Norepinephrine-Induced Human Platelet Activation In Vivo Is Only Partly Counteracted by Aspirin

P.T. Larsson, MD, PhD; N.H. Wallén, MD; P. Hjemdahl, MD, PhD

Background  Epinephrine and mental stress may, via platelet stimulation, enhance the risk of thrombus formation. Norepinephrine is more likely than epinephrine to activate platelets in vivo because of higher levels in plasma but is less well studied in this respect. The antiplatelet drug of choice for patients with coronary artery disease, aspirin, may be less effective during sympathoadrenal activation. We therefore investigated platelet responses in vivo to exogenous norepinephrine with and without aspirin pretreatment.

Methods and Results  Platelet aggregability in vivo was assessed in 11 healthy male subjects, by filtragometry ex vivo (which reflects platelet aggregability in vivo) and by measurements of plasma β-thromboglobulin (β-TG, which reflects platelet secretion). Norepinephrine infusions elevated venous plasma norepinephrine from 1.5 to 4 and 15 nmol/L, respectively, and enhanced platelet aggregability (filtragometry) concentration dependently (P<.001). Platelet secretion (β-TG levels) increased during high-dose infusion (P<.01). Aspirin pretreatment (500 mg orally 12 hours earlier) reduced the excretion of 11-dehydrothromboxane B2 by 62±5% (P<.001) and attenuated platelet aggregability at rest (P<.05) but not the effect of norepinephrine infusion on platelet aggregability. Conversely, resting plasma β-TG levels and the urinary excretion of high-molecular-weight β-TG were not altered by aspirin pretreatment, whereas the norepinephrine-induced increase in plasma β-TG was abolished.

Conclusions  Norepinephrine, at plasma levels easily attained during exercise, enhances platelet aggregability and platelet secretion in vivo in healthy humans. Aspirin may be less effective as an antithrombotic drug during sympathoadrenal activation in humans. (Circulation. 1994;89:1951-1957.)

Key Words  platelets  norepinephrine  aspirin

Aspirin treatment has been proved to be beneficial in ischemic heart disease.1-3 It is commonly accepted that these favorable effects are due to inhibition of the formation of thromboxane, a compound with vascular and platelet-stimulating properties,4 thus resulting in platelet inhibition and attenuation of thrombus formation. The thromboxane pathway, however, is only one of several mechanisms regulating platelet function, and platelets may be stimulated in vivo by a variety of different agonists, among them catecholamines.5 In this context it is of particular interest that sympathoadrenal activation may be a factor involved in the precipitation of acute coronary syndromes6 and that diurnal similarities between epinephrine levels in plasma, myocardial infarction, and platelet sensitivity to epinephrine in vitro have been observed.7,8 Such observations suggest a link between catecholamine-induced platelet activation and acute manifestations of coronary heart disease.

Using filtragometry ex vivo, a technique that monitors platelet aggregability in vivo,9 we have previously found platelet activation by mental stress and high-dose epinephrine infusion (yielding 4 nmol/L in venous plasma), whereas low-dose infusion (1 nmol/L) had no effect.9 We have also found that high-dose epinephrine infusion enhances platelet secretion in vivo10,11 and sensitizes platelets with regard to ADP-induced fibrinogen binding and platelet degranulation (P-selectin expression), as determined by flow cytometry.12 Furthermore, the platelet-activating effects of high-dose epinephrine infusion are α-adrenergic receptor mediated.10 It should be emphasized, however, that epinephrine levels in plasma seldom exceed 1 nmol/L,13 and it may therefore be hypothesized that the α-adrenergic receptor agonist norepinephrine, which activates platelets similarly to epinephrine in vitro,14,15 is of greater physiological and/or pathophysiological interest than epinephrine in this context. Indeed, norepinephrine levels in plasma often increase more markedly than epinephrine during various kinds of stress,13,16 and norepinephrine levels compatible with hormonal functions may easily be attained during, for example, physical exercise.

Interestingly, experimental studies on human platelets in vitro, as well as animal studies in vivo, have indicated that the antiplatelet effects of aspirin are counteracted by catecholamines.17,18 If such interactions between catecholamines and aspirin also occur in vivo in humans, it is possible that platelet activation by catecholamines may limit the antithrombotic effect of aspirin in situations with sympathoadrenal activation. The present study was performed to characterize the effects of norepinephrine on platelet function in vivo and, in addition, to study the impact of aspirin treatment on these effects. We thus chose to compare the effects of low- to moderate-dose norepinephrine infusion on platelet function markers with and without aspirin pretreatment.

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Methods

Subjects and Procedures

The experiments were approved by the Ethics Committee of the Karolinska Institute, and informed consent was obtained from the 11 male volunteers who participated. The study was originally designed for 12 subjects, but results from 1 volunteer were not included because of lack of compliance (twice as high urinary 11-dehydrothromboxane B2 levels “after aspirin”). The volunteers were healthy, 25 to 34 years old, all nonsmokers (one using snuff), and had easily accessible antecubital veins to ensure good quality of the venipunctures performed. They were instructed not to take drugs containing acetylsalicylic acid during at least 14 days before the experiments. The experiments were performed in the morning, between 8 AM and noon, with the subjects fasting overnight and abstaining from tobacco and caffeine-containing beverages. Urine from the preceding night was sampled by the volunteers immediately upon awakening, in flasks containing 0.5 g Na2S2O5 as antioxidant (to protect catecholamines). Two separate experiments (>14 days apart) were performed with each volunteer, one with and one without pretreatment with 500 mg of aspirin (Magneecyl, ACO AB) taken orally 12 hours before the start of the experiment. The subjects rested in a semireclined position for 60 minutes before each experiment, after which time norepinephrine (Noradrenaline, 1 mg/mL Apoteksbolaget) diluted in cold saline with 0.1 mg/mL ascorbic acid (added as antioxidant) was given at a low and a moderately high dose (0.15 and 0.75 mmol·kg⁻¹·min⁻¹; 20 minutes at each dose level). Blood sampling and platelet function tests were performed after the resting period and at the end of each dose level during the infusion. An Exercise Monitor 1160 (Cirtikon Inc) was used to record blood pressure and heart rate semiautomatically throughout the experiments.

Filtragometry Ex Vivo

Equipment and procedures for measurements with the filtration technique have been described in detail previously.⁹,¹⁰ In brief, the technique has been shown to measure platelet aggregates ex vivo in blood continuously drawn from an antecubital vein. Each reading requires a new venipuncture, performed without stasis, by a 19-gauge butterfly needle. The continuously drawn blood (anticoagulated by heparin; final concentration, 5 IU/mL) passes a nickel filter (2.0×0.2 mm) with a pore size of 20 μm. Occlusion of the filter is assessed by pressure transducers, and the time until 25% filter occlusion is measured (τa). Scanning electron microscopy has shown that filter occlusion in the filtragometer is caused by platelet aggregates with few other entrapped blood cells and no fibrin (as a consequence of coagulation) present.⁸ Thus, τa (which may range from 60 to 800 seconds) reflects platelet aggregability in vivo inversely; i.e., rapid filter occlusion with a low τa value indicates high aggregability. The apparatus may contribute to platelet aggregate formation, but this contribution (via contact with the siliconized tubing system and/or shear forces when blood passes the filter) is identical in all measurements. Thus, the technique probably monitors both circulating platelet aggregates and a state of platelet sensitization. In our hands, the within-day variability (coefficient of variation [CV] for log τa, during placebo infusions) has been shown to be 7.9%, and the between-day variability for resting measurements 5.4%.¹⁹

Plasma and Urinary β-Thromboglobulin

New venipunctures of an antecubital vein were performed without stasis for each sample with an 18-gauge stainless steel needle. The first 2 mL of blood was discarded, after which 8 mL was allowed to drip into ice-cooled sampling tubes containing 0.8 mL of an anticoagulant and platelet-stabilizing solution (final concentrations, 9.0 mmol/L EDTA, 1.0 mmol/L theophylline, and 1.4 μmol/L prostaglandin E1). Samples were used only when free flow was obtained. The samples were placed on ice and immediately centrifuged during 30 minutes at 15000g and 4°C. Plasma (0.5 mL) was carefully removed from the midportion of the supernatant and stored at −80°C. β-Thromboglobulin (β-TG) immunoreactivity was analyzed by radioimmunoassay by use of a commercially available kit (Kryptor, Amersham) with modifications described elsewhere.¹⁰ The between-day variability for log plasma β-TG with the present sampling and assay techniques is 8.8% (CV) for resting measurements.

For measurements of the urinary excretion of high-molecular-weight (HMW) β-TG, which increases the specificity of urinary β-TG measurements, the β-TG fraction in urine was prepared by gel filtration and analyzed by radioimmunoassay as previously described.¹⁰

Urinary 11-Dehydrothromboxane B2

Urinary levels of 11-dehydrothromboxane B2 were measured in urine from the preceding night with a commercially available enzyme immunoassay (Cayman Chemical Co). Before analysis, the urines were extracted over Bond-Elute C18 columns (Varian Sample Preparation Products). The eluate was vacuum-centrifuged to dryness, resuspended, incubated in enzyme immunoassay buffer at room temperature overnight (to convert the thromboxane metabolite into opening form), and then analyzed according to instructions from the manufacturer.

Plasma Catecholamines and Other Assays

Plasma was prepared from 10 mL of venous blood anticoagulated with EDTA (final concentration, 10 mmol/L) and stored at −80°C. The plasma catecholamine concentrations were determined by high-performance cation-exchange liquid chromatography with amperometric detection. Blood cell counts, median platelet volume, and hematocrit were estimated in whole blood anticoagulated with EDTA by a Cell-analyzer CA 460 (Medonic AB).

Statistical Analyses

Data are presented as mean±SEM. Filtragometry and β-TG data were logarithmically transformed before statistical evaluation because of the asymmetrical distribution of the data. Antilogs of mean log values are also given in the text. Percent changes are derived from comparisons of original τa and β-TG values. Two-way ANOVAs, repeated-measures design, including the effects of treatment (with and without aspirin) and all time points, were used to compare treatment conditions. One-way ANOVAs, repeated-measures design including all time points, were used to test changes over time during each treatment condition. The significance level for the interaction term of the two-way ANOVA is given. Pairwise comparisons of the effects of aspirin on resting levels of platelet variables were made by use of Student’s t test. Correlations were tested by linear regression analysis (least-squares method), and a two-tailed test was used to determine whether the slope was significantly different from zero. P<.05 was considered to be statistically significant.

Results

Catecholamines and Cardiovascular Variables

Venous plasma norepinephrine levels increased 2- and 10-fold during low- and high-dose infusion, respectively, whereas epinephrine remained at resting levels (Table; Fig 1). Systolic blood pressure increased by 11±2 and 28±3 mm Hg and diastolic blood pressure by 6±2 and 14±3 mm Hg during low- and high-dose infusion, respectively. Heart rate decreased, reflexogenically, by 7±2 beats per minute at the high dose of norepinephrine. Pretreatment with aspirin did not af-
Plasma Catecholamine Levels and Hemodynamic, Platelet, and Other Hematologic Variables at Rest and During Low- and High-Dose Norepinephrine Infusions Without and With Pretreatment With 500 mg Aspirin in 11 Healthy Male Volunteers

<table>
<thead>
<tr>
<th>Plasma catecholamines</th>
<th>Rest</th>
<th>Low-Dose Norepinephrine</th>
<th>High-Dose Norepinephrine</th>
<th>P, ANOVA</th>
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<td>Norepinephrine, nmol/L</td>
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Hemodynamic variables

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<td>Heart rate, beats per minute</td>
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Platelet variables

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Other hematologic variables

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<th>High-Dose Norepinephrine</th>
<th>P, ANOVA</th>
</tr>
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<td>5.8±0.3</td>
<td>7.4±0.5</td>
<td>&lt;.001</td>
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All values are mean±SEM; statistical evaluation was performed by one- and two-way ANOVAs. The P values derived from analysis by two-way ANOVA were >.1 for all variables (ie, no effect of aspirin) and are not shown.

Filtragometry and Plasma β-TG

Ex vivo filtragometry readings (log t_A, 2.38±0.08; antilog, 240 seconds at rest) were shortened by 22±7% during low-dose and by 30±5% during high-dose norepinephrine infusion (both P<.01) (Fig 1). Aspirin pretreatment prolonged resting filtragometry readings by 78±42% (log t_A, 2.56±0.08; antilog, 363 seconds; P<.05) but did not alter the response to norepinephrine infusion.

Resting plasma β-TG levels (log, 1.34±0.06; antilog, 22 ng/mL) were not altered by low-dose but were elevated by 54±27% during high-dose norepinephrine infusion (P<.05). Aspirin pretreatment did not alter resting levels of plasma β-TG (log, 1.31±0.06; antilog, 20 ng/mL) but abolished the increase in β-TG during norepinephrine infusion. The CV for log β-TG at rest was 6.9% despite the difference in treatment on the 2
Experimental days, supporting the contention that aspirin had no effect on plasma β-TG at rest.

Platelet Counts, Platelet Size, and Other Parameters
Platelet counts in venous whole blood increased dose dependently during norepinephrine infusion. Median platelet volume, conversely, was not altered. Hematocrit and leukocyte count were also increased by norepinephrine infusion. Neither of these parameters was affected by aspirin pretreatment (Table).

Urinary HMW β-TG and 11-Dehydrothromboxane B₂
As shown in Fig 2, aspirin pretreatment reduced 11-dehydrothromboxane B₂ levels by 62±5% (P<.001) in urine sampled after the preceding night. Urinary HMW β-TG levels were not influenced by aspirin pretreatment. Reductions of 11-dehydrothromboxane B₂ after aspirin were significantly related to reductions of plasma β-TG responses (log values) to norepinephrine infusion (r=.67, P<.05, n=11) but not to changes in filtragometry readings (log values) at rest or responses to norepinephrine (r=.06 and r=.19, respectively).

Discussion
The main findings of the present study are that elevation of circulating norepinephrine (at levels easily attained during exercise) causes concentration-dependent platelet activation in vivo and, perhaps more interesting, that aspirin pretreatment only partly attenuates this effect.

In agreement with the α-adrenergic receptor mediation of platelet responses in vivo to epinephrine infusion (see the introduction), elevation of the circulating norepinephrine levels by infusion increased both platelet aggregability (ex vivo filtragometry) and platelet release (plasma levels of β-TG). It is reasonable to believe that norepinephrine, too, exerts its platelet-stimulating properties through α-stimulation. In contrast to the situation with epinephrine infusions, there are few previous studies on the effects of norepinephrine infusion on platelet function. These studies used platelet aggregation in vitro to assess platelet activation in vivo in healthy volunteers. Their results are contradictory, which may be related in part to differences in the circulating norepinephrine levels during infusion. Accordingly, plasma levels of ≈4 nmol/L were shown to reduce aggregation in vitro, somewhat higher levels (≈8 nmol/L for 2 hours) did not influence platelet responses in vitro to several agonists, and higher levels (≈12 nmol/L) augmented platelet responses to ADP in vitro. These studies suggest a concentration-dependent (>10 nmol/L) augmentation of platelet responses in vitro by circulating norepinephrine. One small study (four volunteers) does not fit this explanation. To extrapolate in vitro results to the in vivo situation may be difficult. We have previously reported contradictory results with in vitro and in vivo related techniques regarding platelet responses to sympathoadrenal activation. The present data, however, suggest concentration-dependent platelet activation by norepinephrine in vivo.

In the present study we investigated the effect of a single dose of 500 mg of aspirin. This dose, which is somewhat above what has recently been recommended as a bolus dose in the treatment of acute coronary thrombosis, should reduce platelet-dependent thromboxane production in vitro by more than 95%, but the effect on thromboxane synthesis in vivo is expected to be weaker. Our findings of a 62% decrease in the urinary
excretion of 11-dehydrothromboxane B2 reflect a substantial inhibition of the cyclooxygenase pathway in vivo. It cannot be excluded, however, that even more efficient inhibition of thromboxane formation would lead to stronger antiplatelet effects in vivo.

Aspirin clearly prolonged filtragometry readings at rest, in agreement with previous findings, which indicates a reduced tendency of circulating platelets to aggregate. Basal platelet secretion (β-TG in plasma and urine) however, was unaffected by aspirin in our healthy volunteers. Previous studies on healthy volunteers have reported similar findings, ie, unaltered plasma β-TG levels during basal conditions after oral or intravenous aspirin treatment. The doses and duration of treatment used range from 300 to 600 mg during 1 week to one dose of 650 mg 2 hours before public speaking and one of these studies is double-blind and placebo-controlled. Kaplan and coworkers report a reduction of plasma β-TG 90 minutes after ingestion of 600 mg of aspirin, but a return to pretreatment levels after 3.5 days of continuous treatment (600 mg/d). However, this and many other studies lack descriptions of sampling conditions and standardization of rest. We have observed that plasma β-TG levels may decline during hours after the subject assumes the supine position, possibly because of the long half-life of β-TG in plasma (≈100 minutes). Sampling conditions (ie, variable periods of rest) may have influenced the findings of Kaplan and colleagues. Another problem with measurements of plasma β-TG is artifactual release during sampling and sample handling. The present study was performed according to a well-validated procedure and presents low values of β-TG in plasma (≈20 to 24 ng/mL). The observed lack of aspirin effect on these low plasma β-TG levels is substantiated by unaltered urinary excretion of HMW β-TG. Thus, basal platelet secretion does not seem to be inhibited by aspirin.

The effect of aspirin on platelet secretion in vivo may differ, however, between healthy subjects and patients with atherosclerosis, in whom intravascular platelet stimulation might occur. Accordingly, high plasma β-TG levels in patients with severe peripheral arterial occlusive disease have been shown to be reduced after treatment with 1 g aspirin daily for 10 days. This might reflect similar effects of aspirin to those observed during norepinephrine infusion in the present study. However, no effect of aspirin treatment (1.2 g + 200 mg dipyridamole for 2 weeks) was seen on plasma β-TG levels in patients with coronary artery stenosis.

The effect of aspirin on platelet aggregability (filtragometry) at rest could be overcome by norepinephrine. The concentration-response curve for norepinephrine infusion was displaced in a parallel fashion, and aggregability had returned to basal nontreated levels with a dose that elevated plasma norepinephrine to about 15 nmol/L. Higher levels of norepinephrine might be expected to have further effects on platelet aggregability because of the concentration dependence observed. Apparently, the tendency toward platelet aggregate formation at rest is dependent on thromboxane formation to a certain degree, whereas the relative platelet sensitivity to stimulation by norepinephrine is not. It should also be noted that higher plasma norepinephrine levels than those achieved in the present study can be attained during physical exercise, and levels may be even higher in the coronary circulation than in the periphery, with stress causing high degrees of cardiac sympathetic nerve activation. We found elevations of norepinephrine in coronary sinus plasma to almost 20 nmol/L when arterial levels were ≈10 nmol/L during exercise in pacemaker-treated patients (arterial epi-nephrine levels were ≈0.7 nmol/L). Similar discrepancies have been noted in the kidney. The dynamic physiological range for norepinephrine in venous plasma is from ≈1 nmol/L at rest to ≈30 to 50 nmol/L during exhaustive exercise. Thus, sympatoadrenal activation may overcome the platelet-inhibiting effect of aspirin in humans, as previously demonstrated in a dog model of coronary thrombosis.

In contrast to our findings concerning platelet aggregability (ex vivo filtragometry), the norepinephrine-induced elevation of β-TG in plasma was blunted by aspirin pretreatment. In fact, the effect on β-TG secretion seemed to depend on the degree of inhibition of thromboxane formation by aspirin, since reductions of the two parameters were correlated. Thus, platelet aggregability and secretion may be differentially affected by aspirin pretreatment rather than parallel events in vivo. Dissociation of aggregation and secretion has indeed been described in vitro. Interestingly, flow cytometric measurements have recently shown that aspirin pretreatment does not alter fibrinogen binding (a prerequisite for platelet aggregation) or P-selectin expression (a marker for α-granule secretion) in response to stimulation by either weak or strong agonists ex vivo. These data are at some variance with the aspirin effect on norepinephrine-induced elevations of plasma β-TG shown in this study but strengthen the conclusion that aspirin may have limited antithrombotic effects.

The incidence of acute myocardial infarction shows a clear-cut increase in the morning, coinciding with increased sympathetic activity and an increase in platelet aggregability in vitro. Although no causality has yet been proved, it has been hypothesized that the catecholamine surge may, in part, explain the morning peak of myocardial infarctions via platelet activation. Our findings support the role of catecholamines as significant platelet activators in vivo, since even about 4 nmol/L norepinephrine in plasma appears to enhance platelet aggregability. This is above what is seen during normal daily activities and during mental stress, but levels can be higher locally in the coronary circulation, as noted above. Furthermore, our studies were performed in young healthy subjects, whereas older individuals (more representative of those at risk for myocardial infarction) may have more sensitive platelets and thus respond more vigorously to lower levels of catecholamines. Other predisposing factors may also enhance catecholamine responsiveness of the platelets.

In conclusion, the present study shows that the sympathetic neurotransmitter (and under some circumstances also "stress hormone") norepinephrine activates platelets in vivo at plasma levels attained during physical exercise of moderate intensity. This adds importantly to our previous observations of epinephrine-induced platelet activation in vivo, since norepinephrine levels may be more readily elevated than epinephrine levels. Our study also further elucidates the effects and limitations of aspirin as an antiplatelet drug when catecholamine levels are elevated in vivo. The antiplatelet effects of aspirin
observed in the present study, including an abolished platelet secretory response and an upward displacement of the concentration-response curve for platelet aggregability in vivo during norepinephrine infusion, may, in part, form the basis for the clinical observation that aspirin reduces the incidence of acute myocardial infarctions during the morning waking hours.46 However, the present findings also show that the inhibitory effect of aspirin on platelet aggregability could be overcome by moderately high norepinephrine levels. Thus, the antithrombotic effect of aspirin may be limited in situations associated with significant sympathoadrenal activation. Additional platelet inhibition, over and above that afforded by aspirin, may be beneficial in cardiovascular diseases, in which sympathoadrenal activation of platelets may take place.

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