Epinephrine Sensitizes Human Platelets In Vivo and In Vitro as Studied by Fibrinogen Binding and P-Selectin Expression

Paul Hjemdahl, Nicolas A.F. Chronos, Darren J. Wilson, Pierre Bouloux, Alison H. Goodall

Abstract Epinephrine (Epi) infusion influences platelet activation markers in vivo, but in vitro studies have mainly examined supraphysiological Epi concentrations and have yielded conflicting results. In this study whole-blood flow-cytometric measurements of platelet fibrinogen binding and P-selectin expression were used to compare enhancement of ADP (0.1 to 10 μmol/L)-induced platelet activation by Epi infusion in vivo (0.1 and 0.4 nmol • kg⁻¹ • min⁻¹) and by Epi in vitro (10 and 50 nmol/L) in nine healthy volunteers. ADP caused concentration-dependent increases in the percentage of platelets that bound fibrinogen (from 4.4±0.9% to 69.9±4.2%) and that expressed P-selectin (from 4.5±0.5% to 44.2±3.8%). Fibrinogen and P-selectin binding indices (FgBI and PSBI; calculated from mean fluorescence intensity and percentage of positive cells) also increased from 0.18±0.03 to 11.70±1.99 for FgBI and from 0.22±0.03 to 2.34±0.29 for PSBI. Epi concentration-dependently enhanced fibrinogen binding and P-selectin expression in vitro (by 30% at the midpoint of the ADP curve at 10 nmol/L. Epi; P<.001 for both by ANOVAs). High-dose Epi infusion enhanced FgBI similarly and increased maximal P-selectin expression by 38%. Epi (50 nmol/L in vitro) enhanced platelet activation further, whether samples were taken with or without prior Epi infusion. Total expression of glycoprotein Ib/IIIa was unaffected by Epi infusion, but glycoprotein Ib expression per platelet was reduced (P<.05). These in vivo and in vitro effects of Epi on platelet responses to agonist stimulation indicate a prothrombotic potential for sympathoadrenal activation in humans. (Arterioscler Thromb. 1994;14:77-84.)

Key Words • epinephrine • flow cytometry • ADP • fibrinogen • platelet degranulation

There is mounting evidence that platelet activation and thrombus formation are pathophysiological important in ischemic heart disease,1 and it is well established that platelet inhibition by aspirin reduces manifestations of ischemic heart disease in its various stages, ie, stable angina,2 unstable angina,3,4 and after myocardial infarction.3 ADP is a natural agonist for platelet activation,2 and interestingly, ADP-induced platelet aggregation in vitro has prognostic significance for patients with ischemic heart disease.6,7 Platelet function is, however, extremely complex, and various methods elucidating various aspects of platelet function may yield differing results, even in the same study (see Reference 8, for example). Flow cytometry is a sensitive technique with which several important aspects of platelet function can be evaluated directly, including fibrinogen binding (an essential prerequisite for aggregation) and degranulation (as demonstrated by the expression of various granule membrane antigens on the platelet surface).8

Epinephrine (Epi) per se can activate platelets,10,11 However, direct platelet activation by Epi is usually seen at supraphysiological concentrations (micromolar range) and experimentally only under certain conditions.12,13 Lower, more physiological concentrations of Epi can sensitize platelets to other agonists, such as ADP, in vitro, and experimentally only under certain conditions.12,13 Thus, platelet volume increases,14 serum thromboxane B₂ formation is enhanced,16 platelet release is stimulated,15 and platelet aggregation in vivo is enhanced.8,18 Seemingly paradoxically, platelet activation in vivo may be accompanied by reduced platelet sensitivity to agonist stimulation in vitro.19 Thus, the picture emerges that Epi is a poor platelet activator per se but nevertheless has the ability to enhance the effects of other agonists in vitro or to act in concert with other phenomena in vivo to cause platelet aggregation.

The present study was undertaken to further investigate platelet activation by Epi by using flow-cytometric indices of platelet activation. We were particularly interested in determining whether Epi similarly enhances platelet fibrinogen binding or degranulation when administered in vivo and in vitro (at high physiological concentrations); ie, whether the in vivo effects of Epi infusion noted above might be caused by direct or indirect effects of Epi. Since the effects of physiological levels of Epi have been shown to be dependent on the presence of other agonists in vitro, we investigated the effects of Epi in both the absence and presence of another weak agonist. ADP was chosen for this purpose because of its clinically proven predictive value (see above) and common use as a physiological proaggregatory substance.5 We thus investigated the effects of low- and high-dose Epi infusion (at doses previously investigated with in vivo-related techniques and of Epi (10
and 50 nmol/L) in vitro on platelets from human whole blood. The platelet activation markers under study were fibrinogen binding to activated glycoprotein (GP) IIb/IIIa receptors; expression of P-selectin, a degranulation marker representing the α-granule membrane and formerly known as CD62, GMP-140, or PADGEM; and changes in the total levels of expression of GPIIb/IIIa and GPIb receptor complexes.

Methods

Subjects and Procedures

Nine healthy male volunteers aged 20 to 25 years participated in the studies, which were performed on two occasions separated by 1 to 6 weeks. On one occasion 10 mL venous blood was sampled for in vitro studies. On the other occasion the subjects received intravenous infusions of Epi (0.1 and 0.4 mmol·kg⁻¹·min⁻¹), with each dose step lasting 20 minutes before sampling. For this purpose Epi (Adrenalin; ACO, Solna, Sweden) was diluted to the appropriate concentration in ice-cold saline containing ascorbic acid (as an antioxidant) and infused at a rate of 0.5 mL·min⁻¹ by means of a Perufusor pump (B. Braun, Melsungen, FRG) via an indwelling venous cannula inserted before a period of rest. Heart rate was monitored by electrocardiography and blood pressure was measured by sphygmomanometry.

Blood samples for platelet studies and other variables were obtained from the noninfused arm after the subject had rested for 30 minutes in a reclining position and again at the end of each dose level of Epi. Blood was obtained by venipuncture with a 21-gauge butterfly needle as follows. The first 2 mL was drawn into EDTA for blood counts; the next 5 mL was aspirated into Monovette syringes (Sarstedt, Beaumont Leys, Leicester, UK) containing 1/10th volume 3.8% citrate for separation by 1 to 6 weeks. On one occasion 10 mL venous blood was sampled for in vitro studies. On the other occasion the subjects received intravenous infusions of Epi (0.1 and 0.4 mmol·kg⁻¹·min⁻¹), with each dose step lasting 20 minutes before sampling. For this purpose Epi (Adrenalin; ACO, Solna, Sweden) was diluted to the appropriate concentration in ice-cold saline containing ascorbic acid (as an antioxidant) and infused at a rate of 0.5 mL·min⁻¹ by means of a Perufusor pump (B. Braun, Melsungen, FRG) via an indwelling venous cannula inserted before a period of rest. Heart rate was monitored by electrocardiography and blood pressure was measured by sphygmomanometry.

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All subjects underwent a pretrial examination to verify their health status. No subject had taken any platelet-active drug during the 2 weeks preceding either study day. All subjects gave written consent to participate in the study, which was approved by the Ethics Committee of the Royal Free Hospital.

Flow-Cytometric Assay

Reagents

The platelet population was identified with the use of a monoclonal antibody (MAb) against GPIb, RFGP37, which was raised in this laboratory and coupled to fluorescein isothiocyanate (FITC). GPIIb/IIIa was identified with a FITC-conjugated CD41 MAb, RFGP56, also raised in our laboratory. These MAbs were purified from ascites by ammonium sulfate precipitation followed by ion-exchange chromatography on DEAE using standard techniques and coupled to FITC at a FITC to protein ratio of 3 to 4:1. Expression of the GPIb-IX complex was measured with RFGP37, purified as above, but coupled to biotin and detected with streptavidin-phycocerythrin (ST-PE) obtained from Becton Dickinson Ltd, Oxford, UK. Platelet-bound fibrinogen was detected with polyclonal rabbit anti-human fibrinogen coupled to FITC (Dako Ltd, High Wycombe, UK). This was used at a final concentration of 0.1 g/mL. P-selectin expression was determined with a CD62 MAb, CLB-thromb/6, kindly provided in the form of culture supernatant by Dr Willem Ouwerhand, Department of Transfusion Medicine, Cambridge Medical School, Cambridge, UK, and identified with rabbit anti-mouse immunoglobulin G (rabbit anti-mouse IgG) coupled to FITC (Dako Ltd). Negative controls were mouse IgG-FITC (Coulter Immunology, Luton, UK) at a dilution of 1:10 or the appropriate second-layer reagents. All antibodies were used at optimum concentrations that gave maximum fluorescence with minimum nonspecific binding.

The dilution buffer was always N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)—buffered saline (145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgSO₄, and 10 mmol/L HEPES; pH 7.4) that had been passed through a 0.22-μm filter to remove dust particles. This was always supplemented with 0.1 mmol/L ascorbic acid (BDH Chemicals Ltd, Poole, Dorset, UK), which was used as an antioxidant to protect Epi. All reagents were analytical grade or above. ADP and Epi were from Sigma Chemical Co Ltd, Poole, Dorset, UK. Recombinant hirudin was from Accurate Chemical and Scientific Corp, Westbury, NY.

Flow-Cytometric Analysis Procedure

The flow-cytometric analysis of platelets from whole blood has been described and is based on the methods of Shattil et al. and Warkentin et al. Briefly, within 10 minutes of collection 5 μL of whole blood was added to 50 μL of filtered HEPES-buffered saline to which 5 μL of an appropriate dilution of antibody and 5 μL of appropriate concentrations of ADP (final concentrations of 0, 0.1, 0.3, 1, 3, or 10 μmol/L) and Epi (final concentrations of 0, 10, or 50 mmol/L) had been added. The samples were incubated without stirring at 22°C to 26°C and then mixed with 500 μL of 0.2% formyl saline (0.2% [vol/vol] formaldehyde in 0.9% [wt/vol] NaCl) to stop further activation. The incubation time for samples containing directly conjugated antibodies was 20 minutes. For indirect assays the samples were incubated with the primary antibody for 15 minutes followed by another 15-minute incubation after the addition of 5 μL rabbit anti-mouse IgG-FITC or 20 μL ST-PE.

The fixed samples were analyzed in a Coulter EPICS Profile II flow cytometer (Coulter Electronics Ltd, Luton, UK) within 2 hours of collection. The flow cytometer, which produces a laser beam at 488 nm, was aligned daily with 10-μm Immuno Check and Standard Brite beads (Coulter Immunology) to calibrate the light-scatter and fluorescence parameters, respectively. The platelet population was identified by its light-scatter characteristics and enclosed in an electronic bit map. Platelets (5000 per sample) were analyzed, and the results represent the means of duplicate samples. Samples from each subject were labeled with RFGP37-FITC to confirm that more than 98% of the analyzed particles in each were GPIb-positive. The negative cutoff for each antibody was set by using the appropriate negative controls, such that samples gave a value of 2% positive. Typical data from these flow-cytometric analyses are illustrated in Fig 1.

The percentage of platelets positive for the marker and the mean fluorescence intensity (MFI) for each sample were used to calculate the binding index (BI) for the marker from the following equation:

\[
BI = \frac{\text{Percent Positive} \times \text{MFI}}{100}
\]

In Vivo Studies. Concentration-response curves for ADP-induced fibrinogen binding and P-selectin expression were established with and without exogenous Epi (10 or 50 mmol/L). In addition the calcium dependence of Epi-induced enhancement of fibrinogen binding was investigated in four subjects. For this analysis samples of blood were collected into citrate (3.8%) or hirudin (5 U/mL) and analyzed in parallel. Analysis of hirudin samples was carried out in HEPES-buffered saline supplemented with 2 mmol/L CaCl₂.

In Vivo Studies. The three samples obtained during Epi infusion experiments were analyzed as follows. For samples taken at rest (ie, before Epi infusion) and after high-dose Epi, fibrinogen binding was measured over the full concentration range of ADP (0.1 to 10 μmol/L) alone or with 50 mmol/L Epi. In samples taken after low-dose Epi infusion, fibrinogen binding was measured only with 0 and 10 μmol/L ADP and without exogenous Epi. In all samples, P-selectin expression
was measured with 0 and 10 μmol/L ADP without exogenous Epi. Expression of GPIIb/IIIa and GPIb was determined in all three samples without exogenous agonists.

Other Measurements

Platelet counts and mean platelet volume (MPV) determinations were performed on a Coulter SL blood analyzer. Fibrinogen concentrations in plasma were determined by the diagnostic laboratory staff of the Haemophilia Centre, Royal Free Hospital, with a standard thrombin-clotting-time assay. Catecholamine concentrations in venous plasma were determined by high-performance cation-exchange chromatography with electrochemical detection as described and validated.21

Data Presentation and Statistics

Data are presented as mean and SEM; each value was derived from duplicate measurements by flow cytometry. Concentration-effect curves were compared by two-factor repeated-measures ANOVAs calculated by the statistical package SUPERANOVA (Abacus Concepts, Berkeley, Calif) on a Macintosh Fx. Individual data points (eg, resting values) were compared by Student’s t test for paired comparisons using STATVIEW 4.0 (Abacus Concepts).

Results

General Responses to Epi Infusion (Table 1)

Epi infusions evoked the expected increases in venous plasma Epi concentrations, heart rate, and systolic blood pressure and decreases in diastolic blood pressure. Platelet counts increased concentration-dependently during infusion, whereas MPV and plasma fibrinogen were unaltered. Venous plasma norepinephrine (NE) concentrations did not change (from 1.19±0.19 nmol/L at rest). The subjects perceived palpitations and occasionally, slight finger tremor at the high-dose level. One subject developed a migraine headache at the end of high-dose Epi infusion. The sample was taken and the infusion stopped. He rapidly recovered, and his data did not differ from those of the other subjects.

Fibrinogen Binding

In Vitro Studies

In unstimulated samples the percentage of platelets that bound fibrinogen was low (4.4±0.9%). This increased in a concentration-dependent manner with ADP to a maximum of 69.9±4.2% in the absence of Epi and to 79.4±3.6% in the presence of 50 nmol/L Epi in vitro. There was a parallel increase in the MFI so that FgBI was increased 49-fold by ADP in the absence of Epi and 62- to 67-fold in the presence of Epi in vitro (Table 2 and Fig 2). The threshold concentration for enhancement of fibrinogen binding by ADP per se was =0.1 μmol/L (Table 2). Epi alone had no effect on fibrinogen binding when added in vitro (Table 2). The effect of Epi in vitro was to enhance the entire dose-response curve for ADP in a concentration-dependent manner (P<.001 for 10 nmol/L Epi versus control and for 50 versus 10 nmol/L Epi; Fig 2). The enhancement of FgBI increments by 10 nmol/L Epi in vitro was greatest at the lowest concentrations of ADP (290% at 0.1 μmol/L, 90% at 0.3 μmol/L, 31% at 1 μmol/L ADP [ie, at the midportion of the dose-response curve], and 16% at the highest concentration of ADP).

When fibrinogen binding was compared in low and normal extracellular calcium environments, the percentage of positive cells increased in a dose-dependent manner, from 3.3±0.7% to 73.6±3.8% with citrate and from
**TABLE 2. Fibrinogen Binding Index Data for Epinephrine Infusions and In Vitro Experiments**

<table>
<thead>
<tr>
<th>ADP Concentration, μmol/L</th>
<th>0</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro experiments</strong></td>
<td></td>
<td></td>
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<tr>
<td>No Epi in vitro</td>
<td>0.23±0.03</td>
<td>0.34±0.06</td>
<td>1.40±0.26†</td>
<td>4.95±0.80</td>
<td>8.24±1.36</td>
<td>11.22±1.38</td>
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<tr>
<td>+ Epi, 10 nmol/L</td>
<td>0.21±0.03</td>
<td>0.64±0.15*</td>
<td>2.43±0.45</td>
<td>6.41±1.03</td>
<td>10.42±1.08</td>
<td>13.00±1.15</td>
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<tr>
<td>+ Epi, 50 nmol/L</td>
<td>0.22±0.03</td>
<td>0.91±0.21†</td>
<td>2.93±0.54</td>
<td>7.98±1.09</td>
<td>11.75±1.65</td>
<td>14.67±1.33</td>
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<td><strong>Infusion experiments</strong></td>
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</tr>
<tr>
<td>Before infusion</td>
<td>0.18±0.03</td>
<td>0.32±0.06*</td>
<td>1.51±0.37</td>
<td>4.79±1.07</td>
<td>8.85±1.79</td>
<td>11.70±1.99</td>
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<td>+ Epi, 50 nmol/L</td>
<td>0.17±0.04</td>
<td>0.58±0.15*</td>
<td>2.70±0.64</td>
<td>7.37±1.37</td>
<td>11.87±1.96</td>
<td>14.63±2.08</td>
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<td>Low-dose Epi</td>
<td>0.15±0.03</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>11.87±2.15</td>
<td></td>
</tr>
<tr>
<td>High-dose Epi</td>
<td>0.09±0.03‖</td>
<td>0.40±0.09†</td>
<td>2.02±0.51</td>
<td>6.07±1.42</td>
<td>9.72±1.94</td>
<td>12.71±2.20</td>
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<tr>
<td>+ Epi 50 nmol/L</td>
<td>0.12±0.02</td>
<td>0.89±0.32*</td>
<td>3.41±0.99</td>
<td>8.09±1.86</td>
<td>12.09±1.92</td>
<td>15.02±2.34</td>
</tr>
</tbody>
</table>

Epi indicates epinephrine. Values are mean±SEM, n=9. The lowest concentrations of ADP eliciting significant increases in fibrinogen binding (above the respective basal levels) are indicated for in vitro experiments and before and after high-dose Epi infusion (†P<.05, †P<.01). In addition, high-dose Epi infusion reduced basal fibrinogen binding (<P<.05). For ANOVAs comparing concentration-effect curves, see Fig 2 and "Results."

4.4±0.7% to 68.5±8.0% with hirudin. Fig 3 shows that ADP-induced fibrinogen binding was somewhat lower in hirudin-treated samples than in citrated samples, but the enhancing effect of 50 nmol/L Epi was essentially unchanged at physiological extracellular calcium concentrations. Epi enhanced the maximal ADP effects by 39% and 31% on average under low and normal calcium conditions, respectively.

**In Vivo Studies**

Infusion of high-dose Epi in vivo reduced basal levels of fibrinogen binding (Table 2) but enhanced the dose-response curve for ADP-induced fibrinogen binding (FgBI and percentage of positive cells) significantly (P=.02). Low-dose Epi infusion did not alter basal or stimulated fibrinogen binding (Table 2). There was no difference between individual dose-response curves for ADP-induced FgBI in the basal state on the two experimental days (P=.83 for actual values and .97 for the slope of the ADP curve).

The enhancing effect of high-dose Epi in vivo on ADP-induced fibrinogen binding was comparable to that of 10 nmol/L Epi in vitro (P=.24 for actual values and .83 for the slope of the ADP curve). Enhancement was thus slightly but not significantly lower with infusion compared to 10 nmol/L Epi in vitro; at the midportion of the ADP curve (1 μmol/L ADP) the enhancing effects were quite similar (30% versus 31%).

Epi at 50 nmol/L in vitro caused the same degree of enhancement of ADP-induced fibrinogen binding when analyzed in the absence or presence of Epi infusion (P=.32 for actual values and .62 for the slope of the ADP curve).
curves before and after infusion). The concentration-effect curves for ADP plus 50 nmol/L Epi were also very similar on the two experimental days (P=.70 for actual values and .997 for the slope of the curve). Thus, exposure to Epi in vivo did not attenuate further enhancement of ADP-induced fibrinogen binding by a high concentration of Epi added in vitro.

**P-Selectin Expression**

**In Vitro Studies**

P-selectin expression (percentage of positive cells) was 4.5±0.5% in unstimulated samples and increased in a concentration-dependent manner with ADP to 44.2±3.8% in the absence of Epi, to 52.7±3.4% with 10 nmol/L Epi, and to 56.2±3.6% with 50 nmol/L Epi added in vitro. The MFI was not substantially altered, and therefore PSBI showed a similar concentration-dependent increase (=10-fold with ADP alone and =15-fold with ADP+10 nmol/L Epi; Table 3 and Fig 4). The effects of ADP were discernible at 0.3 μmol/L ADP, especially in the presence of Epi, but were not significant until levels of 1 μmol/L ADP or greater had been reached. P-selectin expression was not influenced by Epi per se (Table 3). Epi caused a concentration-dependent shift of the ADP curve for P-selectin expression (P<.001 for 10 nmol/L versus basal and P<.05 for 50 versus 10 nmol/L Epi; Fig 4), with 10 nmol/L Epi causing 44% enhancement of the increment in PSBI evoked by 1 μmol/L ADP and 37% enhancement at 10 μmol/L ADP.

**In Vivo Studies**

Epi infusion enhanced ADP-stimulated P-selectin expression (as shown by an increase in the percentage of positive cells) without affecting basal levels (Table 3 and Fig 4). PSBI increased by 40% on average with high-dose Epi (P<.005). Low-dose Epi infusion did not alter basal or stimulated P-selectin expression. There was no significant difference between either basal or ADP (10^-5 mol/L)-stimulated PSBIs on the two experimental days, and high-dose Epi in vivo and 10 nmol/L Epi in vitro had very similar enhancing effects on ADP-stimulated PSBIs (40% and 37%, respectively).

![Graphs showing ADP-induced fibrinogen binding to platelets in whole blood drawn into citrate (left) and hirudin (right) with or without 50 nmol/L epinephrine (Epi). The buffer was supplemented with 2 mmol/L calcium in the hirudin experiments. Values are mean±SEM, n=4.](image)

**TABLE 3. P-Selectin Binding Index Data for Epinephrine Infusions and In Vitro Experiments**

<table>
<thead>
<tr>
<th>In vitro experiment</th>
<th>0</th>
<th>0.1</th>
<th>0.3</th>
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<th>3</th>
<th>10</th>
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<tbody>
<tr>
<td>ADP only</td>
<td>0.22±0.03</td>
<td>0.22±0.04</td>
<td>0.26±0.04</td>
<td>0.76±0.16*</td>
<td>1.52±0.28</td>
<td>2.34±0.29</td>
</tr>
<tr>
<td>+ Epi, 10 nmol/L</td>
<td>0.22±0.02</td>
<td>0.24±0.03</td>
<td>0.35±0.05</td>
<td>1.00±0.20*</td>
<td>2.11±0.35</td>
<td>3.12±0.35</td>
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<tr>
<td>+ Epi, 50 nmol/L</td>
<td>0.25±0.04</td>
<td>0.28±0.04</td>
<td>0.36±0.05</td>
<td>1.16±0.16†</td>
<td>2.37±0.28</td>
<td>3.34±0.33</td>
</tr>
</tbody>
</table>

**Infusion experiment**

<table>
<thead>
<tr>
<th></th>
<th>0.18±0.03</th>
<th>...</th>
<th>...</th>
<th>...</th>
<th>...</th>
<th>2.64±0.45</th>
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</thead>
<tbody>
<tr>
<td>Low-dose Epi</td>
<td>0.19±0.01</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>2.73±0.48</td>
</tr>
<tr>
<td>High-dose Epi</td>
<td>0.20±0.02</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>3.64±0.63</td>
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</tbody>
</table>

Epi indicates epinephrine. Values are mean±SEM, n=9. The lowest concentrations of ADP eliciting elevations of P-selectin expression above the respective basal value are indicated for in vitro experiments (*P<.01, †P<.001). For ANOVAs comparing concentration-effect curves, see Fig 4.
Expression of GPIIb/IIIa and GPIb

The percentage of platelets expressing GPIIb/IIIa and GPIb was unchanged by Epi infusion, remaining at greater than 99% in all samples. The level of GPIIb/IIIa per platelet (GPIIb/IIIa BI) tended to decrease during low-dose Epi infusion (from 19.0±1.1 to 18.2±0.9; P=.08); no further decrease was found after high-dose infusion (GPIIb/IIIa BI, 18.6±3.0). There was, however, a significant decrease in GPIb expression (GPIb BI) from 25.1±3.2 to 20.5±3.4 (P<.05, n=7) during high-dose Epi infusion. Low-dose Epi had no such effect.

Discussion

The present study clearly demonstrates that high physiological levels of Epi sensitize platelet responses to another weak agonist, as determined by flow-cytometric analyses of fibrinogen binding and P-selectin expression in whole blood, and that the effects of Epi are similar whether administered in vivo or in vitro. However, in agreement with previous in vitro studies, we found that low concentrations of Epi per se did not activate platelets in vitro. We have previously shown that Epi infusion in vivo enhances platelet aggregability at a dose of 0.4 nmol · kg⁻¹ · min⁻¹. This does not contradict the present findings that show no increase in fibrinogen binding or P-selectin expression during Epi infusion in the absence of ADP, as flow cytometry analyzes single platelets. Fibrinogen binding in vivo should lead to platelet aggregation and loss from the blood, which is not observed because P-selectin is not maximally expressed by ADP. The present data show that single platelets are sensitized by Epi infusion so that they bind more fibrinogen and express more P-selectin in response to further stimulation by ADP. When evaluated by flow cytometry in whole blood, platelets are sensitive to stimulation by ADP, especially with regard to fibrinogen binding. The threshold for ADP-induced enhancement of fibrinogen binding has been found to be 0.1 μmol/L, which agrees with previous data and with proaggregatory effects of similar or even lower concentrations of ADP with aggregometry with whole blood in vitro. The threshold for ADP-induced P-selectin expression is somewhat higher (≅0.3 mol/L), but the maximum expression of this variable is also lower. Considerably higher concentrations of ADP are, however, required to induce aggregation with conventional aggregometry in platelet-rich plasma. The present data strengthen the idea that ADP is physiologically important for platelet function. When taken together with the predictive power of ADP-induced platelet aggregation with regard to cardiac events, it appears that ADP is indeed a suitable weak agonist for studies of platelet function.
In the present study low-dose Epi infusion did not sensitize the platelets, which agrees with previous findings that there is no proaggregatory response in vivo at this dose.\textsuperscript{30} High-dose Epi infusion, on the other hand, elicited changes in platelet sensitivity to ADP that were similar to those elicited by 10 nmol/L Epi in vitro. This effect of Epi infusion was not solely due to Epi in the blood sample, as the plasma concentration during high-dose infusion was \( \approx 3 \) nmol/L and the sample was diluted by more than 1/10 in the flow-cytometric assay. Approximately 40\% to 50\% of the Epi in arterial plasma is extracted during one passage through the forearm,\textsuperscript{28} indicating that the arterial concentration of Epi to which platelets have been exposed in vivo is \( \approx 5 \) to 6 nmol/L. It is interesting to note that such concentrations of Epi in vivo had platelet-sensitizing effects almost identical to 10 nmol/L Epi in vitro. Thus the synergy between Epi and ADP in vitro seems representative of events that occur in the more complex in vivo setting.

The enhancement of ADP responsiveness by a high concentration of Epi (50 nmol/L) was similar whether samples were taken with or without prior Epi infusion, suggesting that platelet \( \alpha_2 \)-adrenoceptor sensitivity was not altered. This accords with our previous findings that in vivo proaggregatory effects of infused Epi persist for at least 3 hours\textsuperscript{8} and that platelet \( \alpha_2 \)-adrenoceptors are unaltered by such long-lasting Epi infusions.\textsuperscript{29}

The flow-cytometric analyses were performed under conditions of low extracellular calcium. However, our comparison of fibrinogen binding in blood samples drawn into citrate and hirudin and assayed in 2 mmol/L calcium showed that Epi-mediated enhancement of ADP-induced fibrinogen binding was independent of extracellular calcium levels. This agrees well with the similarity between Epi infusion data and the effects of Epi in vitro. The controversy concerning the calcium dependence of platelet responses to Epi is apparently related to the concentration range studied. At supra-physiological concentrations (\( \geq \)1 \( \mu \)mol/L) Epi per se has been reported to induce aggregation in citrated but not hirudin-treated plasma.\textsuperscript{12,13,30} Enhancement of platelet responsiveness to these high concentrations of Epi in citrated plasma may\textsuperscript{4} or may not\textsuperscript{13} be dependent on enhanced thrombin formation. However, with whole-blood flow cytometry and single-cell counting, Shattil et al\textsuperscript{11} have demonstrated that fibrinogen receptor exposure and fibrinogen binding are enhanced by high Epi concentrations under both low- and high-calcium conditions.

The present study differs from those in which the agonistic effects of Epi alone have been studied. At lower physiological concentrations Epi did not stimulate the platelets directly, but it did enhance the agonistic effects of ADP. This enhancement was consistent, unlike the direct agonistic effects of Epi that show marked interindividual variability. In the present study the enhancing effects of physiological levels of Epi were independent of the concentration of extracellular ionized calcium. The slightly lower response of platelets to ADP in the hirudin samples has two possible causes. ADP-induced platelet activation is enhanced under low-calcium conditions\textsuperscript{31}; thromboxane formation results in a feedback mechanism that leads to secondary-wave aggregation and, in the presence of close cell-cell contact, degranulation.\textsuperscript{32} However, platelet contact and aggregation are avoided in the flow-cytometric assay.\textsuperscript{25} It is possible that the thromboxane generation that occurs in citrated blood could enhance the effects of ADP, but we have shown that ADP-induced fibrinogen binding is unaffected by aspirin in citrated samples.\textsuperscript{33} Alternatively, hirudin may inhibit low-level thrombin generation that augments the effects of ADP. In either case the present data suggest that the facilitating effects of physiological levels of Epi on platelet activation are independent of extracellular calcium (and thus presumably also of thromboxane formation) and are similar under in vivo and in vitro conditions.

The flow-cytometric analysis, which shows increased fibrinogen binding in response to Epi, indicates an increase in the tendency of platelets to aggregate. Increases in bound fibrinogen have been shown to be directly correlated with activation of the GPIIb/IIIa complex,\textsuperscript{24} as determined by binding of the PAC1 MAb to the fibrinogen-binding site on activated GPIIb/IIIa. It was independent of any increase in the total amount of GPIIb/IIIa on the platelets, which was essentially unchanged throughout. The present flow-cytometric data that show enhanced fibrinogen binding to single cells thus agree with our previous studies with flow cytometry ex vivo, which monitors the tendency of circulating platelets to aggregate.\textsuperscript{9}

ADP caused expression of the granule membrane antigen, P-selectin, on \( \approx 50\% \) of platelets. This partial expression of granule membrane antigens has been reported.\textsuperscript{34,35} It is independent of platelet aggregation and platelet fibrinogen receptor occupancy\textsuperscript{36} and is unaffected by inhibitors of the cyclooxygenase pathway.\textsuperscript{37} This partial aggregation is thus unrelated to the full aggregation with ADP, which occurs only under conditions of low extracellular calcium that enhance thromboxane formation.\textsuperscript{31,32}

Whereas Epi infusion had no effect on the total expression of GPIIb/IIIa on platelets, the levels of GPIb per platelet fell significantly with high-dose Epi. The GPIb complex has been shown to be internalized by the platelet after agonist stimulation,\textsuperscript{37,28} and decreased expression of GPIb has been observed in subjects after strenuous exercise.\textsuperscript{39} Thus, our finding of an Epi-induced fall in the surface expression of GPIb provides additional evidence that Epi infusion indeed activates platelets in vivo.

The present findings have potential physiological and pathophysiologic relevance. The venous plasma levels of Epi were elevated from 0.1 nmol/L to 0.7 and 3 nmol/L (corresponding to 5 to 6 nmol/L in arterial plasma, as discussed previously) after infusion. These Epi levels are within the physiological range,\textsuperscript{40} which can be surpassed, for example, during intense exercise.\textsuperscript{41} We chose to study Epi infusion, as this has been well studied with other techniques. However, the results obtained with high physiological concentrations of Epi can probably be generalized to the effects of NE as well, as platelet responses to Epi in vivo are mediated by \( \alpha_2 \)-adrenoceptor stimulation,\textsuperscript{9} and NE infusion, not surprisingly, also enhances platelet aggregability in vivo.\textsuperscript{42} NE increases considerably more than Epi in plasma during physiological activation.\textsuperscript{40,41} Furthermore, local NE levels in plasma from a target organ such as the heart may increase more than in the systemic circulation during stress because of the differentiated pattern with
which sympathetic activation occurs. The α-agonist NE may thus contribute significantly to the platelet-activating properties of sympathoadrenal activation in vivo. The present and previous in vivo-related data are in good agreement and clearly support the idea that sympathoadrenal activation may have a role in thrombosis via direct catecholamine-mediated sensitization of circulating platelets.

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