The inhibitory effect of \textit{Opuntia humifusa} Raf. ethyl acetate extract on platelet aggregation

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This study was designed to investigate the activity of ethyl acetate extract from \textit{Opuntia humifusa} Raf. (OH-EAE) in ligand-activated platelet aggregation. Platelet aggregation was induced either by ADP, a potent agonist to platelet G protein-coupled P2Y receptor, by collagen, a potent ligand that activates platelet integrin \(\alpha_2\beta_1\) and glycoprotein VI, or thrombin, a platelet protease-activated receptors subtype I and IV. The OH-EAE inhibited platelet aggregation induced by ADP (10 \(\mu\)M) in a dose dependent manner. In addition, OH-EAE significantly and dose-dependently inhibited collagen (2.5 \(\mu\)g/ml)- and thrombin (0.05 U/ml)-induced platelet aggregation. Moreover, the downstream signaling analysis revealed that the extract potently inhibited ADP-induced intracellular calcium mobilization ([Ca\(^{2+}\)]\textsubscript{i}). Since degranulation is a marker of platelet activation, the extract effect on the dense granule secretary activity was evaluated. As such, OH-EAE strongly suppressed ADP-induced ATP release. This preliminary result suggests that \textit{O. humifusa} may be taken as a candidate lead natural compound to be considered in the search for natural products with beneficial effects on aberrant platelet activation mediated cardiovascular disorders.

Key words: \textit{Opuntia humifusa} extract, calcium, rat platelets, platelet agonists.

INTRODUCTION

It is well known that platelets play an important role in the physiology of primary hemostasis and in pathophysiological processes 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 on plasma membrane, bioactive substances (e.g. calcium, growth factor, and aggregation-related secretory biomolecules in granules are released in an energy-dependent process that requires ionized calcium (Leclerc, 2002; Savage et al., 2001). The signaling of these endogenous and exogenous molecules is the main factor of platelet aggregation (Huo and Ley, 2004). \textit{In vitro} platelet aggregation can be induced by platelet-activating ligands, such as ADP, collagen, and thrombin. ADP can mainly bind to G protein-coupled P2Y1 receptor and activates phospholipase C, and thus resulting in the elevation of intracellular calcium concentration [Ca\(^{2+}\)]. Collagen, the subendothelial matrix, can bind to integrin \(\alpha_2\beta_1\) and glycoprotein VI (GP VI). GP VI and \(\alpha_2\beta_1\) binding to collagen lead to an increase in cytosolic calcium levels and protein kinase C activation. Thrombin, a serine protease, also takes a critical role in both coagulation cascade and platelet activation. The activation of platelet by thrombin is mediated through two protease-activating receptors (PAR), PAR 1 and PAR 4, belonging to G protein-coupled receptors. Both PAR1 and PAR4 couple...
to phospholipase Cβ (PLCβ) via Gq in human platelets. Upon activation, PLCβ hydrolyses phosphatidylinositol 4, 5-bisphosphate to inositol-3-phosphate, which contributes to calcium release from internal stores, and diacylglycerol (DAG), which activates protein kinase C (PKC). PAR1 and PAR4 also couple to G12/13 to activate Rho/Rho kinase (Woulfe, 2005). The activation of downstream signaling by these ligands leads to shape change and granule secretion. Thus, the activation of integrin αIIbβ3 results in complete platelet aggregation.

On the other hand, in vivo platelet aggregation can be a cause of serious cardiovascular diseases, including atherosclerosis, ischemia, thrombosis, infarction, stroke (Huo and Ley, 2004). On the other hand, the search for natural products with the potential of having anti-platelet activity while reducing adverse side effects is currently the main target of research in the field of food and medicine science.

Opuntia humifusa Raf. (O. humifusa Raf.) is a member of the Cactaceae family, and is widely distributed in semiarid countries throughout the world, especially in the Mediterranean and Central America (Acuna et al., 2002; Goldstein and Nobel, 1994; Lee et al., 2002). In Korea, O. humifusa Raf. has been cultivated for a long time. Concerning the pharmacological profile of Opuntia spp., the total phenols in an ethanolic extract from South Korea’s O. ficus-indica var. saboten has been responsible for the radical scavenging activity toward superoxide and hydroxyl anions (Hyang et al., 2003; Lee et al., 2002). In addition, an ethanol extract of O. ficus-indica var. saboten was reported to have analgesic effects in a writhing test which were induced by acetic acid and anti-inflammatory effect against gastric lesions (Park et al., 1998).

Whereas O. ficus-indica var. saboten and other Opuntia spp. have been extensively studied for their biological effects, such as therapeutic properties against arthritis and cancer (Butera et al., 2002; Hyang et al., 2003; Galati et al., 2002; Park and Chun, 2001), little is known about the pharmacological effect of O. humifusa Raf. In our previous studies, we reported that solvent-extracted fractions have anti-oxidative and anti-inflammatory properties (Cho et al., 2006). Since it is known that the natural products with potent anti-oxidative characteristics can be potential sources of anti-platelet agents (Hung et al., 2005; Kang et al., 2001; Lee et al., 2005; Olas et al., 2005; Son et al., 2004), we examined whether the ethyl acetate extract of (OH-EAE) modulates agonist-induced platelet aggregation. We here report a preliminary in vitro result on anti-platelet activity of OH-EAE in agonist-induced rat platelets activation.

MATERIALS AND METHODS

Materials

ADP, thrombin and fura-2/AM were obtained from the Sigma Co (St. Louis, MO, USA). Collagen was procured from the Chronolog Co (Havertown, PA, USA). All other chemicals were of reagent grade.

Preparation of the extract

O. humifusa Raf. was collected in October 2005 from the province of Asan (Korea). The voucher specimen (PLOH-1001) is deposited in the herbarium of Kyungpook National University, Laboratory of Physiology and cell signaling. The preparation of ethyl acetate extract was described previously (Cho et al., 2006). The crude extracts were stored in −20°C until use.

Animals

Male Sprague-Dawely rats weighing from 240 to 250 g were obtained from Orient Co. (Seoul, Korea) and maintained in a standard laboratory animal facility with free access to feed, water and acclimated for at least two weeks before use. The experiments were carried out in accordance with internationally accepted guidelines on the use of laboratory animals and the protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Kyungpook National University.

Platelet preparation and aggregation assay

The preparation of the platelets has been described previously (Kim et al., 2006). Rat blood (8 ml) was collected from abdominal aorta via the venipuncture, using a 23-g needle, and transferred to a 15 ml test tube containing 1 ml of a citrate phosphate dextrose solution (CPD; 90 mM of Na3C6H5O7·2H2O, 14 mM of C6H8O7·H2O, 128.7 mM of NaH2PO4·H2O, 2.55 g/100 ml dextrose). Blood was centrifuged at 1,000 rpm for 7 min in order to achieve platelet-rich plasma. In order to remove residual erythrocytes, the PRP samples were again centrifuged at 500 rpm for 7 min. Again, to remove the CPD solution, PRP was centrifuged twelve at 2,500 rpm for 10 min and the supernatant was allocated to platelet-poor plasma (PPP), which is used as a reference solution for aggregation assay. The platelets of the precipitance were adjusted to the proper number concentration of the vehicle was kept at less than 0.5% so as to exclude the artificial effect.

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Determining the [Ca2+]

The [Ca2+] was determined with fura-2/AM as described previously (Kamruzzaman et al., 2010). 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 (105/ml) were then pre-incubated with OH-EAE for 2 min at 37°C in the presence of 1 mM CaCl2. Next, the platelets were stimulated with ADP for 3 min. Fura-2 fluorescence was measured in a spectrofluorometer (F-2500, Hitachi, Japan) with an excitation wavelength that ranges from 340 to 380 nm, changing every 0.5 s, and with the emission wavelength of 510 nm. The [Ca2+] was calculated by the method of (Schaeffer and Blaustein, 1989): [Ca2+] = 224 nM × (F−Fₘᵢₙ)/(Fₘₐₓ−Fₘᵢₙ), where 224 nM is the dissociation constant of the fura-2-Ca²⁺ complex, and
**RESULTS**

**The effects of *O. humifusa* Raf. on ADP-induced platelet aggregation**

Since we have previously reported that the ethyl acetate extracts of *O. humifusa* Raf. displayed potent antioxidative and anti-inflammatory properties (Cho et al., 2006), we intended to determine whether this extract affects platelet function. Washed rat platelets were pre-incubated with OH-EAE and then, exposed to ADP (10 µM) in order to examine the inhibitory effect of *O. humifusa* Raf. on rat platelet aggregation. Under ADP treatment, we also obtained an appropriate amount of platelet aggregation up to 98.0 ± 4.5%, which was effectively inhibited by U73122 (10 µM), a specific phospholipase C inhibitor (data not shown). As shown in Figure 1, the extract inhibited the platelet aggregation induced by ADP in a concentration-dependent manner. Its maximal inhibition rate at the dose of 200 µg/ml was 85.2%.

**Effects of *O. humifusa* Raf. on collagen-induced platelet aggregation**

We next determined whether the OH-EAE affects the collagen (2.5 µg/ml)-induced rat platelet aggregation. As shown in (Figure 2), the ethyl acetate extracts inhibited platelet aggregation in a dose-dependent manner with IC$_{50}$ values of 75.3 ± 4.4 µg/ml.

**Effects of *O. humifusa* Raf. on thrombin-induced platelet aggregation**

To further investigate the anti-aggregatory activity of the extract on protease activated receptor-meditated platelet activation, we examined the response of platelets to the extract pretreatment in thrombin-stimulated platelets. OH-EAE dose dependently attenuated thrombin-induced...
platelet aggregation (Figure 3).

Effects of *O. humifusa* Raf. on cytosolic Ca\(^{2+}\) levels

Free cytosolic Ca\(^{2+}\) concentrations in rat platelets were measured by the fura-2/AM loading method. As shown in (Figure 4), ADP (10 \(\mu\)M) evoked a marked increase in [Ca\(^{2+}\)], and this increase was markedly inhibited in the presence of the extract. At maximal dose of the extract (100 \(\mu\)g/ml), cytosolic calcium levels reversed to the basal levels.

The effects of *O. humifusa* Raf. on ATP granule secretion

Similar to the extent of platelet aggregation, ADP provoked the release of endogenous ATP, from platelet dense granules. The amount of ATP released from platelets, determined at 5 min after application of ADP, was increased by four folds from resting state. As shown in (Figure 5), the ethyl acetate extracts of the plant significantly suppressed the ADP-induced ATP release, which was comparable to the treatment of U73122 (4 \(\mu\)M).

DISCUSSION

Natural products of plant origin with anti-platelet activity can be important sources of lead compounds and novel therapeutics. Because of their relative effectiveness, limited side effects, and low cost, natural products are widely prescribed even when their biologically active compounds are unknown. The active constituent(s) of some herbs with nutritional and medicinal values are still unknown. Further studies should be carried out to elucidate these compounds and determine their pharmacological activities as they may represent major but yet largely unknown source of new pharmaceutical products.

Feugang et al. reviewed that cactus pear (*Opuntia* spp.) have not only been used as a source of food but also as medicinal resource (Feugang et al., 2006). The medicinal properties of cactus have been confirmed through an intensive research conducted worldwide. The extractable ingredients of cactus were incorporated in products and were claimed to improve biological function of human body (Feugang et al., 2006). Previous studies suggested that flavonoids such as quercetin, (+)-dihydroquercetin isolated from *Opuntia ficus-indica* var. *saboten* possess neuroprotective effects (Hyang et al., 2003), and attenuates neuronal injury in *in vitro* and *in vivo* models of cerebral ischemia (Kim et al., 2006a). In addition, antioxidant activities of *Opuntia ficus indica* fruit extracts (Butera et al., 2002) and radical scavenging effect of flavonol glycosides from *Opuntia dillenii* have been reported. Moreover, Sreekanth et al. reported apoptotic effects of betanin purified from fruits of *Opuntia ficus-indica* in human chronic myeloid leukemia cell line-K562 (Sreekanth et al., 2007). As a result, studies on medicinal *Opuntia* spp. have attracted special attention in recent years due to their potential biological and pharmacological activities including neuroprotective, antitumor and immunomodulating, and antioxidant effects (Feugang et al., 2006). However, anti-platelet activity of *Opuntia* spp is not reported yet.

We previously reported that *O. humifusa* Raf extract exhibited anti-oxidant, free radical scavenging and...
anti-inflammatory activities (Cho et al., 2006). However, information on the effect of this plant on platelet function is limited.

In this study, we report the preliminary in vitro findings on anti-platelet activity of *O. humifusa* ethyl acetate extract in agonist-induced platelet activation. In the present study, the decrease in agonist-induced platelet aggregations by OH-EAE suggests that the extract influenced the signaling pathways triggered by both agonists. Therefore, the OH-EAE mediated inhibition of platelet aggregation induced by various agonists in the present study suggests that the extract effect is
downstream of the agonist-receptor interactions. In addition, the extract inhibited [Ca\(^{2+}\)], suggesting that it may involve inhibition of cytoplasmic calcium increase. This conclusion is supported by the fact that calcium plays a central role for granule secretions such as ATP release, which is inhibited by the extract in this study. More importantly, platelet α\textsubscript{IIb}β\textsubscript{3} is activated downstream of adhesion receptors GPVI and GPlb-IX–V, or G-protein-coupled receptors, for example, thrombin (PAR-1 or PAR-4) or ADP receptors (P\textsubscript{2}Y\textsubscript{1} or P\textsubscript{2}Y\textsubscript{12}) that reinforce α\textsubscript{IIb}β\textsubscript{3}-dependent platelet aggregation and inside–out activation. The integrin α\textsubscript{IIb}β\textsubscript{3} activation is Ca\(^{2+}\)-dependent and involves changes in the conformation of both the ligand-binding extracellular region and the cytoplasmic tails (Xiong et al., 2003). Following ligand binding, outside–in signals and altered interactions with cytoskeletal proteins, such tyrosine kinases (Calderwood et al., 2002), control post-adhesion events, such as spreading and contraction in platelet aggregation.

**Conclusion**

In *in vitro* assays using freshly isolated rat platelets, the OH-EAE showed significant inhibition of collagen, thrombin and ADP-induced platelet aggregation, ATP secretion and [Ca\(^{2+}\)] mobilization. The main findings of this study suggest that the inhibitory effects of OH-EAE in platelet aggregation possibly involve [Ca\(^{2+}\)], inhibition in activated platelets. Thus, these results suggest that anti-platelet activity of this plant extract may be considered as a lead source candidate in the search of anti-platelet agents. Further study using isolated compound(s) of the extract together with the possible mechanisms of action is required.

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**REFERENCES**


