Warm-up exercise suppresses platelet-eosinophil/neutrophil aggregation and platelet-promoted release of eosinophil/neutrophil oxidant products enhanced by severe exercise in men

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Summary
Heterotypic platelet-eosinophil/neutrophil aggregation and subsequent release of eosinophil/neutrophil oxidant products contribute to pathogenesis of conditions such as asthma and inflammatory bowel diseases. This study investigates whether warm-up exercise (WUE) affects platelet-eosinophil/neutrophil interaction mediated by high-intensity exercise (HIE). Twenty-three healthy sedentary men performed on three occasions light-intensity exercise (LIE, 40%VO₂max for 40 min) and HIE (80%VO₂max for 40 min) with and without WUE (40%VO₂max for 20 min). Before and immediately after exercise, platelet-eosinophil and platelet-neutrophil aggregation (PEA and PNA), reactive oxygen species production of eosinophils and neutrophils (EROS and NROS) enhanced by platelets, and adhesion molecule expression on platelets, eosinophils, and neutrophils were measured. The results of this study demonstrated that HIE enhanced PEA, PNA, and platelet-induced EROS and NROS, was accompanied by increased expressions of Mac-1 on eosinophils and neutrophils and P-selectin on platelets at 5 dyne/cm² of shear stress, 100 µg/ml lipopolysaccharide, and 1 µM N-formylmethionyl-leucyl-phenylalanine treatments, whereas the enhancement of HIE on platelet-eosinophil/neutrophil interaction was suppressed by WUE. Conversely, LIE significantly reduced PEA and PNA, suppressed platelet-induced EROS and NROS, and down-regulated eosinophil/neutrophil Mac-1 and platelet P-selectin expressions under various stimuli and shear flow conditions. Moreover, these effects were more pronounced in platelet interaction with eosinophils than with neutrophils. It is concluded that HIE enhances hetero-aggregation, adhesion molecules expressions, and subsequent oxidative bursts mediated by platelets and eosinophils/neutrophils, this effect diminishes after WUE. However, LIE minimizes the risk of thromboinflammation.

Keywords
Warm-up, eosinophil, platelet, inflammation, thrombosis

Introduction
Eosinophilic inflammation is a principal factor in the pathogenesis of allergic diseases such as asthma (1, 2). Neutrophils play an important role not only in bacterial infection but also in acute respiratory distress syndrome (3), idiopathic pulmonary fibrosis (4), and bronchial asthma (5). Activated eosinophils and neutrophils produce oxygen metabolites, such as superoxide (6), which act as disinfectants and cause airway injury at the inflamed lesion. Platelets contribute to the secondary tethering processes of eosinophils and neutrophils to activated endothelium. Such heterotypic adhesive interactions promote thrombotic formation and vascular occlusion, thereby decreasing blood flow and exacerbating tissue ischemia (7–9). Moreover, activated platelets also induce reactive oxygen species (ROS) release by polymorphonuclear leukocytes (PMNs), resulting in subsequent oxidative damage (10). Several reports have shown that hypereosinophilic patients also had thrombotic disorders and were likely to develop cardiovascular disease with associated mural thrombi (11–13). Conversely, in an animal study platelet depletion by injecting anti-platelet serum reduced eosinophil infiltration of the bronchoalveolar lavage and suppressed airway hyperresponsive-ness after allergen challenge (14).

Exercise-induced bronchoconstriction (EIB) is extremely prevalent in asthmatic patients, whereas severity of exercise-induced bronchoconstriction is associated with airway eosin-
ophilic/neutrophilic inflammation in asthmatic patients (15, 16). However, warm-up exercise (WUE) can induce refractiveness to EIB without inducing significant bronchoconstriction (17, 18). In fact, clinical investigations reported that EIB only occurred in 3% of healthy sedentary persons and 8 to 17% of endurance and power athletes (19). However, for 30% to 50% of persons suffering from allergic diseases, exercise provides a potent stimulus for bronchoconstriction and airway inflammation (20). Endotoxin or lipopolysaccharide (LPS) causes especially neutrophil/eosinophil-related events of asthma, including airway hyperreactivity, inflammation, and remodeling (21, 22). Therefore, this study intends to simulate allergic or inflammatory environments in vitro, elucidating whether WUE affects eosinophil/neutrophil-related thrombosis and ROS production induced by HIE under these inflammatory conditions in healthy sedentary men.

A multistep, sequential process of adhesive interactions has been elucidated for the leukocyte-platelet thrombus formation (9, 23). Under a condition of dynamic flow, interaction of platelet P-selectin with leukocyte PSGL-1 mediates the initial tethering and rolling of leukocytes, and then upregulates the binding affinity of integrins (i.e., platelet GPIIb/IIIb and leukocyte Mac-1, respectively) converting transient rolling interactions into stable leukocyte adhesion (9, 23). Furthermore, platelets associating with leukocytes can induce activation of NADPH oxidase in leukocytes through a multistep signal transduction pathway, thereby causing inflammatory response (24). Although various studies, including previous studies by the authors, identified increased platelet activation (25, 26) or eosinophilic/neutrophilic inflammation (15, 16) following high-intensity exercise (HIE), the effects of WUE on heterotypic platelet-eosinophil and platelet-neutrophil interactions modulated by HIE remain unclear. Accordingly, we hypothesize that WUE suppresses heterotypic platelet-eosinophil and platelet-neutrophil aggregation (i.e. PEA and PNA) and subsequent platelet-promoted eosinophil and neutrophil ROS production (i.e. EROS and NROS) induced by HIE by modulating the expression of adhesion molecules on eosinophils and platelets.

In light of the discussion above, this study elucidates whether light-intensity exercise (LIE, 40% VO_{2max} for 40 min) and HIE (80% VO_{2max} for 40 min) with or without WUE (40% VO_{2max} for 20 min) influence the following mechanisms: i) PEA and PNA; ii) platelet-promoted EROS and NROS; and iii) expression of adhesion molecules on platelets and eosinophils/neutrophils under various stimuli and shear flow conditions. This study should provide new insights into the possible protective effects of WUE against risk of eosinophil/neutrophil-related thrombosis and ROS production caused by vigorous exercise.

### Methods

#### Subjects

The Ethics Committee of Chang Gung Memorial Hospital reviewed and approved the protocol for this study. Each subject provided informed consent. Twenty-three healthy sedentary men were enrolled in this study. The physical characteristics of these 23 subjects, expressed as means ± SEM, were as follows: age 24.5±0.5 years; height, 170.2±1.6 cm; and body weight, 69.5±1.3 kg. No subject had engaged in regular physical activity for at least 1 year prior to the study. All subjects were nonsmokers, did not use medication/vitamins, were infection or cardiopulmonary risks-free subjects, and abstained from all medication for at least 2 weeks before the study. Subjects fasted for at least 8 hours prior to this study and were instructed to refrain from exercise for at least 24 hours prior to blood sampling. All subjects arrived at the testing center at 9:00 AM to avoid a possible diurnal influence.

#### Exercise and blood collection protocol

Subjects performed 4 exercise protocols on a bicycle ergometer in the laboratory on 4 days (Lode, Corival 400). The first protocol comprised 2 min of unloaded pedaling; the workload was then increased in increments of 20 to 30 Watts every 3 min until the subject reached exhaustion. This exercise test determined a subject’s maximal oxygen consumption (VO_{2max}), as previously described (25). The mean VO_{2max} was 34.9±2.7 ml/min/kg. For the second and the third protocols, subjects rode the bicycle at 40% (LIE) and 80% (HIE) of the predetermined VO_{2max} for 40 min, respectively. For the fourth test, subjects initially rode at 40% VO_{2max} for 20 min (WUE), then recovered at a sitting position for 30 min (WUE-Rc) and finally exercised strenuously (HIE) for 40 min. Second, third, and fourth protocols were randomized in a counterbalanced order and performed at 2-week intervals to ensure complete recovery between trials. Immediately and 5, 10, 20, and 30 min following these 4 tests, no subject presented an EIB sign, namely, FEV1/FVC reduced by ≥10% compared with pre-exercise levels (Table 1).

At rest, WUE-Rc, and immediately after randomly selected exercise tests, blood samples were collected from a forearm vein to measure hematomal parameters and platelet-eosinophil and platelet-neutrophil interactions. Blood cell counts were analyzed using a Sysmax SF-3000 cell counter.

#### Table 1: Comparisons of dynamic respiratory function following LIE, HIE, and WUE/HIE.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>LIE</th>
<th>HIE</th>
<th>WUE/HIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.56±0.23</td>
<td>4.47±0.2</td>
<td>4.46±0.28</td>
</tr>
<tr>
<td>5</td>
<td>4.55±0.25</td>
<td>4.53±0.28</td>
<td>4.54±0.29</td>
</tr>
<tr>
<td>10</td>
<td>4.58±0.30</td>
<td>4.57±0.35</td>
<td>4.56±0.32</td>
</tr>
<tr>
<td>20</td>
<td>4.02±0.27</td>
<td>4.00±0.31</td>
<td>3.95±0.30</td>
</tr>
<tr>
<td>30</td>
<td>4.00±0.28</td>
<td>4.02±0.25</td>
<td>3.85±0.27</td>
</tr>
</tbody>
</table>

Data were expressed as mean±SEM; LIE indicates light-intensity exercise; HIE, high-intensity exercise; WUE/HIE, high-intensity exercise with warm-up exercise; FVC, forced vital capacity; FEV1, the 1st-second forced expiratory volume.
**Eosinophil, neutrophil, and platelet separation**

Peripheral blood plasmaphenomonuclear leukocytes (PMNs) were isolated from 40 ml whole venous blood by dextran sedimentation followed by density separation over Ficoll-Hypaque and hypotonic lysis (27). Then, eosinophils were prepared from the PMN suspensions with a MACS negative immunomagnetic selection technique; neutrophils were isolated positively by human CD16 immunomagnetic microbeads (approximately 50 nm in size and biodegradable), as described previously (28). Both eosinophils and neutrophils were resuspended in Hank’s buffered salt solution (HBSS) (Sigma), pH 7.4, and adjusted to $1 \times 10^7$ cells/ml. Platelets were washed by repeated centrifugation with an albumin cushion, and then adjusted with HBSS solution to $2 \times 10^8$ cells/ml, as described previously (25). Analysis of eosinophil, neutrophil, and platelet functions was completed within 2 hours after cell purification.

**Heterotypic platelet-eosinophil (or neutrophil) aggregation**

Platelet suspension was incubated with a saturating concentration of monoclonal anti-human CD42b antibody conjugated with PE (CD42b-PE) (eBioscience) in darkness for 20 min at 37°C. Then, 50 µl platelets labeled with CD42b-PE ($2 \times 10^8$ cells/ml) and 50 µl eosinophils (or neutrophils) ($1 \times 10^7$ cells/ml) were mixed on 1 mg/ml albumin (Sigma)-coated glass (32 mm diameter) and sheared at controlled levels of shear stress at 37°C for 5 min using a rotational viscometer (CAP2000, Brookfield). The cell mixture was exposed to either static condition (SC, 0 dyne/cm$^2$) or constant physiological shear stress (CSS, 5 dyne/cm$^2$) for 5 min at 37°C. Additionally, we also examined the ability of platelets to bind to eosinophils (or neutrophils) at a mimicked stenotic arterial flow using an alternating shear stress (ASS, 1–3 bouts of 80 dyne/cm$^2$ for 10 seconds and reduced to 5 dyne/cm$^2$ within 10 seconds at 37°C) after CSS for 5 min after CSS for 5 min (29). In some experiments, various stimuli, such as 100 µg/ml lipopolysaccharide (LPS) (Sigma), 1 µM N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma), and 1 µM phorbol myristate acetate (PMA) (Sigma), were added to the cell mixture, which was then warmed to 37°C for 30 min at the SC. These cell mixtures were transferred into polypropylene tubes containing 2% formaldehyde (Sigma) in PBS immediately following exposure to various shear stresses and stimuli. Then, the fluorences from 5,000 events, representing the CD42b-PE-labeled platelets bound to eosinophils (or neutrophils) were calculated with a FACScan flow cytometer (Becton Dickinson). In brief, when the eosinophils (or neutrophils) were gated separately from the platelets on basis of forward/sideward scatter, then the PE stained events found in the eosinophils (or neutrophils) were gated as the percentage of definition eosinophil or neutrophil/platelet complexes. This result shows the percentage of eosinophils or neutrophils that bind platelets.

**Platelet-promoted eosinophil (or neutrophil) ROS production**

Eosinophils (or neutrophils) ($1 \times 10^7$ cells/ml) were incubated in 495 µl of Hanks’ solution, pH 7.4 with 5 µl dihydrodramine 123 working solution (DHR 123; final concentration, 10 µM) (Sigma), in a polypropylene test tube for 20 min at 37°C (30). Following incubation, eosinophils (or neutrophils) were washed and resuspended in HBSS, pH 7.4 ($1 \times 10^7$ cells/ml). Then, the mixture of 50 µl platelets without labeling fluorescent dye ($2 \times 10^6$ cells/ml) and 50 µl eosinophils (or neutrophils) with loading DHR ($1 \times 10^7$ cells/ml), or alone the suspension of 100 µl eosinophils (or neutrophils) with loading DHR ($5 \times 10^6$ cells/ml) was exposed to SC and CSS for 5 min and incubated with 1 µg/ml LPS, 1 µM fMLP, and 1 µM PMA for 30 min at 37°C. The non-fluorescent DHR 123 following loading was converted to the fluorescent product rhodamine 123 (R123) by interaction with reactive oxygen intermediates (31). The R123 fluorences obtained from 5,000 events, which represented the eosinophils (or neutrophils) interaction with platelets and the eosinophils (or neutrophils) alone, were measured by flow cytometry.

In some experiments, various monoclonal antibodies, including 10 µg/ml monoclonal anti-human CD62b antibody (clone number: CBTHromb/6, Immunotech) or 10 µg/ml monoclonal anti-human CD41 antibody (clone number: clone P2, Immunotech) was added to platelet suspension as well as 10 µg/ml monoclonal anti-human CD11b antibody (clone number: ICRF44, Biologend) was added to eosinophil or neutrophil suspension at

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### Table 2: Comparisons of blood leukocyte and platelet counts following LIE, HIE, and WUE/HIE.

<table>
<thead>
<tr>
<th>Leukocyte (10$^3$/µl)</th>
<th>Rt</th>
<th>Rc</th>
<th>Ex</th>
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<tbody>
<tr>
<td><strong>LIE</strong></td>
<td></td>
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<td></td>
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<tr>
<td>5.65±0.27</td>
<td>6.53±0.33</td>
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<tr>
<td>5.7±0.31</td>
<td>8.0±0.43$^*$</td>
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<tr>
<td><strong>WUE/HIE</strong></td>
<td>5.66±0.30</td>
<td>4.89±0.36</td>
<td>7.15±0.44$^{**}$</td>
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<tr>
<td><strong>Neutrophil (10$^3$/µl)</strong></td>
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<tr>
<td>3.14±0.23</td>
<td>3.35±0.23</td>
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<tr>
<td>3.16±0.25</td>
<td>4.30±0.27$^*$</td>
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<tr>
<td><strong>WUE/HIE</strong></td>
<td>3.15±0.21</td>
<td>3.06±0.29</td>
<td>3.79±0.30$^{**}$</td>
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<tr>
<td><strong>Eosinophil (10$^3$/µl)</strong></td>
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<tr>
<td>0.18±0.02</td>
<td>0.18±0.01</td>
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<tr>
<td>0.19±0.03</td>
<td>0.19±0.02</td>
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<tr>
<td><strong>WUE/HIE</strong></td>
<td>0.18±0.03</td>
<td>0.16±0.03</td>
<td>0.17±0.04</td>
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<tr>
<td><strong>Basophil (10$^3$/µl)</strong></td>
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<tr>
<td>0.03±0.01</td>
<td>0.03±0.01</td>
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<tr>
<td>0.03±0.00</td>
<td>0.04±0.01</td>
<td></td>
<td></td>
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<tr>
<td><strong>WUE/HIE</strong></td>
<td>0.03±0.01</td>
<td>0.03±0.01</td>
<td>0.03±0.00</td>
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<tr>
<td><strong>Lymphocyte (10$^3$/µl)</strong></td>
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<tr>
<td>2.10±0.20</td>
<td>2.42±0.16</td>
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<tr>
<td>2.12±0.23</td>
<td>3.03±0.19$^*$</td>
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<tr>
<td><strong>WUE/HIE</strong></td>
<td>2.11±0.21</td>
<td>1.8±0.10</td>
<td>2.78±0.21$^{**}$</td>
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<tr>
<td><strong>Monocyte (10$^3$/µl)</strong></td>
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<tr>
<td>0.31±0.02</td>
<td>0.32±0.02</td>
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<tr>
<td>0.32±0.03</td>
<td>0.45±0.02$^*$</td>
<td></td>
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<tr>
<td><strong>WUE/HIE</strong></td>
<td>0.31±0.02</td>
<td>0.25±0.02</td>
<td>0.38±0.03</td>
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<tr>
<td><strong>Platelet (10$^3$/µl)</strong></td>
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<tr>
<td>207±8</td>
<td>221±12</td>
<td></td>
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<tr>
<td>210±12</td>
<td>248±16$^*$</td>
<td></td>
<td></td>
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<tr>
<td><strong>WUE/HIE</strong></td>
<td>209±11</td>
<td>222±12</td>
<td>225±15$^*$</td>
</tr>
</tbody>
</table>

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Data were expressed as mean±SEM. LIE indicates light-intensity exercise; HIE, high-intensity exercise; WUE/HIE, high-intensity exercise with warm-up exercise; Rt, rest; Rc, recovery 30 min after warm-up exercise, Ex, immediately after LIE or HIE. P <0.05, R vs. E; +P <0.05, HIE vs. HIE/WUE.
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37°C for 20 min prior to measuring PEA, PNA, and platelet-promoted EROS and NROS, clarifying whether the P-selectin and GPIIb/IIIa on platelets and the Mac-1 on eosinophils/neutrophils mediated their hetero-aggregation and the ROS production.

Expressions of adhesion molecules on platelet, eosinophil, and neutrophil

The platelet suspension (1 x 10^8 cells/ml), the eosinophil (or neutrophil) (5 x 10^6 cells/ml) suspension, or the mixture of platelets (2 x 10^6 cells/ml) and eosinophils (or neutrophils) (1 x 10^7 cells/ml) was exposed to SC, CSS, and various stimuli. Aliquots were then incubated in darkness for 20 min at 4°C with a saturation concentration of FITC-conjugated anti-CD62P (P-selectin) (Serotec), FITC-conjugated anti-PAC-1 (activated form of GP IIb/IIIa) (Pharmingen), FITC-conjugated anti-CD11b (Mac-1) (eBioscience), or FITC-conjugated anti-rabbit IgG control antibodies (Serotec). After fixation with 2% formaldehyde in PBS, the fluorescence obtained from 5,000 events, representing platelets, eosinophils, neutrophils, or platelet-eosinophil (neutrophil) aggregates, were measured by flow cytometry.

To assess the reliability of PEA, PNA, platelet-promoted EROS and NROS, and adhesion molecules expressions on platelets and eosinophils/neutrophils, blood samples of the subjects (n=23) were tested twice at one day intervals. PEA, PNA, platelet-promoted EROS and NROS, and adhesion molecules expressions on platelets and eosinophils/neutrophils were highly reproducible from day to day, and single measure intra-class correction were from 0.786 to 0.854 for the test-retest reliability in this study.

Statistics

Data were expressed as means±SEM. StatView IV statistical software was employed for data analyses. Comparisons of cell counts, leukocyte-platelet aggregation, cellular ROS production, and expression of adhesion molecules on platelets and leukocytes at rest, WUE-Rc, and immediately after the exercise tests, were analyzed by repeated measure ANOVA and Tukey’s multiple range test. These results obtained from this present study have high power values (0.871–0.998) in statistical analysis. Statistical significance was set at P<0.05.

Results

The changes of platelet and WBC counts by exercise

HIE increased platelet, neutrophil, lymphocyte, and monocyte counts (Table 2) (P<0.05), whereas the enhancement of neutrophil and monocyte counts by HIE were hindered by WUE. Blood platelet count and total leukocyte counts including neutrophil, eosinophil, basophil, lymphocyte, and monocyte were not changed by LIE (Table 2).

Heterotypic platelet-eosinophil/neutrophil aggregation mediated by exercise

These CSS-, LPS- or fMLP-induced PEA (Fig. 1A) (P<0.05) or PNA (Fig. 1D) (P<0.05) decreased immediately after LIE.
versely, immediately following HIE, subjects had increased amounts of PEA (Fig. 1B, P<0.05) and PNA (Fig. 1E, P<0.05) induced by CSS, LPS, or fMLP. However, WUE suppressed the HIE-induced heterotypic cell aggregation under CSS and various stimuli (Fig. 1C, 1F). Additionally, there were no significant changes to PMA-induced PEA or PNA following LIE and HIE with and without WUE (Fig. 1A–F). Moreover, basal PEA and PNA at SC also remained unchanged in response to LIE and HIE with and without WUE (Fig. 1A–F).

The results demonstrated that 1–3 bouts of ASS enhanced PEA (Fig. 2A) (P<0.05) and PNA (Fig. 2B) (P<0.05) induced by CSS at rest (P<0.05). Conversely, immediately after HIE, the ASS condition dissociated the PEA (Fig. 2A) and PNA (Fig. 2B) induced by CSS (P<0.05). However, performing WUE prior to HIE reduced the dissociation of PEA (Fig. 2A) and PNA (Fig. 2B) by HIE. Additionally, there was no significant change in ASS-mediated PEA (Fig. 2A) or PNA (Fig. 2B) caused by LIE.

Platelet-enhanced eosinophil/neutrophil ROS production mediated by exercise

Under CSS, LPS or fMLP treatments, platelets significantly elevated cellular ROS concentrations of eosinophils and neutrophils (Fig. 3A–F). Levels of EROS (Fig. 3A) (P<0.05) and NROS (Fig. 3D) (P<0.05) promoted by platelets were suppressed by LIE, while HIE enhanced platelet-induced EROS (Fig. 3B) (P<0.05) and NROS (Fig. 3E) (P<0.05). However, WUE suppressed these effects in HIE under CSS and various stimuli (Fig. 3C, 3F).

Figure 3: Effects of LIE (A,D) and HIE without (B,E) and with (C,F) WUE on basal and platelet-promoted ROS production of eosinophil (A-C) and neutrophil (D-F) under SC, CCS, LPS, fMLP, and PMA conditions. Rt, rest; Rc, recovery 30 min after WUE; Ex, immediately after LIH and HIE without and with WUE; Leu, leukocyte alone; Leu on Plt, leukocyte binding on platelet; MFI, mean fluorescence intensity. "P<0.05, Rt vs. E or Rc.

Figure 4: Effects of CD1b monoclonal antibody (mAb), CD62P mAb, and CD41 mAb on platelet-eosinophil aggregation (A), platelet-neutrophil aggregation (B), platelet-induced ROS production of eosinophil (C) and neutrophil (D) under SC, CCS, LPS, fMLP, and PMA conditions. N=6, *P<0.05, Vehicle vs. CD11mAb, CD62P mAb, or CD41 mAb.
Additionally, PMA-induced ROS production of leukocytes with or without platelets was not significantly influenced by LIE or HIE with and without WUE (Fig. 3A–F). Moreover, LIE and HIE with and without WUE did not influence the extent of platelet-induced EROS and NROS at SC (Fig. 3A–F).

**Adhesion molecules-mediated hetero-aggregation and ROS production of platelet and eosinophil/neutrophil**

Treating platelets with CD62P monoclonal antibody as well as treating eosinophils or neutrophils with CD11b monoclonal antibody significantly reduced PEA (Fig. 4A), PNA (Fig. 4B), and platelet-promoted EROS (Fig. 4C) and NROS (Fig. 4D) under shear flow and various stimuli. Although treating platelets with CD41 monoclonal antibody significantly reduced PMA-induced PEA, PNA, and platelet-promoted EROS and NROS, this antibody only slightly suppressed CSS-induced EROS and NROS and iMLP-induced NROS promoted by platelets and did not influence PEA and PNA at shear flow and various stimuli conditions (Fig. 4A–D).

Additionally, under CSS and LPS, and iMLP treatments, platelets enhanced expression of Mac-1 (Fig. 5; P<0.05) on eosinophils and neutrophils, whereas eosinophils and neutrophils simultaneously increased significantly expression of P-selectin (Fig. 6) but not affected activation of GP IIb/IIIa on platelets (PAC-1) (Fig. 7) (P<0.05).

**Expression of adhesion molecules on platelet and eosinophil/neutrophil mediated by exercise**

LIE suppressed platelet-enhanced expression of eosinophil (Fig. 5A)/neutrophil Mac-1 (Fig. 5D) (P<0.05) and eosinophil/neutrophil-enhanced expression of platelet P-selectin (Fig. 6A) (P<0.05) under shear flow and various stimuli. Although HIE enhanced expressions of eosinophil/neutrophil Mac-1 and platelet P-selectin on the platelet-eosinophil and platelet-neutrophil aggregates (Fig. 5B, 5E, 6B), WUE suppressed the HIE-promoted expressions of these adhesion molecules on these hetero-cell aggregates (Fig. 5C, 5F, 6C). The GPIIb/IIIa activation on platelets alone and platelets that bound with leukocytes did not significantly change following LIE or HIE with and without WUE (Fig. 7A–C) under shear flow and various stimuli. Additionally, basal (i.e. at SC) and PMA-induced expressions of these adhesion molecules on eosinophils, neutrophils and platelets did not significantly change following LIE or HIE with and without WUE (Figs. 5–7).
Discussion

In this study, all subjects were cardiopulmonary risks-free subjects and had not presented an EIB sign. Moreover, basal platelet-eosinophil/neutrophil interactions were also unchanged in response to all exercise tests. However, when simulating inflammatory conditions in vitro, this investigation is the first clearly demonstrating the following findings: i) the intensity of exercise is an important factor affecting PEA and PNA and subsequent EROS and NROS induced by platelets under shear flow and various inflammatory stimuli, i.e., these responses are desensitized by Lie and enhanced by HIE; and, ii) WUE attenuates enhancement of platelets adhesion to eosinophils and neutrophils and subsequently evoked oxidant production of eosinophils and neutrophils during HIE performed by healthy sedentary men. Moreover, these changes are accompanied by changes in cell adhesion molecules, in which HIE enhanced- and Lie and WUE decreased-expressions of both P-selectin on platelets and Mac-1 on eosinophils and neutrophils.

Accumulation and activation of eosinophils and neutrophils and their subsequent interaction with other cells are critical for determining the severity of allergic and inflammatory conditions (1-5). Recent investigations showed that platelets are secondary tethering platforms which recruit fast-flowing polymorphonuclear leukocytes (PMNs) to activate vascular endothelial cells in asthmatic patients (7). The results from this study demonstrated that although HIE increased the number of platelets binding to eosinophils/neutrophils under condition mimicking a venous circuit (5 dyne/cm²), PEA and PNA were dissociated by ASS mimicking a stenotic arterial flow (80 dyne/cm²). Some studies found that after HIE, agonists enhanced platelet-leukocyte aggregation under a lack of shear flow control or static conditions (32), whereas the ability of leukocytes to adhere to surface-adherent platelets decreased under high shear flow (27). The results in this study were consistent with those findings (27, 32). With respect to how strenuous exercise exhibits the bidirectional modulation to platelet interaction with eosinophil/neutrophil at different experimental conditions, our previous studies revealed that PMN-dependent inhibition of platelet activation and PMN-derived nitric oxide (NO) metabolite level increased (27), while soluble P-selectin release from platelet (27) and membrane bound P-selectin expression (26) on platelet were enhanced following HIE. PMN-derived NO can inhibit platelet activation by increasing platelet cGMP content, subsequently suppressing PMN interaction with platelet (33). Additionally, P-selectin exerts a dual influence on platelet-leukocyte interactions, namely, it stimulates this interaction when P-selectin is membrane-associated (34) and inhibits this interaction when it is present in the fluid phase (35). Therefore, the reduction of the capacity of platelet-eosinophil/neutrophil aggregates to withstand high shear flow observed for HIE is possibly a result of increasing releases of soluble P-selectin from platelets or NO from eosinophils/neutrophils. The paradoxical effects of shear-mediated platelet-eosinophil/neutrophil interaction by HIE may be the negative feedback pathway against the risk of vigorous exercise-induced eosinophils/neutrophil-related thrombosis. However, elucidation of the underlying mechanisms of this reciprocal modulation requires further investigation.

By simulating inflammatory conditions using pretreatment of LPS (a principal component of the outer membrane of Gram-negative bacteria) and FMLP (a chemotactic peptide), these stimuli were shown to modulate bronchoconstriction and eosinophil/neutrophil functions under asthma or other pulmonary diseases (36, 37). The results revealed that in the static condition, HIE also increased PEA and PNA induced by LPS and FMLP, which was accompanied by increased expression of Mac-1 on the platelet-leukocyte aggregates. LPS and FMLP induced only an eosinophil and neutrophil activation and did not influence platelet activity, whereas HIE may directly activate eosinophils, thereby increasing its surface Mac-1 content and, then, indirectly enhancing platelet P-selectin expression by activated eosinophils/neutrophils. Thus, the ability of platelets binding to eosinophils/neutrophils at these mimicked inflammatory conditions was enhanced. The results also showed that eosinophil/neutrophil interaction with activated platelets generated substantial amounts of ROS under shear flow and inflammatory conditions, while HIE increased the degree of platelet-promoted oxidative bursts of eosinophil and neutrophil.

Wang, et al. demonstrated that strenuous exercise sensitizes platelet reactivity (25, 26), whereas exercise at moderate intensity (60% VO\textsubscript{2max} for 40 min) directly suppressed platelet adhesion and aggregation induced by physical shear forces and chemical agonists (38). These reactions of moderate-intensity exercise (MIE) were accompanied by a decrease in von Willebrand factor binding to platelets and P-selectin expression on platelets (38). Moreover, MIE also suppressed platelet-PMN interaction by increasing PMN anti-oxidative capacity (38). Notably, exercise intensity lowered to 40% VO\textsubscript{2max} for same the duration (40 minutes) in this study suppressed platelets binding to eosinophils/neutrophils and promoted ROS production of eosinophils/neutrophils; as with the change of platelet-PMN interaction by MIE (38). Moreover, Lie also decreased the expressions of Mac-1 on eosinophils/neutrophils and P-selectin on platelets under CSS, LPS, and fMLP treatments. These findings imply that LIE effectively reduces eosinophil/neutrophil-induced thrombi growth and oxidative damage.

The results indicated that recovery for 30 min following WUE inhibits platelet-eosinophil/neutrophil functions, whereas HIE preceded by WUE did not significantly induce PEA and PNA, platelet-promoted EROS and NROS, and expression of adhesion molecules on platelets and eosinophils/neutrophils under various stimuli and shear flow conditions. A previous investigation showed that patients with ischemic heart disease developed a warm-up phenomenon during repeated exercise testing, characterized by a delay in angina pain onset and diminished electrocardiographic evidence of myocardial ischemia, this effect being a form of preconditioning (39). Ischemic preconditioning can benefit tissue ischemia-reperfusion injury by preserving the mitochondrial redox state, which protects tissue against reperfusion injury caused by leukocyte-derived oxidants (40). A recent study demonstrated that strenuous exercise diminished both GSH content and mitochondrial transmembrane potential, whereas moderate exercise improved anti-oxidative capacity and reduced lipid peroxidation in leukocytes (41). In light of these findings, LIE to MIE may i) upregulate antioxidative capacity of leukocytes to inhibit oxidant production of leukocytes

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induced by HIE, and ii) suppress platelet reactivity to limit the extent of HIE-enhanced platelet activation. By influencing reciprocal modulation of platelet and eosinophil/neutrophil activities, WUE might protect individuals against risk of eosinophil/neutrophil-related thrombosis and inflammation caused by vigorous exercise.

A recent clinical investigation also confirmed that warm-up exercise abrogated ADP-induced platelet-leukocyte aggregation recovery for 60 min after patients with claudication performed exhaustive exercise (42). However, the study did not precisely control WUE intensity according to an individual's level in physical fitness, such as relative to the number of % VO\textsubscript{peak} performed, and did not evaluate specifically interactions between platelets and eosinophils/neutrophils or their underlying mechanisms. In the present study, the effects of exercise were more pronounced in eosinophil than in neutrophil interaction with platelets, particularly in production of ROS and expressions of adhesion molecules. The PMNs' interaction with activated platelets can evoke the activation of respiratory burst, correlating with the rise in the phosphorylation of protein kinase C (PKC) and the translocation of cytosolic p47\textsuperscript{phox} to the plasma membrane in PMNs (24, 30). However, an atypical PKC, PKC\textgreek{c}, regulates human eosinophil and neutrophil functions in a differential manner. A previous study revealed that a myristoylated specific PKC\textgreek{c} inhibitor peptide significantly blocked fMLP-induced ROS generation by eosinophils, whereas this inhibitor did not influence the ROS production of neutrophil induced by fMLP (43). Recently, Perrini, et al. also found that exercise at approximately 70% VO\textsubscript{peak} increased the PKC\textgreek{c} content and phosphorylation in human skeletal muscle (44). Therefore, further investigations are needed to clarify the roles of various PKC isoforms and NADPH oxidase in signaling events, associated with the functional responses of eosinophil and neutrophil mediated by different exercise regimes.

As in numerous other investigations, a limitation of this study is that the subjects were predominantly young and healthy. Therefore, further clinical evidence was required to extrapolate results to patients with abnormal or diseased respiratory or hemostatic systems, such as for those with asthmatic or idiopathic hypereosinophilic symptoms. Additionally, the neutrophil positively selected by CD16 immunomagnetic microbeads might influence itself bioactivity or function in this study. Therefore, a negative selection by secondary antibody coated beads is necessary for purification of blood neutrophils in further investigation with relation to the platelet-neutrophil interaction (45).

In conclusion, HIE enhances platelet-eosinophil/neutrophil hetero-aggregation and platelet-promoted ROS production of eosinophils/neutrophil under conditions that mimicked pathophysiological shear flow and inflammation. These effects diminished after WUE. Specific findings provide a novel interpretation of how warm-up exercise reduces vigorous exercise-induced risks of thrombosis associated with eosinophils/neutrophils and platelets under inflammatory conditions. Additionally, LIE decreases eosinophil/neutrophil-platelet interaction, suppressing respiratory bursts of eosinophil/neutrophil caused by platelets. This finding provides further confirmation that mild exercise is a “safe” exercise dosage for minimizing the risk of eosinophil/neutrophil- and platelet-related disorders by eliciting beneficial physiological changes.

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References