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Mechanisms Underlying the Morning Increase in Platelet Aggregation: A Flow Cytometry Study

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Objectives. **Mechanisms underlying the morning increase in platelet aggregation produced by arising and assuming the upright posture were studied by examining 1) the expression on the platelet surface of activation-dependent markers; 2) platelet aggregation in whole blood; and 3) hematologic factors likely to influence aggregation.**

Background. **The morning increase in thrombotic cardiovascular events has been attributed, in part, to the morning surge in platelet aggregability, but its mechanisms are poorly understood.**

Methods. **Expression of seven platelet surface antigens (including P-selectin, activated GPIIb-IIIa and GPIb-IX), whole-blood platelet aggregation, platelet count and hematocrit were measured before and after arising in 17 normal volunteers. The fibrinolytic variables, tissue-type plasminogen activator, plasminogen activator inhibitor 1 and catecholamine levels were also measured.**

Results. **On arising and standing, platelet aggregation increased by 71% ($p < 0.01$) and 27% ($p < 0.03$) in response to collagen and adenosine diphosphate, respectively. However, there was no change in any of the activation-dependent platelet surface markers. Whole-blood platelet count and hematocrit increased by 15% and 7% (both $p < 0.0001$), respectively. Norepinephrine and epinephrine levels increased by 189% ($p < 0.0001$) and 130% ($p < 0.01$), respectively. Tissue-type plasminogen activator antigen increased (31%, $p < 0.01$), but there was no significant increase in plasminogen activator inhibitor 1, suggesting an overall increase in fibrinolysis on standing. Prothrombin fragment 1.2 increased by 28% ($p < 0.02$), indicating a small increase in thrombin generation. The increases in hematocrit and platelet count that occurred on standing were carefully mimicked in vitro and resulted in a 115% ($p < 0.05$) increase in platelet aggregation in response to adenosine diphosphate.**

Conclusions. **These data demonstrate that the morning increase in platelet aggregation is not accompanied by expression of activation-dependent platelet surface receptors and suggest that the increase in whole-blood aggregation may be primarily due to the increases in catecholamine levels, platelet count and hemoconcentration.**

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Myocardial infarction, stroke and sudden death occur more commonly in the morning than at other times (1–3). The circadian variation in hemostatic factors that promote thrombus formation or discourage its dissolution is widely believed to be responsible for this increased incidence of thrombotic events. In the early morning hours there is a trough in fibrinolysis, and on subjects' awakening and assuming the upright posture (arising), most but not all investigators have reported an increased tendency of platelets to aggregate in platelet rich plasma (4–11). The mechanism for this increase in aggregation has remained obscure; some investigators have postulated that the catecholamine surge that accompanies arising directly activates platelets; however, in vitro data suggest that catecholamine levels on arising are insufficient to directly affect platelet aggregation (12–16).

The assessment of platelet activation by whole-blood flow cytometry is a recent development and is potentially a much more specific and reproducible technique than traditional aggregation studies (17). The technique depends on the detection of surface antigens (usually glycoproteins [GPs] or their ligands) that become expressed during platelet activation. Antibodies that detect surface expression of activated GPIIb-IIIa, the alpha-granule membrane protein P-selectin or surface-bound ligands, such as fibrinogen or von Willebrand factor (vWF), have been used in most studies. Recently, the decrease in expression of GPIb-IX and the increase in expression of GPIV have been proposed as more sensitive markers of platelet activation (18,19).

The present study was performed to investigate 1) whether the increase in platelet aggregation on arising is accompanied by expression on the platelet surface of markers of activation; 2) to determine whether the increase in aggregation occurs in the more physiologic milieu of whole blood; 3) to delineate the changes in hematologic factors on standing that can influence platelet aggregation; and 4) to further examine the effects on fibrinolytic factors of standing.

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Abbreviations and Acronyms

ADP = adenosine diphosphate
FITC = fluorescein isothiocyanate
GP = glycoprotein
PPP = platelet-poor plasma
PRP = platelet-rich plasma
t-PA = tissue-type plasminogen activator
vWF = von Willebrand factor

Methods

Subjects. Seventeen normal subjects (12 men, 5 women; mean [±SEM] age 33 ± 2 years, range 23 to 51) were admitted to hospital the day before the study. Subjects avoided aspirin and other agents known to alter platelet function for 14 days before the study and refrained from caffeine-containing beverages the day before and during the study. A 16-gauge venous cannula was inserted into a forearm vein, and saline was continuously infused. Free-flowing blood withdrawals were confirmed in each subject. No heparin was used. Subjects were provided with a quiet, darkened room and slept undisturbed until 7 AM, when a blood sample was obtained by the two-syringe technique, and immediately afterward, blood pressure and heart rate were recorded. A sphygmomanometer cuff at 30 mm Hg was used to assist with blood withdrawal. After 10 min, subjects arose and remained standing until a second sample was obtained a mean of 26 ± 4 min later. Samples were analyzed for whole-blood flow cytometry, impedance aggregometry, platelet count and hematocrit. Plasma was assayed for catecholamine, fibrinogen, tissue-type plasminogen activator antigen (t-PA), plasminogen activator inhibitor antigen and prothrombin fragment 1.2 levels. Written informed consent was obtained from subjects, and the study was approved by the Investigational Review Board of the National, Heart, Lung, and Blood Institute.

Whole-blood flow cytometry. The technique of whole-blood flow cytometry allows assessment of platelet activation within minutes of sampling and avoids centrifugation or stirring, which can cause ex vivo platelet activation. Samples were taken into polypropylene syringes and immediately placed in tubes containing 32% sodium citrate (diluted 99:1). Within 5 min, samples were diluted in modified HEPES–Tyrode's buffer. Ten 20-μl aliquots were then each incubated with a saturating concentration of one of nine monoclonal antibodies (two directed at GPIb-IX; two at platelet-bound vWF; and one each at platelet-bound fibrinogen, activated GPIIb-IIIa, GPIV, P-selectin and a lysosomal surface-expressed antigen) conjugated with fluorescein isothiocyanate (FITC), a saturating concentration of a biotinylated antibody (directed at complex GPIIb-IIIa) to identify platelets and phycoerythrin–streptavidin. The samples were analyzed within 2 h on an EPICS 753 flow cytometer (Coulter Corporation). Platelets were identified by both phycoerythrin–streptavidin positivity and characteristic light scatter. Platelet binding of monoclonal antibodies was determined by analyzing both for the proportion with fluorescence (percent positivity) and intensity of fluorescence (arbitrary units). This latter measurement reflects relative changes in the number of antigens expressed per platelet.

Monoclonal antibodies directed against GPIb-IX (thrombin site, colony 12.4), platelet-bound fibrinogen (colony 26.8) and vWF (colonies 22.9 and 30.7), a lysosomal surface-expressed antigen (colony 32.9) and P-selectin (colony 12.2) were produced in the Hematology Service, Clinical Center, National Institutes of Health (20–22). An antibody directed against GPIb-IX (vWF site) was obtained from Pharminogen; the antibody against activated GPIIb-IIIa (PAC-1) was provided by Sanford J. Shattil, MD, University of Pennsylvania; GPIV (OKM5) antibody was puchased from Ortho Diagnostic Systems, and GPIIb-IIIa complex antibody was obtained from Immunotech.

Platelet aggregation. Aggregation was assessed with a mobile four-channel impedance aggregometer (Chronolog-Log Corporation), which allowed measurement of aggregation beginning 1 min after blood sampling. Blood (2.25 ml) was collected into preheated plastic syringes containing 0.25 ml of sodium citrate (3.8%, pH 7.4). Samples were diluted 1:1 in physiologic saline, stirred at 1,200 rpm and kept at 37°C. Aggregation was induced by adenosine diphosphate (ADP) (final concentration 2.5 to 20 μmol/liter) and collagen (final concentration 1.0 to 3.0 μg/ml). For each aggregating agent, two aggregation curves were recorded. The aggregation curve was recorded for 5 min and quantified by calculating the area under the curve relating electrical impedance to time (Ohms. seconds [Ω.s]). This single measurement is sensitive to changes in any of the standard aggregation curve variables: lag time (time from addition of aggregating agent to onset of curve), slope and maximal amplitude. On the day before the study, aggregation curves to a range of concentrations of ADP and collagen were obtained and used to determine the concentrations required to achieve intermediate curves that would be responsive to both increases and decreases in platelet aggregation.

Hemostatic and fibrinolytic factors. Platelet count and hematocrit samples were collected in EDTA and analyzed by standard clinical methods (Coulter counter model STKS). Fibrinogen and prothrombin fragment 1.2 levels were determined in citrated plasma by a standard clinical photooptical clot detection method and by enzyme immunoassay (Enzgnost F1+2 micro, Behring Diagnostics Inc.), respectively. Samples for determination of plasma t-PA and plasminogen activator inhibitor 1 antigen levels were collected into tubes containing sodium citrate, theophylline, adenosine and dipyridamole (Diatube H, Diagnostica Stago, Asnieres-Sur-Seine, France) and were determined by enzyme immunoassay (TintElize tPA, Biopool and Asserachrom PAI-1, Diagnostica Stago). To allow for the hemoconcentrating effect of standing, plasma concentrations of both rest and standing samples were converted to whole-blood concentrations

Table 1. Catecholamines and Fibrinolytic and Procoagulant Factors

	Supine (mean ± SEM)	Standing (mean ± SEM)	p Value
Catecholamines			
Epinephrine (pg/ml)	14 ± 2	33 ± 6	< 0.01
Norepinephrine (pg/ml)	177 ± 17	512 ± 50	< 0.001
Fibrinolytic factors			
PAI-1 antigen (ng/ml)	11.4 ± 3.4	13.8 ± 4.8	0.03
t-PA antigen (ng/ml)	6.0 ± 0.9	7.7 ± 1.1	< 0.01
Procoagulant factors			
Fibrinogen (mg/dl)	203 ± 14	241 ± 30	< 0.03
Fibrinogen (whole blood)*	117 ± 7	133 ± 16	0.02
PT fragment 1.2 (nmol/liter)	0.69 ± 0.11	0.86 ± 0.12	0.01

*Calculated whole-blood concentration. PAI = plasminogen activator inhibitor; PT = prothrombin; t-PA = tissue-type plasminogen activator.

$$\text{Whole blood} = \text{Plasma} \times (1 - \text{Hematocrit}),$$

and statistical comparisons were then repeated. Plasma epinephrine and norepinephrine levels were determined by high performance liquid chromatography, as previously described (23).

In vitro studies. Nine in vitro aggregation studies were performed on blood samples obtained from four normal volunteers (two men), three of whom also participated in the in vivo study. All blood samples were obtained from supine, rested subjects in an identical manner to the in vivo study. Citrated blood was serially centrifuged to obtain platelet-rich plasma (PRP) (760 g for 3 min) and platelet-poor plasma (PPP) (1,500 g for 10 min). Fresh whole blood was collected for impedance aggregometry, but instead of dilution with physiologic saline 1:1 (see above), it was diluted to obtain a control and a hemoconcentrated sample. The hemoconcentrated sample was achieved by diluting whole blood with PRP and saline (ratio 1.1:0.1:0.8, respectively) and the control sample by dilution with PPP and saline (ratio 1:0.2:0.8). Aggregation curves were started within 3 min of sampling. Platelet count and hematocrit were measured in both samples. Duplicate aggregation curves in response to ADP were obtained for six control and six hemoconcentrated samples. Collagen curves were obtained from three control and three hemoconcentrated samples.

Statistical analysis. Mean values for duplicate aggregation curves for each aggregating agent were obtained. The effects of standing were compared by a paired Student *t* test. A p value <0.05 (two-tailed test) was considered significant for *t* tests of the primary end points (platelet measurements) and $p < 0.01$ for secondary end points (plasma factors). This approach also allowed for the effects of multiple comparisons. The Pearson coefficient was used to test for correlations. Results are expressed as mean value ± SEM.

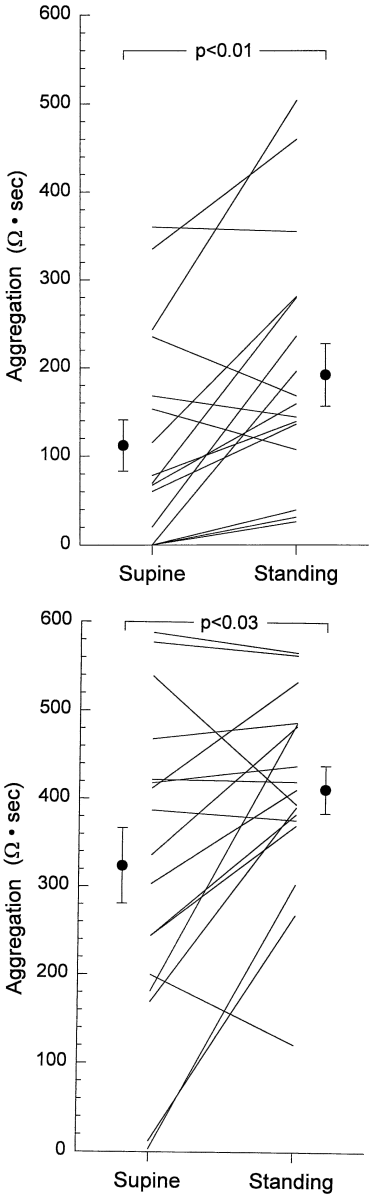

Figure 1. Effect of standing on whole-blood platelet aggregation in response to ADP (**top**) and collagen (**bottom**). **Circles** and **vertical bars** = mean values ± SEM, respectively.

Results

Before arising, mean heart rate was 60 ± 2 beats/min, and this increased to 87 ± 3 beats/min on standing. Rest epinephrine and norepinephrine levels increased on standing by 189% ($p < 0.01$) and 130% ($p < 0.001$), respectively (Table 1).

Platelet aggregation (Fig. 1). In whole blood, ADP-induced platelet aggregation increased markedly on arising and standing in the morning. The area under the ADP aggregation curve increased by 71% ($p < 0.01$), and collagen-induced aggregation increased by 27% ($p < 0.03$).

Flow cytometry studies (Table 2). Nine activation-dependent antibodies were studied in whole blood before and after arising. Supine measurements demonstrated very low levels of expression

Table 2. Whole-Blood Flow Cytometry

Membrane Antigen	% Positive		Intensity (arbitrary units)	
	Supine	Standing	Supine	Standing
GPIb-IX (vWF)	98 ± 1	98 ± 1	132 ± 3	132 ± 2
GPIb-IX (thrombin)	97 ± 1	97 ± 0	103 ± 5	103 ± 5
GPIV	97 ± 1	97 ± 1	121 ± 41	119 ± 4
Activated GPIIb-IIIa	0.2 ± 0.1	0.2 ± 0.1	57 ± 11	68 ± 18
Fibrinogen	0.0 ± 0.0	0.1 ± 0.0	62 ± 9	74 ± 10
P-selectin	0.1 ± 0.0	0.2 ± 0.1	47 ± 7	46 ± 5
vWF (colony 22.9)	0.2 ± 0.1	0.3 ± 0.1	56 ± 7	59 ± 14
vVF (colony 30.7)	0.1 ± 0.1	0.2 ± 0.0	54 ± 11	58 ± 9
Lysosomal protein	1.2 ± 0.5	1.1 ± 0.5	37 ± 7	40 ± 7

Data presented are mean value ± SEM. GP = glycoprotein; vWF = von Willebrand factor.

(mean positivity, all ≤1.2%) of antibodies directed against P-selectin, activated GPIIb-IIIa, vWF and fibrinogen, indicating that no significant ex vivo activation occurred as a result of our methodology. On standing, the expression of these markers did not change (mean positivity, all ≤1.1%). Furthermore, the intensity of fluorescence of antibodies against GPIb-IX receptors did not decrease and that against GPIV receptors did not increase on arising.

Hemostatic factor levels (Fig. 2, Table 1). Increases in platelet count and hematocrit, which can promote both whole-blood and PRP aggregation, accompanied standing. On arising, platelet count increased by 15 ± 3% and hematocrit by 7 ± 1% (both $p < 0.001$). There was a trend toward an increase in plasma fibrinogen levels that became nonsignificant when allowance was made for changes in hematocrit (+12 ± 7%, $p > 0.2$). Prothrombin fragment 1.2, a marker of thrombin generation, increased by 28 ± 6% and remained significant after adjustment for changes in hematocrit (+22 ± 6%, $p < 0.01$).

Fibrinolytic factor levels (Table 1). The t-PA factor antigen levels increased on arising (31 ± 7%), even after adjustment for changes in hematocrit (25 ± 7%, $p < 0.01$). Plasminogen activator inhibitor antigen levels did not change significantly ($p = 0.3$).

In vitro aggregation studies. To mimic the effect of standing on platelet count and hematocrit, rest whole-blood samples were enriched with PRP, and dilution with saline was reduced before induction of aggregation. This process increased platelet count and hematocrit by 17 ± 2% and 10 ± 1%, respectively. Aggregation in response to ADP increased from 104 ± 27 Ω.s in the control sample to 224 ± 48 Ω.s in the hemoconcentrated and platelet-enriched sample (+115%, $p < 0.01$). In response to collagen, aggregation increased from 86 ± 14 to 222 ± 4 Ω.s, respectively. Duplicate ADP aggregation curves for six samples from the same subject, each with different red cell and platelet concentrations, demonstrated strong correlations between increasing platelet count and aggregation ($y = 4.3x - 873$, $r = 0.879$, $p < 0.03$) and between hematocrit and aggregation ($y = 22x - 855$, $r = 0.68$, $p = 0.14$). Similarly, collagen aggregation curves for four samples from another subject demonstrated strong correlations with both platelet count ($y = 4.0x - 762$, $r = 0.93$) and hematocrit ($y = 33x - 1{,}222$, $r = 0.97$).

Discussion

The present study clarifies several aspects of the effects on platelet aggregation of arising and assuming the upright posture in the morning: 1) On arising, increased platelet aggregation can readily be observed in whole blood; 2) this increased aggregation is not accompanied by platelet activation, as evidenced by changes in activation-dependent markers on the platelet surface; 3) the observed increase in aggregation in whole blood may be partly explained by increases in platelet count and hematocrit that accompany arising. In addition, the study confirmed previous reports of increased fibrinolysis on standing and provided new evidence of an opposing increase in thrombin generation on standing.

Comparison with previous studies. Studies reporting the effects of arising in the morning on platelet aggregation (7–11,13) have exclusively studied aggregation in PRP, and to our knowledge, the present study is the first to report the effects of arising on platelet aggregation in whole blood. Of the previous studies on PRP, the majority have reported a significant increase in aggregation with standing, but interestingly, the only study that adjusted for the increase in platelet count on standing reported nonsignificant results (11).

In whole blood, an increase in platelet count promotes the platelet release reaction, and a large increase in hematocrit promotes platelet aggregation (24–26). Furthermore, in PRP, exercise-induced increases in aggregation may be explained by increases in platelet count alone (27). Because we observed significant increases in platelet count and hematocrit on standing, we investigated the effects of this phenomenon on whole-blood platelet aggregation in vitro. When these postural effects of standing on platelet count and hematocrit were carefully mimicked in vitro, whole-blood aggregation increased to a similar extent as that observed in vivo when arising. Furthermore, for samples from the same subject, in vitro increases in platelet count and to lesser extent hematocrit correlated strongly with increases in aggregation. Thus, an increase in platelet count and hematocrit with assumption of the upright

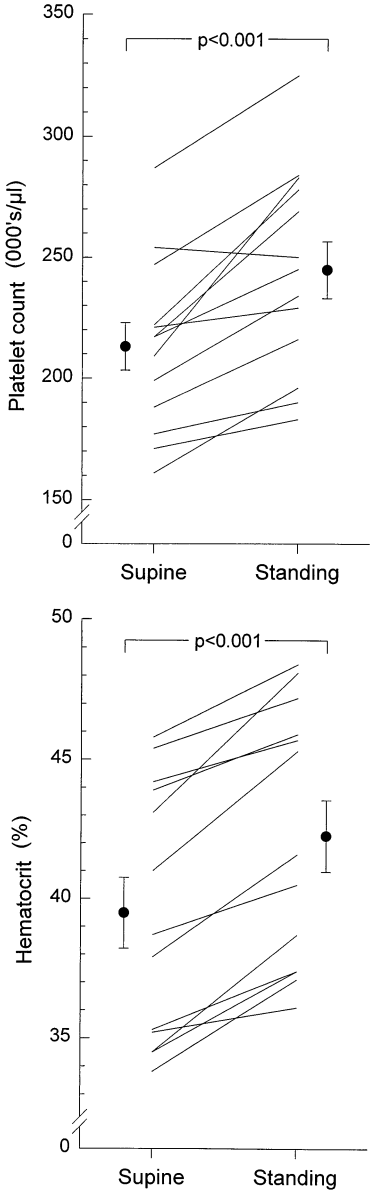

Figure 2. Effect of standing on platelet count **(top)** and hematocrit **(bottom)**. Symbols as in Figure 1.

posture may provide at least a partial explanation for our apparently paradoxic observation of increased aggregation in the absence of platelet activation.

The effect of standing on whole-blood platelet count and hematocrit is believed to be due in part to increased epinephrine levels (10). Epinephrine infusions and exercise cause a rapid but transient increase in platelet count and, importantly, in platelet size (27–33). The spleen is the likely source of these platelets because it is the only organ that contains sufficient sequestrated platelets (~30% of the normal circulating pool) of the right size (splenic platelets are larger than their circulating counterparts) that could be rapidly released into the circulation (34,35). Given the increase in catecholamine levels that accompany standing and because larger platelets are known to aggregate more readily, it is likely that the increase in platelet count observed on standing is a result of the splenic release of larger, more aggregable platelets (35,36).

Previous investigators have postulated that the morning increase in aggregation is due to direct stimulation by epinephrine of platelet alpha$_2$-receptors. However, the lowest reported threshold for potentiation by epinephrine of ADP aggregation is 600 pg/ml (approximately sevenfold higher than the highest level recorded in our study), and the majority of studies have reported thresholds of at least 1,800 pg/ml (12–15). One could postulate that the increase in thrombin generation (see below) combined with the increase in epinephrine could have contributed to the observed morning increase in aggregation. However in a study in which thrombin generation was increased fourfold, no change in the epinephrine threshold was observed (12).

Strenuous exercise in sedentary subjects has been reported (19) to cause platelet activation, as measured by a flow cytometric method. Kestin et al. (19) reported downregulation of GPIb-IX after exercise, but the most consistent effect of exercise was the sensitization of platelets to activation by thrombin. In another flow cytometric study (37), infusion of high physiologic concentrations of epinephrine in normal subjects sensitized platelets to activation by ADP when assessed by fibrinogen binding and P-selectin expression. However, epinephrine did not affect either of these markers of activation in the absence of ADP. These studies are consistent with the present one, and together they suggest that in vivo physiologic increases in epinephrine do not directly activate platelets but can sensitize platelets to the action of aggregating agents.

Advantages of whole blood-platelet aggregometry and flow cytometry. The technique of whole-blood impedance aggregometry has been demonstrated (38) to correlate with PRP optical aggregometry in a large population-based study. Furthermore, characteristic features of specific aggregating agents, such as the lag time between addition of collagen and onset of aggregation, are seen with both techniques (39–41), which suggests that impedance aggregometry is indeed a measure of platelet aggregability and provides information similar to that of traditional turbidometry (39–42). Impedance aggregometry has the advantage of being a whole-blood technique, and it can be performed immediately after blood sampling, thus avoiding the spontaneous activation of platelets that can occur with time in citrated blood. The technique also avoids both the activation of platelets and the loss of subpopulations of platelets that can occur during the preparation of platelet rich plasma.

In addition to the aforementioned advantages of the whole blood-technique, flow cytometric platelet analysis provides direct measurement of platelet activation by analyzing GP changes on the platelet surface. In samples that have not been stimulated ex vivo with aggregating agents, flow cytometric platelet analysis is unaffected by variables, such as platelet count, size and density. The technique is very sensitive and can detect populations of activated platelets as small as 1% of all platelets in the sample (9). In pilot experiments (data not

shown), we confirmed the recent reports demonstrating that a decrease in intensity of fluorescence of antibody to GPIb-IX may be used as a marker of platelet activation (18).

In our study, platelet aggregation, flow cytometry, platelet count, hematocrit and all plasma factors were measured on the same blood samples from subjects before and after arising in the morning. In particular, aggregation and flow cytometric studies used the same anticoagulant (sodium citrate), and both studies were initiated within 5 min of blood sampling. Flow cytometric analysis was performed promptly after adding the platelet antibodies, thus obviating the need for fixing agents. The low levels of expression of activation-dependent platelet markers at baseline, compared with most whole-blood flow cytometric platelet studies (18,19,37), clearly suggest that minimal ex vivo activation did occur with our technique. We studied a comprehensive array of surface markers of activation, including two recently reported markers (changes in antibody fluorescence intensity of GPIb-XI and GPIV) (18,19). Despite these efforts, on the same blood samples that showed marked increases in whole-blood impedance aggregometry on standing, there was no suggestion of any activation-dependent changes in platelet surface markers.

Postural changes in fibrinolysis and thrombin generation. A circadian rhythm in fibrinolysis with a trough in the early morning has long been recognized (4,5). A recent study (43) demonstrated that standing increases fibrinolysis, but because this effect was consistent throughout the day, it did not affect the underlying circadian rhythm. The results of the present study suggest that the increase in fibrinolysis on standing is at least partly explained by an increase in t-PA in the absence of an increase in plasminogen activator inhibitor. These results are in agreement with those of Winther et al. (10).

Prothrombin fragment 1.2 levels increased on standing even after allowing for the increase in hematocrit. Prothrombin fragment 1.2 is cleaved from prothrombin when thrombin is generated by the action of factors Xa and Va. In conditions such as disseminated intravascular coagulation, values equal to 10 times the upper limit of normal are seen, whereas in venous thromboembolism, 2-fold increases typically occur (44,45). Mild elevations of prothrombin fragment 1.2 levels have been associated with hypertension, male gender, smoking and increasing age, all of which are risk factors for atherosclerosis (46,47). Thus, our finding of a modest increase in prothrombin fragment 1.2 levels with standing suggest that thrombin generation occurs on standing and adds a further factor that contributes to the prothrombotic state associated with arising. However, until the risk of arterial thrombosis associated with such small increases in prothrombin fragment 1.2 has been defined, the relevance of our finding remains unclear.

Conclusions. Platelet aggregation in whole blood increases on arising in the morning, but this increase is not associated with evidence of platelet activation as detected by changes in expression of activation-dependent platelet surface markers. Increases in platelet count, hematocrit and catecholamine levels that accompany assumption of the upright posture may be primarily responsible for the observed effects.

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