

# Platelets Are Highly Activated in Patients of Chronic Thromboembolic Pulmonary Hypertension

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**Objective**—Chronic thromboembolic pulmonary hypertension (CTEPH) is a fatal disease that is distinct from pulmonary arterial hypertension (PAH). Although CTEPH is characterized by obstruction of major pulmonary artery because of chronic thrombus, it remains unclear whether CTEPH is associated with prothrombotic condition.

**Approach and Results**—In addition to conventional markers, GTP-bound levels of Rap1, RhoA, RalA, Rac1, and Ras in platelets, which are implicated for platelet activation, were measured in patients without pulmonary hypertension (non-PH, n=15), patients with PAH (n=19), and patients with CTEPH (n=25). Furthermore, the responsiveness to ex vivo thrombin stimulation was also evaluated. The ratios of the P-selectin positive platelets in the non-PH patients, patients with PAH, and patients with CTEPH were 1.40% (median and interquartile range, 0.83–1.82), 2.40% (1.80–3.39), and 2.63% (1.90–8.22), respectively (non-PH versus CTEPH,  $P<0.01$ ). The activated GPIIb/IIIa-positive platelets were 6.01% (1.34–7.87), 11.39% (5.69–20.86), and 9.74% (7.83–24.01), respectively (non-PH versus CTEPH,  $P=0.01$ ). GTP-bound RhoA was 1.79% (0.94–2.83), 4.03% (2.01–5.14), and 2.01% (1.22–2.48), respectively (non-PH versus PAH,  $P=0.04$ ), and GTP-bound RalA was 1.58% (1.08–2.11), 3.02% (2.03–3.54), and 2.64% (1.42–4.28), respectively (non-PH versus PAH,  $P=0.023$ ; non-PH versus CTEPH,  $P=0.048$ ). In contrast, Rac1, Rap1, or Ras was not activated in any groups. The platelets of patients with CTEPH exhibited hyperresponsiveness to ex vivo thrombin stimulation compared with those of non-PH patients when evaluated for the surface markers. Either D-dimer or fibrin degradation product level was not increased in patients with CTEPH.

**Conclusions**—These results provide the first direct evidence that platelets of patients with CTEPH are highly activated and exhibit hyperresponsiveness to thrombin stimulation. (*Arterioscler Thromb Vasc Biol.* 2014;34:2486-2494.)

**Key Words:** blood platelets ■ chronic thromboembolic pulmonary hypertension ■ GTP-binding proteins ■ pulmonary hypertension

Chronic thromboembolic pulmonary hypertension (CTEPH) is a fatal disease that is distinct from pulmonary arterial hypertension (PAH) including idiopathic pulmonary artery hypertension and collagen disease-related pulmonary hypertension in the Dana Point Classification.<sup>1</sup> CTEPH is characterized by obstruction of the major pulmonary arteries because of organized thrombus and distal pulmonary vasculopathy.<sup>2,3</sup> Usually, the severity of CTEPH is gradually accelerated without drastic embolic events,<sup>4</sup> causing elevated pulmonary vascular resistance, increased pulmonary arterial pressure, and finally right heart failure and death.

There are 2 systems for hemostasis and thrombosis in our body: one is the coagulation system and the other is the platelet system. As the final product of the activated coagulation system by a series of protease cascade of coagulation factors, fibrin clot is formed. On the other hand, activated platelets secrete granules containing bioactive substances such as ADP and

P-selectin, aggregate each other mediated by activated integrin  $\alpha$ IIb $\beta$ 3 (GPIIb/IIIa). These cellular functions are regulated by intracellular molecular switches such as small GTPases. It has been demonstrated that Rap1B<sup>5</sup> and RhoA<sup>6,7</sup> regulate aggregation while Rab27<sup>8</sup> and Ral<sup>9</sup> control granule secretion. Furthermore, RhoA<sup>10,11</sup> and Rac1<sup>12</sup> are implicated in sharp change through the regulation of cytoskeletal reorganization.

Although CTEPH is considered as a thromboembolic disorder, it remains unclear whether it is associated with prothrombotic condition. The association of coagulation states with CTEPH has been shown only in specific conditions such as lupus anticoagulant/antiphospholipid antibodies.<sup>13</sup> However, most of patients with CTEPH are free from these disorders. The association between CTEPH and the activation of platelets is suggested based on the findings that platelet activating states such as splenectomy or thyroid hormone replacement are a risk factor of CTEPH in a few reports.<sup>14,15</sup> Nevertheless,

Received on: February 3, 2014; final version accepted on: August 13, 2014.

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The online-only Data Supplement is available with this article at <http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.114.304404/-/DC1>.

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*Arterioscler Thromb Vasc Biol* is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.114.304404

| Nonstandard Abbreviations and Acronyms |   |
|--|---|
| <b>CTEPH</b>                           | chronic thromboembolic pulmonary hypertension |
| <b>FDP</b>                             | fibrin degradation product                    |
| <b>GAP</b>                             | GTPase activity protein                       |
| <b>PAH</b>                             | pulmonary arterial hypertension               |
| <b>PAR</b>                             | protease-activated receptor                   |
| <b>PH</b>                              | pulmonary hypertension                        |
| <b>VCAM-1</b>                          | vascular cell adhesion molecule-1             |

there have been few reports linking directly CTEPH to platelets activation, to date.

In the present study, we thus investigated whether the coagulation and platelet systems were activated in patients without pulmonary hypertension (non-PH), patients with PAH, and patients with CTEPH. In addition to the conventional markers reflecting the activation states of coagulation and platelets, we analyzed the activation levels of several small GTPases, such as Rap1, RhoA, RalA, Rac1, and Ras in platelets, because

**Table 1. Clinical Characteristics of the Non-PH Patients, Patients With PAH, and Patients With CTEPH**

|  | Non-PH<br>(n=15) | PAH<br>(n=19) | CTEPH<br>(n=25) | P Value |
|--|------------------|---------------|-----------------|---------|
| Age, y   | 55.2±12.9        | 52.9±14.6     | 59.9±12.6       | 0.22    |
| Female, %  | 11 (73.3)        | 16 (84.2)     | 22 (84.6)       | 0.51    |
| White blood cell, 10 <sup>3</sup> /μL                      | 5.59±1.38        | 5.53±1.54     | 5.44±1.00       | 0.94    |
| Hemoglobin, g/dL   | 13.1±1.9         | 12.2±1.5      | 12.4±1.7        | 0.28    |
| Platelet, 10 <sup>9</sup> /μL                              | 224±77           | 197±61        | 213±53          | 0.46    |
| Mean platelet volume, fL                                   | 9.33±0.92        | 8.61±1.15     | 8.68±0.84       | 0.09    |
| Platelet distribution width, %                             | 14.6±2.7         | 15.6±2.4      | 16.4±0.6*       | 0.03    |
| Total bilirubin, mg/dL                                     | 0.83±0.29        | 0.86±0.40     | 0.68±0.25       | 0.15    |
| Total cholesterol, mg/dL                                   | 181.2±42.7       | 172.6±40.7    | 196.7±44.2      | 0.18    |
| LDL cholesterol, mg/dL                                     | 99.85±30.6       | 96.4±26.0     | 111.3±34.1      | 0.28    |
| HDL cholesterol, mg/dL                                     | 54.7±24.0        | 54.8±16.6     | 60.6±19.4       | 0.54    |
| Uric acid, mg/dL   | 4.9±1.3          | 5.2±1.5       | 5.4±1.3         | 0.51    |
| Fibrin degradation product, μg/mL                          | 2.93±0.94        | 3.25±1.24     | 2.69±0.63       | 0.17    |
| D-dimer, μg/mL   | 0.88±0.50        | 1.63±3.28     | 0.68±0.31       | 0.26    |
| Diabetes mellitus, %                                       | 1 (6.7)          | 3 (15.8)      | 1 (4.0)         | 0.43    |
| History of acute coronary syndrome, %                      | 1 (6.7)          | 1 (5.2)       | 0               | 0.33    |
| Glomerular filtration rate, mL/min per 1.73 m <sup>2</sup> | 74.0±18.3        | 68.7±20.7     | 68.4±16.3       | 0.61    |
| Body mass index, kg/m <sup>2</sup>                         | 23.3±3.9         | 23.0±3.6      | 24.3±4.1        | 0.51    |
| NYHA Class, %  |                  |               |                 | <0.01   |
| I  | 12 (80.0)        | 4 (21.1)      | 6 (24.0)        |         |
| II   | 3 (20.0)         | 14 (73.7)     | 17 (68.0)       |         |
| III  | 0                | 1 (5.3)       | 2 (8.0)         |         |

Data are presented as mean±SD. CTEPH indicates chronic thromboembolic pulmonary hypertension; HDL, high-density lipoprotein; LDL, low-density lipoprotein; non-PH, patients without pulmonary hypertension; NYHA, New York Heart Association; and PAH, pulmonary artery hypertension.

\*P<0.05 vs non-PH with Tukey honestly significant difference test.

they were implicated as the novel factors for platelet activation. Furthermore, we also evaluated the responsiveness of platelets to ex vivo thrombin stimulation.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

#### Baseline Characteristics

We confirmed the principles outlined in the Declaration of Helsinki. The study protocol was approved by the Ethical Committees of Tohoku University, and all patients provided written informed consent. The baseline characteristics of the enrolled patients are shown in Table 1. This study enrolled the non-PH patients as control patients (n=15: hypertension n=7, hyperlipidemia n=5, hypertrophic cardiomyopathies n=2, and paroxysmal atrial fibrillation n=1), patients with PAH (n=19: idiopathic pulmonary hypertension n=10, collagen diseases with pulmonary hypertension n=8, and congenital heart disease with pulmonary hypertension n=1), and patients with CTEPH (n=25). All patients with PAH and CTEPH and 10 among 15 patients of non-PH patients underwent cardiac catheterization in our institute from October, 2012, to June, 2013. The diagnosis of patients with PAH and CTEPH was made according to the European Society of Cardiology (ESC)/ European Respiratory Society (ERS) Guideline.<sup>16</sup> The ratios of diabetes mellitus, history of acute coronary syndromes, glomerular filtration rate, and obesity were comparable among 3 groups (Table 1). No patients had undergone splenectomy. The drugs used for the enrolled patients are shown in Table 2. The ratio of use of unfractionated heparin was comparable among 3 groups.

The hemodynamic parameters of the enrolled patients are shown in Table 3. Mean pulmonary artery pressure (mm Hg) of

**Table 2. Drugs of the non-PH Patients, Patients With PAH, and Patients With CTEPH**

|   | Non-PH<br>(n=15) | PAH<br>(n=19) | CTEPH<br>(n=25) | P Value |
|---|------------------|---------------|-----------------|---------|
| ARB/ACE, %  | 3 (20.0)         | 3 (15.8)      | 8 (32.0)        | 0.47    |
| Ca-blocker, %   | 7 (46.7)         | 3 (15.8)      | 5 (20.0)        | 0.10    |
| β-Blocker, %  | 5 (33.3)         | 0             | 1 (4.0)         | <0.01   |
| Statin, %   | 7 (46.7)         | 1 (5.3)       | 5 (20.0)        | 0.01    |
| Aspirin, %  | 2 (13.3)         | 0             | 3 (12.0)        | 0.30    |
| ADP receptor blocker, %                                   | 0                | 0             | 0               | -       |
| Epoprostenol, %   | 0                | 6 (31.6)      | 1 (4.0)         | <0.01   |
| Oral PGI <sub>2</sub> analogue, %                         | 0                | 8 (42.1)      | 14 (56.0)       | <0.01   |
| Endothelin receptor antagonist, %                         | 0                | 14 (73.7)     | 8 (32.0)        | <0.01   |
| PDE V inhibitor, %  | 0                | 16 (84.2)     | 18 (72.0)       | <0.01   |
| Warfarin, %   | 2 (13.3)         | 7 (36.8)      | 23 (92.0)       | <0.01   |
| Low molecular weight heparin or unfractionated heparin, % | 3 (20.0)         | 8 (42.1)      | 12 (48.0)       | 0.20    |

Data are presented as n (%). ACE indicates angiotensin-converting enzyme; ARB, angiotensin receptor blocker; CTEPH, chronic thromboembolic pulmonary hypertension; non-PH, patients without pulmonary hypertension; PAH, pulmonary artery hypertension; PDE, phosphodiesterase; and PGI<sub>2</sub>, prostaglandin I<sub>2</sub>.

**Table 3. Hemodynamic Characteristics of the Non-PH Patients, Patients With PAH, and Patients With CTEPH**

|                              | Non-PH (n=10) | PAH (n=19)    | CTEPH (n=25) | P Value |
|------------------------------|---------------|---------------|--------------|---------|
| Mean RAP, mmHg               | 4.5±2.1       | 5.5±2.8       | 7.1±2.9      | 0.06    |
| Systolic PAP, mmHg           | 29.1±3.9      | 55.4±19.8*    | 48.8±18.2†   | <0.01   |
| Diastolic PAP, mmHg          | 11.5±3.9      | 25.2±10.1*    | 18.0±5.6‡    | <0.01   |
| Mean PAP, mmHg               | 17.9±2.8      | 37.1±13.4*    | 28.8±8.7‡    | <0.01   |
| PCWP, mmHg                   | 9.6±3.6       | 8.4±2.9       | 11.2±3.2‡    | 0.03    |
| Systolic AoP, mmHg           | 143.1±29.2    | 100.0±15.3*   | 119.4±20.3*‡ | <0.01   |
| Diastolic AoP, mmHg          | 71.9±12.6     | 58.5±12.6*    | 63.1±11.9†   | <0.01   |
| Mean AoP, mmHg               | 104.5±15.7    | 74.3±13.4*    | 83.8±16.2*   | <0.01   |
| PVR, dyn·s·cm <sup>-5</sup>  | 141.2±28.9    | 407.2±158.7*  | 373.5±196.5* | <0.01   |
| SVR, dyn·s·cm <sup>-5</sup>  | 1770.5±454.6  | 1227.6±340.3* | 1545.8±488.5 | <0.01   |
| CO, L/min                    | 4.71±1.54     | 4.95±0.97     | 4.17±0.78‡   | 0.05    |
| CI, L/min per m <sup>2</sup> | 2.99±0.66     | 3.24±0.58     | 2.62±0.49‡   | <0.01   |
| HR, /min                     | 64.0±12.6     | 72.6±10.9†    | 63.9±7.2‡    | <0.01   |

Data are presented as mean±SD. Data of non-PH patients were 10 among 15 patients of non-PH patients who underwent cardiac catheterization. AoP indicates arterial pressure; CI, cardiac index; CO, cardiac output; CTEPH, chronic thromboembolic pulmonary hypertension; HR, heart rate; non-PH, patients without pulmonary hypertension; PAH, pulmonary artery hypertension; PAP, pulmonary artery pressure; PCWP, pulmonary wedge pressure; PVR, pulmonary vascular resistance; RAP, right atrial pressure; and SVR, systemic vascular resistance.

\**P*<0.01 vs non-PH, †*P*<0.05. ‡*P*<0.05 vs PAH with Tukey honestly significant difference test.

the non-PH patients undergoing cardiac catheterization (n=10), patients with PAH, and patients with CTEPH was 17.9±2.8 (mean±SD), 37.1±13.4, and 28.8±8.7 (non-PH versus PAH,

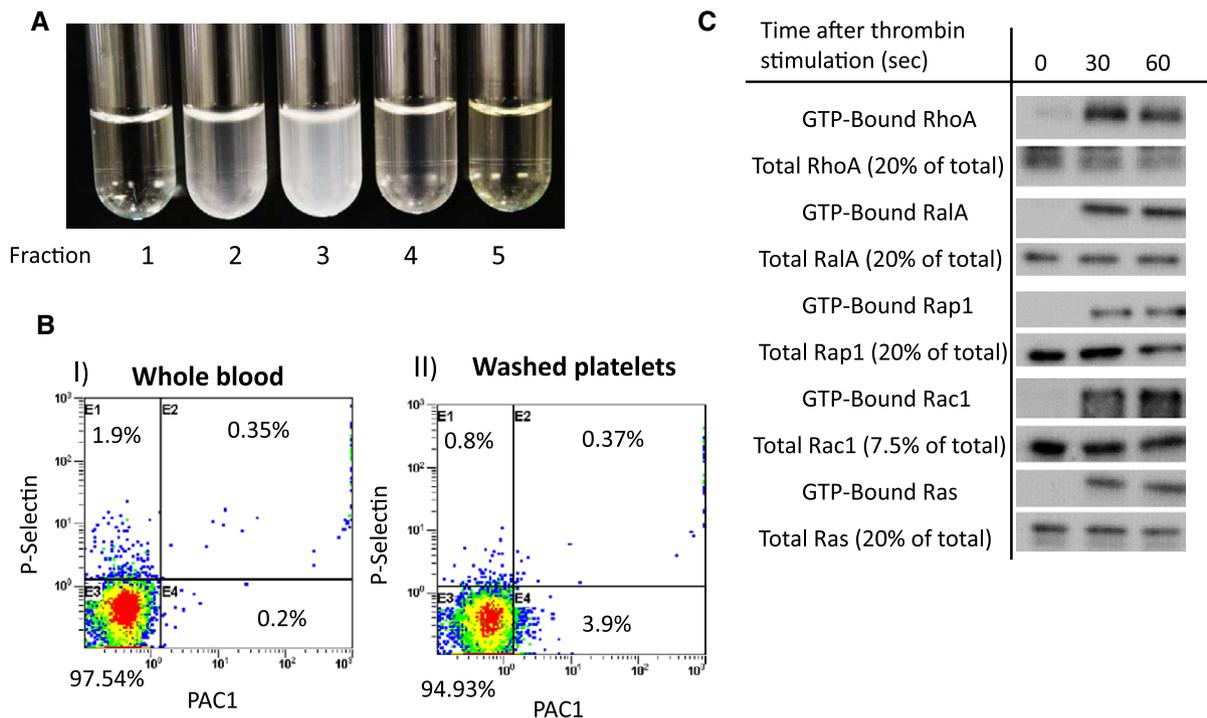
*P*<0.01; non-PH versus CTEPH, *P*=0.01; PAH versus CTEPH, *P*=0.04), respectively. The rest 5 patients who did not undergo cardiac catheterization in the non-PH group exhibited no pulmonary hypertension evaluated by echocardiography (peak pressure gradient of tricuspid regurgitation 21.7±3.8 mmHg [mean±SD]). However, mean aortic pressure (mmHg) of the non-PH patients, patients with PAH, and patients with CTEPH was 104.5±15.7, 74.3±13.4, and 83.8±16.2 (non-PH versus PAH, *P*<0.01; non-PH versus CTEPH, *P*<0.01), respectively.

**D-Dimer or Fibrin Degradation Product Level Was Not Increased in Patients With CTEPH**

D-dimer level (µg/mL) of non-PH patients, patients with PAH, and patients with CTEPH was 0.88±0.50, 1.63±3.28, and 0.68±0.31 (non-PH versus PAH, *P*=0.54; non-PH versus CTEPH, *P*=0.95), respectively (Table 1). Fibrin degradation product (FDP) level (µg/mL) was 2.93±0.94, 3.25±1.24, and 2.69±0.63 (non-PH versus PAH, *P*=0.63; non-PH versus CTEPH, *P*=0.75), respectively. Both D-dimer and FDP are degraded products of fibrin and are used as the markers of the acute thrombosis.<sup>17,18</sup> Although D-dimer and FDP levels tended to be increased in patients with PAH, these markers were not elevated in patients with CTEPH.

**Platelet Preparation and Establishment of a Novel Method Evaluating the Activation States of Small GTPases in Platelets**

Platelet rich plasma was prepared from the whole blood, and 1 mL platelet rich plasma was separated on Sepharose2B gel filtration column, where each 1 mL fraction was collected. The washed



**Figure 1.** Isolation of washed platelets and their characterization. **A**, Platelet rich plasma was separated by Sepharose 2B gel filtration column, and each 1 mL fraction was collected as described in the Methods section in the online-only Data Supplement. In the fractions 2 and 3, washed platelets were eluted while serum was eluted after the fraction 5. **B**, The surface expression of P-selectin and PAC-1 binding of platelets with flow cytometric analysis in whole blood (left) and the isolated washed platelets (right). **C**, The washed platelets were stimulated by 0.25 U/mL thrombin at 30°C for indicated time, and various GTP-bound GTPases were analyzed by the pull down assay as described in the Methods section in the online-only Data Supplement.

platelets, appearing cloudy, were efficiently separated from the serum appearing yellow (Figure 1A, fractions 2 and 3 compared with fraction 5). The analysis with flow cytometry revealed that there was no difference in P-selectin expression or PAC-1 binding between the platelets in whole blood and the washed platelets (Figure 1B), indicating that the platelets were not activated during this isolation process. Therefore, we used the washed platelets (fractions 2 and 3) in the following analysis of platelet activity.

To confirm that the pull down assays could evaluate activity of various GTPases (RhoA, RalA, Rac1, Rap1, and Ras), the washed platelets were stimulated with 0.25 U/mL thrombin at 30°C, and their time-dependent activations were analyzed by the pull down assay (see the Methods section in the online-only Data Supplement). Activities of all the GTPases were clearly elevated after the thrombin stimulation (Figure 1C), indicating that the pull down assay worked well.

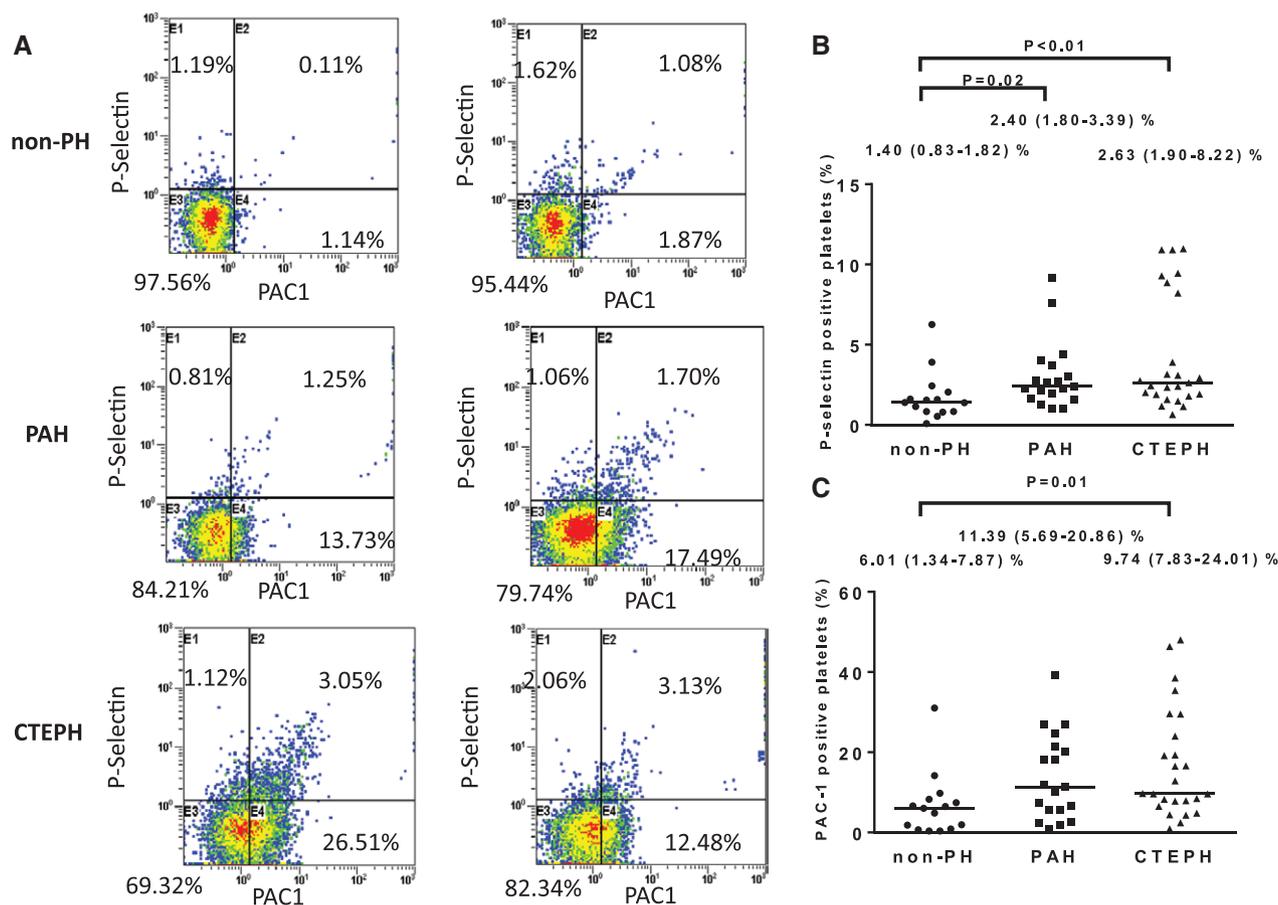
### Surface Expression Levels of P-Selectin and PAC-1 Binding Were Elevated in Platelets of Patients With CTEPH and PAH

We first analyzed activated states of platelets by flow cytometry. As shown in Figure 2A, the platelets of patients with PAH and CTEPH were highly activated compared with those

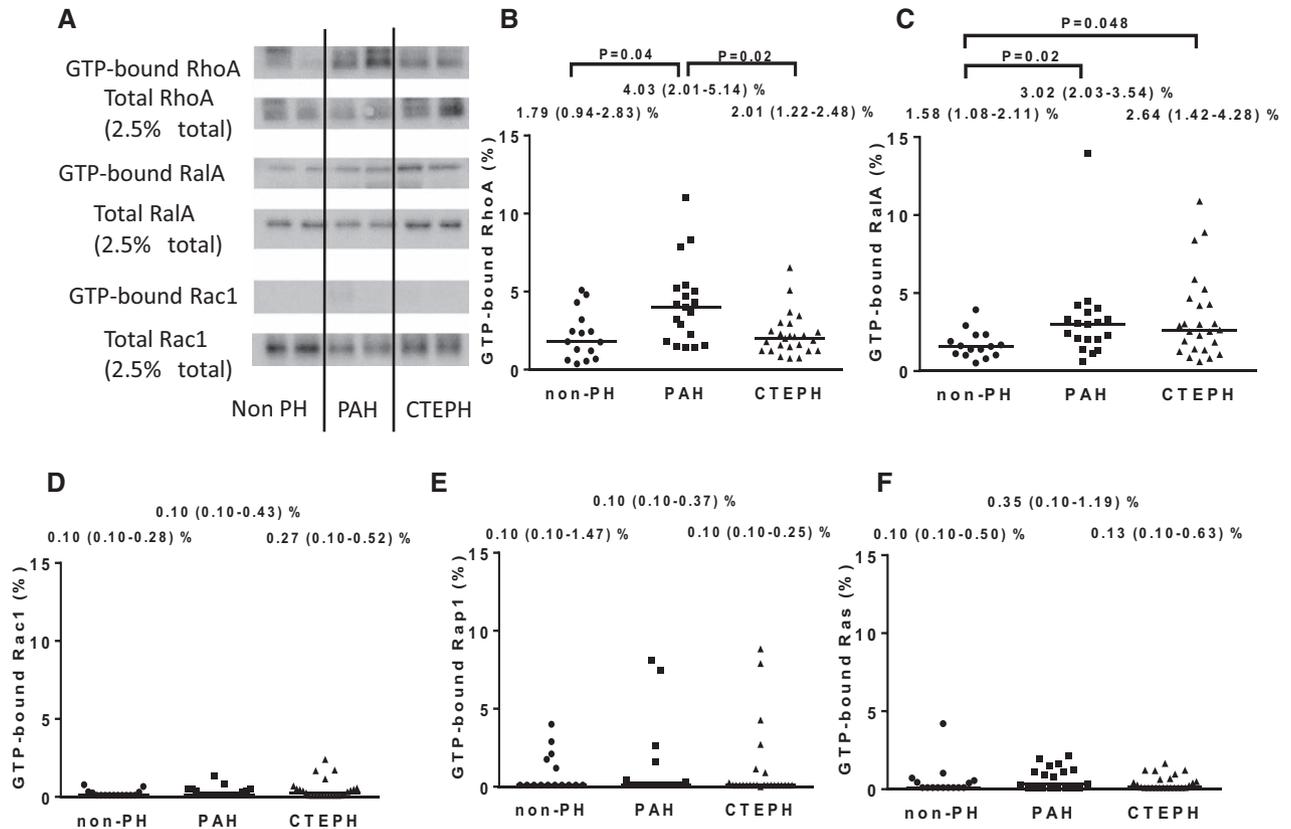
of non-PH patients when evaluated for surface expression levels of P-selectin (a marker of granule secretion) and PAC-1 binding (a marker of GPIIb/IIIa activation). The P-selectin-positive platelets of non-PH patients, patients with PAH, and patients with CTEPH were 1.40% (0.83–1.82; median [interquartile range]), 2.40% (1.80–3.39), and 2.63% (1.90–8.22), respectively (Figure 2B; non-PH versus PAH,  $P=0.02$ ; non-PH versus CTEPH,  $P<0.01$ ). The PAC-1-positive platelets of non-PH patients, patients with PAH, and patients with CTEPH were 6.01% (1.34–7.87), 11.39% (5.69–20.86), and 9.74% (7.83–24.01), respectively (Figure 2C; non-PH versus PAH,  $P=0.07$ ; non-PH versus CTEPH,  $P=0.01$ ). Thus, the platelets were highly activated in patients with PAH and CTEPH compared with non-PH patients.

### RalA Was Activated in Platelets of Patients With PAH and CTEPH While RhoA Was in Those of Patients With PAH

GTP-bound RhoA of the non-PH patients, patients with PAH, and patients with CTEPH was 1.79% (0.94–2.83), 4.03% (2.01–5.14), and 2.01% (1.22–2.48), respectively (Figure 3A and 3B; non-PH versus PAH,  $P=0.04$ ). The GTP-bound RalA of the non-PH patients, patients with PAH, and patients with



**Figure 2.** Evaluation of platelet activation by flow cytometry. Surface expressions of P-selectin and PAC-1 binding were evaluated by flow cytometry as described in the Methods section in the online-only Data Supplement. **A**, Typical results by the flow cytometric analysis. Analysis for P-selectin expression (**B**) and PAC-1 binding (**C**). The line represents the median value. The ratios of P-selectin- or PAC-1-positive platelets of each patient group were expressed as median (interquartile range). Statistical significance was determined with the Steel–Dwass test. CTEPH indicates chronic thromboembolic pulmonary hypertension; PAH, pulmonary arterial hypertension; and PH, pulmonary hypertension.



**Figure 3.** Activation of various small GTPases in the platelets evaluated with pull down assay. **A**, Typical results of immunoblotting for GTP-bound RhoA, RalA, and Rac1 and total RhoA, RalA, and Rac1 (2.5% total) are shown. The individual data of RhoA (**B**), RalA (**C**), Rap1 (**D**), Rac1 (**E**), and Ras (**F**) were analyzed. The line represents the median value. The percentage of GTP-bound GTPases of each group is expressed as median (interquartile range). Statistical significance was determined with the Steel-Dwass test. CTEPH indicates chronic thromboembolic pulmonary hypertension; PAH, pulmonary arterial hypertension; and PH, pulmonary hypertension.

CTEPH was 1.58% (1.08–2.11), 3.02% (2.03–3.54), and 2.64% (1.42–4.28), respectively (Figure 3C; non-PH versus PAH,  $P=0.02$ ; non-PH versus CTEPH,  $P=0.048$ ). However, Rap1, Rac1, and Ras were not activated in any group (Figure 3D–3F). Thus, in platelets, RhoA was significantly activated in patients with PAH and RalA in both patients with PAH and CTEPH compared with non-PH patients.

### Reduced Expression of RalGAP $\beta$ in Platelets of Patients With CTEPH Might Mediate the Elevated RalA Activity

Activation levels of small GTPases are regulated by stimulatory regulators, guanine nucleotide exchange factors, and inhibitory regulators, GTPase activity proteins (GAPs). Although each GTPase generally has many kinds of its guanine nucleotide exchange factors and GAPs, only 2 molecules are known to have RalGAP activity in human cells that are RalGAP-1 (a heterodimer consisting of  $\alpha 1$  and  $\beta$  subunits) and RalGAP-2 (that consisting of  $\alpha 2$  and  $\beta$  subunits). Because complex formation of  $\alpha$  and  $\beta$  subunits is essential for the GAP activity, as well as mutual protein stability, the RalGAP activity could be monitored by evaluating the amount of common RalGAP $\beta$  subunit.<sup>19,20</sup>

To address the cause of elevated activation of RalA in patients with PAH and CTEPH, we evaluated the expression levels of RalGAP $\beta$  subunit in platelets. As shown in

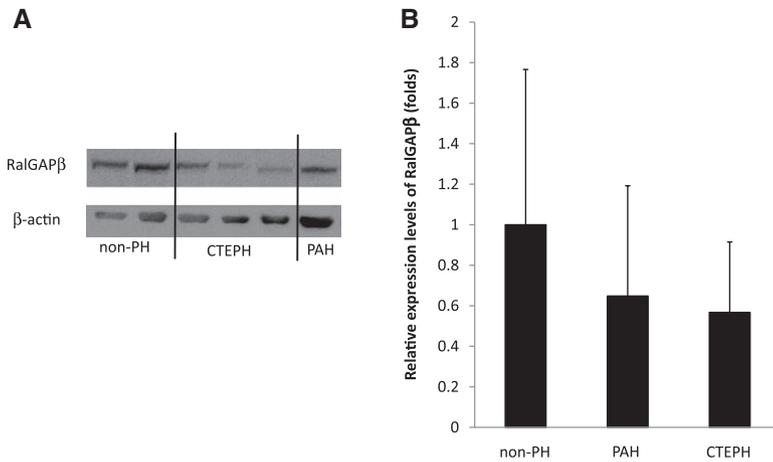
Figure 4, relative expression levels of platelets of non-PH patients ( $n=9$ ), patients with PAH ( $n=11$ ), and patients with CTEPH ( $n=17$ ) were  $1\pm 0.76$ ,  $0.65\pm 0.54$ , and  $0.57\pm 0.35$  folds, relatively (Figure 4). Thus, RalGAP $\beta$  in platelets tended to be decreased in patients with PAH and CTEPH. Reduced expression levels of RalGAP might cause elevated RalA activity.

### Multivariate Regression Analysis for the Activation of Platelets in Patients With PAH and CTEPH

The activation of platelets is influenced by the drugs used for the treatment of PH, especially epoprostenol.<sup>21</sup> We analyzed whether the difference of the activation of RhoA and RalA or the ratio of P-selectin- and PAC-1-positive platelets was influenced by the drugs.

The multiple regression analysis with backward elimination stepwise variable selection was used to explore an optimal set of the drugs which involves starting with the initial set of PH drugs, including epoprostenol, oral prostaglandin I<sub>2</sub> analogue, endothelin receptor antagonist, and phosphodiesterase V inhibitor. The final models are listed in Table 4.

The activation of RhoA was positively influenced by patients with PAH (2.592,  $P<0.001$ ) and negatively influenced by epoprostenol ( $-1.853$ ,  $P=0.026$ ). The activation of RalA was positively influenced by patients with CTEPH (1.723,  $P=0.033$ ).



**Figure 4.** The expression levels of RalGAP $\beta$  in the platelets. **A**, Representative results of the expression of RalGAP $\beta$  in the platelets of non-pulmonary hypertension (PH) patients, patients with pulmonary arterial hypertension (PAH), and patients with chronic thromboembolic pulmonary hypertension (CTEPH). **B**, Quantification of the RalGAP $\beta$  expression levels in the platelets of non-PH patients (n=9), patients with PAH (n=11), and patients with CTEPH (n=17). The RalGAP $\beta$  expression levels were adjusted with  $\beta$ -actin levels, and the relative levels (mean $\pm$ SD) were expressed using RalGAP $\beta$  expression levels in control group as 1.0.

The ratio of P-selectin positive platelets was positively influenced by patients with CTEPH (3.315,  $P=0.005$ ). The ratio of PAC-1 positive platelets was positively influenced by patients with CTEPH (10.122,  $P=0.010$ ).

Thus, the RalA was activated in platelets of patients with CTEPH, and the platelets were more activated in patients with CTEPH than non-PH patients.

After the stimulation of thrombin, the ratio of PAC-1 positive platelets was positively influenced by patients with PAH

**Table 4. Multivariate Regression Analysis Examining Factors Affecting the Expression Levels of RhoA, RalA, P-Selectin-Positive Platelets, and PAC-1-Positive Platelets**

|  | Estimate | P Value |
|--|----------|---------|
| <b>RhoA</b>  |          |         |
| Patients with PAH  | 2.592    | <0.001  |
| Patients with CTEPH  | 0.117    | 0.845   |
| Epoprostenol   | -1.853   | 0.026   |
| <b>RalA</b>  |          |         |
| Patients with PAH  | 1.534    | 0.070   |
| Patients with CTEPH  | 1.723    | 0.033   |
| <b>P-selectin-positive platelets</b>                           |          |         |
| Patients with PAH  | 1.866    | 0.146   |
| Patients with CTEPH  | 3.315    | 0.005   |
| Epoprostenol   | 2.753    | 0.049   |
| Oral prostaglandin I <sub>2</sub> analogue                     | 1.913    | 0.048   |
| PDE V inhibitor  | -2.689   | 0.013   |
| <b>PAC-1-positive platelets</b>                                |          |         |
| Patients with PAH  | 7.094    | 0.081   |
| Patients with CTEPH  | 10.122   | 0.010   |
| <b>P-selectin-positive platelets with thrombin stimulation</b> |          |         |
| Patient with PAH   | 15.482   | 0.049   |
| Patients with CTEPH  | 11.718   | 0.117   |
| <b>PAC-1-positive platelets with thrombin stimulation</b>      |          |         |
| Patients with PAH  | 26.13    | 0.017   |
| Patients with CTEPH  | 30.900   | 0.004   |

Adjusted for patients group, epoprostenol, oral prostaglandin I<sub>2</sub> analogue, endothelin receptor antagonist, and PDE V inhibitor. CTEPH indicates chronic thromboembolic pulmonary hypertension; PAH, pulmonary arterial hypertension; and PDE, phosphodiesterase.

(26.13,  $P=0.017$ ) and CTEPH (30.90,  $P=0.004$ ). Thus, the platelets of patients with PAH and CTEPH were more hyper-responsive than non-PH patients to thrombin.

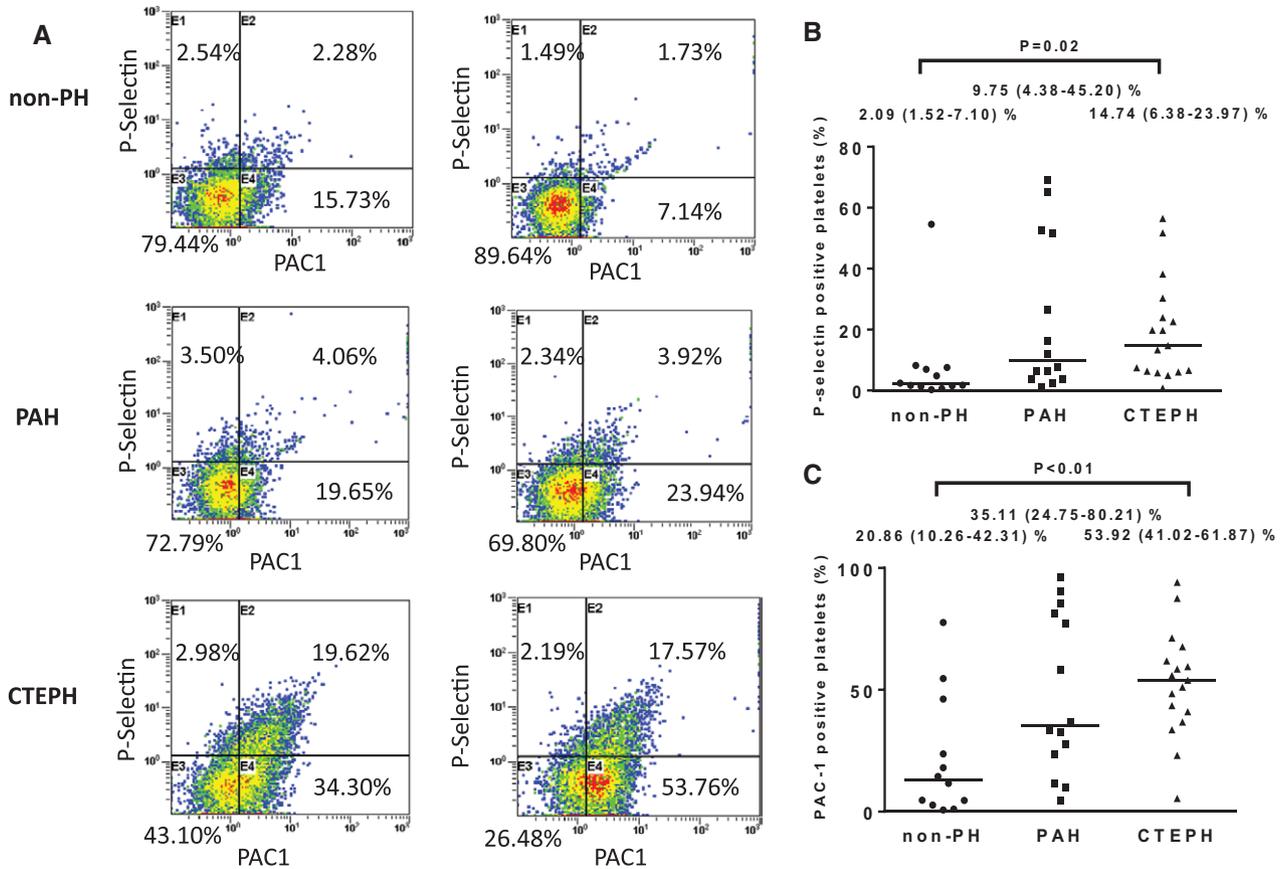
**Platelets of Patients With CTEPH Were Hypersensitive to Thrombin Stimulation, Mediated by Protease-Activated Receptor 1**

The responsiveness of platelets was evaluated after stimulation by 0.05 U/mL thrombin. P-selectin-positive platelets of the non-PH patients, patients with PAH, and patients with CTEPH were 2.09% (1.52–7.10), 9.75% (4.38–45.20), and 14.74% (6.38–23.97), respectively (Figure 5B; non-PH versus PAH,  $P=0.07$ ; non-PH versus CTEPH,  $P=0.02$ ). PAC-1-positive platelets of the non-PH patients, patients with PAH, and patients with CTEPH were 20.86% (10.26–42.31), 35.11% (24.75–80.21), and 53.92% (41.02–61.87), respectively (Figure 5C; non-PH versus PAH,  $P=0.06$ ; non-PH versus CTEPH,  $P<0.01$ ). Thrombin stimulation at 0.1 U/mL induced similar response to thrombin stimulation at 0.05 U/mL (Figure I in the online-only Data Supplement). Thus, the platelets of patients with CTEPH were significantly activated by the thrombin stimulation than those of non-PH patients (Figure 5A).

Protease-activated receptor (PAR) 1 and PAR4 are known to mediate thrombin signaling in platelets.<sup>22</sup> PAR1 agonist induced similar response to thrombin in patients with PAH and CTEPH while PAR4 agonist hardly stimulated platelets (Figure I in the online-only Data Supplement), suggesting that PAR1 played a dominant role in the hyperresponsive to thrombin stimulation in patients with PAH and CTEPH.

**Thrombin Hyperresponsive Platelets in Patients With PAH and CTEPH Induced the Expression of Vascular Cell Adhesion Molecule-1 in Human Lung Microvascular Endothelial Cells**

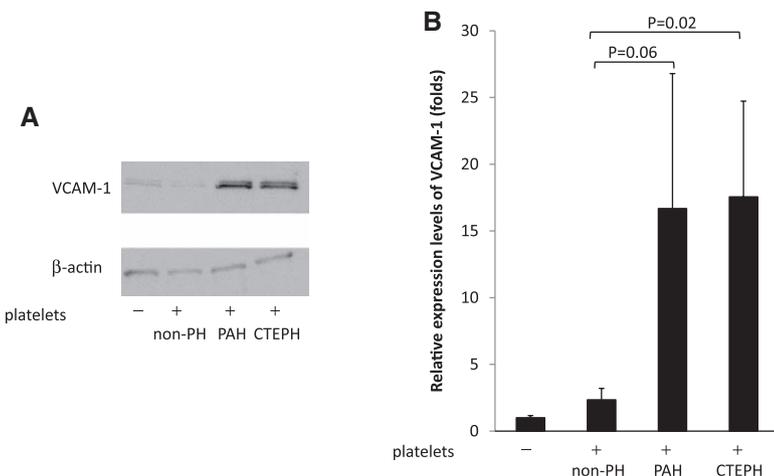
We evaluated functional relevance of the thrombin hyperresponsiveness of platelets in patients with PAH and CTEPH. Many chemical mediators would be released from activated platelets and induce the expression of inflammatory proteins such as vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells.<sup>23</sup> We examined the expression levels of VCAM-1 in human lung microvascular endothelial cells, after incubation with conditioned media of platelets of non-PH patients



**Figure 5.** Responsiveness of washed platelets to thrombin stimulation. **A**, Representative results of the surface expression of P-selectin and PAC-1 binding on washed platelets of non-pulmonary hypertension (PH) patients, patients with pulmonary arterial hypertension (PAH), and patients with chronic thromboembolic pulmonary hypertension (CTEPH) after stimulation with 0.05 U/mL thrombin at 25°C, 20 minutes. Individual data of the ratios of P-selectin (**B**) and PAC-1 binding (**C**) in non-PH patients, patients with PAH, and patients with CTEPH. The line represents the median value. The percentage of P-selectin- or PAC-1-positive platelets of each group was expressed as median (interquartile range). Statistical significance was determined with the Steel–Dwass test.

(n=3), patients with PAH (n=4), and patients with CTEPH (n=3) activated with 0.05 U/mL thrombin for 20 minutes at 25°C. We also measured it after incubation with the same concentration of thrombin without platelets as the control. Relative expression levels of VCAM-1 by platelets of non-PH patients, patients with PAH, and patients with CTEPH to

the controls were 2.35±0.85, 16.68±10.12, and 17.56±7.17 folds, respectively (non-PH versus PAH, *P*=0.06; non-PH versus CTEPH, *P*=0.02; Figure 6). Thus, the hyperstimulated platelets of patients with PAH and CTEPH have potentials to induce the VCAM-1 in the endothelial cells compared with non-PH patients.



**Figure 6.** Responsiveness of endothelial cells to thrombin-stimulated platelets. **A**, Representative results of the expression of vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells after incubation at 37°C for 12 hours with conditioned media of platelets prepared by stimulation with 0.05 U/mL thrombin at 25°C for 20 minutes in non-pulmonary hypertension (PH) patients, patients with pulmonary arterial hypertension (PAH), and patients with chronic thromboembolic pulmonary hypertension (CTEPH). The control experiments were performed with the same concentration of thrombin, but without platelets. **B**, Quantification of VCAM-1 expression in endothelial cells after the incubation in control (n=3), non-PH patients (n=4), patients with PAH (n=3), and patients with CTEPH (n=4). The VCAM-1 expression was adjusted with β-actin levels, and the relative expressive levels (mean±SD) were expressed using that VCAM-1 expression levels in the control group as 1.0. Statistical significance was determined with the Student *t* test.

## Discussion

In the present study, we demonstrated for the first time that the platelets, but not the coagulation system, are activated in patients with CTEPH, by showing that (1) P-selectin- and PAC-1-positive platelets were increased, (2) RalA in platelets was activated, (3) platelets were hyperresponsive to thrombin stimulation, and (4) the D-dimer or FDP level was not elevated in patients with CTEPH compared with non-PH patients.

CTEPH is a thrombosis-related disease. Although CTEPH is considered to link to venous thrombosis and pulmonary embolism,<sup>2</sup> our data indicated that platelets, but not coagulation system, are activated in patients with CTEPH. In the multivariate regression analysis, the activation of RalA was influenced by patients with CTEPH, but not PAH patients, whereas the activation of RhoA was influenced by patients with PAH, but not patients with CTEPH. Furthermore, elevation of PAC-1-positive platelets and P-selectin-positive platelets was detected in patients with CTEPH, but not in patients with PAH in the multivariate regression analysis, although pulmonary artery pressure was higher in patients with PAH than patients with CTEPH. Thus, elevation of RalA activity, PAC-1-positive platelets, and P-selectin-positive platelets would be rather specific to CTEPH and would not be dependent on high pulmonary artery pressure in CTEPH. These difference between PAH and CTEPH suggested the difference in the pathogenesis between PAH and CTEPH.

However, it cannot absolutely be excluded that the activated platelets of patients with CTEPH are secondary phenomena caused by surrounding circumstances such as pulmonary hypertension per se because platelets of patients with PAH were also activated in previous studies<sup>24,25</sup> and this study. The platelet activation in patients with PAH is considered because of exposure to high shear in the pulmonary arteries and elevated levels of various cytokines.<sup>26</sup> Once the platelets are activated, they release serotonin and other chemical mediators that are involved in the progression of pulmonary hypertension.<sup>27</sup> In any case, activated platelets could contribute to the progression of the severity of CTEPH.

We showed that the platelets of patients with CTEPH exhibited higher levels of the surface expression of P-selectin and PAC-1 binding than those of non-PH patients in response to the ex vivo thrombin stimulation. Thus, the platelets of patients with CTEPH are not only in hyperactive states but also in hyperresponsive states to exogenous thrombin stimulation. Platelets of patients with PAH were also in hyperresponsive states to thrombin.

Many cytokines and endothelial dysfunction are involved in the pathogenesis of PH.<sup>28</sup> We showed that the hyperresponsiveness of platelets in patients with CTEPH and PAH induced VCAM-1 expression in endothelial cells, which was a key molecule of inflammation in endothelial cells.<sup>23</sup> These data suggest that the hyperresponsiveness of platelets in patients with CTEPH and PAH enhances inflammatory responses of the endothelial cells of pulmonary artery and contribute to the pathogenesis and progression of CTEPH and PAH.

Several small GTPases are also the key regulators of platelet functions. In the present study, we have successfully established the method to evaluate directly activated states

of various small GTPases in platelets isolated from patients. With this method, we were able to demonstrate that RalA, which is implicated for granule secretion, was significantly activated in patients with CTEPH and PAH compared with non-PH patients and that RhoA, which is implicated for aggregation and shape change of platelets, was significantly activated in patients with PAH. RalA and RhoA in platelets could be sensitively activated compared with other G proteins. The difference in the mode of increased activity of small GTPases between CTEPH and PAH might reflect the different pathophysiology of these diseases. However, we found no increase in the activity of Rac1, Rap1, or Ras in platelets of patients with CTEPH, suggesting that the activities of these small GTPases are strictly regulated.

There are only 2 molecules, RalGAP-1 and RalGAP-2, which inactivate RalA.<sup>19,20</sup> We showed that the expression levels of RalGAP tended to be reduced in the platelets of patients with PAH and CTEPH. These results suggest that RalGAP might be a molecule involved in the activation of RalA and the hyperresponsiveness of platelets in patients with CTEPH. It is possible that Ral-guanine nucleotide exchange factors contribute to the RalA activation. As for Ral-guanine nucleotide exchange factors, 6 molecules have been identified to date, 4 of which are activated directly by GTP-bound Ras and 2 of which contain Pleckstrin-homology domains and activated by elevated Ca<sup>2+</sup> concentration.<sup>29</sup> Thus, the stimulatory regulation of Ral activity is so complicated that further examination is necessary to determine their contribution. It is also the case for RhoA. Because both stimulatory and inhibitory regulations of RhoA are as complicated, even more complicated, as that of RalA, further investigation is required in the future.

To prevent the progression of CTEPH, patients with CTEPH are at present treated by anticoagulation treatment. Our data suggest that platelets in patients with CTEPH are activated. Therefore, antiplatelet treatment might be effective to prevent the progression of CTEPH. Further investigation is required in the future.

D-dimer and FDP are degraded products of fibrin fibers generated by fibrinolysis, reflecting the presence of newly generated fibrin fibers and plasmin activity.<sup>17</sup> Thus, these markers depend on newly produced fibrin clot and the activation of coagulation. In the present study, D-dimer and FDP levels were not elevated in patients with CTEPH compared with non-PH patients, a consistent finding with the previous study.<sup>30</sup> These results indicate that the coagulation system is at a basal level in the chronic stage of CTEPH.

Our study has several limitations. First, the number of the patients was relatively small. Second, because most of the patients were in New York Heart Association class I or II, we were unable to evaluate the association between platelet activation and clinical symptoms of patients with CTEPH.

In conclusions, the present study provides the first direct evidence that the platelets of patients with CTEPH are highly activated and more hyperreactive to thrombin stimulation compared with non-PH patients.

## Acknowledgments

We thank Hikari Satoh for her excellent technical assistance.

## Sources of Funding

This work was supported, in part, by the MEXT and JSPS KAKENHI grants (to H. Horiuchi and R. Shirakawa), grants from the Senshin Medical Research Foundation (to H. Horiuchi), and the Bayer Scholarship for Cardiovascular Research (to R. Shirakawa).

## Disclosures

None.

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## Significance

Chronic thromboembolic pulmonary hypertension (CTEPH) is one of the distinct disease entities of pulmonary hypertension, characterized by obstruction of the major pulmonary arteries by organized thrombosis and pulmonary vascular remodeling. However, it remains to be elucidated whether or not CTEPH is associated with a prothrombotic states. In the present study, we examined activation states of platelets of patients with CTEPH systematically. We showed that (1) platelets of patients with CTEPH were at a high-activation state, (2) among several small GTPases, RalA, which is implicated for platelet granule secretion, was activated in platelets of patients with CTEPH when evaluated by the novel assay that we newly established, (3) platelets of patients with CTEPH exhibited hyperresponsiveness to ex vivo thrombin stimulation. Thus, the present study provides the first direct evidence that the platelets of patients with CTEPH are highly activated and more hyper-reactive to thrombin stimulation compared with nonpulmonary hypertension patients.

# Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

## Platelets Are Highly Activated in Patients of Chronic Thromboembolic Pulmonary Hypertension

Nobuhiro Yaoita, Ryutaro Shirakawa, Yoshihiro Fukumoto, Koichiro Sugimura, Satoshi Miyata, Yutaka Miura, Kotaro Nochioka, Masanobu Miura, Shunsuke Tatebe, Tatsuo Aoki, Saori Yamamoto, Kimio Satoh, Tomohiro Kimura, Hiroaki Shimokawa and Hisanori Horiuchi

*Arterioscler Thromb Vasc Biol.* 2014;34:2486-2494; originally published online August 28, 2014;

doi: 10.1161/ATVBAHA.114.304404

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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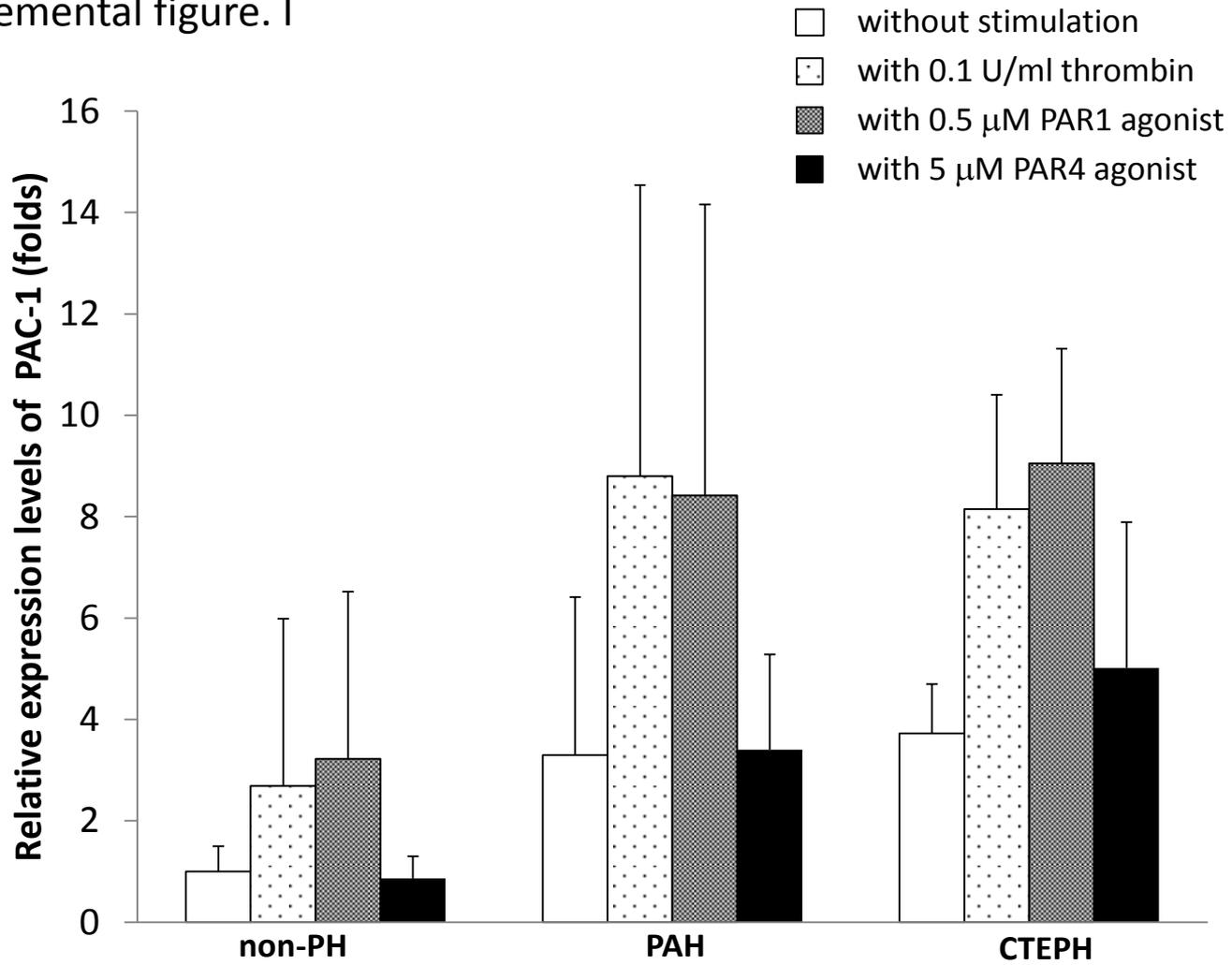
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# Supplemental Figure

Supplemental figure. I



## **Supplemental figure legends**

### **Platelets are highly activated in patients of chronic thromboembolic pulmonary hypertension (CTEPH)**

Nobuhiro Yaoita, MD<sup>1</sup>, Ryutaro Shirakawa, PhD<sup>2</sup>; Yoshihiro Fukumoto, MD, PhD<sup>1</sup>; Koichiro Sugimura, MD, PhD<sup>1</sup>; Satoshi Miyata, PhD<sup>1</sup>; Yutaka Miura, MD, PhD<sup>1</sup>; Kotaro Nochioka, MD, PhD<sup>1</sup>; Masanobu Miura, MD, PhD<sup>1</sup>; Shunsuke Tatebe, MD, PhD<sup>1</sup>; Tatsuo Aoki, MD, PhD<sup>1</sup>; Saori Yamamoto, MD, PhD<sup>1</sup>; Kimio Satoh, MD, PhD<sup>1</sup>; Tomohiro Kimura, PhD<sup>2</sup>; Hiroaki Shimokawa, MD, PhD<sup>1</sup>; Hisanori Horiuchi, MD, PhD<sup>2</sup>.

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**Supplemental figure I. Responsiveness of washed platelets to various stimulation.**

The relative ratios of PAC-1 positive platelets in washed platelets of indicated patients after the stimulation at 25°C for 20 min in the absence (□) and presence of 0.1 U/ml thrombin (◻), 0.5μM PAR1 agonist (▣) and 5μM PAR4 agonist (■) in non-PH (n=5), PAH (n=4) and CTEPH (n=6). The relative levels (mean±SD) were expressed using non-PH patients without stimulation as 1.0.

## **MATERIALS AND METHODS**

### **Platelets are highly activated in patients of chronic thromboembolic pulmonary hypertension (CTEPH)**

Nobuhiro Yaoita, MD<sup>1</sup>, Ryutaro Shirakawa, PhD<sup>2</sup>; Yoshihiro Fukumoto, MD, PhD<sup>1</sup>; Koichiro Sugimura, MD, PhD<sup>1</sup>; Satoshi Miyata, PhD<sup>1</sup>; Yutaka Miura, MD, PhD<sup>1</sup>; Kotaro Nochioka, MD, PhD<sup>1</sup>; Masanobu Miura, MD, PhD<sup>1</sup>; Shunsuke Tatebe, MD, PhD<sup>1</sup>; Tatsuo Aoki, MD, PhD<sup>1</sup>; Saori Yamamoto, MD, PhD<sup>1</sup>; Kimio Satoh, MD, PhD<sup>1</sup>; Tomohiro Kimura, PhD<sup>2</sup>; Hiroaki Shimokawa, MD, PhD<sup>1</sup>; Hisanori Horiuchi, MD, PhD<sup>2</sup>.

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## Materials and Methods

### *Study Population*

The study enrolled the patients without pulmonary hypertension (non-PH) (n=15, hypertension n=7, hyperlipidemia n=5, hypertrophic cardiomyopathies n=2 and paroxysmal atrial fibrillation n=1), the patients with pulmonary artery hypertension (PAH) (n=19, idiopathic pulmonary hypertensions n=10, collagen diseases with pulmonary hypertension n=8, congenital heart disease with pulmonary hypertension n=1) and the patients with chronic thromboembolic pulmonary hypertension (CTEPH) (n=25). All patients underwent cardiac catheterization. The diagnosis of PAH and CTEPH was made according to the ESC/ERS Guideline.<sup>1</sup> Among those patients, platelets of 12 non-PH, 14 PAH and 17 CTEPH patients were stimulated with ex vivo 0.05U/ml thrombin.

### *Preparation of the washed platelets*

At the beginning of the right cardiac catheterization, the blood samples (4 ml) were collected in a tube containing a final concentration of 0.313% sodium citrate. Platelet rich plasma (PRP) was prepared by centrifugation of blood at 200 G at 25°C for 10 min. Then, the washed platelets were prepared by gel filtration column chromatography, as described<sup>2</sup> with slight modification. Briefly, 1 ml PRP was separated by 6 ml Sepharose 2B with Hepes-Tyrode Buffer without Ca<sup>2+</sup> (10 mM Hepes, 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.7 mM MgCl<sub>2</sub>, 11.9 mM NaHCO<sub>3</sub>, 5 mM glucose) with 0.3% bovine serum albumin. Washed platelets were obtained in 2 ml in the elution volume 2-4 ml as shown in Fig. 1A, within 1 hour after blood collection.

### *The flow cytometric analysis of the platelets*

Washed platelets (1.25 µl) were immediately incubated with the monoclonal antibodies against PAC-1 FITC, CD62P-PE (Beckton-Dickinson) and CD61-PerCP (Beckton-Dickinson) in a tube for 20 min at 25°C in the dark. Mouse IgG1-PE control antibody (Beckton-Dickinson) and RGDS peptides (Sigma) were used to evaluate for non-specific binding. After the incubation, the samples were fixed by 500 µl of 1% paraformaldehyde in phosphate-buffered saline at 4°C. Within 24 hours, the samples were analyzed with FC-500 Flow Cytometer (Beckman Coulter). The platelets were identified by size on forward and side scatter plots and the presence of the CD61 antigen. PAC-1 binding and

P-selectin expression were calculated as % of total platelet numbers (n=5,000).

We also performed the flow cytometric analysis to examine the effect of weak stimulation with thrombin on platelets activation. Washed platelets of 10 non-PH, 14 PAH and 17 CTEPH patients were stimulated by 0.05 U/ml thrombin (Sigma) and 5 mM CaCl<sub>2</sub> at 25°C for 20 min and the flow cytometric analysis was performed. Washed platelets of non-PH (n=5), PAH (n=4), CTEPH patients (n=6) were also stimulated by 0.1 U/ml thrombin, 0.5 μM PAR1 agonist (SFLLRN) (Sigma) or 5 μM PAR4 agonist (AYPGFK) (Sigma) at 25°C for 20 min and the flow cytometric analysis was performed.

#### *Preparation of the recombinant proteins for the pull down assay*

The glutathione-S transferase (GST) fusion proteins for the pull down assay to evaluate GTP-bound GTPases were produced and purified as described previously<sup>3</sup> using the following plasmids. PGEX2T Rap1 binding domain of Ral GDS (amino acid residues of 801-883) for Rap1 that was cloned from cDNA of the brain of human, PGEX2T Ral binding domain of Sec5 for RalA,<sup>4</sup> PGEX2T Ras binding domain of Raf for Ras,<sup>5</sup> PGEX2T N-terminal fragment of human Daam1 for RhoA,<sup>6</sup> and PGEX2T p21 binding domain of PAK1 PBD for Rac1.<sup>6</sup> All of the purified recombinant proteins were extensively dialyzed against Buffer A (50 mM Hepes/KOH, pH 7.2, 78 mM KCl, 4 mM MgCl<sub>2</sub>, 0.2 mM CaCl<sub>2</sub>, 2 mM EGTA, 1 mM dithiothreitol) and stored at -80°C until use. Protein concentrations were determined by the intensities of the bands on Coomassie Brilliant Blue-stained SDS-polyacrylamide gels using bovine serum albumin as a standard.

#### *The pull down assay for the washed platelets*

Washed platelets were incubated with 0.5% Triton X-100 at 4°C for 5 min. After centrifugation of the samples at 300,000 G at 4°C for 5 min, the supernatants were obtained as the platelet lysates. Then, the platelet lysates were incubated with glutathione-Sepharose beads coated with 200 μg of each GST fusion protein at 4°C for 30 min. After washing the beads by Buffer A three times, beads-associated RalA, RhoA, Rap1, Rac1 and Ras proteins were analyzed by immunoblotting with anti-RalA (BD transduction laboratory), anti-Rap1 (BD transduction laboratory), anti-Rac1 (BD transduction laboratory) and anti-Ras (Millipore) monoclonal antibodies and anti-RhoA polyclonal antibodies (Santa Cruz Biotechnology), respectively, and were visualized with chemiluminescence

(ECL Prime, GE Healthcare). Densitometric analysis was performed on the blots using Image J 1.38x software (National Institutes of Health). The data were expressed as the percentage of GTP-bound GTPases of total amount.

*Endothelial cell culture and stimulation with conditioned media of thrombin-activated platelets*

Human lung microvascular endothelial cells (HMVECs) were purchased from Lonza and cultured with EGM™-2MV SingleQuots™ Kit (EGM-2) (Lonza). EGM-2 was exchanged to EBM™-2MV Basal Medium (EBM-2) (Lonza) 10 hours before stimulation. Washed platelets were stimulated by 0.05 U/ml thrombin (Sigma) and 5 mM CaCl<sub>2</sub> at 25°C for 20 min and were centrifuged at 20,400 G at 4°C for 10 min and the supernatant was collected as conditioned media. The control conditioned media was prepared in the same way with thrombin in the absence of platelets.

Subconfluent HMVECs were washed with PBS and added with 1.5 ml EBM-2 and 0.5 ml either conditioned medium. The HMVECs were cultured at 37°C for 12 hours. The cells were solubilized with 1% Triton-X after washing once with PBS. Then, the supernatant was collected after centrifugation at 20,400 G for 1 min.

The expression of VCAM-1 or  $\beta$ -actin in endothelial cells was analyzed by immunoblotting with anti-VCAM-1 antibody (Cell Signaling) and anti- $\beta$ -actin antibody (Sigma) and was visualized with chemiluminescence (ECL Prime, GE Healthcare). Densitometric analysis was performed on the blots using Image J 1.38x software (National Institutes of Health).

*Western blotting for RalGAP $\beta$*

The RalGAP $\beta$  protein or  $\beta$ -actin in the lysate of washed platelets was analyzed by immunoblotting with anti-RalGAP $\beta$  antibody<sup>5 7</sup> and anti- $\beta$ -actin antibody (Sigma) and was visualized with chemiluminescence (ECL Prime, GE Healthcare). Densitometric analysis was performed on the blots using Image J 1.38x software (National Institutes of Health).

*Statistical analysis.*

The ratios of P-selectin or PAC-1 positive platelets and GTP-bound GTPases are expressed as median (interquartile ranges [IQR]) because of these were not normal distribution. All other continuous variables were expressed as the mean  $\pm$

SD. Multiple group comparison of continuous variables was analyzed by ANOVA followed by Tukey's HSD (honest significant difference) test or Kruskal-Wallis test followed by Steel-Dwass test, as appropriate. Categorical variables were presented as frequency counts and percentages, and intergroup comparisons of categorical variables were analyzed by Fisher's exact test.

To determine an optimal set of independent factors for the activation of RhoA and RalA, the ratios of P-selectin positive platelets or PAC-1 positive platelets, multiple regression analysis with backward elimination stepwise variable selection was utilized, which involves starting with all PH drugs, epoprostenol, oral prostaglandin I<sub>2</sub> analogue, endothelin receptor antagonist, phosphodiesterase V inhibitor.

All statistical analyses were performed using JMP Pro (SAS Institute Inc., Cary, NC) and R version 3.1.1, and P values <0.05 were considered to be statistically significant.

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