Evidence for Prothrombotic Effects of Exercise and Limited Protection by Aspirin

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Background—Exercise may activate platelets and leukocytes and promote thrombosis. The effects of aspirin treatment on the prothrombotic effects of exercise have not been established.

Methods and Results—A total of 15 healthy men performed exhaustive exercise without and with 1 week of pretreatment with aspirin (500 mg/day). Before and immediately after exercise, platelet aggregability ex vivo was measured by filtragometry, and venous blood samples were obtained. Whole-blood flow cytometry was used to determine platelet and leukocyte activation and platelet-leukocyte aggregates. Exercise increased platelet P-selectin expression, CD11b expression in neutrophils and lymphocytes, and platelet and leukocyte responses to thrombin, ADP, platelet activating factor, and N-formyl-methionyl-leucyl-phenylalanine (fMLP) in vitro. Consistent with enhanced platelet and leukocyte activation, more circulating platelet-platelet and platelet-leukocyte aggregates were detected after exercise (P < 0.001 for both). Filtragometry readings were shortened, and plasma soluble P-selectin and prothrombin fragment 1+2 were elevated. Aspirin markedly reduced the urinary excretion of 11-dehydrothromboxane B2, decreased P-selectin expression in single platelets at rest (P < 0.05), and inhibited fMLP-induced neutrophil CD11b expression, but it did not attenuate exercise-induced increases in platelet aggregability, platelet P-selectin expression, leukocyte CD11b expression, platelet-leukocyte aggregate formation, soluble P-selectin, or prothrombin fragment 1+2.

Conclusions—Exercise induced platelet and leukocyte activation and platelet-leukocyte aggregation in vivo, and it increased platelet and leukocyte responsiveness to in vitro stimulation. Aspirin treatment attenuated certain signs of platelet activity in vivo at rest and fMLP-induced neutrophil activation in vitro, but it did not attenuate the prothrombotic effects of exercise. (Circulation. 1999;100:1374-1379.)

Key Words: exercise ▪ aspirin ▪ platelets ▪ leukocytes

Strenuous exertion promotes a prothrombotic state1 and may trigger acute myocardial infarction.2 Exercise may induce thrombocytosis and platelet activation in vivo and enhanced platelet reactivity in vitro, but results concerning exercise-induced platelet activation are far from unanimous.1,3,4 Strenuous exercise also induces leukocytosis and leukocyte activation, especially in patients with ischemic heart disease.5 Previous studies focused on either platelet or leukocyte activation, and little is known about their interaction during exercise, although the prothrombotic state involves multicellular activation. Therefore, it is of interest to evaluate how exercise influences multicellular activation in vivo, the responsiveness of the cells to in vitro stimulation, and platelet-leukocyte aggregate formation in vivo and in vitro.

Aspirin is a widely accepted antiplatelet drug that lowers cardiovascular mortality and morbidity.6 Aspirin exerts its antithrombotic effects mainly through inhibition of thromboxane formation, which may be overemphasized when platelet aggregation is evaluated under conditions of low extracellular calcium concentrations.7 Aspirin treatment does not influence the activation of single platelets, when evaluated by whole-blood flow cytometry,8,9 suggesting that thromboxane becomes important only during platelet aggregation. Aspirin has little or no effect on exercise-10 or norepinephrine-induced11 platelet aggregability in vivo. The antithrombotic effect of aspirin may, however, also involve other mechanisms, such as attenuation of erythrocyte-mediated activation of platelets,12 inhibition of platelets via a neutrophil-mediated, nitric oxide/cGMP dependent mechanism,13 and reduced thrombin generation.14,15 Moreover, aspirin may attenuate superoxide anion generation by neutrophils,16 inhibit adhesion molecule expression of monocytes,17 and inhibit lipid body and eicosanoid generation in leukocytes.18 Therefore, further investigation of the possible influence of aspirin treatment on platelet-leukocyte interactions and on the prothrombotic effects of exercise is of interest.

Methods

Subjects
A total of 16 healthy, nonsmoking men aged 28±4 years (height, 183±1 cm; weight, 78±2 kg), gave informed consent to participate...
in the study, which was approved by the Ethics Committee of the Karolinska Institute. One subject was excluded before the data analysis due to an infection 1 week before his second visit.

**Study Design**

The study was an open crossover study comparing no treatment and aspirin treatment (500 mg/day for 7 days) during exercise. The interval between experiments was ≥2 weeks; aspirin preceded control in 8 subjects. The volunteers were instructed to refrain from caffeine intake ≥12 hours before experiments, to take the medication between 7 and 8 AM, to collect all their morning urine, and to have a light lunch 2 hours before their visit. On arrival (at 1 PM), they rested for 30 minutes in the supine position. Thereafter, blood samples were taken, and a resting filtragometry measurement was performed on the other arm. After another 30 minutes of rest (to process resting samples), exercise commenced on a computerized bicycle ergometer (Siemens-Elema AB), with a starting workload of 30 W and increments of 10 W/min. Blood pressure and heart rate were monitored, and fatigue was estimated by the 20-grade Borg scale; exercise was terminated on exhaustion. Sampling and a resting filtragometry measurement were performed immediately thereafter.

**Flow Cytometric Analysis**

Platelets were identified with the fluorescein isothiocyanate (FITC)-conjugated anti-CD42a (GPIIX) monoclonal antibody (MAb) Beb1 (Becton Dickinson); leukocytes were identified with the R-phycocerythrin (RPE)-conjugated pan-leukocyte CD45-MAb T29/33 (Dakopatts AB). Platelet P-selectin expression was determined by the RPE-conjugated anti-P-selectin MAb AC1.2 (Becton Dickinson), and leukocyte CD11b expression by the FITC-conjugated MAb BEAR 1 (Immunotech). FITC- and RPE-conjugated isotypic MAb DAK-GO1 were used as negative controls. The agonists used were ADP, human α-thrombin, platelet activating factor (PAF), and N-formyl-methionyl-leucyl-phenylalanine (fMLP). When thrombin was used, clotting was prevented by the peptide GPRP (reagents were from Sigma).

Samples were prepared as described previously. Within 3 minutes of collection, 5 μL of citrated whole blood was added to 45 μL of HEPES-buffered saline containing appropriately diluted antibodies and agonists; the mixture was then incubated at room temperature for 20 minutes. Samples were diluted and mildly fixed with formaldehyde saline (0.2% for platelet analyses and 0.5% for leukocytes and aggregate measurements) before analysis using a Coulter EPICS XL-MCL flow cytometer.

**Platelet P-Selectin Expression**

The flow cytometric analysis of platelets in whole blood has been described previously. We made this minor modification: a RPE-CD62P MAb was used to monitor P-selectin positive cells in the platelet population.

**Platelet-Platelet Aggregates**

The method of determining platelet-platelet aggregates (PPAs) was adapted from previous methods. Briefly, the cytometer was triggered by FITC-CD42a fluorescence. Samples were diluted so that <50 FITC-positive events/s were detected to minimize the risk of coincidence in the flow chamber. Events were subjected to 2-parameter (forward scatter versus side scatter) analysis, and FITC-positive events larger than single platelets were regarded as PPAs; the percentage of PPAs in the total platelet population was calculated.

**Platelet-Leukocyte Aggregates**

Platelet-leukocyte aggregates (PLAs) were determined as described previously. The percentages of platelet-conjugated leukocytes among total leukocytes, lymphocytes, monocytes, and neutrophils were obtained.

**Leukocyte CD11b Expression**

The protocol for leukocyte CD11b analysis was modified from the method for PLA analysis. Total leukocytes, lymphocytes, monocytes, and neutrophils were gated and subjected to single-color (FITC-CD11b) analysis. Mean fluorescence intensities in leukocytes and subpopulations were determined.

**Filtragometry Ex Vivo**

Filtragometry was used to measure platelet aggregates ex vivo. Blood was drawn from an antecubital vein and anticoagulated by heparin before passing through a nickel filter (pore size, 20 μm). Rapid filter occlusion with a low t50 value (ie, aggregation time) indicates high platelet aggregability.

**Urinary 11-Dehydrothromboxane B2**

Urinary 11-dehydrothromboxane B2 was determined by enzyme immunoassay (SPI-BIO) using a sample work-up procedure developed in our laboratory. Aliquots of morning urine were stored at −80°C. After thawing, samples were centrifuged (1400g at 4°C for 5 minutes). The supernatant was diluted 1:2 with 63 mmol/L ammonium bicarbonate buffer (pH 8.6) and incubated 3 hours to convert 11-dehydrothromboxane B2 to its open ring form before extraction with Bond-Elute Certify II columns (Varian) and elution of the analyte with 2% formic acid in methanol. The eluate was cryoevaporated, resuspended (pH 8.6), and incubated 6 hours before analysis. Data are expressed in relation to urinary creatinine.

**Plasma Variables and Cell Counting**

**Physiological Responses to Exercise**

Physiological responses to exercise were also similar on the 2 occasions (data without treatment are given). Exercise increased heart rate (from 60±2 to 199±5 bpm), systolic blood pressure (from 120±3 to 190±4 mm Hg), and plasma nor-adrenaline.

| Hematologic Measurements Before and After Exercise Without Aspirin Treatment |
|-----------------------------|-----------------------------|-----------------------------|
| Erythrocytes, 1012/L        | 4.6±0.0 5.1±0.1             | 10±1                        |
| Hematocrit, %              | 40±1 46±1                   | 14±1                        |
| Leukocytes, 109/L          | 4.8±0.3 9.3±0.4             | 101±10                      |
| Neutrophils, 109/L         | 2.7±0.3 4.1±0.3             | 56±7                        |
| Lymphocytes, 109/L         | 1.5±0.1 4.0±0.3             | 178±18                      |
| Monocytes, 109/L           | 0.3±0.0 0.7±0.0             | 108±13                      |
| Platelets, 1012/L          | 230±9 288±10                 | 26±2                        |

Data were similar with aspirin and are expressed as mean±SEM. *P<0.01, compared to resting values for all parameters.
epinephrine (from 1.49±0.08 to 14.28±1.01 nmol/L) and epinephrine levels (from 0.14±0.02 to 1.09±0.31 nmol/L) (P<0.001 for all). Exercise also increased blood cell counts (Table).

Platelet Activation
Exercise increased P-selectin–positive single platelets in unstimulated samples (Figure 1) and enhanced responses to thrombin and ADP (Figure 2). Aspirin decreased P-selectin–positive platelets at rest (from 1.1±0.2% to 0.8±0.2%; P<0.05), but it failed to protect against the exercise-induced activation of single platelets (Figure 1), and it did not attenuate responses to agonist stimulation in vitro (data not shown). Exercise increased soluble P-selectin (n=14) from 55.3±4.7 to 69.4±6.4 µg/L without aspirin and from 53.7±2.7 µg/L to 71.4±3.7 µg/L with aspirin (P<0.001 for exercise effect; no aspirin effect).

Aspirin decreased the urinary excretion of 11-dehydrothromboxane B₂ from 50.4±3.9 to 10.9±0.9 ng/mmol creatinine (P<0.001) or by 77±2% (range, 60 to 91%), indicating good compliance.

Leukocyte Activation
Exercise increased leukocyte counts (Table). Increased CD11b expression was found in neutrophils (mean fluores-
cence intensities increased from 0.63±0.07 to 0.72±0.07; \( P<0.05 \) and lymphocytes (from 0.32±0.01 to 0.39±0.02; \( P<0.01 \)). Exercise enhanced agonist-stimulated CD11b expression in neutrophils (Figure 3) and lymphocytes (\( P<0.01 \)) but not in monocytes.

Aspirin did not influence basal CD11b expression before or after exercise; it did attenuate fMLP-induced CD11b expression in neutrophils (Figure 3) and lymphocytes (\( P<0.01 \)) but not in monocytes.

Plasma elastase (\( n=15 \)) increased from 30.9±2.6 to 75.1±7.0 ng/mL after exercise without aspirin and from 35.9±3.3 to 75.2±6.3 ng/mL after exercise with aspirin treatment (\( P<0.001 \) for exercise effect; \( P=0.47 \) for aspirin effect).

**Platelet-Leukocyte Aggregates**

Exercise increased PLAs from 2.8±0.3% to 3.9±0.5% (\( P<0.001 \)); PLA counts increased markedly, with responses in all leukocyte subpopulations (Figure 4). Aspirin treatment had no effect on this (PLAs were 2.5±0.2% before and 3.6±0.3% after exercise).

Thrombin, ADP, and PAF enhanced heterotypic aggregate formation dose-dependently, but to different extents. PLAs increased from 2.8±0.3% to 7.3±1.6% and 34.4±3.0% with 3×10\(^7\) and 10\(^8\) mol/L ADP, respectively, and to 26.6±4.5% and 57.6±2.1% with 0.02 and 0.08 U/mL thrombin, respectively. At 10\(^9\) mol/L PAF, PLAs increased to 7.4±2.6% (\( P<0.01 \)). Despite maximal leukocyte activation (Figure 3D), 10\(^6\) mol/L fMLP only increased PLAs to 3.5±0.3% (\( P<0.01 \)). The propensity to form PLAs differed among leukocyte subpopulations. For example, with 0.08 U/mL thrombin, lymphocytes had a 10% propensity to form PLAs, monocytes had a 60% propensity, and neutrophils had a 90% propensity.

Consistent with platelet and leukocyte sensitization, agonist-induced PLA formation in vitro increased after exercise (\( P<0.001 \)), as illustrated for thrombin in Figure 5. Similar results were obtained with ADP and PAF, whereas fMLP had minor effects (data not shown).

**Other Variables**

Exercise increased plasma von Willebrand factor antigen (\( n=13 \)) from 0.91±0.06 to 1.60±0.10 U without aspirin and from 1.03±0.07 to 1.61±0.11 U with aspirin (\( P<0.001 \) for exercise effect; no effect of aspirin). Plasma \( F_{1+2} \) (\( n=14 \)) was 0.60±0.05 nmol/L at rest on both occasions and, after exercise, it increased to 0.70±0.06 nmol/L without aspirin and 0.82±0.07 nmol/L with aspirin (\( P<0.001 \) for exercise effect; no effect of aspirin).

**Discussion**

Strenuous exercise induced in vivo activation and sensitization of platelets, leukocytes, and endothelial cells (as re-
Exercise-induced platelet activation may be related to several mechanisms. Anaerobic metabolism may be involved.\(^1\) Catecholamines in plasma increased \(\approx 10\)-fold, and they might act synergistically with other agonists to promote platelet activation.\(^{11,27}\) Norepinephrine is more likely to be involved than epinephrine.\(^{31}\) Furthermore, the cardiovascular responses to exercise (ie, increases in cardiac output and blood pressure and alterations in blood flow resulting in increased shear forces) and enhanced thrombin generation may contribute to exercise-induced platelet activation.\(^{28}\)

Exercise induced leukocytosis, but responses differed among leukocyte subpopulations. Leukocytosis may be due to the release and demarginalization of leukocytes from the bone marrow, the spleen, and other organs; elevations of cardiac output and catecholamines may contribute to it.\(^{29}\) Interestingly, exercise markedly enhanced the resting and agonist-stimulated expression of CD11b in neutrophils and lymphocytes, with only slight increases in monocytes. Plasma elastase also increased, supporting the contention that exercise activates neutrophils in vivo. Elastase is secreted by activated neutrophils, and it may influence platelets by promoting fibrinogen binding to platelet glycoprotein IIb/IIIa receptors and attenuating thrombin effects by cleaving the platelet glycoprotein Iib subunit.\(^{30}\) Elastase attacks elastic fibers of the vascular extracellular matrix and may contribute to arterial wall aging.\(^{31}\) Moreover, elastase activity is positively correlated to cardiovascular risk factors, such as hypertension and diabetes.\(^{32}\)

Exercise enhanced PLA formation, both in the absence and presence of in vitro stimulation. Although the implications of this are presently unclear, evidence suggests that there are functional consequences of PLAs. Conjugated platelets may facilitate leukocyte rolling, adhesion, and migration into the vessel walls and enhance leukocyte accumulation at inflammatory sites and, thus, tissue damage. Conjugated platelets may also help to clear activated leukocytes from the circulation.\(^{34}\) Moreover, platelet-monocyte aggregates may enhance thrombin generation by bringing together tissue factor expressed on monocytes, platelet-released coagulation factor Va, and a catalytic surface on the platelet phospholipid bilayer membrane.

Taken together, the present data support the idea that exercise causes multicellular activation, which may promote thrombosis. However, exercise also activates fibrinolysis,\(^{1,35}\) and a disturbed fibrinolytic response to exercise seems to have prognostic implications.\(^{36}\) The thrombotic risk during exercise will depend on the balance between platelet activity, fibrin formation, and fibrinolysis.

The protective effect of aspirin treatment involves complex mechanisms, some of which were evaluated in the present study. We found that aspirin inhibited P-selectin expression on single platelets in vivo at rest, although this was not seen by previous investigators.\(^{8,9}\) However, we found no effect of aspirin on thrombin generation in vivo or soluble P-selectin, circulating PPAs, or PLAs at rest. Furthermore, aspirin did not attenuate the responses to exercise of any of these parameters, despite good compliance, as verified by consistent decreases of urinary 11-dehydrothromboxane B\(_2\) excretion.

Interestingly, aspirin inhibited fMLP-induced neutrophil CD11b expression, both at rest and after exercise. Aspirin and other nonsteroidal antiinflammatory drugs may inhibit neutrophil adhesion,\(^{37}\) transmigration,\(^{38}\) superoxide anion generation,\(^{16}\) and adhesion-molecule expression.\(^{17}\) The mechanisms involved in the present findings are not clear, and further investigation is warranted.

In conclusion, the present data demonstrate that strenuous exercise induces multicellular activation in vivo, enhances the in vitro responsiveness of both platelets and leukocytes, and promotes a prothrombotic state. Novel findings are that exercise increases platelet-leukocyte aggregation and that aspirin treatment may reduce resting platelet P-selectin ex-
pression in vivo and agonist-induced neutrophil CD11b expression. However, the prothrombotic effects of exercise were not attenuated by aspirin.

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References
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