Effect of Strenuous Exercise on Platelet Activation State and Reactivity

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Background. It has been hypothesized that platelets are activated, or made more activatable, by strenuous exercise and that these changes may play a role in the genesis of exercise-induced coronary ischemia. Previous studies have yielded conflicting results but have used assays (eg, platelet aggregation, plasma platelet factor 4, and plasma β-thromboglobulin) that are subject to methodological problems.

Methods and Results. In the present study, a whole blood flow cytometric method was used to study the platelet activation state and reactivity of 12 physically active and 12 sedentary individuals before and after standardized treadmill exercise testing. The peptide gly-pro-arg-pro (GPRP) was included in this assay to prevent fibrin polymerization and platelet aggregation, thus allowing the measurement of the reactivity to thrombin of individual platelets in the physiological milieu of whole blood. A panel of fluorescent-labeled monoclonal antibodies was used to monitor activation-dependent platelet surface changes: downregulation of glycoprotein (GP) Ib (6D1) and upregulation of GMP-140 (S12), the GPIIb-IIIa complex (PAC1), and GPIV (OKM5). In samples obtained before exercise, platelets not exposed to thrombin showed no evidence of in vitro activation. In the sedentary subjects, exercise caused a consistent and significant augmentation of the platelet activation state and reactivity as judged by the binding of 6D1 in the presence of thrombin 0.05 U/mL (P<.001), 0.05 U/mL (P=.001), and 0 U/mL (P=.004) and by the binding of OKM5 in the presence of thrombin 0.05 U/mL (P<.001), 0.005 U/mL (P=.029), and 0 U/mL (P=.035). Exercise increased the binding of PAC1 at only a single thrombin concentration (0.005 U/mL, P=.027) and did not alter the binding of S12 at any thrombin concentration. In contrast, in the physically active subjects, exercise failed to cause a consistent alteration in either platelet activation state or platelet reactivity. No significant differences were found between the 12 male and 12 female volunteers.

Conclusions. Strenuous exercise in sedentary subjects but not physically active subjects resulted in both platelet activation and platelet hyperreactivity. These changes were more readily detected with monoclonal antibodies directed against GPIb (6D1) and, to a lesser extent, GPIV (OKM5) rather than those directed against the GPIIb-IIIa complex (PAC1) and GMP-140 (S12). Platelet activation by thrombin, generally regarded as the most physiologically important agonist, can be studied in whole blood in a clinical setting through the use of the peptide GPRP. (Circulation. 1993;88[part 1]:1502-1511.)

Key Words • thrombin • antibodies • platelets • exercise • peptides • flow cytometry

Epidemiological studies have described a complex relation between exercise and coronary ischemic syndromes. Habitual, sustained exercise has been postulated to reduce the incidence of ischemic heart disease and may reduce cardiovascular mortality as well. The beneficial effects of regular exercise have been observed both in healthy individuals and in patients with known coronary artery disease. However, acute exertion has also been reported to be a cardiovascular stressor. The risk of cardiac arrest in men not known to have coronary artery disease rises acutely and transiently during acute exercise, particularly in men who are sedentary. The clinical observation that strenuous exercise can precipitate coronary ischemia forms the basis for the widespread use of treadmill exercise testing as a means of provoking ischemia for diagnostic or prognostic purposes.

Several lines of evidence suggest that platelets may play a role in the pathogenesis of coronary ischemia. Angiography and angioscopy during acute coronary ischemic syndromes frequently reveal intracoronary arterial thrombi, which pathological studies have found to be rich in platelets. Large therapeutic trials of antiplatelet agents support the concept of an association between platelets and cardiovascular ischemia: Antiplatelet agents have been shown to reduce the incidence of cardiovascular ischemic events. Furthermore, some investigators have found biochemical markers of platelet activation to be elevated during episodes of acute cardiac ischemia. Platelets normally circulate in the resting state and can form platelet thrombi only when activated after...
FIG 1. Effect of platelet activation on monoclonal antibody binding is illustrated. Binding of monoclonal antibodies (in italics) to resting platelets and relative change in the binding of these antibodies after thrombin activation is depicted. S12 is directed at the α-granule membrane protein GMP-140\(^6\) (also referred to as P-selectin\(^6\) and PADGEM protein\(^6\)). GMP-140 is not detectable on the surface of resting platelets. After thrombin activation, GMP-140 is translocated to the platelet plasma membrane; thus, S12 only binds to the surface of activated platelets. PAC1 is directed at the fibrinogen binding site on the GPIb-IIIa complex.\(^6\) This fibrinogen binding site is not exposed on resting platelets. Thrombin stimulation results in a conformational change in the GPIIb-IIIa complex that exposes the fibrinogen binding site. Thus, PAC1 only binds to the surface of activated platelets. OKM5 is directed at an epitope on GPIb that may be a thrombospondin binding site.\(^7\) OKM5 binds to resting platelets, but binding is increased after thrombin stimulation.\(^8\) 6D1 is directed at the von Willebrand factor binding site on GPIb.\(^7\) In distinct contrast to the other monoclonal antibodies used in this study, binding of 6D1 is markedly reduced after thrombin stimulation.\(^6\)\(^2\)\(^7\)

exposure to an appropriate agonist. Thus, the state of activation and the activatability of platelets may be an important determinant of platelet thrombus formation.

The effect of exercise on platelet activation is controversial\(^6\) due in part to the fact that the methods used to detect platelet activation during exercise are complicated by various factors.\(^6\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\) (eg, platelet aggregation and radioimmunoassays of β-thromboglobulin [βTG] and/or platelet factor 4 [PF4]) are bedeviled by major methodological problems.\(^6\)\(^5\)\(^6\)\(^7\)\(^8\) In the present study, we used a whole blood flow cytometric assay\(^9\)\(^10\)\(^11\) to quantify the state of platelet activation and platelet reactivity before and after standardized treadmill exercise testing. This flow cytometric assay circumvents many of the problems associated with assays of βTG, PF4, and platelet aggregation by directly studying platelet activation in the physiological milieu of whole blood.\(^6\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\) Inclusion in the assay of an inhibitor of fibrin polymerization (glycyl-L-prolyl-L-arginyl-L-proline [GPRP])\(^9\) enabled us to study platelet reactivity in whole blood to human α-thrombin, which has been shown recently to be the preeminent platelet agonist mediating arterial thrombotic processes.\(^6\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\) A panel of monoclonal antibodies was used to study a number of activation-dependent changes in platelet surface glycoproteins (Fig 1).\(^6\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\) We used these methods to test the hypothesis that strenuous exercise activates or alters the reactivity of the platelets of healthy individuals.

Methods

Study Population

Twelve physically active and 12 sedentary volunteers matched for sex (Table 1) were enrolled in this study after approval of the protocol by the Human Studies Committee of the Medical Center of Central Massachusetts. These volunteers gave written informed consent for their participation in the study. Volunteers were excluded from participation if, within the last 10 days, they had ingested medications known to affect platelet function. The volunteers had been contacted by phone to remind them not to ingest over-the-counter medications for the 10 days before the study. On the day of the study, a trained research nurse assisted all participants in completing a questionnaire designed to elicit information about prior medical events, medical symptoms, medications, and habitual physical activity. No volunteer had a history of diabetes, coronary artery disease, myocardial infarction, stroke, malignancy, or renal failure, and all denied episodic chest discomfort, undue dyspnea on exertion, palpitations, and syncope. Subjects were classified as physically active (Table 1) if they habitually engaged in strenuous exercise (30 minutes or more at least three times per week)\(^14\) and if they also demonstrated an ability to engage in strenuous and prolonged exercise on the treadmill (women, ≥17 minutes; men, ≥19 minutes). Subjects classified as sedentary exercised no more than once every 2 weeks and exhibited a clearly diminished capacity for treadmill exercise (≤14 minutes). The mean age of the physically active subjects (39.3±9.2 years) did not differ significantly from that of the sedentary subjects (37.9±9.1 years). Similarly, the mean ages of the male (37.6±2.2 years) and female (39.3±3.0 years) subjects did not differ statistically.
Exercise Protocol

To avoid the reported increase in platelet reactivity between 6 AM and 9 AM,75 all exercise tests were performed after 9 AM. Before the exercise study, an indwelling 22-gauge intravenous line was inserted with a minimum of trauma into an arm vein. Blood samples were drawn from the intravenous line before treadmill testing (pre), immediately after descending from the treadmill (post), and after a 15-minute rest period (rest). The intravenous access line was gently flushed with 10 mL of normal saline, and a 21-gauge needle was used to withdraw samples from the line using a two-syringe technique. The first 5 mL at each time point was discarded. Samples were analyzed only if blood flowed freely and rapidly into the syringe. Subjects exercised on a motor-driven treadmill (Marquette Case 12, Horseham, Pa) using a standard Bruce protocol,76 which consists of sequential 3-minute stages of successively increasing stage and grade. All subjects were instructed to exercise to volitional fatigue. None of the 24 subjects developed exercise-induced symptoms or signs of coronary insufficiency. Blood pressure and 12-lead electrocardiographic data were recorded in standard fashion; these parameters were analyzed by a cardiologist (A.M.E.) who was blinded to the results of the platelet analysis. No subject demonstrated evidence of exercise-induced ischemia as judged by standard criteria.76

A subgroup of 6 of the physically active subjects (3 men and 3 women matched for age with the sedentary group) were restudied. Exercise in the physically active men was stopped early, such that their duration of exercise was identical to the mean duration of exercise of the sedentary men. Exercise in the physically active women was stopped early, such that their duration of exercise was identical to the mean duration of exercise of the sedentary women. The post and rest time points were as previously defined.

Monoclonal Antibodies

S1266 (provided by Dr Rodger P. McEver, University of Oklahoma), PAC169 (provided by Dr Sanford J. Shattil, University of Pennsylvania, Philadelphia), OKM570 (provided by Dr Patricia Rao, Ortho Diagnostic Systems, Raritan, NJ), and 6D172 (provided by Dr Barry S. Coller, SUNY, Stony Brook, NY) are described in Fig 1. AK3 (provided by Dr Michael C. Berndt, University of Sydney, Australia) is directed at the macroglycopeptide portion of the α-chain of GPIb.77

Whole Blood Flow Cytometric Assay of Platelet Activation State and Reactivity

The whole blood flow cytometric method has been described in detail previously.62 To avoid in vitro platelet activation, there were no centrifugation, gel filtration, vortexing, or stirring steps. Briefly, the method was as follows: Blood was drawn directly into a polypropylene syringe containing acid-citrate-dextrose and, within 5 minutes, diluted in modified HEPES-Tyrode's buffer.62 The samples were then incubated with a saturating concentration of a biotinylated activation-dependent monoclonal antibody (PAC1, S12, OKM5, or 6D1), 2.5 mmol/L GPRP, purified human α-thrombin (provided by Dr John W. Fenton II, New York Department of Health, Albany) or buffer only, a saturating concentration of fluorescein isothiocyanate (FITC) conjugated monoclonal antibody OKM5, and phycocerythrin-streptavidin. The samples were then fixed in 1% formaldehyde, diluted, and analyzed within 24 hours in an

<table>
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<th>TABLE 1. Characteristics of Sedentary and Physically Active Volunteers Participating In the Study</th>
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To be classified as "physically active," subjects provided a history of habitual exercise and demonstrated an ability to engage in prolonged exercise on the day of the study (women, ≥17 minutes; men, ≥19 minutes). Sedentary subjects did not engage in regular exercise and were unable to tolerate more than 14 minutes on the treadmill.
EPICS Profile flow cytometer (Coulter Cytometry, Hialeah, Fla).

After identification of platelets by gating on both FITC positivity and characteristic light scatter, binding of biotinylated monoclonal antibody was determined by analyzing 5000 individual platelets for phycoerythrin fluorescence. In addition to platelets, OKM5 binds to monocytes, but these cells were completely gated out by size (forward light scatter). The fact that platelet activation results in increased platelet surface expression of GPIV, as reflected by increased platelet binding of OKM5, does not affect the ability of OKM5 to identify platelets.

For OKM5-biotin assays, platelets were identified by the FITC-conjugated GPIb-specific monoclonal antibody AK3. Because GPIb is not present on any circulating blood cell except platelets, the activation-induced decrease in the platelet surface expression of GPIb, as reflected by decreased platelet binding of AK3, does not result in fluorescence below the threshold used to distinguish platelets from other cells. Thus, no subpopulations of platelets are excluded. This finding has been independently confirmed by both Shattil et al and Ault et al.

At all thrombin concentrations used (0.005, 0.05, 0.1, and 10 U/mL), thrombin resulted neither in a fibrin clot nor, as determined by light scatter, platelet-to-platelet or platelet-to-monocyte aggregates, thus enabling quantitation of activation-dependent changes per platelet. This method results in no significant differences in fluorescence intensity between samples analyzed immediately and samples analyzed within 24 hours.

In whole blood mixing studies designed to measure the sensitivity of the whole blood flow cytometric assay, aliquots of diluted whole blood were incubated (15 minutes, 22°C) with either 0.05 or 10 U/mL human α-thrombin in the presence of 2.5 mmol/L GPRP, fixed in 1% formaldehyde, and then mixed in different proportions with diluted whole blood not exposed to thrombin. Samples were then diluted and analyzed as described above.

Statistical Analysis

The SAS general linear model (GLM) procedure was used to relate the activation state and reactivity of platelets to the exercise state (pre, post, or rest). Since the distributions of platelet reactivity were positively skewed, a log (x + 1) transformation was used to encourage normality. Separate analyses were performed for each combination of monoclonal antibody (S12, 6D1, OKM5, PAC1) and thrombin dose (0, 0.005, 0.05, and 0.1 U/mL). In these analyses, exercise state and subject were treated as class variables; differences between exercise states were analyzed using the least significant difference method.

Results

Sensitivity of the Whole Blood Assay

Previously reported mixing studies with gel-filtered platelets have demonstrated that as few as 0.8% fully activated platelets could be detected. To quantify the sensitivity of detection of subpopulations of activated platelets in whole blood, we performed mixing studies by adding, in varying proportions, whole blood samples whose platelets had been activated to whole blood samples containing resting platelets (no added thrombin). As we were interested in detecting both fully activated and partially activated platelets in whole blood, we performed these mixing studies both with blood exposed to 10 U/mL thrombin (Fig 2) and with blood exposed to 0.05 U/mL thrombin (Table 2). As determined by the GMP-140-specific monoclonal antibody S12, subpopulations of fully activated (S12-positive) platelets could be clearly visualized in the resultant histograms (Fig 2). Furthermore, a subpopulation of as few as 1% of partially activated platelets could be detected in whole blood by these methods (Table 2).

Whole Blood Assay Does Not Cause In Vitro Activation

Previous methods used to quantify platelet activation (eg, platelet aggregation, assays of plasma PF4, and βTG) have been criticized primarily because platelet activation can be introduced easily in vitro. We therefore examined the effect of our whole blood assay on the activation state of platelets. In whole blood
Sedentary subjects. Sedentary subjects demonstrated consistent, highly statistically significant exercise-induced alterations in platelet activation and activatability, as determined by antibody 6D1 and, to a lesser extent, by antibody OKM5 (Table 3 and Fig 3). Exercise resulted in an immediate (post compared with pre) and significant augmentation of the platelet activation state of sedentary subjects as judged by the binding of 6D1 in the presence of thrombin 0.05 U/mL  

\[ P < .001 \]  

and 0.005 U/mL  

\[ P = .029 \]  

and 0 U/mL  

\[ P = .007 \]  

(Table 3). Exercise also resulted in significant augmentation of the platelet activation state of sedentary subjects as judged by the binding of antibody OKM5 in the presence of thrombin 0.05 U/mL  

\[ P < .001 \]  

0.005 U/mL  

\[ P = .029 \]  

and 0 U/mL  

\[ P = .035 \]  

(Table 3). However, exercise in sedentary subjects resulted in a significant augmentation of the binding of PAC1 at only a single thrombin concentration (0.005 U/mL  

\[ P = .027 \]  

and did not alter the binding of S12 at any thrombin concentration (Table 3).

Comparison of rest with pre samples also demonstrated that exercise in sedentary subjects resulted in platelet activation and platelet hyperreactivity, as judged by the binding of 6D1 in the presence of thrombin 0.05 U/mL  

\[ P < .001 \]  

0.005 U/mL  

\[ P = .007 \]  

and 0 U/mL  

\[ P < .001 \]  

OKM5 in the presence of thrombin 0.05 U/mL  

\[ P = .001 \]  

but not by the binding of S12 or PAC1 at any thrombin concentration (data not tabulated).

Physically active subjects. The binding of all monoclonal antibodies to the platelets in the pre whole blood sample of the physically active subjects was indistinguishable from that of the sedentary subjects (data not shown). Thus, before the start of exercise, platelets from physically active and sedentary subjects were comparable. However, in contrast to our findings with sedentary subjects, exercise had no effect on the activation state or activatability of platelets of physically active subjects, as determined by comparison of post samples to pre samples (Table 3). Similar results were obtained when rest samples were compared with pre samples (data not shown).

To address the possibility that the differences between the physically active subjects and the sedentary subjects could be attributed to the longer duration of exercise by the physically active subjects, additional experiments were performed. We restudied 6 of the physically active subjects (3 men and 3 women) who were matched for age with the sedentary group. Exercise in the physically active men was stopped early, such that their duration of exercise was identical to the mean duration of exercise of the sedentary men. Similarly, exercise in the physically active women was stopped early, such that their duration of exercise was identical to the mean duration of exercise of the sedentary women. The post and rest time points were as previously defined. These studies in physically active individuals demonstrated that exercise resulted in neither platelet activation nor increased platelet reactivity, as determined by the binding of monoclonal antibodies 6D1, OKM5, S12, and PAC1 after incubation of the samples with thrombin 0.005, 0.05, or 0.1 U/mL (data not shown). Thus, the observed differences in exercise-induced platelet activation between the sedentary and physically active subjects was not the result of differences in the duration of exercise.

### Table 2. Whole Blood Mixing Study to Distinguish Partially Thrombin-Activated Platelets From Resting Platelets

<table>
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<th>Added</th>
<th>Detected</th>
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<tr>
<td>100.0</td>
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<td>50.0</td>
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<td>25.0</td>
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<td>10.0</td>
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<td>2.5</td>
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<td>1.0</td>
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Diluted whole blood incubated at 22°C for 15 minutes with 0.05 U/mL thrombin in the presence of 2.5 mmol/L GPRP resulted in platelets with an S12 fluorescence intensity 25.8% of those in whole blood incubated with 10 U/mL thrombin. Aliquots of these partially activated platelets in whole blood were fixed and then mixed in different proportions with diluted whole blood samples without added thrombin. Binding of the activation-dependent antibody S12 was determined as described in "Methods." Percent of detected platelets was determined by S12 positivity, as defined by the percentage of platelets that had a phycoerythrin fluorescence >98% of resting platelets. Unmixed whole blood samples incubated with or without 0.05 U/mL thrombin were assigned 100% and 0%, respectively. Experiment is representative of three so performed.

Samples obtained from the sedentary volunteers before exercise (pre), binding of both S12 and PAC1 (directed at GMP-140 and the GPIIb-IIIa complex, respectively) to platelets was found to be insignificant when no exogenous thrombin was added (Fig 3, open circles). In fact, none of the 24 subjects were found to demonstrate significant binding of these two antibodies before exercise (data not shown), thereby demonstrating that activation of platelets had not been introduced in vitro during the assay.66,69 When we deliberately attempted to activate platelets by exposing the blood to increasing concentrations of exogenous thrombin, we found increases in the platelet surface binding of monoclonal antibodies S12 (GMP-140-specific), PAC1 (GPIIb-IIIa complex-specific), and OKM5 (GPIV-specific) and decreased platelet surface binding of monoclonal antibody 6D1 (GPIb-specific) (Fig 3), thereby confirming that platelet reactivity to thrombin could be studied in vitro in whole blood.

**Effect of Exercise on Platelet Activation State and Reactivity**

To test the hypothesis that strenuous exercise activates and/or alters the reactivity of platelets of healthy individuals, 24 subjects underwent standardized treadmill exercise testing. Blood samples were drawn before treadmill testing (pre), immediately after treadmill testing (post), and after a 15-minute rest period (rest). The state of platelet activation in blood samples exposed in vitro to thrombin 0, 0.005, 0.05, 0.1, and 10 U/mL was determined by flow cytometric assay of monoclonal antibody binding to platelets in whole blood.
Effect of sex. Independent analysis of the data from male and female subjects showed that exercise did not cause a consistent, statistically significant alteration in the state of activation or reactivity of the platelets in either group (data not shown).

Exercise does not result in the formation of platelet subpopulations. Despite the ability of our flow cytometric assay to detect a subpopulation of as few as 1% partially activated platelets (Table 2), analysis of the histograms generated from each subject (n=24) showed no distinct platelet subpopulations (data not shown).

Discussion
The following conclusions can be drawn from this study: (1) Strenuous exercise in sedentary subjects but not physically active subjects resulted in both platelet activation and platelet hyperreactivity. (2) These changes were more readily detected with monoclonal antibodies directed against GPIb (6D1) and GPIV (OKM5) rather than the GPIIb-IIIa complex (PAC1) and GMP-140 (S12). (3) Platelet activation by thrombin, generally regarded as the most physiologically important agonist,63-65 can be studied in whole blood in a clinical setting through the use of the peptide GPRP. Although strenuous exercise has been consistently reported to result in an increase of 10% to 40% in the platelet count,21,23,38,39,41,81,82 the effects of strenuous exercise on platelet function are controversial. Strenuous exertion has been found by some investigators38 but not by others39,41 to augment the adhesion of platelets to glass beads. Whereas some studies have reported that strenuous exercise results in increased platelet aggregation in response to ADP,38,39,47,51,53,54 collagen,39,51 and epinephrine,51 other studies have reported that strenuous exercise has no effect on platelet aggregation21,40,44,48,52 with these agonists. Radioimmunoassays of the plasma concentrations of βTG and PF4, platelet-specific proteins liberated from α-granules during activation, have been used as indicators of the degree of platelet activation.56,57 Some studies21,43,46,53,54 but not others23,49,50,52 report that strenuous exercise causes an increase in the plasma concentration of βTG. Likewise, some studies45,46,50 but not others23,42,49 report that strenuous exercise causes an increase in the plasma concentration of PF4.

The inconsistency of these results illustrates the widely recognized methodological problems associated with the use of platelet aggregation and measurements of βTG and PF4 in vivo studies.55-61 Platelet aggregation studies are semiquantitative and subject to standardization problems.55,61 As a result of the plasma separation procedures required, radioimmunoassays of plasma βTG and PF4 concentrations are particularly vulnerable to artificial in vitro platelet activation.56,57 Platelet aggregometry can measure exercise-induced changes in platelet reactivity but cannot determine whether exercise directly activates platelets. Radioimmunoassays of plasma βTG and PF4 concentrations can indirectly determine whether exercise
Table 3. Effect of Exercise on Platelet Activation State and Reactivity in Healthy Volunteers

| Antibody Binding, | Thrombin, | Antibody Binding, |
|------------------| U/mL      | arbitrary units of | arbitrary units of |
|                  | Pre       | fluorescence       | fluorescence       |
|                  | Post      |                  |                  |
|                  |           |                  |                  |
| 6D1              | 0.000     | 100.0 ±5.92       | 100.0 ±1.48       |
|                  | 0.005     | 88.6 ±3.32        | 91.0 ±2.48        |
|                  | 0.050     | 73.3 ±6.65        | 71.8 ±6.48        |
|                  | 0.100     | 51.4 ±5.14        | 61.8 ±6.48        |
| OKM5             | 0.000     | 100.0 ±12.9       | 100.0 ±10.2       |
|                  | 0.005     | 124.8 ±14.9       | 125.8 ±4.1        |
|                  | 0.050     | 173.8 ±19.2       | 172.5 ±10.0       |
|                  | 0.100     | 231.6 ±24.9       | 197.7 ±20.3       |
| S12              | 0.000     | 4.7 ±0.97         | 4.5 ±1.60         |
|                  | 0.005     | 8.0 ±1.7          | 8.0 ±3.2          |
|                  | 0.050     | 25.1 ±3.9         | 22.1 ±5.7         |
|                  | 0.100     | 67.2 ±5.1         | 50.2 ±8.8         |
| PAC1             | 0.000     | 1.1 ±0.3          | 1.1 ±0.3          |
|                  | 0.005     | 0.8 ±0.2          | 1.6 ±0.4          |
|                  | 0.050     | 2.1 ±0.5          | 6.1 ±1.4          |
|                  | 0.100     | 18.0 ±5.6         | 24.1 ±8.3         |

Healthy volunteers underwent standardized treadmill exercise testing as described in "Methods." Criteria for "sedentary" subjects and "physically active" subjects are specified in "Methods." Blood samples were drawn before treadmill testing (pre), immediately after treadmill testing (post), and after a 15-minute rest period (data not shown). State of platelet activation in blood samples exposed in vitro to 0, 0.005, 0.05, and 0.1 U/mL thrombin was determined by flow cytometric assay of the binding to platelets in whole blood of monoclonal antibodies 6D1 (GPIV-specific), OKM5 (GPIV-specific), S12 (GMP-140-specific), and PAC1 (GPIb-IIa complex-specific). For assays with 6D1 and OKM5, the fluorescence of platelets in pre samples without added thrombin was assigned 100 units. For assays with S12 and PAC1, fluorescence of platelets in pre samples that were maximally activated (thrombin, 10 U/mL) was assigned 100 units. Data are mean±SEM, n=12. P values were determined by paired t test analysis. Only values <.05 are listed. Arrows indicate whether a change was in the direction of increased (↑) or decreased (↓) activation or activatability. NS indicates no change.

activates platelets but cannot measure exercise-induced changes in platelet reactivity. Furthermore, none of these assays can measure the extent of activation of individual platelets, nor can they detect distinct subpopulations of platelets. Clinical studies that use flow cytometric assays of washed platelets or intact platelets may also be susceptible to artifactual changes in vitro platelet activation as a result of the washing procedures.

The whole blood flow cytometric assay used in the present study circumvents many of these methodological problems. In this flow cytometric assay, platelets are directly analyzed in their native milieu of whole blood with minimal manipulation of samples, thereby preventing artifactual changes in vitro activation and potential loss of state of platelet subpopulations. The flow cytometric method permits the detection of a spectrum of specific activation-dependent modifications in the platelet membrane surface. There is a high degree of sensitivity for the detection of platelet subpopulations, and only minuscule volumes (2 μL) of whole blood are required. The other whole blood flow cytometric assays described in the literature use a GPIb-specific monoclonal antibody to identify platelets because GPIb is not present on any other circulating blood cell. The results of the present study demonstrate that the activation-dependent downregulation of the platelet surface expression of GPIb is itself a very sensitive marker of platelet activation in vivo. To analyze platelet surface GPIb in whole blood, we used monoclonal antibody OKM5 (GPIV-specific) as a platelet-specific marker. Although OKM5 binds to monocytes as well as platelets, monocytes can be excluded from analysis because their light-scattering properties are readily differentiated from those of platelets. Another novel feature of our flow cytometric assay, the inclusion of the peptide GPRP, an inhibitor of fibrin clot formation and fibrinogen binding to platelets, permitted the measurement of reactivity to α-thrombin of individual platelets in the physiological milieu of whole blood. Thus, we were able to use the whole blood flow cytometric assay to test the hypothesis that strenuous exercise activates platelets and/or alters platelet reactivity to thrombin.

This study illustrates the importance of analyzing activation-dependent alterations by use of a panel of...
monoclonal antibodies directed against different platelet surface antigens. If the present study had relied on a GMP-140-specific monoclonal antibody (S12) and/or a monoclonal antibody that recognizes exposure of the fibrinogen binding site on the GPIIb-IIIa complex (PAC1), the consistent, highly statistically significant differences in platelet activation state and reactivity in sedentary subjects would have been overlooked. In this study, the exercise-induced changes in platelet activation state and reactivity of sedentary individuals were most easily observed with monoclonal antibodies directed at GPIb and, to a lesser extent, GPIV.

Recently, there has been great interest in the concept that upregulation of the platelet surface expression of GMP-140 or the fibrinogen receptor on the GPIIb-IIIa complex may serve as useful probes for monitoring the state of platelet activation in clinical settings.35,39,41,59,65,66 such as cardiopulmonary bypass,66,68-90 angioplasty,94,95 deep vein thrombosis,65,95 essential thrombocytosis,83,95 and burns.94 However, there is in vitro and in vivo evidence that platelet activation is not an all-or-none phenomenon. For example, in vitro ADP can downregulate platelet surface GPIb without significant exposure of GMP-140 on the surface of the same platelet.62 In addition to the present study, other studies of platelet activation in vivo have found that activation-dependent changes in the binding of monoclonal antibodies are not always concordant.90,93,95 For example, a preliminary report of patients with deep venous thrombosis described significant reductions in the binding of an antibody directed at GPIb but little or no alteration in the binding of antibodies directed at GMP-140, GPIIb/IIIa, or lysosomal membrane proteins.95 In addition to the fact that platelet activation is not an all-or-none phenomenon, it is likely that the extent of platelet activation affects their survival.96-98 Platelet surface GMP-140 has been shown to mediate the adhesion of activated platelets to neutrophils96,97,99 and monocyes,96,99 Although the significance of this heterotypic adhesion is not fully understood, adhesion of a platelet to another cell may affect its survival in the circulation. Furthermore, as our whole blood assay was deliberately designed to analyze single platelets, platelets adherent to monocytes or neutrophils would be specifically excluded from analysis. Thus, in the present study, it is possible that exercise did induce the platelet surface exposure of GMP-140 and/or the fibrinogen receptor on the GPIIb-IIIa complex but that these highly activated platelets were preferentially and rapidly cleared from the circulation.

In this study, we have demonstrated that exercise results in both platelet activation and platelet hyperreactivity in sedentary individuals but not in physically active individuals. Any exercise-induced change in the platelet activation state as a result of the endogenous release of ADP and/or epinephrine was detected in the samples with no added exogenous agonist. Our findings may therefore be explained on the basis of a greater exercise-induced release into the plasma of catecholamines in sedentary individuals compared with physically active individuals.100

Given the evidence linking platelet activation to ischemia, it is possible that the alterations we have described may play a role in the particular vulnerability of sedentary individuals to death during exercise.101 The differences in platelet activation found in this study also may play a role in the reduction of cardiovascular risk attributed to athletic training.1-8,10-12 It is important to emphasize that the individuals described in this study as "physically active" were not extraordinary athletes but rather exhibited a level of fitness well within the reach of many normal individuals.

The presently described methodology should be widely applicable to other clinical settings in which platelet activation is hypothesized to play a role, eg, coronary artery disease,27 hyperlipoproteinemia,101 cigarette smoking,102 and emotional stress,103

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