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Activation of Platelets in Bronchial Asthma*

Chie Moritani, MD; Shinichi Ishioka, MD; Yoshinori Haruta, MD; Masayuki Kambe, MD; and Michio Yamakido, MD, FCCP

**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\(^{2+}\)]\(_i\)) 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\(^{2+}\)]\(_i\) in platelets were similar among the three groups, the [Ca\(^{2+}\)]\(_i\) 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\(^{2+}\)]\(_i\)=intracellular free calcium concentration; cAMP=cyclic adenosine monophosphate; EGTA=ethyleneglycol-bis-(β-aminoethylether)-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; P1=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 α-granules, which are chemo- kines such as platelet factor 4 (PF4)\(^2,4\) 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. For example, platelets from asthmatics were found not to undergo the second wave of aggregation following stimulation with platelet agonists in vitro, and a number of studies in vitro demonstrated that circulating platelets aggregated and platelet-specific proteins, PF4 and β-thromboglobulin, were released into the circulation and 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 from the cell surface as a result of mediator release from α-granules.

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, β2-agonists, anticholinergics, disodium cromoglycate), and the other (group B) comprised 18 patients who took oral theophylline preparation but did not use any medications known to interact with theophylline. Values are expressed as means ± SE.

Table 1—Characteristics of Normal Subjects and Asthmatic Patients

<table>
<thead>
<tr>
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<th>Normal Subjects</th>
<th>Group A</th>
<th>Group B</th>
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<tbody>
<tr>
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</tr>
<tr>
<td>Male</td>
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</tr>
<tr>
<td>Female</td>
<td>11</td>
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<td>8</td>
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<tr>
<td>Age, yr Mean ± SE</td>
<td>38.3 ± 2.6</td>
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<td>24-67</td>
<td>20-65</td>
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<tr>
<td>Nonatopic</td>
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<tr>
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</tr>
<tr>
<td>Moderate</td>
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<td>0</td>
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</tr>
<tr>
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<td>0</td>
</tr>
<tr>
<td>Inhalation, No.</td>
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<td>Corticosteroids</td>
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<tr>
<td>β2-Agonists</td>
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<td>Pulmonary function</td>
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<td>FVC, L</td>
<td>3.81 ± 0.8</td>
<td>3.63 ± 1.08</td>
<td>3.71 ± 1.11</td>
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<td>% predicted</td>
<td>108.9 ± 10.1</td>
<td>106.6 ± 14.7</td>
<td>104.9 ± 17.0</td>
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<tr>
<td>FEV1, L</td>
<td>3.25 ± 0.69</td>
<td>2.79 ± 1.01</td>
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<tr>
<td>% predicted</td>
<td>104.6 ± 14.9</td>
<td>91.0 ± 15.0</td>
<td>84.4 ± 13.5</td>
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</table>

*a* 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.

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 750 g for 5 min. Platelets were separated from plasma by gel filtration, diluted with Ca2+-free Na1-hepes-ethanesulfonic acid (HEPES) buffer (145 mM NaCl, 5 mM KCl, 1 mM MgSO4, 10 mM HEPES, and 5 mM glucose; pH 7.4) to a concentration of 107 cells per milliliter. The platelet preparations were >99.9% pure as judged by a hematology analyzer (Micro Diff II; Coulter Electronics Limited; Beds, UK). Subsequently, the platelet-rich buffer was incubated with 1 μM fura-2/acetoxymethyl ester (fura-2/AM; Molecular Probes; Eugene, Ore) and 0.02% (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 107 cells per milliliter were incubated with 1 mM CaCl2 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-
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 mM ethyleneglycol-bis(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA). 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 \([Ca^{2+}]_i\)s were calculated using the equation of Grynkiewicz et al. 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 \([Ca^{2+}]_i\)s were calculated using the equation of Grynkiewicz et al. Thrombin (Sigma Chemical Co, St Louis), PAF (Sigma Chemical Co), and thromboxane A\(_2\) (TXA\(_2\)) analog, 9,11-epithia-11,12-methano-TXA\(_2\) (STA\(_2\), 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 TXA\(_2\) are deeply associated with bronchial asthma and thrombin is generally used in activation of platelets. The optimal concentrations of the stimulants (thrombin, 1.0 U/mL; PAF, 10 nM; STA\(_2\), 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-μL aliquots of whole blood were put into polystyrene tubes containing 40 μL isotonic HEPES buffer, and 5 μL monoclonal antibody, fluorescein isothiocyanate (FITC) labeled anti-CD62P (CD62P/FITC; Serotec Ltd; Oxford, UK), and the required agonist were added to each tube. After incubation at 37°C for 5 min without stirring, the reactions were stopped by addition of 500 μL of 1% (w/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 IgG\(_1\)/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 10,000 g 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 \([Ca^{2+}]_i\)s 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±12.0, 445.1±15.1, and 422.1±14.5 nM, and after PAF stimulation were 130.5±4.7, 125.8±7.4, and 130.2±5.9 nM (Fig 1). There were no significant differences between any two groups. However, the \([Ca^{2+}]_i\) in group A after STA\(_2\) stimulation (109.7±7.4 nM) was significantly higher than that of the normal subjects (82.9±7.3 nM, p<0.05). The \([Ca^{2+}]_i\)s in the normal subjects and group B

![Figure 1](https://example.com/figure1.png)
(87.0±6.7 nM) after STA2 stimulation did not differ significantly, whereas the [Ca$^{2+}$]$_i$ in group B after STA2 stimulation was significantly lower than that in group A (p<0.05). In contrast, the respective [Ca$^{2+}$]$_i$s in the absence of external Ca$^{2+}$ 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 [Ca$^{2+}$]$_i$s in the absence of external Ca$^{2+}$ 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 [Ca$^{2+}$]$_i$s in the absence of external Ca$^{2+}$ after PAF stimulation of any two groups (Fig 1). In addition, we made a comparison between atopic and nonatopic asthmatics of each group, but the respective values did not differ significantly (data not shown). Neither values between $\beta_2$-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 they 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 ins-

![Figure 2. 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).](chestjournal.org)
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 \( \beta_2 \)-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 \( \beta_2 \)-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 \([\text{Ca}^{2+}]_{\text{c}}\)s after thrombin or STA2 stimulation in the absence of external \( \text{Ca}^{2+} \) in group A were significantly higher than those in the normal subjects, indicating that the amount of \( \text{Ca}^{2+} \) 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).](image)
second messengers, inositol 1,4,5-triphosphate (IP3), which mobilizes Ca\(^{2+}\) from internal Ca\(^{2+}\) stores, and diacylglycerol, which activates protein kinase C (PKC). This chain of systematic responses is called the PI pathway and, therefore, increased Ca\(^{2+}\) release from internal stores may suggest potentiation of the PI pathway. Block et al\(^{24}\) 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 [Ca\(^{2+}\)]i, elevation in aggregation of and secretion by human platelets have not been elucidated.\(^{26-30}\)

With respect to secretion, Walker and Watson\(^{31}\) recently reported that the signaling events required for mediator secretion are [Ca\(^{2+}\)]i, 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 [Ca\(^{2+}\)]i 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 [Ca\(^{2+}\)]i of their platelets in the presence of external Ca\(^{2+}\) were not elevated significantly compared with those of normal subjects. We suspected that this may be due to a mechanisms regulating Ca\(^{2+}\) entry into platelets. In human platelets, the Ca\(^{2+}\) entry mechanisms are still unclear, and conflicting results have been reported. For instance, several studies have indicated the existence of a Ca\(^{2+}\) entry pathway secondary to Ca\(^{2+}\) depletion of store, the store-regulated influx pathway,\(^{32,33}\) whereas receptor-evoked entry pathways have been demonstrated by others.\(^{34}\) In any case, Ca\(^{2+}\) in the cytoplasm is cytotoxic, so it is necessary to have a mechanism that regulates [Ca\(^{2+}\)]i to avoid overload. Therefore, after increase of Ca\(^{2+}\) release from internal Ca\(^{2+}\) stores due to PI pathway potentiation, the Ca\(^{2+}\) influx into platelets is possibly reduced to regulate [Ca\(^{2+}\)]i.

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 Ca\(^{2+}\) signal-

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**References**


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