Activation of Platelets in Bronchial Asthma*

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Study objectives: To investigate whether platelets are activated in asthmatics with increased release of preformed mediators and to investigate the influence of oral administration of theophylline on them.

Design: Comparison of the intracellular free calcium concentration ([Ca\(^{2+}\)]\textsubscript{i}) in platelets as an indicator of platelet activation, CD62P expression on platelets, and the chemokine regulated upon activation in normal T cells expressed and presumably secreted (RANTES) level in platelet-rich buffer supernatants between asthmatics and normal subjects.

Setting: The respiratory outpatient clinics, Hiroshima University, Japan.

Participants: Twenty-five normal volunteers, 19 asthmatics taking no oral drugs associated with asthma treatment (group A), and 18 asthmatics taking oral theophylline (group B).

Measurements and results: While the resting [Ca\(^{2+}\)]\textsubscript{i}s in platelets were similar among the three groups, the [Ca\(^{2+}\)]\textsubscript{i}s in group A were significantly higher than those in normal subjects (p<0.05) and group B (p<0.01) after thrombin or 9,11-epithia-11,12-methano-thromboxane A\(_2\) (STA\(_2\)) stimulation in the absence of external Ca\(^{2+}\). The CD62P expression level and RANTES level in group A after STA\(_2\) stimulation were significantly higher than those in normal subjects and group B (p<0.05).

Conclusions: We conclude that agonist-mediated activation of platelets is augmented in asthmatics resulting in enhanced release of chemokine such as RANTES, which could be suppressed by oral administration of theophylline.

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Key words: asthma; CD62P; intracellular free calcium concentration; platelet; RANTES; theophylline

Abbreviations: [Ca\(^{2+}\)]\textsubscript{i}, intracellular free calcium concentration; cAMP, cyclic adenosine monophosphate; EGTA, ethyleneglycol-bis-(\(\beta\)-aminoethyl) ether-N,N,N',N'-tetraacetic acid; FITC, fluorescein isothiocyanate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP\(_3\), inositol 1,4,5-triphosphate; PF4, platelet factor 4; PAF, platelet activating factor; PI, phosphatidylinositol; PKC, protein kinase C; PLC, phospholipase C; RANTES, regulated upon activation in normal T cells expressed and presumably secreted; STA\(_2\), 9,11-epithia-11,12-methano-thromboxane A\(_2\); TXA\(_2\), thromboxane A\(_2\)

In recent years, asthma has become recognized as a chronic inflammatory disease associated pathologically with eosinophilic infiltration and airway epithelial damage.\(^1\) It is clear that eosinophils, mast cells, and T lymphocytes play key roles in these inflammatory events.\(^1\) However, platelets may also play a role in this allergic inflammatory process, because they are a rich source of a wide range of biologically active materials capable of inducing or augmenting allergic inflammatory responses.\(^2,3\) Such materials have been demonstrated to be preformed mediators stored in \(\alpha\)-granules, which are chemokines such as platelet factor 4 (PF4) and regulated upon activation in normal T cells expressed and presumably secreted (RANTES).\(^2,3\) These chemokines are released from platelets after stimulation with potent anaphylactic mediators such as platelet activating factor (PAF)\(^2,4\) and cause eosinophilic chemotaxis,\(^5\) providing additional evidence for a contribution of platelets to bronchial asthma. Actu-
ally, some evidences for platelet abnormalities in asthmatic patients have been reported.\textsuperscript{5-16} For example, platelets from asthmatics were found not to undergo the second wave of aggregation following stimulation with platelet agonists in vitro,\textsuperscript{10} and a number of studies in vivo demonstrated that circulating platelets aggregated\textsuperscript{11} and platelet-specific proteins, PF4 and β-thromboglobulin, were released into the circulation\textsuperscript{11,12} and BAL fluid\textsuperscript{13} during provoked or spontaneous asthmatic attacks. Moreover, platelets were found to accumulate in the pulmonary microvasculature and were detected in BAL fluid of patients undergoing bronchial provocation with allergens,\textsuperscript{14,15} and many abnormal megakaryocytes were reportedly observed in lung tissues removed at autopsy from patients who died of status asthmaticus.\textsuperscript{16} These previous reports concerning platelets in bronchial asthma have focused on their aggregation, release of PF4 and β-thromboglobulin, or accumulation in lung, whereas few reports have investigated intracellular calcium movement in platelets or release of RANTES.

In this study, we attempted to determine whether platelets are activated in asthmatics by measuring the resting and agonist-evoked intracellular free calcium concentrations ([Ca\textsuperscript{2+}]\textsubscript{i}) in platelets. In addition, we measured the resting and agonist-evoked CD62P expressions on platelets and the RANTES levels in platelet-rich buffer supernatants, assuming that the release of preformed mediators from platelets could be enhanced when they are activated. CD62P is an α-granules membrane glycoprotein and redistributed to the cell surface as a result of mediator release from α-granules.\textsuperscript{17}

### MATERIALS AND METHODS

#### Study Population

Twenty-five normal volunteers (14 male, 11 female) who had no history of respiratory disease and allergic disease (normal subjects), and 37 patients with stable asthma (20 male, 17 female) participated in the study (Table 1). The asthmatic patients were divided into two groups: one (group A) comprised 19 patients, who used no drugs for their asthma other than by inhalation (eg, corticosteroids, β\textsubscript{2}-agonists, anticholinergics, disodium cromoglycate), and the other (group B) comprised 18 patients who took oral theophylline preparations (200 to 600 mg/d for 1 to 110 months, mean 20.5±6.7 months), but did not use oral corticosteroids, antiallergic drugs, or medications known to interact with theophylline. We excluded asthmatics suffering attacks because it was difficult to withdraw blood from them before starting emergency therapy and afterwards, they were deeply influenced by various drugs, such as corticosteroids. The three groups were age and sex matched. The patients were classified as having atopic or nonatopic asthma by IgE value, radioallergosorbent testing response, and history of allergic disease. Their disease severity was classified according to the criteria from the Global Initiative for Asthma and no patient was classified as having severe disease. All the subjects underwent pulmonary function tests, and the percent predicted FEV\textsubscript{1} values of the asthmatics were significantly lower than those of the normal subjects (group A, \(p<0.05\); group B, \(p<0.01\)), but there was no significant difference between the values of groups A and B.

#### Measurement of [Ca\textsuperscript{2+}]\textsubscript{i}

Venous blood (9 mL) was drawn under informed consent using two-syringe technique with a 19-gauge needle, and anticoagulated with 1 mL of 3.8% (w/v) trisodium citrate. Platelet-rich plasma was obtained by centrifugation of whole blood at 750g for 5 min. Platelets were separated from plasma by gel filtration, diluted with Ca\textsuperscript{2+}-free Na\textsuperscript{+}–4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (145 mM NaCl, 5 mM KCl, 1 mM MgSO\textsubscript{4}, 10 mM HEPES, and 5 mM glucose; pH 7.4) to a concentration of 10\textsuperscript{6} cells per milliliter. The platelet preparations were >99.9% pure as judged by a hematoma analyzer (Micro Diff II; Coulter Electronics Limited; Beds, UK). Subsequently the platelet-rich buffer was incubated with 1 mM fura-2/acetoxymethyl ester (fura-2/AM; Molecular Probes; Eugene, Ore) and 0.025% (w/v) Pluronic F-127 (Molecular Probes) for 30 min at 37°C, followed by gel filtration again to remove the extracellular dye. The platelets adjusted to 10\textsuperscript{7} cells per milliliter were incubated with 1 mM CaCl\textsubscript{2} for 7 min at 37°C to de-esterify the fura-2/AM before fluorescence recording. Fluorescence was measured using a spectrofluorometer (DM3000CM; SPEX In-

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<th>Table 1—Characteristics of Normal Subjects and Asthmatic Patients*</th>
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<td>Normal Subjects</td>
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*Group A=asthmatic patients who used no drugs for their asthma other than by inhalation; group B=asthmatic patients who took oral theophylline preparation but did not use any medications known to interact with theophylline. Values are expressed as means±SE.

[1] Significantly different from normal group (\(p<0.05\)).

[2] Significantly different from normal group (\(p<0.01\)).
dustries Inc; Edison, NJ) equipped with a stirring apparatus and a thermostated cuvette holder. First, the fluorescence in the resting state was measured and then the required agonist was added to determine the \([Ca^{2+}]_i\) response. The fluorescence recordings were corrected for extracellular fura-2/AM that leaked from the cytosol using 10 nM ethyleneglycol-bis-(\(\beta\)-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA).\(^{16}\) In order to measure the \([Ca^{2+}]_i\) response to agonists in the absence of extracellular \(Ca^{2+}\), EGTA was added after recording the fluorescence in the resting state, the required agonist was added to the cell suspension 30 s later, and the fluorescence was recorded again. The \([Ca^{2+}]_i\) were calculated using the equation of Grynkiewicz et al.\(^{19}\) Thrombin (Sigma Chemical Co, St Louis), PAF (Sigma Chemical Co), and thromboxane A2 (TXA2) analog, 9,11-epithio-11,12-methano-TXA2 (STA2, a gift from Dr. A. Kawasaki of the Research and Development Division, Ono Pharmaceutical Co, Ltd, Osaka, Japan) were selected as agonists, because PAF and TXA2 are deeply associated with bronchial asthma\(^{1,20}\) and thrombin is generally used in activation of platelets. The optimal concentrations of the stimulants (thrombin, 1.0 U/mL; PAF, 10 nM; STA2, 10 nM) were determined after constructing a concentration-response curve of agonist against \([Ca^{2+}]_i\) for each agonist (data not shown).

**CD62P Expression Level Measurement**

Within 5 min of collection, 5-\(\mu\)L aliquots of whole blood were put into polystyrene tubes containing 40 \(\mu\)L isotonic HEPES buffer, and 5 \(\mu\)L monoclonal antibody, fluorescein isothiocyanate (FITC) labeled anti-CD62P (CD62P/FITC; Serotec Ltd; Oxford, UK), and the required agonist was added to each tube. After incubation at 37°C for 5 min without stirring, the reactions were stopped by addition of 500 \(\mu\)L of 1% (v/v) paraformaldehyde in HEPES buffer, and the samples were analyzed using a flow cytometer (FACStar; Becton-Dickinson; Mountain View, Calif) with software ( Consort 30; Becton-Dickinson). The fluorescence intensity of 10,000 platelets per sample was analyzed. A mouse monoclonal antibody, FITC-labeled mouse immunoglobulin G1 (mouse IgG1/FITC; Becton-Dickinson Immunocytometry Systems; San Jose, Calif), was used as an isotype-specific control to set the threshold value.

**RANTES Level Measurement**

One milliliter aliquots of platelet-rich buffer adjusted to 10\(^7\) cells per milliliter were put into polystyrene tubes and incubated at 37°C in a water bath for 30 min with required agonist. After centrifugation at 10,000g for 10 min at 4°C, the supernatant samples were stored at -80°C. The RANTES levels of the supernatants were assayed in duplicate using an enzyme-linked immunosorbent assay kit for RANTES (R&D Systems; Minneapolis), without repeated freeze-thaw cycles of the samples.

**Statistical Analysis**

The data are expressed as means±SE. Analysis of variance was performed using the Mann-Whitney U test for nonpaired samples. Statistical analysis was performed using a personal computer program (Statview; Apple Macintosh, Apple Computer Inc; Cupertino, Calif). Differences at p<0.05 were considered significant.

**RESULTS**

**\([Ca^{2+}]_i\) Responses to Agonists**

The resting \([Ca^{2+}]_i\) in the normal subjects and groups A and B were 11.8±0.6, 12.1±0.7, and 11.8±0.7 nM, respectively. The respective values after thrombin stimulation were 440.1±210.0, 445.1±151.1, and 422.1±14.5 nM, and after PAF stimulation were 130.5±4.7, 125.8±7.4, and 130.2±6.9 nM (Fig 1). There were no significant differences between any two groups. However, the \([Ca^{2+}]_i\) in group A after STA2 stimulation (444.1±32.7 nM) was significantly higher than that of the normal subjects (62.9±7.3 nM, p<0.05). The \([Ca^{2+}]_i\) in the normal subjects and group B

**Figure 1.** The \([Ca^{2+}]_i\) of platelets from normal subjects and groups A and B. Platelets were stimulated with 1.0 U/mL thrombin (top), 10 nM PAF (center), and 10 nM STA2 (bottom). They were evaluated when external \(Ca^{2+}\) existed (open bar) or not (filled bar). Values are expressed as means±SE. Statistically significant differences are indicated by Mann-Whitney U test (asterisk: p<0.05 vs normal subjects; dagger: p<0.05 vs group A; two daggers: p<0.01 vs group A).

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(87.0±6.7 nM) after STA₂ stimulation did not differ significantly, whereas the [Ca²⁺]ᵢ in group B after STA₂ stimulation was significantly lower than that in group A (p<0.05). In contrast, the respective [Ca²⁺]ᵢ in the absence of external Ca²⁺ in the normal subjects and groups A and B after thrombin stimulation were 116.5±5.1, 140.3±8.1, and 113.8±6.9 nM, and after STA₂ stimulation they were 64.3±5.0, 85.5±5.9, and 65.6±4.6 nM. Thus, the value in group A after stimulation by each of these agonists was significantly higher than that in the normal subjects (p<0.05). The [Ca²⁺]ᵢ in the absence of external Ca²⁺ of normal subjects and group B after thrombin or STA₂ stimulation did not differ significantly, whereas the group B values were significantly lower than the group A values (p<0.05 and p<0.01, respectively). There were no significant differences between the [Ca²⁺]ᵢ in the absence of external Ca²⁺ of atopic and nonatopic asthmatics of each group, but the respective values did not differ significantly (data not shown). Neither values between β₂-agonist users and nonusers nor those between corticosteroid inhalation users or nonusers also showed significant differences (data not shown).

**CD62P Expression Level**

CD62P expression on platelets was assessed by determining the binding of FITC-labeled antibodies to platelets, measuring the mean fluorescence intensity. We evaluated the relative CD62P expression level as the ratio of the mean fluorescence intensity of CD62P/FITC and the negative control (mouse IgG₁/FITC). The CD62P expression levels in the normal subjects and groups A and B without stimulation were 2.10±0.17, 2.65±0.32, and 1.89±0.19, respectively, and there were no significant differences between any two groups (Fig 2). The respective CD62P expression levels after thrombin stimulation were 3.18±0.32, 4.73±0.66, and 2.65±0.25, and after PAF stimulation were 2.19±0.17, 3.06±0.37, and 2.04±0.20. The CD62P expression levels in group A after thrombin or PAF stimulation appeared to be higher than those in the normal subjects, but the differences were not significant. After STA₂ stimulation, the CD62P expression levels in the normal subjects and groups A and B were 2.13±0.16, 3.22±0.43, and 2.04±0.26, respectively, and the group A value was significantly higher than that of the normal subjects (p<0.05). In contrast, the CD62P expression level in group B after stimulation by each agonist was significantly lower than the corresponding level in group A (p<0.05; Fig 2). The CD62P expression levels between corticosteroid in-

![Figure 2.](image-url) Levels of CD62P expression on platelets before and after 1.0 U/mL thrombin (top), 10 nM PAF (center), and 10 nM STA₂ (bottom) stimulation in normal subjects (squares) and groups A (triangles) and B (circles). The expression levels are assessed by CD62P/FITC binding to platelets, measuring the mean fluorescence intensity. Values are expressed as means±SE. Statistically significant differences are indicated by Mann-Whitney U test (asterisk: p<0.05 vs normal subjects; dagger: p<0.05 vs group A).
halation users and nonusers did not show significant differences in each group A, group B, and groups A and B. Difference of other conditions, for example atopic or nonatopic and β₂-agonist users or nonusers also did not affect the respective values.

**RANTES Level**

The RANTES levels without stimulation in the normal subjects and groups A and B were 789±157, 933±111, and 688±151 pg/mL, respectively, and there were no significant differences between any two groups. The respective levels after thrombin stimulation were 1,543±397, 1,883±227, and 1,139±284 pg/mL, and after PAF stimulation they were 1,150±252, 1,676±230, and 937±170 pg/mL (Fig 3). After thrombin or PAF stimulation, the RANTES level in group A was higher than that in the normal subjects, but the differences were not statistically significant, and the levels in group B were significantly lower than those in group A (p<0.05). The RANTES levels in the normal subjects and groups A and B after STA₂ stimulation were 816±149, 1,595±203, and 785±162 pg/mL, respectively. The RANTES levels in the normal subjects and group B were not affected by STA₂, whereas those in group A increased and were significantly higher than those of both the normal subjects and group B (p<0.05; Fig 3). There were no significant differences in the RANTES levels between corticosteroid inhalation users and nonusers in each group A, group B, and groups A and B. Difference of other conditions such as atopic or nonatopic and β₂-agonist users or nonusers did not affect the respective values.

**DISCUSSION**

In this study, we demonstrated that asthmatic platelets were easily activated by agonist resulting in the increased release of RANTES, and that this release was likely inhibited by theophylline administration. The [Ca²⁺]iS after thrombin or STA₂ stimulation in the absence of external Ca²⁺ in group A were significantly higher than those in the normal subjects, indicating that the amount of Ca²⁺ released from the endoplasmic reticulum to the cytosol of platelets is significantly increased in patients with bronchial asthma, possibly through potentiation of phosphatidylinositol (PI) pathway. The transduction of human platelets by the actions of agonists such as thrombin, PAF, and TXA₂ involves the activation of receptor-associated guanosine triphosphate-binding proteins and the subsequent stimulation of phospholipase C (PLC).²¹-²³ PLC induces the hydrolysis of PI 4,5-bisphosphate and subsequent production of the

![Figure 3. RANTES levels in the platelet-rich buffer before and after 1.0 U/mL thrombin (top), 10 nM PAF (center), and 10 nM STA₂ (bottom) stimulation. The buffer was obtained from whole blood of normal subjects (squares) and groups A (triangles) and B (circles) by gel filtration. They were incubated for 30 min at 37°C with each agonist. The RANTES levels of the supernatants were assayed in duplicate using an enzyme-linked immunosorbent assay kit. Values are expressed as means±SE. Statistically significant differences are indicated by Mann-Whitney U test (asterisk: p<0.05 vs normal subjects; dagger: p<0.05 vs group A).](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21759/ on 04/26/2017)
second messengers, inositol 1,4,5-triphosphate (IP₃), which mobilizes Ca²⁺ from internal Ca²⁺ stores, and diacylglycerol, which activates protein kinase C (PKC). This chain of systematic responses is called the PI pathway and, therefore, increased Ca²⁺ release from internal stores may suggest potentiation of the PI pathway. Block et al²⁴ also observed elevated IP₃ levels in platelets from asthmatics.

Both the CD62P expressions and RANTES levels after stimulation of platelets by agonist, such as thrombin, PAF, or STA₂, in group A were higher than those in the normal subjects, and using STA₂ the difference was statistically significant (p<0.05). These results suggest that mediator secretion by platelets stimulated with agonists is enhanced in asthmatics in comparison to normal subjects. As a consequence of the generation of two second messengers, IP₃ and diacylglycerol, many proteins change their phosphorylation state in association with platelet aggregation and secretion responses.²⁵

The roles of PKC and [Ca²⁺], elevation in aggregation of and secretion by human platelets have not been elucidated.²⁶-³⁰ With respect to secretion, Walker and Watson³¹ recently reported that the signaling events required for mediator secretion are [Ca²⁺], elevation and synergistic activation of PKC.³¹

Thus, it is possible that secretion by platelets after agonist stimulation is enhanced in bronchial asthma as a result of PI pathway potentiation, which will result in not only [Ca²⁺], elevation but also increase of PKC activation. The CD62P expression and RANTES level changes we observed lend support to this hypothesis.

Although our results suggest PI pathway potentiation in group A, the [Ca²⁺] of their platelets in the presence of external Ca²⁺ were not elevated significantly compared with those of normal subjects. We suspected that this may be due to a mechanisms regulating Ca²⁺ entry into platelets. In human platelets, the Ca²⁺ entry mechanisms are still unclear, and conflicting results have been reported. For instance, several studies have indicated the existence of a Ca²⁺ entry pathway secondary to Ca²⁺ depletion of store, the store-regulated influx pathway,³²,³³ whereas receptor-evoked entry pathways have been demonstrated by others.³⁴ In any case, Ca²⁺ in the cytoplasm is cytotoxic, so it is necessary to have a mechanism that regulates [Ca²⁺], to avoid overload. Therefore, after increase of Ca²⁺ release from internal Ca²⁺ stores due to PI pathway potentiation, the Ca²⁺ influx into platelets is possibly reduced to regulate [Ca²⁺].³⁵

In contrast, in group B, platelet activation and secretion were significantly inhibited compared with group A, and these inhibitory effects were likely attributed to theophylline. Inhibition of Ca²⁺ signal-

ing after treating platelets with various cyclic adenosine 3′,5′-monophosphate (cAMP) elevating agents, such as theophylline and prostacycline, has been observed through inhibition of phosphodiesterase, resulting in accumulation of cAMP.³ⁱ,³³ Several investigators have found that cyclic nucleotides inhibit PLC-induced phospholipid hydrolysis and inositol phosphate production,³⁶,³⁷ and others have proposed that cAMP-dependent phosphorylation may impair the activity of a PLC regulatory protein³⁸ or reduce Ca²⁺ release involving IP₃ receptors.³⁹ Furthermore, increased cAMP level has been suggested to activate resequstration of Ca²⁺ into the internal stores and/or extrusion of Ca²⁺ across plasma membranes.⁴⁰ Our results that in asthmatics taking oral theophylline the [Ca²⁺], elevations in, CD62P expression on, and RANTES release from platelets in response to stimulation by various agonists are inhibited, are consistent with the above reports. Recently, theophylline was shown to exhibit many anti-inflammatory effects.⁴¹,⁴² Our data suggest that these anti-inflammatory effects may involve the reduction of the release of chemokines, such as RANTES, from platelets.

In conclusion, our results suggest that platelets in asthmatics are easily activated upon stimulation resulting in the increased release of RANTES, which could play a role in bronchial asthma by attracting eosinophils, and that theophylline inhibit RANTES release from platelets as a part of anti-inflammatory effect.

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