collagen (2.5 μg/ml). The phosphorylation of the 60 and 85 kDa proteins was differentially inhibited by davallialactone (54 and 108 μM). The exact target protein for which davallialactone effectively suppressed collagen-induced tyrosine phosphorylation has not been elucidated yet.

4. Discussion

The present study provides evidence regarding the novel inhibitory effect of davallialactone isolated from the mushroom *I.*

xeranticus on platelet function. Davallialactone displayed a broad-spectrum inhibitory activity on platelet aggregation, independent of ligands such as collagen, thrombin, and ADP that act on different types of platelet receptors. These results suggest that davallialactone did not block ligand binding on the plasma membrane, but that it did cause the modification of signaling downstream of the membrane receptor. In other words, its effects were explained by the inhibition of cytoplasmic calcium mobilization and by the inhibition of MAPK protein phosphorylation, such as that of ERK2 or p38 MAPK.

$[Ca^{2+}]_i$ is an important factor that is involved in platelet aggregation and is induced by the exogenous or endogenous activation of a membrane receptor with thrombin, collagen, or ADP (Holmsen, 1994; Rink and Sage, 1990). An increase of $[Ca^{2+}]_i$ comes from two sources: internal stores (i.e., dense granules) and Ca^{2+} entry through plasma membrane channels (Holmsen, 1994). The activation of Gq protein-coupled receptors, such as thrombin receptors PAR1 and PAR3 or a P2Y ADP receptor, leads to the activation of phospholipase (PLC)-β. In addition, collagen binds to glycoprotein VI on the platelet surface and increases PLC activity through the phosphorylation of PLC_γ-2 on a tyrosine residue. Both types of receptors, therefore, stimulate inositol trisphosphate (IP3) release from phosphatidylinositol bisphosphate (PIP2). IP3 binds to type 2 IP3 receptors in the secretory granules of human platelets, resulting in calcium mobilization (Daniel et al., 1986; Rosado and Sage, 2002; Smith et al., 1992). This up-regulation of intracellular calcium ions stimulates downstream target proteins, including calcium/calmodulin kinase, myosin light chain kinase and protein kinase C. At this point, we suggest that davallialactone's anti-platelet activity is due to the inhibition of calcium mobilization in the collagen-activated platelet.

MAPKs, including ERK, p38 MAPK and JNK, can be activated in platelets by a number of agonists such as collagen and thrombin (Bugaud et al., 1999; Garcia et al., 2007; Mazharian et al., 2005; Roger et al., 2004). Although the roles of ERK and p38 MAPK in platelet activation are controversial (Kuliopulos et al., 2004; Li et al., 2006; McNicol and Jackson, 2003; Nadal et al., 1997; Shankar et al., 2006; Thisoda et al., 2006), evidence regarding the critical role of these MAPKs in platelet aggregation is accumulating. Although the specific roles of p38 MAPK in the activation of platelets and shape changes are unclear, agonists (e.g., collagen and thrombin) phosphorylated this kinase, and SB203580, a specific inhibitor of p38 MAPK, was shown to inhibit platelet activation that was induced by collagen and thrombin (Kramer et al., 1995; Mazharian et al., 2005; Saklatvala et al., 1996). It has been demonstrated that ERKs are activated after stimulation by collagen and thrombin (Borsch-Haubold et al., 1995; Mazharian et al., 2005), and ERK2 activation is involved in collagen-induced platelet aggregation and secretion (Roger et al., 2004). In human platelets, an ERK cascade is required in the activation of store-mediated Ca^{2+} entry (Rosado and Sage, 2001, 2002). Although agonists, including collagen and thrombin, induced calcium mobilization, it has been shown that the additional entry of Ca^{2+} is necessary in order to reach sufficiently high Ca^{2+} levels to allow platelet activation. Therefore, the inhibition of ERK2 and p38 MAPK phosphorylation by davallialactone seems to contribute, in part, to the anti-platelet activity of this compound in collagen-induced aggregation. Both ERK1 and ERK2 are present in platelets, and we found that ERK2 was activated upon stimulation by collagen and thrombin.

Collagen induced tyrosine phosphorylation in platelets via the integrin receptor α2β1 and glycoprotein VI, with different modes of action (Hers et al., 2000). In addition, the dramatic increase of tyrosine phosphorylation plays an important role in platelet activation that is induced by collagen, which seems to be the regulated step upstream of MAPKs. In this study, we found that davallialactone inhibited the tyrosine phosphorylation of the 60 and 85 kDa proteins that were activated by collagen. In platelets, a wide variety of phosphotyrosines are phosphorylated by agonists: receptor tyrosine phosphorylation and non-receptor tyrosine phosphorylation (Hers et al., 2000; Jackson et al., 2004; Rosado and Sage, 2002; Stojanovic et al., 2006). We assume that Akt, $pp60^{src}$ and p85 phosphatidylinositol-3-kinase are candidates for being the 60 and 85 kDa proteins. This is due to previous reports regarding their specific roles in collagen-induced platelet aggregation (Chen et al., 2004; Jackson et al., 2004; Stojanovic et al., 2006). Therefore, the identity of the davallialactone target will be further explored in the next experiments.

In addition, the mechanism of davallialactone's anti-platelet action could not rule out the following. Davallialactone's inhibitory activity, toward platelet aggregation, could be due to its anti-oxidative activitiy. It has been reported that platelet agonists (e.g., collagen, ADP, thrombin) activate NAD(P)H oxidase, which results in the intracellular production of reactive oxygen species (ROS), $\alpha_{IIb}\beta_3$ activation, and platelet activation (Begonja et al., 2005; Begonja et al., 2006; Raghavan et al., 2003). In addition, it has been demonstrated that hydrogen peroxide and peroxynitrite enhances Ca^{2+} mobilization and platelet aggregation (Loiko et al., 2003; Redondo et al., 2005). Considering that davallialactone has been reported as a potent anti-oxidatant, and that strong antioxidants (flavonoids and vitamin E) decreased platelet aggregation by inhibiting the intracellular production of hydrogen peroxide or peroxynitrite (Olas et al., 2004; Pignatelli et al., 2000; Pignatelli et al., 1999), therefore, modulation of cellular redox system by davallialactone may be regarded as one of potential anti-platelet aggregation mechanisms. However, in our system, potent antioxidant ascorbate did not inhibit agonist-induced platelet aggregation even at 300 μM (data not shown). In addition, davallialactone showed significant inhibitory effects on LPS-induced phosphorylation and kinase activity of Src kinase, implying that Src may be a potential pharmacological target of davallialactone in macrophage cell line (Cho & Rhee, submitted). PP2, a specific Src kinase inhibitor, potently inhibited agonist-induced platelet aggregation (data not shown). This possibly suggested that davallilactone's anti-platelet activity is mediated via modulating intracellular signaling pathway rather than via its simple scavenging effect.

In summary, we first report in this study that davallialactone potently suppressed rat platelet aggregation induced by collagen, thrombin and ADP. The inhibitory mechanism of davallialactone toward platelet aggregation seems to be the inhibition of $[Ca2+]_i$, tyrosine phosphorylation, and the ERK2 and p38 MAPK pathways. Therefore, our data suggest that this

compound may be considered a potent therapeutic agent against platelet aggregation-mediated diseases such as thrombosis and arteriosclerosis.

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References

Al-Fatimi, M., Wurster, M., Kreisel, H., Lindequist, U., 2005. Antimicrobial, cytotoxic and antioxidant activity of selected basidiomycetes from Yemen. Pharmazie 60, 776–780.

Awadh Ali, N.A., Mothana, R.A., Lesnau, A., Pilgrim, H., Lindequist, U., 2003. Antiviral activity of *Inonotus hispidus*. Fitoterapia 74, 483–485.

Begonja, A.J., Gambaryan, S., Geiger, J., Aktas, B., Pozgajova, M., Nieswandt, B., Walter, U., 2005. Platelet NAD(P)H-oxidase-generated ROS production regulates alphaIIbbeta3-integrin activation independent of the NO/cGMP pathway. Blood 106, 2757–2760.

Begonja, A.J., Teichmann, L., Geiger, J., Gambaryan, S., Walter, U., 2006. Platelet regulation by NO/cGMP signaling and NAD(P)H oxidase-generated ROS. Blood Cells Mol. Diseases 36, 166–170.

Borsch-Haubold, A.G., Kramer, R.M., Watson, S.P., 1995. Cytosolic phospholipase A2 is phosphorylated in collagen- and thrombin-stimulated human platelets independent of protein kinase C and mitogen-activated protein kinase. J. Biol. Chem. 270, 25885–25892.

Bugaud, F., Nadal-Wollbold, F., Levy-Toledano, S., Rosa, J.P., Bryckaert, M., 1999. Regulation of c-jun-NH2 terminal kinase and extracellular-signal regulated kinase in human platelets. Blood 94, 3800–3805.

Burczyk, J., Gawron, A., Slotwinska, M., Smietana, B., Terminska, K., 1996. Antimitotic activity of aqueous extracts of *Inonotus obliquus*. Boll. Chim. Farm. 135, 306–309.

Chen, J., De, S., Damron, D.S., Chen, W.S., Hay, N., Byzova, T.V., 2004. Impaired platelet responses to thrombin and collagen in AKT-1-deficient mice. Blood 104, 1703–1710.

Cho, H.J., Cho, J.Y., Rhee, M.H., Lim, C.R., Park, H.J., 2006. Cordycepin (3′-deoxyadenosine) inhibits human platelet aggregation induced by U46619, a TXA2 analogue. J. Pharm. Pharmacol. 58, 1677–1682.

Daniel, J.L., Dangelmaier, C.A., Selak, M., Smith, J.B., 1986. ADP stimulates IP3 formation in human platelets. FEBS Lett. 206, 299–303.

Garcia, A., Shankar, H., Murugappan, S., Kim, S., Kunapuli, S.P., 2007. Regulation and functional consequences of ADP receptor-mediated ERK2 activation in platelets. Biochem. J. 404, 299–308.

Grenache, D.G., Coleman, T., Semenkovich, C.F., Santoro, S.A., Zutter, M.M., 2003. Alpha2beta1 integrin and development of atherosclerosis in a mouse model: assessment of risk. Arterioscler. Thromb. Vasc. Biol. 23, 2104–2109.

Hers, I., Berlanga, O., Tiekstra, M.J., Kamiguti, A.S., Theakston, R.D., Watson, S.P., 2000. Evidence against a direct role of the integrin alpha2beta1 in collagen-induced tyrosine phosphorylation in human platelets. Eur. J. Biochem. 267, 2088–2097.

Holmsen, H., 1994. Significance of testing platelet functions in vitro. Eur J Clin Invest. 24, 3–8.

Huo, Y., Ley, K.F., 2004. Role of platelets in the development of atherosclerosis. Trends Cardiovasc. Med. 14, 18–22.

Jackson, S.P., Yap, C.L., Anderson, K.E., 2004. Phosphoinositide 3-kinases and the regulation of platelet function. Biochem. Soc. Trans. 32, 387–392.

Jarosz, A., Skorska, M., Rzymowska, J., Kochmanska-Rdest, J., Malarczyk, E., 1990. Effect of the extracts from fungus *Inonotus obliquus* on catalase level in HeLa and nocardia cells. Acta. Biochim. Pol. 37, 149–151.

Kau, J.H., Sun, D.S., Tsai, W.J., Shyu, H.F., Huang, H.H., Lin, H.C., Chang, H.H., 2005. Antiplatelet activities of anthrax lethal toxin are associated with suppressed p42/44 and p38 mitogen-activated protein kinase pathways in the platelets. J. Infect. Dis. 192, 1465–1474.

Kim, S.D., Park, S.K., Cho, J.Y., Park, H.J., Lim, J.H., I., Y.H., Park, S.C., Lee, K.Y., Kim, S.K., Rhee, M.H., 2006a. Surfactin C inhibits platelet aggregation. J. Pharmac. Pharmacol. 58, 867–870.

Kim, Y.O., Park, H.W., Kim, J.H., Lee, J.Y., Moon, S.H., Shin, C.S., 2006b. Anti-cancer effect and structural characterization of endo-polysaccharide from cultivated mycelia of *Inonotus obliquus*. Life Sci. 79, 72–80.

Kramer, R.M., Roberts, E.F., Strifler, B.A., Johnstone, E.M., 1995. Thrombin induces activation of p38 MAP kinase in human platelets. J Biol Chem. 270, 27395–27398.

Kuliopulos, A., Mohanlal, R., Covic, L., 2004. Effect of selective inhibition of the p38 MAP kinase pathway on platelet aggregation. Thromb. Haemost. 92, 1387–1393.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Leclerc, J.R., 2002. Platelet glycoprotein IIb/IIIa antagonists: lessons learned from clinical trials and future directions. Crit. Care Med. 30, S332–S340.

Lee, I.K., Seok, S.J., Kim, W.K., Yun, B.S., 2006. Hispidin derivatives from the mushroom *Inonotus xeranticus* and their antioxidant activity. J. Nat. Prod. 69, 299–301.

Lee, I.K., Yun, B.S., 2006. Hispidin analogs from the mushroom *Inonotus xeranticus* and their free radical scavenging activity. Bioorg. Med. Chem. Lett. 16, 2376–2379.

Li, Z., Zhang, G., Feil, R., Han, J., Du, X., 2006. Sequential activation of p38 and ERK pathways by cGMP-dependent protein kinase leading to activation of the platelet integrin alphaIIb beta3. Blood 107, 965–972.

Loiko, E.N., Samal, A.B., Shulyakovskaya, S.M., 2003. H2O2-induced platelet aggregation and increase in intracellular Ca2+ concentration are blocked by inhibitors of intracellular signaling. Biochemistry (Mosc.) 68, 1210–1216.

Lull, C., Wichers, H.J., Savelkoul, H.F., 2005. Antiinflammatory and immunomodulating properties of fungal metabolites. Mediat. Inflamm. 2005, 63–80.

Mazharian, A., Roger, S., Maurice, P., Berrou, E., Popoff, M.R., Hoylaerts, M.F., Fauvel-Lafeve, F., Bonnefoy, A., Bryckaert, M., 2005. Differential involvement of ERK2 and p38 in platelet adhesion to collagen. J. Biol. Chem. 280, 26002–26010.

McNicol, A., Jackson, E.C., 2003. Inhibition of the MEK/ERK pathway has no effect on agonist-induced aggregation of human platelets. Biochem. Pharmacol. 65, 1243–1250.

Nadal, F., Levy-Toledano, S., Grelac, F., Caen, J.P., Rosa, J.P., Bryckaert, M., 1997. Negative regulation of mitogen-activated protein kinase activation by integrin alphaIIbbeta3 in platelets. J. Biol. Chem. 272, 22381–22384.

Olas, B., Nowak, P., Kolodziejczyk, J., Wachowicz, B., 2004. The effects of antioxidants on peroxynitrite-induced changes in platelet proteins. Thromb. Res. 113, 399–406.

Park, Y.M., Won, J.H., Kim, Y.H., Choi, J.W., Park, H.J., Lee, K.T., 2005. In vivo and in vitro anti-inflammatory and anti-nociceptive effects of the methanol extract of *Inonotus obliquus*. J. Ethnopharmacol. 101, 120–128.

Pignatelli, P., Pulcinelli, F.M., Celestini, A., Lenti, L., Ghiselli, A., Gazzaniga, P.P., Violi, F., 2000. The flavonoids quercetin and catechin synergistically inhibit platelet function by antagonizing the intracellular production of hydrogen peroxide. Am. J. Clin. Nutr. 72, 1150–1155.

Pignatelli, P., Pulcinelli, F.M., Lenti, L., Gazzaniga, P.P., Violi, F., 1999. Vitamin E inhibits collagen-induced platelet activation by blunting hydrogen peroxide. Arterioscler. Thromb. Vasc. Biol. 19, 2542–2547.

Raghavan, S.A., Sharma, P., Dikshit, M., 2003. Role of ascorbic acid in the modulation of inhibition of platelet aggregation by polymorphonuclear leukocytes. Thromb. Res. 110, 117–126.

Redondo, P.C., Jardin, I., Hernandez-Cruz, J.M., Pariente, J.A., Salido, G.M., Rosado, J.A., 2005. Hydrogen peroxide and peroxynitrite enhance Ca2+ mobilization and aggregation in platelets from type 2 diabetic patients. Biochem. Biophys. Res. Commun. 333, 794–802.

Rink, T.J., Sage, S.O., 1990. Calcium signaling in human platelets. Annu. Rev. Physiol. 52, 431–449.

Roger, S., Pawlowski, M., Habib, A., Jandrot-Perrus, M., Rosa, J.P., Bryckaert, M., 2004. Costimulation of the Gi-coupled ADP receptor and the Gq-coupled TXA2 receptor is required for ERK2 activation in collagen-induced platelet aggregation. FEBS Lett. 556, 227–235.

Rosado, J.A., Sage, S.O., 2001. Role of the ERK pathway in the activation of store-mediated calcium entry in human platelets. J. Biol. Chem. 276, 15659–15665.

Rosado, J.A., Sage, S.O., 2002. The ERK cascade, a new pathway involved in the activation of store-mediated calcium entry in human platelets. Trends Cardiovasc. Med. 12, 229–234.

Saklatvala, J., Rawlinson, L., Waller, R.J., Sarsfield, S., Lee, J.C., Morton, L.F., Barnes, M.J., Farndale, R.W., 1996. Role for p38 mitogen-activated protein kinase in platelet aggregation caused by collagen or a thromboxane analogue. J. Biol. Chem. 271, 6586–6589.

Savage, B., Cattaneo, M., Ruggeri, Z.M., 2001. Mechanisms of platelet aggregation. Curr. Opin. Hematol. 8, 270–276.

Schaeffer, J., Blaustein, M.P., 1989. Platelet free calcium concentrations measured with fura-2 are influenced by the transmembrane sodium gradient. Cell Calcium 10, 101–113.

Seo, S.Y., Sharma, V.K., Sharma, N., 2003. Mushroom tyrosinase: recent prospects. J. Agric. Food Chem. 51, 2837–2853.

Shankar, H., Garcia, A., Prabhakar, J., Kim, S., Kunapuli, S.P., 2006. P2Y12 receptor-mediated potentiation of thrombin-induced thromboxane A2 generation in platelets occurs through regulation of Erk1/2 activation. J. Thromb. Haemost. 4, 638–647.

Shattil, S.J., Kashiwagi, H., Pampori, N., 1998. Integrin signaling: the platelet paradigm. Blood 91, 2645–2657.

Smith, J.B., Selak, M.A., Dangelmaier, C., Daniel, J.L., 1992. Cytosolic calcium as a second messenger for collagen-induced platelet responses. Biochem. J. 288, 925–929.

Stojanovic, A., Marjanovic, J.A., Brovkovych, V.M., Peng, X., Hay, N., Skidgel, R.A., Du, X., 2006. A phosphoinositide 3-kinase-AKT-nitric oxide-cGMP signaling pathway in stimulating platelet secretion and aggregation. J. Biol. Chem. 281, 16333–16339.

Stouffer, G.A., Smyth, S.S., 2003. Effects of thrombin on interactions between beta3-integrins and extracellular matrix in platelets and vascular cells. Arterioscler. Thromb. Vasc. Biol. 23, 1971–1978.

Sullivan, R., Smith, J.E., Rowan, N.J., 2006. Medicinal mushrooms and cancer therapy: translating a traditional practice into Western medicine. Perspect. Biol. Med. 49, 159–170.

Thisoda, P., Rangkadilok, N., Pholphana, N., Worasuttayangkurn, L., Ruchirawat, S., Satayavivad, J., 2006. Inhibitory effect of *Andrographis paniculata* extract and its active diterpenoids on platelet aggregation. Eur. J. Pharmacol. 553, 39–45.

Wasser, S.P., Weis, A.L., 1999. Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: a modern perspective. Crit. Rev. Immunol. 19, 65–96.