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Inhibitory effects of black soybean on platelet activation mediated through its active component of adenosine

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ABSTRACT

Owing to the beneficial health effects on human cardiovascular system, soybeans and soy-related products have been a focus of intensive research. Soy isoflavones are known to be primarily responsible for the soy-related biological effects including anti-platelet activity but its *in vivo* relevancy has not been fully verified. Here we compared the role of adenosine, an active ingredient abundant in black soybean (BB) extract, in the anti-platelet effects of BB, to that of soy isoflavones. At the concentrations existing in BB, isoflavones such as genistein and daidzein could not attenuate collagen-induced platelet aggregation, however, adenosine significantly inhibited platelet aggregation with an equivalent potency to BB, suggesting that adenosine may be the major bioactive component. Consistently, the anti-aggregatory effects of BB disappeared after treatment of adenosine receptor antagonists. The effects of BB are mediated by adenosine through intracellular cAMP and subsequent attenuation of calcium mobilization. Of note, adenosine and BB significantly reduced platelet fibrinogen binding and platelet adhesion, other critical events for platelet activation, which were not affected by isoflavones. Taken together, we demonstrated that adenosine might be the major active ingredient for BB-induced anti-platelet activity, which will shed new light on the roles of adenosine as a bioactive compound in soybeans and soy-related food.

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Introduction

A link between the consumption of soy-related products and low incidence of chronic diseases has long been recognized and supported by several clinical and epidemiological studies [1–4]. To identify the active ingredients for the health benefits of soy products, many efforts have been made both in pharmacological and nutritional field. Various bioactive ingredients, such as isoflavones, soy proteins and soy saponins have been identified in soybeans and suggested to be responsible for the biological activities of soybeans [2,3,5]. Especially, soy isoflavones including genistein and daidzein have been considered as the primary active components for anti-osteoporosis [6,7], anti-diabetes [8,9], anti-cancer effects [10,11] and cardiovascular benefits of soybean [4,12–14].

However, the authenticity of soy isoflavones as a true major active ingredient in soy or soy-related food is being questioned, especially for anti-platelet effects of soybeans. Increased systemic bioavailability of genistein and daidzein did not result in equally enhanced anti-platelet effects [15]. Anti-platelet activities of genistein and daidzein were observed at the concentration range of 10 to 30 μM [16–19], which has a large margin from their actual contents in soybeans. In addition, other active components in soybeans are newly being discovered to be effective in attenuating platelet activation, including anthocyanin or soy saponin [20–23]. Considering the diversity and heterogeneity of soybeans, there is a huge probability that components other than isoflavones may contribute to the anti-platelet effects of soy foods [3,5].

Recently, we demonstrated that black soybean (Glycine max; BB), which has shown superior biological activities to yellow or green soybeans such as anti-oxidant, anti-cancer, and anti-inflammatory effects [24–26], has potent inhibitory effect on platelet aggregation both in vitro human platelets and *in vivo* rat thrombosis models [27]. The active principle of anti-aggregating effect of BB was suggested to be adenosine through activity-guided fractionation and NMR/ESI-MS

Abbreviations: AC, adenylyl cyclase; ACD, acid-citrate-dextrose; anti-GP Ib PE Ab, phycoerythrin-labeled monoclonal antibody against human glycoprotein Ib; BB, black soybean; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; PAC-1 FITC, fluorescein isocyanate labeled PAC-1; PBS, phosphate-buffered saline; PGE_1, prostaglandin E_1; PRP, platelet rich plasma; WP, washed platelets; YB, yellow soybean.

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analyses. However, the contribution of adenosine to the inhibitory effect of BB on platelet activation and the exact mechanism underlying has not been elucidated in details.

Adenosine is an endogenous molecule accumulating in the extracellular space in response to cell damage or metabolic stress [28]. Generally, adenosine plays protective roles including anti-inflammatory [29], anti-ischemic [30], and neuroprotective effects [31]. In cardiovascular system, adenosine increases vascular reactivity [32], promotes angiogenesis [33], decreases vascular adhesion [34], suppresses endothelial tissue factor expression [35] and inhibits platelet aggregation [36,37]. Despite the diverse biological activities of adenosine, the studies regarding the roles of food-based adenosine as an active ingredient in foods or dietary supplements are limited. To our best knowledge, the role of adenosine has not been addressed in the health effects from soybean or soy-related food.

In this study, we compared the role of soy isoflavones and adenosine in the anti-platelet effects of BB. In addition, the effects of BB and adenosine on intra-platelet signaling pathways and other aspects of platelet activation were examined to provide a detailed mechanistic explanation of anti-platelet effect of BB.

Materials and methods

Materials

Trisodium citrate, citric acid, dimethyl sulfoxide (DMSO), ethanol, adenosine, genistein, daidzein, 2′,5′-dideoxyadenosine (DDA), SQ22538, urethane, ferric chloride, HEPES, glucose, NaCl, KCl, $MgCl_2$, $NaHCO_3$, Na_2HPO_4, $CaCl_2$, KH_2PO_4, Tris–HCl, EDTA, triton X-100, prostaglandin E_1 (PGE_1) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). SCH 58261 and 8-(3-Chlorostyryl) caffeine (CSC) were purchased from Tocris Biosciences (Bristol, UK). Collagen was from Chrono-log Co. (Harvertown, PA). Phycoerythrin-labeled monoclonal antibody against human CD42b (anti-CD42b-PE) and fluorescein isocyanate labeled PAC-1 (PAC-1 FITC) was from BD bioscience (San Diego, CA). Fluo-4 AM, calcein-AM and Alexa Fluor 488 conjugated fibrinogen was from Invitrogen (Eugene, OR) and cAMP ELISA kit was from Cayman Chemical Co. (Ann Arbor, MI). Protease inhibitor cocktail was from Calbiochem (San Diego, CA).

Soybean extraction

Black soybean (black seed coat and a green cotyledon) and yellow soybean (yellow seed coat and a yellow cotyledon) were from Boeun and Goesan, Korea. Dry matured soybeans were extracted for 3–5 hr at 50-60 °C with 20% ethanol. The extraction was repeated three times, and left for 12 hr at room temperature. After filtration and concentration under reduced pressure, the extract was lyophilized. The final yield of black soybean and yellow soybean extracts were 7.6% and 8.7%, respectively and the resultant powder was stored at −20 °C. The black soybean extract (BB) consisted of protein (1.0%), lipid (1.2%), carbohydrate (86.8%), ash (10.2%) and water (0.8%), and the content of total phenolic compounds was 1.29%. For *in vitro* experiments soybean extracts were dissolved in water.

Preparation of human platelets

Human blood was collected from healthy male volunteers (18–25 years old) who had not taken any drugs for at least 14 days with an approval from the Ethics Committee of Health Service Center at Seoul National University. Blood was anti-coagulated with acid-citrate-dextrose (ACD; 85 mM trisodium citrate, 71 mM citric acid, 111 mM glucose) for preparation of washed platelets (WP). All procedures were conducted at room temperature and the use of glass containers and pipettes was avoided. Platelet rich plasma was prepared by centrifugation for 15 min at 150 *g*, and platelets were pelleted by centrifugation at 500 *g* for 10 min. Platelets were washed with Tyrode buffer (134 mM NaCl, 2.9 mM KCl, 1.0 mM $MgCl_2$, 10.0 mM HEPES, 5.0 mM glucose, 12.0 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 2 mM $CaCl_2$ and 0.3% BSA, pH 7.4) containing 1 μM PGE_1 and 10% ACD. After centrifugation at 500 *g* 10 min, platelets were resuspended with Tyrode buffer, and the cell number was adjusted to 3×10^8 cells/ml. The final $CaCl_2$ concentration was adjusted to 2 mM prior to use.

Platelet aggregation measurement

Platelet aggregation was determined by turbidometric method using an aggregometer (Chrono-log, Havertown, PA). After incubation with soybean extracts, adenosine, genistein or daidzein for 10 min at 37 °C, WP was loaded on the aggregometer and stimulated with collagen (1–4 μg/ml) for 6 min. The concentration of collagen selected for each individual platelet sample was the lowest showing maximum (>80%) aggregation. Platelet aggregation was measured by light transmission, with 100% calibrated as the absorbance of Tyrode buffer and 0% calibrated as the absorbance of WP.

Measurement of intracellular calcium levels

Intracellular calcium change was determined using fluo-4 AM with flow cytometry. Fluo-4 AM (5 μM) was loaded to platelets in the presence of PGE_1 (1 μM) for 45 min at 37 °C in dark. Then platelets were spun-down by centrifugation at 300 *g* for 10 min, and resuspended with Tyrode buffer. Dye loaded platelets were incubated with soybean extracts or adenosine for 10 min and stimulated with collagen (1–4 μg/ml) for 6 min. The reaction was terminated by dilution with Tyrode buffer. After incubated with anti-CD42b-PE for 20 min as platelet identifiers, platelets were analyzed on the FACSCalibur (BD Biosciences, San Jose, CA) equipped with argon lasor (λ_{ex} 488 nm). Data from 5,000 events were collected and analyzed using CellQuest Pro software (BD Biosciences).

Measurement of cAMP

Platelets were incubated with soybean extracts or adenosine for 10 min and stimulated with collagen (1–4 μg/ml) for 6 min. The reaction was terminated by adding HCl (final 0.1 M) and platelets were lysed by sonication for 60 s in ultrasonic processor (Sonics and Materials Inc., Newtown, CT). The cAMP level in each samples were measured using a commercial cAMP ELISA kit, following the procedure provided by the manufacturer.

Measurement of GP IIb/IIIa activation or fibrinogen binding

GP IIb/IIIa activation was determined with flow cytometry using PAC-1, a GP IIb/IIIa activation specific antibody. Platelets were incubated with soybean extracts, adenosine, genistein or daidzein for 10 min and stimulated with collagen (1–4 μg/ml) for 6 min. The reaction was terminated by dilution with Tyrode buffer. Platelets were incubated with anti-CD42b-PE for 20 min and analyzed on FACSCalibur as described above. To determine fibrinogen binding, Alexa Fluor 488 conjugated fibrinogen was used instead of PAC-1.

Measurement of platelet-collagen adhesion

For measurement of platelet adhesion, 96 well plates were coated with collagen (40 μg/ml) for 1 hr, blocked with phosphate-buffered saline (PBS; 1 mM KH_2PO_4, 154 mM NaCl, 3 mM Na_2HPO_4, pH 7.4) containing 5% BSA for 1 hr, and washed 3 times with PBS. Platelets were stained with calcein-AM (2.5 μM) in for 15 min at 37 °C in dark. Calcein-AM loaded platelets were incubated with soybean extracts, adenosine, genistein or daidzein for 10 min and 50 μl of platelets were placed onto collagen-coated well for 30 min at room temperature.

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