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Inhibitory Effect of Allyl Isothiocyanate on Platelet Aggregation

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Supporting Information

ABSTRACT: Allyl isothiocyanate (AITC) is one of the major components of mustard. The present study for the first time attempted to evaluate the effect of AITC on platelet aggregation. In the in vitro study using platelet-rich plasma (PRP) from rats and humans, AITC at concentrations of 100 and 300 μM significantly inhibited platelet aggregation induced by collagen, thrombin, ADP, and arachidonic acid. AITC also attenuated thromboxane A_2 production and ATP release in rat and human PRP. AITC elicited inhibitory effects on cellular Ca^{2+} increase and platelet shape change in rat PRP. AITC further showed inhibitory effects on the phosphorylation of PKCδ, p38, ERK, and Akt in rat PRP. In the rat ex vivo study, 1 and 3 mg/kg (po) of AITC showed significant inhibitory effect on platelet aggregation. Furthermore, AITC showed a protective effect in thromboembolism attack model in mouse. These results suggest that AITC has remarkable antiplatelet effects and maybe a therapeutic potential for the prevention of aberrant platelet activation-related disorders.

KEYWORDS: *allyl isothiocyanate, platelet, aggregation, thromboxane A_2, ATP*

INTRODUCTION

Platelets play an essential physiological role in hemostasis by permitting the formation of blood clots. However, the aberrant activation of platelets by pathological factors and subsequent thrombogenesis are commonly associated with atherosclerotic lesions. Indeed, thrombosis and embolization contribute to cardiovascular diseases such as ischemic heart disease and stroke.[1] Therefore, a suitable antiplatelet therapy is critical for preventing the progression of cardiovascular diseases.[2] A number of antiplatelet drugs, including clopidogrel, have been developed and are used clinically to prevent thrombosis. However, some of the chemically synthesized antiplatelet agents may be related to adverse effects, such as gastrointestinal bleeding and palpitation. Considering that plant constituents have a nature-friendly image and various safety merits, much effort recently has been devoted toward developing herbal medicines for the prevention of thrombosis and atherosclerotic diseases.[3,4]

Mustard (*Brassica* spp.), one of the first domesticated crops, is a widely consumed vegetable[5] and currently grown on millions of acres in the North America and India. Also, many people of East Asia use mustard oil as a cooking oil. Mustard and mustard oil have been reported to have physiological effects such as anti-inflammation[6] and antitumor.[7] Recently, it has been reported that polyunsaturated fatty acid-rich mustard oil had an antiplatelet effect in an ex vivo study.[8] However, there is little information about the antiplatelet effect of mustard oil in in vitro studies. In addition, its active constituent and inhibitory mechanism are still unknown. Allyl isothiocyanate (3-isothiocyanato-1-propene, AITC) is the chief constituent of mustard oil[9,10] and causes the pungent flavor. AITC has been used as a food additive for its flavor-enhancing, antibacterial, and antifungal effects.[11] In addition, studies using animal models have shown that AITC inhibits bladder cancer growth, attenuates levels of inflammatory markers such as interleukin-1β, and reduces blood glucose levels.[12−14] However, the antiplatelet effects of AITC have not been evaluated.

This study for the first time evaluated the effects of AITC on platelet aggregation and its mechanism of action, focusing on thromboxane A_2 (TXA_2) production and ATP release in platelets from rat and human blood. In addition, its ex vivo antiplatelet effect in rat and its in vivo protective effect against thromboembolism attack in mouse were also investigated.

MATERIALS AND METHODS

Reagents. Allyl isothiocyanate (AITC) at 95% purity, apyrase, acetylsalicylic acid (ASA), bovine serum albumin (BSA), Fura-2/AM, polyethylene glycol (PEG), β-nicotinamide adenine dinucleotide (reduced form, β-NADH), U46619, and pyruvic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Collagen, thrombin, adenosine diphosphate (ADP), arachidonic acid (AA), and luciferin−luciferase reagent were purchased from Chrono-Log Co. (Havertown, PA, USA). Thromboxane B_2 EIA kits were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Antibodies against PLCγ, phosphor-PLCγ, PKCδ, phospho-PKCδ, ERK, phospho-ERK, p38, phospho-p38, Akt, and phospho-Akt were purchased from Cell Signaling (Danvers, MA, USA).

Animals. Animals were purchased from Samtako Laboratory Animal Center (Suwon, Korea). Sprague−Dawley (SD) rats (8 weeks, 220−240 g) and ICR mice (8 weeks, 34−40 g) were used for experiments. They were housed in a conventional animal facility with free access to food and water in a temperature- and relative humidity-controlled environment under a 12/12 h lighting schedule. The animals were acclimatized for at least 7 days before experiments. Animal study protocols conformed to the guidelines in the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and

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were approved by the Committee on Animal Research at Ajou Medical Center, Ajou University.

Experimental Treatment. For in vitro experiments, AITC and ASA were dissolved in 70% PEG−saline. The final concentration of 70% PEG−saline never exceeded 0.1%. For ex vivo and in vivo experiments, AITC and ASA were dissolved in 70% PEG−saline. Seventy percent PEG−saline solution did not influence platelet aggregation. All agents were prepared just before use.

Preparation of Platelet-Rich Plasma (PRP) from Rat and Human Blood. PRP from rat blood was prepared as described previously.[15,16] Briefly, SD rats weighing 200−240 g were lightly anesthetized with diethyl ether. A volume of 8−10 mL of blood was collected from the abdominal aorta into a syringe containing 3.8% sodium citrate. The ratio of blood to 3.8% sodium citrate was adjusted to 1:9 v/v. After centrifugation at 150*g* for 10 min at room temperature, supernatants (PRP) were used for the aggregation study. To prepare washed platelets (WP), blood was collected into a syringe containing acid citrate dextrose (ACD; 2.5% trisodium citrate, 2% dextrose, and 1.5% citric acid) and adjusted to a ratio of 1:9 v/v. After centrifugation at 150*g* for 10 min at room temperature, the platelets were isolated and then centrifuged again at 150*g* for 10 min in washing buffer (Tyrode's solution containing 10% ACD buffer and 0.3% BSA). Platelets were diluted with Tyrode's solution (11.9 mM NaCl, 2.7 mM KCl, 2.1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 11.1 mM glucose, and 3.5 mg/mL bovine serum albumin, pH 7.2). To make platelet-poor plasma (PPP) from rat blood, PRP was centrifuged at 1200*g* for 10 min at room temperature, and supernatants were obtained as PPP. For PRP from human blood, human platelet suspensions were supplied from the Red Cross (Suwon, Korea). Human blood was collected from healthy human volunteers who had not taken medicine as indicated by the Red Cross guide.

In Vitro Platelet Aggregation and Shape Change in Rat PRP. The platelet aggregation study was performed as previously described.[17,18] Briefly, PRP was diluted with Tyrode's solution containing 0.3% BSA and adjusted to about 2×10^8 platelets/mL. PRP was stimulated to aggregate by using different aggregating agents at the following final concentrations: collagen, 2 μg/mL; thrombin, 0.2 U/mL; ADP, 2.5 μM; AA, 100 μM; and U46619, 1 μM. We decided the concentrations of agonists on the basis of the results from preliminary experiments (data not shown). Platelet aggregation was recorded for 5 min after stimulation by using whole blood/optical Lumi-Aggregometer (Chrono-Log). Aggregations were measured and expressed as percent changes in light transmission with respect to PPP. Data of light transmission assay were calculated to percent change by using Chrono-Log software. In the in vitro study, PRP was preincubated with AITC or ASA at 37 °C for 3 min before being stimulated with the aggregating agents. To monitor shape change, a platelet shape change study was performed as previously described.[19] Platelets (2×10^8 platelets/mL) were preincubated with apyrase (2 U/mL), indomethacin (10 μM), and EGTA (1 mM) before the addition of collagen.

Determination of Cytotoxicity in Rat PRP. To determine the cytotoxicity of AITC, lactate dehydrogenase (LDH) release from platelets was measured as previously described.[20] After incubation of PRP (2×10^8 platelets/mL) with 70% PEG−saline or different concentrations of AITC for 3 min at 37 °C, PRP was centrifuged at room temperature at 10000*g* for 1 min. A 25 μL aliquot of the supernatant was mixed with 100 μL of NADH solution (0.03% β-NAD, reduced form of the disodium salt in phosphate buffer) and 25 μL of pyruvate solution (22.7 mM pyruvic acid in phosphate buffer) at room temperature. Reductions in absorbance at 340 nm because of conversion of NADH to NAD^+ were measured to determine LDH activity in supernatants. LDH release is expressed as the percentage of the total enzyme activity in platelets completely lysed with 0.2% Triton X-100.

Measurement of TXA_2 Production and ATP Release in Rat and Human PRP. Measurement of TXA_2 production was conducted using a TXB_2 EIA kit (Cayman Chemical Co, Ann Arbor, MI, USA) as previously described.[21] PRP (2×10^8 platelets/mL) was incubated for 3 min in the presence or absence of samples, and then collagen (2 μg/mL) was added and PRP was incubated at 37 °C for 5 min with stirring. EDTA (10 mM) was added to stop TXA_2 production. After centrifugation at 12000*g* for 3 min, the amount of TXB_2 was measured according to the manufacturer's instructions.

Detection of ATP release was determined as previously described.[22] PRP (2×10^8 platelets/mL) was incubated at 37 °C with stirring at 1200 rpm. After addition of the luciferin−luciferase reagent, collagen (2 μg/mL) was added to initiate the reaction. PRP was incubated with AITC (30, 100, or 300 μM) for 3 min before the addition of luciferin−luciferase reagent.

Determining the $[Ca^{2+}]_i$ in Rat PRP. The intracellular Ca^{2+} was determined with Fura-2/AM as described previously.[23] Briefly, the PRP was incubated with 6 μM Fura-2/AM for 60 min at 37 °C. The Fura-2-loaded washed platelets (10^8 mL^{-1}) were then preincubated with AITC for 3 min with 1 mM $CaCl_2$. Next, WPs were stimulated with collagen (2 μg/mL) for 5 min. Fura-2 fluorescence was measured in a spectrofluorometer with an excitation wavelength that ranged from 340 to 380 nm, changing every 0.5 s, and an emission wavelength of 510 nm. The $[Ca^{2+}]_i$ was calculated as[24]

$$[Ca^{2+}]_i \text{ in cytosol} = 224 \text{ nM} \times (F - F_{min})/(F_{max} - F)$$

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. F_{min} is the fluorescence intensity of the Fura-2−Ca^{2+} complex at 510 nm, after the platelet suspension containing 20 mM Tris/10 mM EGTA had been solubilized by 0.1% Triton X-100. F_{max} is the fluorescence intensity of the Fura-2−Ca^{2+} complex at 510 nm, after the platelet suspension containing 1 mM $CaCl_2$ had been solubilized by 0.1% Triton X-100. F indicates the fluorescence intensity of the Fura-2 complex at 510 nm after WPs were stimulated by collagen, with 1 mM $CaCl_2$ and AITC or vehicle.

Western Blotting. The immunoassay was performed as previously described.[4] The WPs (2×10^8 platelets/mL) were stimulated by collagen (2 μg/mL) in the presence or absence of AITC (300 μM). Reactions were terminated by adding 10 mM EDTA and centrifuged at 12000*g* for 3 min. After the addition of ice-cold platelet lysis buffer (1% NP40, 15 mM HEPES, 75 mM NaCl, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM sodium fluoride (NAF), 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 μg/mL aprotinin; pH 7.4), lysates were centrifuged at 13000*g* for 10 min at 4 °C. The supernatants were collected, and protein concentration was determined using a BCA protein assay (Thermo Scientific, Vernon Hills IL, USA). Equal volumes of protein were resolved using SDS-PAGE (10%) and transferred to PVDF membranes. The PVDF membranes were blocked with 5% nonfat dry milk in TBS and incubated with the primary antibody diluted in TBS buffer (1:1000 ratio, 4 °C overnight). Immunoblots were then incubated again with a horseradish peroxidase (HRP)-conjugated secondary antibody, and then the immune-reactive bands were visualized with a LAS 4000 (Fuji, Honshu, Japan) using a Western blot detection system (WestZol, Intron, Seoul, Korea).

Ex Vivo Platelet Aggregation Study in Rat. Two hours before experiments, SD rats were orally administered 70% PEG−saline, AITC (0.3, 1, 3 mg/kg), or ASA (50 mg/kg). Rat blood samples were collected, and the platelet aggregation study was performed as described above.

In Vivo Acute Pulmonary Thromboembolism Study in Mouse. Acute pulmonary thromboembolism model was performed as previously described.[25] Male ICR mice weighing 35−40 g were fasted for 6 h before experiments. AITC and ASA were dissolved in 70% PEG−saline. Mice were orally administered AITC or ASA 2 h before experiments were performed. Collagen (500 μg/kg) plus epinephrine (25 μg/mL) was injected to the tail vein to induce thrombosis. Each mouse was watched for 15 min to determine whether the mouse was paralyzed, dead, or recovered from the acute thrombotic challenge.

In Vivo Mouse Tail Bleeding Time Assay. Mouse tail bleeding times were recorded as previously described.[26,27] Male ICR mice

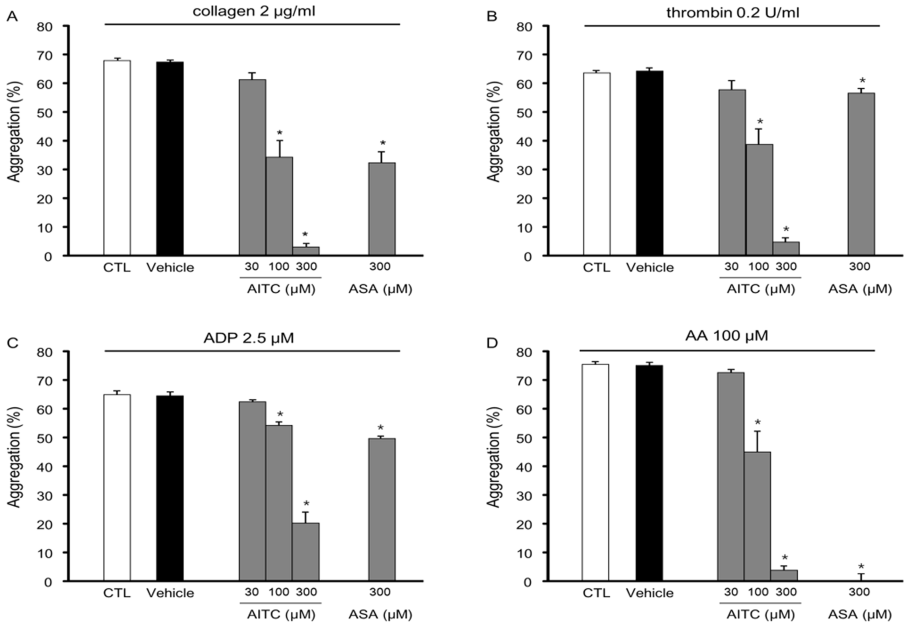

Figure 1. Concentration-dependent inhibitory effect of AITC on in vitro platelet aggregation. Platelets were preincubated with 70% PEG−saline (vehicle group), ASA (300 μM), or AITC (30, 100, or 300 μM) or not (control group) for 3 min at 37 °C. After preincubation, platelets were stimulated by the following agonists: collagen, 2 μg/mL (A); thrombin, 0.2 U/mL (B); ADP, 2.5 μM (C); or arachidonic acid (AA), 100 μM (D). Data are expressed as means ± SEMs (n = 5). (*) P < 0.05 versus vehicle.

weighing 35−40 g were fasted for 16 h before experiments. Two hours after the oral administration of AITC (0.3, 1, or 3 mg/kg) or ASA (50 mg/kg), the mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Thereafter, the mice were individually placed on a hot plate to control body temperature, and tails were transected 3 mm from the tips by using a razor blade. The tail was then immersed in a 15 mL clear conical tube containing warmed saline (37 °C). The time until the cessation of blood flow was then measured. Bleeding time was defined as the time taken for bleeding to stop for 15 s.

Statistical Analysis. In the thromboembolism experiment, the chi-square test was used to determine significant differences between the vehicle and treated groups. Statistical significance was considered at P < 0.05. In other experiments, data were expressed as means ± SEMs of at least three experiments. One-way ANOVA followed by Dunnett's test or the paired t test by using Sigma Stat statistical software (San Rafael, CA, USA) was used for the analysis. Significance was accepted for P values of <0.05.

RESULTS AND DISCUSSION

Effect of AITC on Rat Platelet Aggregation in Vitro. To evaluate the antiplatelet effect of AITC, we first examined its effect on rat platelet aggregation induced by various agonists. During pathological thrombogenesis, platelets are activated after binding to collagen on a vascular lesion. Activated platelets initiate intrinsic pathways, such as arachidonic acid (AA) liberation, to release secondary agonists including ADP, which in turn activate other platelets.[28] Thrombin initiates platelet activation through cleavage of the N-terminal exodomain of the protease-activated receptor.[29] Thrombus formation is induced by multiple physiological ligands and, thus, an antiplatelet agent that affects only one type of ligand-platelet interaction is likely to have a limited preventive effect against thrombogenesis.[27] Therefore, an antiplatelet agent that acts on multiple platelet activation pathways has been suggested to be a more effective compound for treating platelet-involved cardiovascular disease. Indeed, dual antiplatelet therapy, such as a combination of clopidogrel and ASA, has been shown to be more effective in inhibiting pathogenic thrombi.[30] ASA inhibits platelet aggregation through inhibition of cyclooxygenase, and clopidogrel inhibits platelet activation through irreversible binding to the $P2Y_{12}$ ADP receptor on platelet membrane.

In this study, PRP was stimulated by collagen (2 μg/mL), thrombin (0.2 U/mL), ADP (2.5 μM), or AA (100 μM) to induce platelet aggregation. The platelet aggregations induced by collagen, thrombin, ADP, and AA (68, 64, 62, and 74%, respectively) were significantly inhibited by either 100 or 300 μM AITC (6, 8, 18, and 5%, respectively) (Figure 1). In addition, AITC showed lower IC_{50} values than ASA in collagen, thrombin or ADP-induced platelet aggregation (Table 1). Taken together, these results indicate that AITC inhibits platelet aggregations induced by multiple agonists, and thus it may have beneficial potential for the prevention of platelet-related disorders.

Table 1. IC_{50} of AITC and ASA for Platelet Aggregation in Vitro[a]

agonist[b]	AITC IC_{50} (μM)	ASA IC_{50} (μM)
collagen	112.8 ± 16.3	312.7 ± 30.6
thrombin	100.6 ± 10.7	1314.7 ± 76.4
ADP	149.7 ± 17.7	1422.6 ± 129.1
AA	167.2 ± 22.6	27.3 ± 0.9

[a]Results are expressed as means ± SDs. [b]Concentration of agonists were as follows: collagen (2 μg/mL), thrombin (0.2 U/mL), ADP (2.5 μM), and AA (100 μM).

Effect of AITC on Platelet Cytotoxicity. To examine the cytotoxicity of AITC, LDH release from platelets was measured. LDH release is used as an index of cellular injury, including injury to platelets.[31] There was no significant LDH release from platelets treated with either vehicle (70% PEG−saline) or AITC (30, 100, or 300 μM) (Table 2). These findings suggest that the antiplatelet effect of AITC was not the result of cytotoxic effects, such as platelet membrane disruption.

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Table 2. Effect of AITC on LDH Release by Platelets[a]

treatment	LDH release[b]
CTL	11.2 ± 1.6
vehicle	13.1 ± 1.7
AITC 300 μM	11.5 ± 1.6
AITC 100 μM	11.8 ± 2.2
AITC 30 μM	10.1 ± 2.0

[a]Data are expressed as means ± SEMs (n = 5). [b]LDH release was measured after rat platelet-rich plasma was treated with either vehicle or AITC (30, 100, or 300 μM) for 3 min. P < 0.05 versus vehicle.

Effects of AITC on TXA_2 Production, ATP Release, and Ca^{2+} Increase in Rat PRP. Platelets are activated by various agonists including collagen. Activated platelets recruit other platelets to form aggregation. To recruit other platelets, activated platelets secrete TXA_2 and granules containing ATP and ADP, which act as paracrine activators.[22,32] Through interaction with the T_P receptor on the platelet membrane, TXA_2 plays a key role in platelet shape change and fibrinogen receptor (GPIIb/IIIa) activation, subsequently leading to platelet aggregation.[33,34] A parallel process involves release of ATP and ADP, which then bind to $P2X_1$ and $P2Y_1/P2Y_{12}$ receptors, respectively, leading to Ca^{2+} entry and amplification of platelet activation and shape change.[35]

Because TXA_2 production and ATP release follow platelet activation, they are well-known indicators of platelet activation.[36] To evaluate the mechanism of the antiplatelet effect of AITC, TXA_2 production and ATP release were examined in this study. Because TXA_2 is unstable and quickly converted to its stable form, TXB_2, the production of TXB_2 was measured instead. Collagen-induced TXB_2 production (4022 pg/mL) was significantly inhibited by AITC at a concentration of 300 μM (1320 pg/mL), but not by 100 μM (3812 pg/mL) (Figure 2A). Consistently, AITC significantly inhibited platelet aggregation induced by U46619 (77%), a thromboxane A_2 receptor agonist, only at the concentration of 300 μM (11%) (Supporting Information file 1). These findings suggest that the antiplatelet effect of 100 μM AITC (Figure 1) may be attributed to a mechanism other than the TXA_2 inhibition pathway. To investigate the mechanism underlying the effect of 100 μM AITC, the effect of AITC on granule secretion was examined by measuring ATP release, which reflects the release of ADP, because ATP/ADP release from granules is known to be activated independently of TXA_2 pathway.[22,37] AITC, at the concentrations of both 100 and 300 μM, significantly inhibited collagen-induced ATP release by 48 and 84%, respectively (Figure 2B). From these results, it is suggested that the antiplatelet effect of AITC at 100 μM is likely to be attributed to inhibition of ATP/ADP release rather than TXA_2 inhibition, whereas the antiplatelet effect of 300 μM AITC is attributed to inhibition of both ATP/ADP release and TXA_2.

Another biomarker of platelet activation is intracellular Ca^{2+} increase. After collagen binds platelet membrane receptor, intracellular Ca^{2+} increases and plays a key role in TXA_2, ATP/ADP secretion, and shape change.[38] In addition, increasing

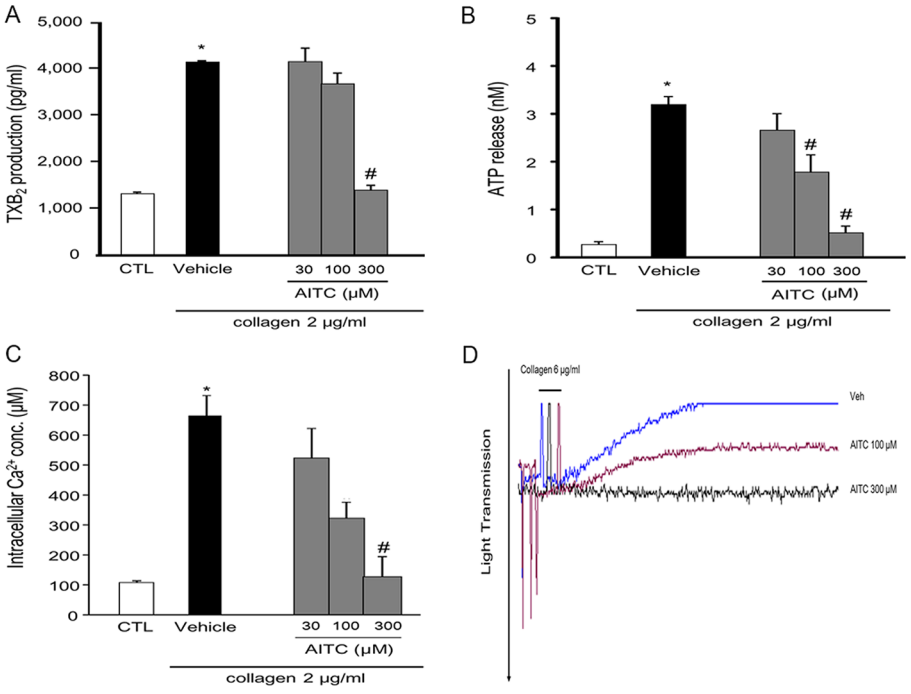

Figure 2. Inhibitory effect of AITC on collagen-induced TXA_2 production (A), ATP release (B), intracellular Ca^{2+} increase (C), and platelet shape change (D). For the TXA_2 assay, platelets were preincubated with AITC (30, 100, or 300 μM) for 3 min, and then platelets were stimulated with collagen (2 μg/mL) for 6 min. The amount of TXA_2 was determined by measuring TXB_2 concentrations using an EIA kit (n = 8). For ATP measurement, platelets were preincubated with AITC (30, 100, or 300 μM) for 3 min before the addition of a luciferin−luciferase reagent. After the luciferin−luciferase reagent was added, platelets were stimulated with collagen (2 μg/mL) for 6 min. ATP release was measured using a lumi-aggregometer (n = 8). For measurement of intracellular Ca^{2+} increase, platelets were loaded with Fura-2/AM. The platelets were preincubated with or without AITC (30, 100, or 300 μM) for 3 min and stimulated with collagen 2 μg/mL (n = 5). For measurement of platelet shape change, platelets were preincubated with apyrase (2 U/mL), indomethacin (10 μM), EGTA (1 mM), and with or without AITC (30, 100, or 300 μM) for 3 min followed by stimulation with collagen (6 μg/mL) and traces recorded. Data are expressed as means ± SEMs (n = 5). (*) P < 0.05 versus control. (#) P < 0.05 versus vehicle.

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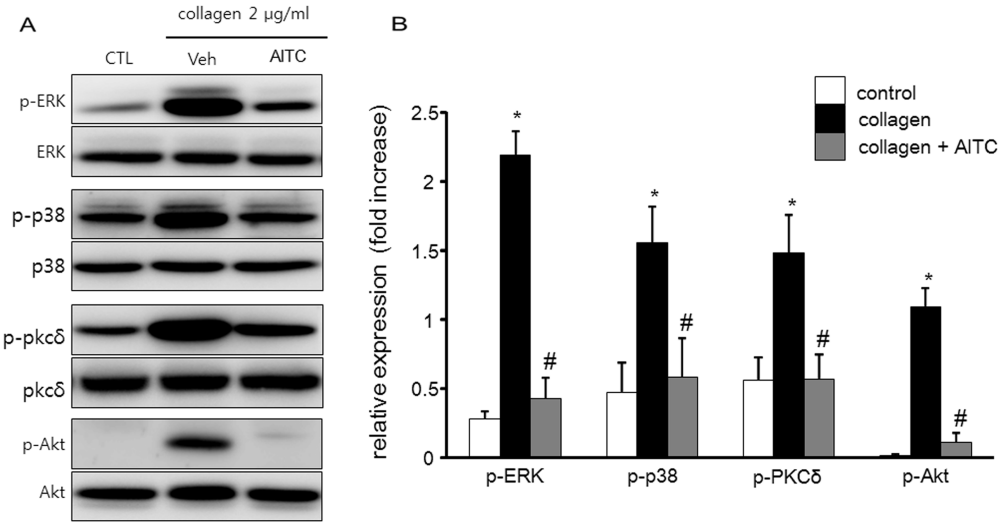

Figure 3. Effect of AITC on protein phosphorylation. (A) AITC attenuated collagen-activated platelet Akt, PKCδ, ERK, and p38 phosphorylation. Washed platelets were preincubated with either vehicle or AITC (300 μM) for 3 min with stirring. After preincubation, washed platelets were stimulated with collagen (2 μg/mL) for 5 min. Platelet lysates were analyzed by a Western blot immunoassay. (B) Relative expression was compared between total expression and phosphorylated expression. Values in bar graphs are means ± SEMs (n = 4). (∗)P < 0.05 versus control. (#) P < 0.05 versus vehicle.

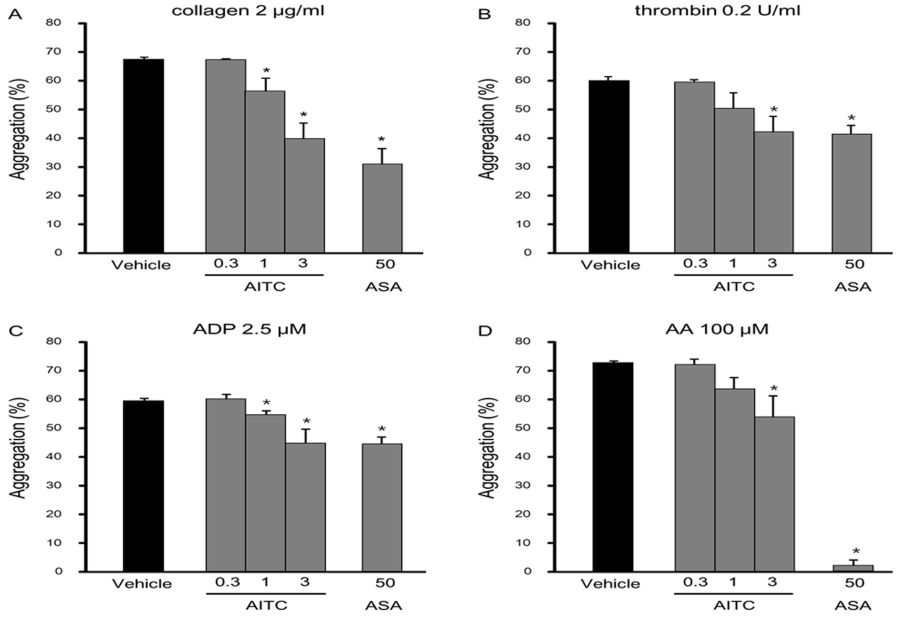

Figure 4. AITC inhibits ex vivo platelet aggregation after oral administration in a dose-dependent manner. AITC (0.3, 1, or 3 mg/kg) or ASA (50 mg/kg) was administered 2 h before blood collection. Platelet aggregation was induced by the following agonists: collagen, 2 μg/mL (A); thrombin, 0.2 U/mL (B); ADP, 2.5 μM (C); AA, 100 μM (D). Data are expressed as means ± SEMs (n = 6). (∗) P < 0.05 versus vehicle.

Ca^{2+} contributes to platelet aggregation through transformation of fibrinogen binding receptor GPIIb/IIIa. Transformed GPIIb/IIIa has high affinity with fibrinogen to aggregate platelets through fibrinogen binding. Furthermore, high intracellular Ca^{2+} contributes to platelet shape change through dissociation of actin−profilin complexes.[39] In this study, the effects of AITC on intracellular Ca^{2+} increase and shape change were examined to elicit its inhibitory mechanism on platelet aggregation. Collagen-induced intracellular Ca^{2+} increase (612 μM) was significantly inhibited by AITC at 100 and 300 μM (350 and 181 μM, respectively) (Figure 2C). In addition, collagen-induced platelet shape change was also inhibited by 100 and 300 μM AITC. These results suggest that the antiplatelet mechanisms of AITC at 100 and 300 μM may involve its inhibition of intracellular Ca^{2+} increase and shape change.

Effects of AITC on Phosphorylation of PLCγ, p38, ERK, PKCδ, and Akt. At the sites of atherosclerotic lesion, collagen is the most important thrombogenic component of the subendothelial matrix. Collagen binds to receptors on the platelet membrane, including the glycoprotein VI (GPVI) receptor, which has recently been considered as a new therapeutic target to prevent platelet aggregation with fewer side effects.[40,41] Recent studies have shown that inhibition of GPVI contributes to protection from thrombi with mild side effects, such as minor bleeding.[42,43] In the process of platelet

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aggregation, collagen binding to GPVI receptor triggers activation of PLCγ/protein kinase C delta (PKCδ)/extracellular signal-regulated kinases (ERK) and/or p38 mitogen-activated protein kinases (p38) signaling pathway and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling.[44] PLCγ is a specific signaling molecule for the collagen-induced platelet activation pathway, whereas Akt, PKCδ, ERK, and p38 are common molecules for all of the collagen-, thrombin- and ADP-induced signaling pathways for platelet activation.[45,46] The phosphorylation of Akt, PKCδ, ERK, and p38 is known to contribute to platelet activation, which can lead to thrombogenesis.[44,46−48] In this context, phosphorylation of proteins in the collagen-activated pathway was examined in this study to investigate the underlying action of signaling pathway mechanism of AITC. AITC was shown to markedly inhibit the phosphorylation of Akt, PKCδ, ERK, and p38 induced by collagen (2 μg/mL) (Figure 3), but not PLCγ phosphorylation (Supporting Information file 2). From these results, it is suggested that the inhibitory effect of AITC on collagen-induced platelet aggregation is, at least partially, mediated through Akt, PKCδ, ERK, and p38 inhibition rather than PLCγ suppression.

Effect of AITC on ex Vivo Platelet Aggregation in Rat. To investigate the ex vivo effect of AITC in rat, AITC was orally administered at 0.1, 0.3, 1, 3, or 10 mg/kg. Compared to vehicle treatment, AITC at 3 mg/kg po significantly inhibited platelet aggregation induced by collagen (2 μg/mL), thrombin (0.2 U/mL), ADP (2.5 μM), and AA (100 μM) (Figure 4).

Table 3. ID_{50} of AITC and ASA for Platelet Aggregation ex Vivo[a]

agonist[b]	AITC ID_{50} (mg/kg)	ASA ID_{50} (mg/kg)
collagen	6.2 ± 0.6	84.0 ± 8.9
thrombin	13.3 ± 0.9	837.0 ± 28.3
ADP	27.9 ± 0.7	450.0 ± 14.1
AA	24.1 ± 1.3	27.3 ± 5.5

[a]Results are expressed as means ± SDs. [b]Concentration of agonists were as follows: collagen (2 μg/mL), thrombin (0.2 U/mL), ADP (2.5 μM), and AA (100 μM).

Also, IC_{50} values of AITC and ASA were suggested at Table 3. ASA at 50 mg/kg was used as positive control. There was no antiplatelet effect of AITC at 0.1 and 0.3 mg/kg, and there was no significant difference in antiplatelet effect between 3 and 10 mg/kg AITC (data not shown). These results indicate that per orally treated AITC has a significant antiplatelet effect in rat, although further information such as pharmacokinetic data may be needed to link in vitro concentrations to in vivo doses of AITC.

Protective Effect of AITC on Pulmonary Thromboembolism Attack in Mouse. The goal of antiplatelet agents is prevention of thrombosis such as venous thromboembolism, which is the third most common vascular disease after heart attack and stroke.[49] The acute pulmonary thromboembolism mouse model, widely used to investigate antithrombotic therapeutic compounds, was evaluated to determine the protective effect of AITC against thromboembolism attack.[50] In the present study, oral administration of 3 mg/kg AITC had a protective effect against mortality (63%) caused by pulmonary thromboembolism (Table 4). This means that the antiplatelet effect of AITC is maintained in vivo.

Table 4. Effect of AITC on Acute Pulmonary Thromboembolism in Mice[a]

treatment	paralyzed or dead animals/total	protection (%)
CTL	8/8	0
vehicle	14/15	7
AITC		
0.3 mg/kg	11/13	15[b]
1 mg/kg	9/15	40[b]
3 mg/kg	6/16	63[b]
ASA		
50 mg/kg	3/15	80[b]

[a]The chi-square test used to examine the difference between the vehicle and treated group. [b]$P < 0.05$ versus vehicle.

Effect of AITC on Mouse Tail Bleeding Times. We investigated whether AITC has a prolonging effect on mouse tail bleeding time. Because the bleeding time reflects normal platelet function during physiologic hemostasis,[26] a prolongation effect of bleeding time has been used as an indicator for the side effect.[20] Because 3 mg/kg AITC and 50 mg/kg ASA showed similar inhibitory effects on collagen-induced platelet aggregation, we estimated bleeding time at those concentrations (3 mg/kg AITC and 50 mg/kg ASA). As shown in Figure 5, the prolonged bleeding time by 3 mg/kg AITC was

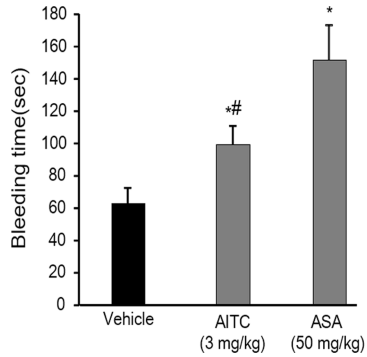

Figure 5. Effect of AITC on mouse tail bleeding time. AITC (3 mg/kg) or ASA (50 mg/kg) was given orally to mice 2 h before experiments. Data are expressed as means ± SEMs ($n = 10$). (*) $P < 0.05$ versus vehicle. (#) $P < 0.05$ versus ASA.

significantly less (40% less) than that by 50 mg/kg ASA, even though AITC still induced prolongation ($P < 0.05$). These results imply that AITC elicits an antiplatelet effect with less bleeding adverse effect.

Antiplatelet Effect of AITC in PRP from Human Blood. To investigate the effect of AITC on human blood, a platelet aggregation experiment was performed with PRP from human blood. Similar to the inhibitory effect of AITC on rat PRP, 100 and 300 μM AITC significantly inhibited platelet aggregation of human PRP induced by collagen, thrombin, ADP, and AA (Figure 6). AITC also inhibited TXB_2 production and ATP release in human platelets.

In conclusion, the present study is the first to demonstrate the antiplatelet activity of AITC in vitro, ex vivo, and in vivo. These effects of AITC seem to be mediated, at least partially, through inhibition of TXA_2 production, ATP/ADP release, and intracellular Ca^{2+} increase. AITC also showed protective effect against thromboembolism attack. These findings suggest that AITC may have therapeutic potential for the prevention of

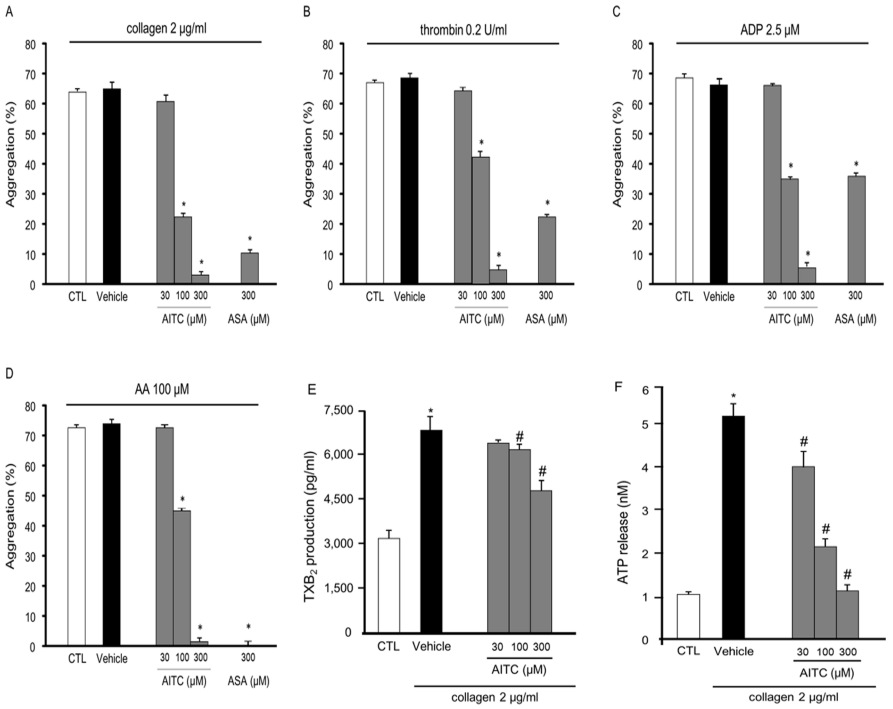

Figure 6. Effect of AITC on human platelet. Platelets were preincubated with 70% PEG−saline (vehicle group), ASA (300 μM), or AITC (30, 100, or 300 μM) or not (control group) for 3 min at 37 °C. After preincubation, platelets were stimulated by the following agonists: collagen, 2 μg/mL (A); thrombin, 0.2 U/mL (B); ADP, 2.5 μM (C); or arachidonic acid (AA), 100 μM (D) (n = 5). For the TXA_2 assay (E), platelets were preincubated with AITC (30, 100, or 300 μM) for 3 min, and then platelets were stimulated with collagen (2 μg/mL) for 6 min. The amount of TXA_2 was determined by measuring TXB_2 concentrations using an EIA kit (n = 4). For ATP measurement (F), platelets were preincubated with AITC (30, 100, or 300 μM) for 3 min before the addition of a luciferin−luciferase reagent. After the luciferin−luciferase reagent was added, platelets were stimulated with collagen (2 μg/mL) for 6 min. ATP release was measured using a lumi-aggregometer (n = 4). Data are expressed as means ± SEMs (n = 6). (*) $P < 0.05$ versus vehicle.

aberrant platelet activation, although further study remains to be performed for the potential of clinical application.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

File 1. Platelets were preincubated with 70% PEG−saline (vehicle group), ASA (300 μM), or AITC (30, 100, or 300 μM) or not (control group) for 3 min at 37 °C. After preincubation, platelets were stimulated by the U46619 (1 μM). Data are expressed as means ± SEM (n = 5). (*) $P < 0.05$ versus vehicle. File 2. Effect of AITC on PLCγ phosphorylation. Washed platelets were preincubated with either vehicle or AITC (300 μM) for 3 min with stirring. After preincubation, washed platelets were stimulated with collagen (2 μg/mL) for 5 min. Platelet lysates were analyzed by a Western blot immunoassay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

AA, arachidonic acid; ADP, adenosine diphosphate; AITC, allyl isothiocyanate; Akt, protein kinase B; ASA, acetylsalicylic acid; ATP, adenosine triphosphate; ERK, extracellular signal-regulated kinases; GPVI, glycoprotein VI; p38, p38 mitogen-activated protein kinases; PI3K, phosphoinositide 3-kinase; PKCδ, protein kinase C delta; PPP, platelet-poor plasma; PRP, platelet-rich plasma; $TXA(B)_2$, thromboxane $A(B)_2$; WP, washed platelet

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