Neither endogenous nor inhaled nitric oxide influences the function of circulating platelets in healthy volunteers

Johanna ALBERT*, N. Håkan WALLÉN†, Nailin LI†, Claes FROSTELL‡ and Paul HJEMDAHL†

*Department of Surgical Sciences, Section of Anesthesiology and Intensive Care, Karolinska Hospital, S-171 76 Stockholm, Sweden, †Department of Laboratory Medicine, Division of Clinical Pharmacology, Karolinska Hospital, S-171 76 Stockholm, Sweden, and ‡Pediatric Intensive Care Programme, Karolinska Hospital, S-171 76 Stockholm, Sweden

ABSTRACT

Experimental models have indicated prothrombotic effects of inhibition of nitric oxide (NO) production, and anti-thrombotic effects of inhaled NO, but the influence of NO on platelet function ex vivo in humans is not well established. We therefore investigated the effects of systemic inhibition of NO synthesis by N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) and of NO inhalation on platelet function in vivo. On two occasions, L-NMMA (13.5 mg/kg) or saline infusion was administered to 14 healthy volunteers in a double-blind cross-over study. After a 30 min infusion of L-NMMA or placebo, NO inhalation (30 p.p.m) was added during the remaining 30 min of infusion, on both occasions. Measurements included filtragometry ex vivo (reflecting platelet aggregability), flow-cytometric evaluation of platelets in whole blood (fibrinogen binding and P-selectin expression), plasma \(\beta\)-thromboglobulin (reflecting platelet secretion), cGMP in platelets and plasma, thrombin generation markers (thrombin fragment 1+2 and thrombin–antithrombin complexes) in plasma, and bleeding time. L-NMMA increased blood pressure and decreased heart rate. NO inhalation did not influence blood pressure or heart rate, but caused a 3-fold elevation in plasma cGMP levels (\(P<0.001\)). Neither L-NMMA nor NO influenced filtragometry readings or flow-cytometric determinations of platelet fibrinogen binding and P-selectin expression. Furthermore, plasma \(\beta\)-thromboglobulin, platelet cGMP and thrombin generation markers were not influenced by either treatment. Bleeding time was not influenced by L-NMMA compared with placebo, but was increased by \(\approx 25\%\) during NO inhalation (\(P<0.01\)), whether NO synthesis had been inhibited or not. The prolongation of bleeding time by inhaled NO was not accompanied by any effect on the platelet variables assessed. The present results indicate that circulating platelets are not influenced by endogenous or inhaled NO, presumably due to the rapid inactivation of NO in the blood. This does not exclude possible effects of endothelial NO in the interface between the blood and the vessel wall.

INTRODUCTION

Endogenous nitric oxide (NO) produced by endothelial cells is a vasodilator which is produced continuously in the circulatory system [1], and which may inhibit platelet function [2,3]. Animal studies have shown prothrombotic effects due to the inhibition of endogenous NO production [4,5] and anti-thrombotic effects of inhaled NO [6]. However, studies showing effects of NO on platelet function have mainly been performed in vitro, and it is
not well established to what extent NO influences the function of platelets in vivo in humans. NO has a very short half-life in blood, due to inactivation by oxygen or haemoglobin [7], but prolonged effects may be seen if NO is stabilized by carrier molecules in plasma [8]. Platelet function has been evaluated in humans during administration of NO donors [9–13] or of inhaled NO [14–19], and during inhibition of NO synthesis by Nω-monomethyl-L-arginine (L-NMMA) [14,20,21]. The results of these studies are, however, conflicting, as NO administration was associated with reduced platelet activity in some studies [9,10,12,15,17], but not in others [11,13,14,16,18]. Similarly, infusion of L-NMMA did [20], or did not [14,21], increase platelet aggregation. These conflicting results may, in part, be related to the use of different methods to assess platelet function. It is important to minimize in vitro manipulation of samples when evaluating platelet function in vivo; thus whole-blood methods involving little or no sample preparation, and minimal delays before measurements, are preferable [22].

Patients with acute respiratory distress syndrome (ARDS) and infants with pulmonary hypertension may be treated with inhaled NO, but bleeding complications have been reported following such treatment [23]. Interestingly, inhaled NO has been reported to increase bleeding time in healthy volunteers [16,24], but not in ARDS patients [15].

The primary aim of the present study was to investigate whether inhibition of endogenous NO production influences platelet function in vivo. We therefore administered L-NMMA or saline in a double-blind, placebo-controlled study, using different well documented methods to assess platelet function in vivo. The dose of L-NMMA used has been shown to have clear-cut haemodynamic effects and to decrease NO in nasal air by approx. 70% [25]. A secondary aim was to study if inhaled NO inhibits platelet function in humans when the endogenous production of NO is reduced. Platelet function was therefore also studied during NO inhalation, with continued infusion of L-NMMA or placebo.

METHODS

Subjects and study design

Fourteen healthy male volunteers (mean age 22 years; range 18–25 years) were included in the study. All participants were non-smokers and had not taken any drugs during the 2 weeks before experiments. Young healthy males were selected in order to minimize possible risks associated with L-NMMA. They all had clearly visible antecubital veins, allowing repeated venipuncture for blood sampling. The study was approved by the Ethics Committee of the Karolinska Hospital and by the Swedish Medical Products Agency. All subjects gave their informed consent before participating.

The study had a randomized, double-blind, cross-over design with regard to the effects of L-NMMA. Experiments were performed in the morning, after a light breakfast without caffeine, and the two studies were separated by at least 2 weeks. The volunteers received L-NMMA (Clinalfa AG, Läufelfingen, Switzerland) or placebo (0.9% NaCl) intravenously, via an ankle catheter. The antecubital veins were used for sampling. The dose of L-NMMA was 0.3 mg·min⁻¹·kg⁻¹ for 30 min, followed by 0.15 mg·min⁻¹·kg⁻¹ for a further 30 min, based on our pilot study which showed significant cardiovascular responses to L-NMMA and a marked decrease of NO in nasal air [25]. The subjects also inhaled NO (30 p.p.m.; a dose in the high clinical dose range), during continued infusion of L-NMMA or placebo, for the last 30 min of infusion. NO inhalation procedures have been described in detail previously [16].

Haemoglobin saturation was measured continuously with a pulse oximetry finger probe (Datex, OSP-200 Helsinki, Finland). Blood pressure and heart rate were monitored (Finapress 2300; Ohmeda Monitoring Systems). Baseline samples and measurements were obtained after 30 min of rest. Thereafter the infusion of L-NMMA or placebo was started, and blood pressure and heart rate were registered after 5, 10 and 15 min. Sampling and measurements were repeated between 20 and 30 min of L-NMMA/placebo infusion, and again between 20 and 30 min of inhalation of NO. A local anaesthetic cream (EMLA®; Astra, Södertälje, Sweden) was used to minimize discomfort during venipunctures.

Flow-cytometric assay of platelets in whole blood

Platelets were identified by a monoclonal antibody against GPIb (CD42b; RFGP37), coupled to fluorescein isothiocyanate (FITC) at a FITC/protein ratio of 3:4:1 (Cymbus Biotechnology Ltd., Chathersford, Hants., U.K.). Platelet-bound fibrinogen was detected with a polyclonal rabbit anti-(human fibrinogen) antibody coupled to FITC (Rαfγn–FITC; Dakopatts AB, Stockholm, Sweden). Antibodies were used at concentrations that gave maximum fluorescence with minimum non-specific binding. ADP, human α-thrombin and Gly-Pro-Arg-Pro were from Sigma Chemical Co. (St Louis, MO, U.S.A.). All reagents were analytical grade or above.

The flow-cytometric analysis of platelets in whole blood has been described previously [26], and is based on the methods of Shattil et al. [27] and Warkentin et al. [28]. Within 5 min of collection, 5 μl of citrated whole blood was added to 50 μl of Hepes-buffered saline, 5 μl of the appropriate agonist (thrombin at 0.02–0.08 NIH unit·ml⁻¹ or ADP at 10⁻⁷–10⁻⁵ mol·l⁻¹; final concen-
and 5 μl of an appropriate dilution of antibody. When thrombin was used, 2 μl of Gly-Pro-Arg-Pro (0.8 mmol·l⁻¹; final concentration) was added to inhibit fibrin cross-linking [29]. Samples were incubated without stirring for 20 min at room temperature, and diluted with 500 μl of saline containing 0.2% formaldehyde to stop activation. Samples were further diluted 7-fold in formyl saline, before analysis in a Coultier Epics XL flow cytometer (Coulter Corp., Hialeah, FL, U.S.A.). Analyses were performed in duplicate.

The flow cytometer was aligned daily with ‘Immuno-check’ and ‘Standard Brite’ beads (Coulter Immunology) to calibrate light scatter and fluorescence parameters respectively. The platelet population was identified by its light-scattering characteristics and enclosed in an electronic bit map, and 5000 such cells per sample were analysed. Samples from each subject were labelled with antibody RFGP37 to confirm that more than 97% of the analysed particles were GPIib-positive. The percentage of platelets positive for the marker and the mean fluorescence intensity (MFI) for each sample were used to calculate a binding index for the platelet activation marker from the following equation:

\[
\text{Binding index} = \left( \frac{\% \text{ positive cells} \times \text{MFI}}{100} \right)
\]

**Filtragometry measurements**

The *ex vivo* filtragometry technique has been described in detail previously [30,31]. It measures platelet aggregates in blood drawn continuously from an antecubital vein. Each reading requires a new venipuncture, performed without stasis, by a 19 G butterfly needle. Heparin (final concentration 5 units·ml⁻¹) is infused into the tubing system leading the blood to the apparatus, to prevent coagulation. The aggregation time (\(t_a\)) is the time taken to occlude 25% of the pore area of a nickel filter (pore size 20 μm), and is inversely related to platelet aggregability. Previous validation has shown that the filter occlusion is caused by retained platelets [30]; aspirin prolongs filtragometry readings [10,16,30,32]. Filtragometry readings vary markedly between individuals, but are reproducible within individuals, with a coefficient of variation for log\(_t_a\) of < 10% in our hands [31].

**β-Thromboglobulin (β-TG) and markers of thrombin generation in plasma**

An antecubital vein was punctured without stasis, using a 21 G needle (Vacutainer system). A 9 ml sample of blood was drawn into ice-cooled sampling tubes containing 1 ml of an anticoagulant and platelet-stabilizing solution (final concentrations: 9 mmol·l⁻¹ EDTA, 1 mmol·l⁻¹ theophylline and 0.2 mg·l⁻¹ iloprost). Samples were immediately centrifuged at 15000 g (4 °C) for 30 min. Plasma was carefully removed from the mid-portion of the supernatant, divided into aliquots, and stored at −80 °C. Plasma β-TG was analysed by radioimmunoassay (IM-88; Amersham), with modifications described previously [33].

For measurement of the thrombin-generation markers prothrombin fragment 1+2 (F₁⁺₂) and thrombin–antithrombin complexes (TAT), blood was collected into 5 ml Vacutainer tubes with sodium citrate. Samples were centrifuged within 1–2 min (2000 g at 4 °C for 10 min). Aliquots of plasma were stored at −80 °C until analysed for F₁⁺₂ and TAT by enzyme immunoassay (Enzygnost F₁⁺₂ and Enzygnost TAT micro; Behringwerke AG, Marburg, Germany).

**cGMP in plasma and platelets**

Blood for measurements of cGMP in plasma was dispensed into pre-chilled tubes containing EDTA (final concentration 10 mmol·l⁻¹). After centrifugation (2000 g, 4 °C, 10 min), plasma was stored at −80 °C. Plasma cGMP was determined after ethanol extraction, by enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI, U.S.A.).

For determination of cGMP in platelets, a previously published method [34] was modified. Briefly, 20 ml of blood was collected into Vacutainer tubes containing EDTA, indomethacin and isobutylmethylxanthine (final concentrations 5 mmol·l⁻¹, 0.1 mmol·l⁻¹ and 0.1 mmol·l⁻¹ respectively). The blood was immediately centrifuged at 190 g for 15 min to obtain platelet-rich plasma, which was further centrifuged at 450 g for 20 min. The platelet pellet was washed with PBS containing 5 mmol·l⁻¹ EDTA and 0.1 mmol·l⁻¹ isobutylmethylyxanthine, re-centrifuged, and finally resuspended to a platelet count of 250 × 10⁶ cells·l⁻¹. After platelet lysis (1% Triton X-100, 30 min), and centrifugation (12000 g, 30 min), the supernatant was divided into aliquots and stored at −80 °C until analysed by enzyme immunoassay (Cayman Chemical Co.), after ethanol extraction.

**Bleeding time and blood cell counts**

Standardized transverse incisions were made on the volar aspect of the forearm, at a venous pressure of 40 mmHg, using a disposable device (Surgicut II; Ortho Diagnostics, Raritan, NJ, U.S.A.). Each incision was made 10–15 mm distally to the previous incision, and blood was collected at 30 s intervals on filter papers, which were weighed to determine total bleeding volume and peak bleeding rate [35]. The intra-individual coefficient of variation was 14% for bleeding time with the present technique and operator [16].

Platelet counts, platelet size distribution, haemoglobin and white blood cell counts were determined in blood anticoagulated with EDTA (final concentration...
Statistics

Descriptive statistics are presented as mean values ± S.E.M. Percentage changes were calculated. A significant interaction term indicates that the treatment given (L-NMMA or placebo) influenced the response variable during the experiment. If this was the case, a Scheffé post hoc test was used for further analysis. Statistical significance was accepted at the 99% level (P < 0.01), and 0.01 < P < 0.05 was considered to indicate borderline significance, in order to compensate for multiple statistical testing.

A few flow-cytometric data were missing due to haemolysis or technical difficulties, and a few filtragometry measurements were missing for technical reasons (two subjects during L-NMMA infusion and one subject during placebo infusion); in these instances, case-wise deletion of missing data was used. Decisions to exclude data were made before unblinding of the study. Analyses were performed with STATISTICA for Windows (StatSoft Inc., Tulsa, OK, U.S.A.).

RESULTS

Haemodynamic measurements

The effects of L-NMMA infusion on heart rate and on systolic and diastolic blood pressures are shown in Table 1. Heart rate decreased by 15% during L-NMMA infusion, compared with a 7% fall during placebo infusion (P < 0.05 for difference between treatments). Systolic blood pressure increased by 24% during L-NMMA infusion compared with 6% during placebo infusion (P < 0.001), and diastolic blood pressure increased by 30% during L-NMMA infusion compared with 13% during placebo infusion (P < 0.001). Inhalation of NO during continued infusion of L-NMMA or placebo did not influence heart rate or blood pressures.

Flow cytometry and filtragometry ex vivo

In unstimulated samples, 0.8 ± 0.1% of platelets expressed P-selectin and 1.9 ± 0.2% bound fibrinogen before interventions. None of the treatments studied influenced these markers of basal platelet activity. P-selectin expression increased dose-dependently upon stimulation with ADP or thrombin, but this effect was not influenced by infusion of L-NMMA or placebo. Inhalation of NO tended to increase P-selectin expression at the highest ADP concentration, during either infusion (Figure 1). Fibrinogen binding to platelets also showed dose-dependent increases upon stimulation with ADP or thrombin, but this was not influenced by either L-NMMA infusion or NO inhalation (results not shown). The binding indices for P-selectin expression and fibrinogen binding, which take mean fluorescence intensity into account, yielded similar results, with no significant effect of either intervention (results not shown). Filtragometry readings, reflecting platelet aggregability, did not change significantly during either L-NMMA infusion or NO inhalation (Table 2).

Bleeding time

Bleeding time did not differ significantly before infusions of L-NMMA and placebo (P = 0.09), but tended to decrease during infusion of L-NMMA (−7 ± 8%) and to be prolonged on placebo infusion (+15 ± 10%) (Table 2). However, there was no significant effect of L-NMMA treatment. NO inhalation increased bleeding time (P < 0.001), with similar and significant effects during infusions of both L-NMMA and placebo (P < 0.01 for both). Peak bleeding rate and integrated bleeding volume did not differ during infusion of L-NMMA compared with placebo, but showed general increases over time.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Cardiovascular responses to infusion of L-NMMA or placebo, before and during NO inhalation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO was inhaled at a dose of 30 p.p.m. Values are expressed as means ± S.E.M. Significance of differences due to the infusion of L-NMMA compared with placebo: *P &lt; 0.05; **P &lt; 0.001. The effects of NO inhalation were not significant for all parameters.</td>
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<tr>
<td>Substance infused</td>
<td>Baseline</td>
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<tr>
<td>------------------</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>L-NMMA</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>L-NMMA</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>L-NMMA</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
</tr>
</tbody>
</table>
Figure 1  P-selectin expression on platelets during infusion of L-NMMA (upper panels) or placebo (lower panels), before and during inhalation of NO
Increasing concentrations of ADP (left-hand panels) or thrombin (right-hand panels) are displayed on the x-axis. P-selectin expression is defined as the percentage of positive cells. Results are means \( \pm \) S.E.M. There were no statistically significant effects of the treatments given.

which were slightly more pronounced during placebo infusion \( (P = 0.02 \) and \( P = 0.008 \) respectively).

**Platelet secretion and thrombin generation**
Plasma \( \beta \)-TG was not elevated by L-NMMA compared with placebo infusion (Table 2). There was a slight increase in \( \beta \)-TG levels over time \( (P = 0.03 \) by ANOVA), which was somewhat greater during placebo infusion. Plasma TAT and F\( _{1+2} \) did not change significantly during the experiments (Table 2).

**cGMP in plasma and platelets**
Plasma cGMP did not change during infusion of L-NMMA or placebo, but increased in both cases during NO inhalation (Table 2). The response to NO inhalation was greater during L-NMMA infusion than during placebo infusion \( (P < 0.001 \) for difference). Platelet cGMP was not influenced by either L-NMMA infusion or NO inhalation (Table 2).

**White blood cell and platelet counts**
White blood cell counts showed similar slight increases with time during both L-NMMA and placebo infusion. NO inhalation induced no additional changes (Table 2).

Platelet counts (Table 2) and mean platelet volume (results not shown) did not change over time or differ between the treatments given. Haemoglobin values were also stable during the experiments.

**DISCUSSION**
The present study investigated whether systemic inhibition of NO synthesis by L-NMMA increases platelet activity, and whether inhalation of NO inhibits platelet function *in vivo*. Surprisingly, we found no effect of L-NMMA, despite clear-cut cardiovascular responses. In agreement with our previous findings [16], inhaled NO increased bleeding time, without causing any measurable alteration in the function of circulating platelets; we presently extend these observations to show that this is the case even when the synthesis of endogenous NO is inhibited by L-NMMA.

Neither L-NMMA infusion nor NO inhalation influenced platelet aggregation, as assessed by two independent methods, i.e. fibrinogen binding to platelets...
The latter finding is surprising, as the dose of L-NMMA was inhaled at a dose of 30 p.p.m. Values are expressed as means ± S.E.M. Significance of differences due to NO inhalation compared with infusion values: *P < 0.01; **P < 0.0001. Significance of difference in plasma cGMP levels upon NO inhalation with simultaneous infusion of L-NMMA compared with infusion of placebo: †P < 0.01. The effects of L-NMMA infusion compared with placebo infusion (in the absence of NO inhalation) were not significant for all parameters.

### Table 2 Responses to infusion of L-NMMA or placebo, before and during NO inhalation

<table>
<thead>
<tr>
<th>Substance infused</th>
<th>Baseline</th>
<th>Infusion only</th>
<th>NO inhalation + infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding time (min)</td>
<td>L-NMMA</td>
<td>5.5 ± 0.6</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>4.5 ± 0.4</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>Filtragometry (log/g)</td>
<td>L-NMMA</td>
<td>2.11 ± 0.09</td>
<td>2.18 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>2.03 ± 0.06</td>
<td>2.01 ± 0.07</td>
</tr>
<tr>
<td>β-TG (ng/ml)</td>
<td>L-NMMA</td>
<td>16.9 ± 1.9</td>
<td>18.6 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>14.7 ± 1.5</td>
<td>16.8 ± 1.8</td>
</tr>
<tr>
<td>Plasma cGMP (nmol/l)</td>
<td>L-NMMA</td>
<td>4.0 ± 0.5</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>3.8 ± 0.3</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>Platelet cGMP (pmol/10⁹ cells)</td>
<td>L-NMMA</td>
<td>7.4 ± 2.0</td>
<td>6.9 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>6.1 ± 0.9</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>Haemoglobin (g/l)</td>
<td>L-NMMA</td>
<td>153 ± 2</td>
<td>154 ± 4</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>158 ± 3</td>
<td>155 ± 2</td>
</tr>
<tr>
<td>Platelet count (10⁹/l)</td>
<td>L-NMMA</td>
<td>210 ± 8</td>
<td>204 ± 10</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>195 ± 5</td>
<td>203 ± 5</td>
</tr>
<tr>
<td>Leucocyte count (10⁹/l)</td>
<td>L-NMMA</td>
<td>6.1 ± 0.8</td>
<td>7.0 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>5.8 ± 0.3</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td>TAT (μg/l)</td>
<td>L-NMMA</td>
<td>1.05 ± 0.15</td>
<td>1.59 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>1.25 ± 0.31</td>
<td>1.61 ± 0.35</td>
</tr>
<tr>
<td>F₁,₂ (nmol/l)</td>
<td>L-NMMA</td>
<td>0.44 ± 0.05</td>
<td>0.45 ± 0.05</td>
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<tr>
<td></td>
<td>Placebo</td>
<td>0.44 ± 0.07</td>
<td>0.48 ± 0.08</td>
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</tbody>
</table>

in whole blood, which requires activation of the platelet GPIIb/IIIa complex, and filtragrometry *ex vivo*, which reflects platelet aggregability [22,30]. Previous studies in humans reported either unchanged [21] or increased [20] platelet aggregation *in vitro* after L-NMMA infusion. The latter finding is surprising, as the dose of L-NMMA was < 1% of the present dose, and no haemodynamic effects were observed [20]. Previous findings with inhaled NO are also conflicting, as decreased platelet aggregation has been reported in adults with ARDS [15,19] and in critically ill neonates [17], whereas no effect was found in infants with pulmonary hypertension [18] or in our study of healthy volunteers [16]. The clinical studies were performed with *in vitro* techniques, and in patients in whom effects may be related to clinical improvement, rather than studying the direct anti-platelet effects of the inhaled NO. In agreement with this interpretation, Gries et al. [19] excluded clinical non-responders to the inhaled NO before finding attenuation of platelet activity in ARDS patients. Methodological differences may also contribute to the conflicting findings. The present results were obtained with whole-blood methods, which minimize delays (NO is rapidly inactivated in blood) and the risk of artifacts related to manipulation of the sample for *in vitro* studies.

Platelet secretion, as assessed by measurements of plasma β-TG and P-selectin expression on platelets in whole blood, was also uninfluenced by L-NMMA infusion or NO inhalation. As with platelet aggregation, previous results from studies on the effects of L-NMMA on platelet secretion are conflicting. Bodzenta-Lukaszyk et al. [20] reported that very low doses of L-NMMA elevated the plasma levels of platelet secretion products, and that this effect was reversed by L-arginine. However, Krejcy et al. [14] found no effects on platelet secretion of higher doses of L-NMMA, in agreement with the findings of the present placebo-controlled study. There is little reason to believe that L-NMMA would be less effective at high doses. Plasma β-TG increased slightly with time in the present study, a result consistent with our previous study of inhaled NO [16]. This may be related to repeated sampling, which increases the risk of sampling artifacts. Nonetheless, there was no tendency towards attenuated platelet secretion with inhalation of NO in either study; this was the case regardless of whether or not the endogenous synthesis of NO was reduced. Thus there is little or no support for the contention that either endogenous or inhaled NO influences platelet secretion in healthy volunteers.

Bleeding time tended to be shortened by infusion of L-NMMA compared with placebo, but we were not able to confirm the significant decrease found in our previous uncontrolled study [25]. Others have reported that bleeding time was decreased by 24% [21] or unchanged [14] after intravenous infusion of L-NMMA. The prolongation of bleeding time during NO inhalation is in
agreement with previous findings [16,24], although negative results also exist [14]. Inhaled NO yielded similar bleeding time responses whether the endogenous production of NO was intact or not, and the effect was not associated with platelet inhibition in either the present or previous [16] studies. The moderate increases in bleeding time that we have observed are probably of limited clinical relevance.

We found that inhaled NO elevated cGMP levels in plasma, suggesting activation of guanulate cyclase in some cells in vivo, whereas neither l-NMMA nor inhaled NO influenced cGMP levels in platelets. The findings with inhaled NO support our previous findings of dose-dependent elevations of cGMP in plasma [16], and results from a recent study of patients with ARDS [19]. The source of cGMP in plasma is unknown, but may include endothelial cells (especially in the pulmonary circulation) and blood cells. However, platelets are not likely to make an important contribution, in view of the present results. We have no explanation for the finding that the plasma cGMP response to inhaled NO was greater after infusion of l-NMMA compared with placebo.

The fact that we did not find any effects of l-NMMA on circulating platelets was unexpected, as studies in vitro and in animal models have indicated that endothelium-derived NO inhibits platelet function [3,4,36–39]. In addition, platelets are capable of synthesizing NO which may also modulate platelet function [40]. However, we assessed several different aspects of platelet function: platelet aggregation (filtragometry, fibrinogen binding), platelet secretion (β-TG, P-selectin expression) and bleeding time, which is an integrated measure of haemostasis. These methods have, in our hands, previously readily detected platelet activation [11,22,31,32,41,42] or platelet inhibition [16,32,41,43]. We took great care to minimize artifacts created by sampling and/or sample handling, and we achieved low levels of β-TG, TAT and P-selectin-positive platelets in the flow-cytometric assay were low in the absence of agonist stimulation. It is unlikely that the dosage of l-NMMA used was too low, as we observed similar elevations of blood pressure as in our previous study, which also showed a marked (≥ 70%) reduction of NO in nasal air [25]. Thus the present findings suggest that endogenously produced NO has little effect on circulating platelets in healthy individuals.

It has been shown that inhibition of NO synthesis by N’-nitro-arginine methyl ester (l-NAME) increases platelet adhesion in experimentally injured arteries, but not in intact vessels, in rats [5]. Interestingly, cGMP in platelets increased after vascular injury, and this effect was reversed by l-NAME [5]. In agreement with the present findings, platelet cGMP was not influenced by l-NAME in uninjured animals [5]. Thus inhibition of NO synthesis might decrease platelet cGMP levels and influence platelet function in individuals with endothelial lesions, although we were unable to show such effects in healthy individuals.

There is no doubt that NO inhibits platelet function under certain experimental conditions [3,36,39]. Exposure of human platelet-rich plasma to > 100 p.p.m. NO in vitro stabilizes platelets [3,36,39]. The moderate inactivation of NO in blood [1,7,8] limits the access of the circulating platelets to endothelium-derived or inhaled NO. The use of NO donors may circumvent this problem, but results may be influenced by continued release of NO in vitro after sampling. Treatment with different NO donors has [9,10,12] or has not [11,13] been associated with reduced platelet secretion or platelet aggregation. Thus effects of NO on circulating platelets are difficult to demonstrate, presumably due to the rapid inactivation of NO in blood. Most importantly, one must separate effects related to clinical improvement from direct effects of manipulating the NO system in patient studies. This is not a problem in studies of healthy volunteers.

The present finding that thrombin generation was uninfluenced by l-NMMA infusion and/or inhalation of NO does not rule out the possibility that the NO system might influence thrombin generation under pathological conditions. Activated platelets provide a catalytic surface on which thrombin generation occurs, and anti-platelet treatment may attenuate this process.

We studied young healthy male subjects, as high-dose infusion of l-NMMA might cause vascular complications in patients with arteriosclerosis. It is conceivable that endogenous NO might influence circulating platelets differently in patients with haemostatic activation. We measured several, but not all, possible markers of platelet activation in the study. For example, we chose not to evaluate excretion of stable thromboxane metabolites in urine, as this would have required longer experimental periods. It would have been of interest to specifically evaluate platelet adhesion in vivo, which may more closely reflect platelet–vessel-wall interactions, but our bleeding time measurements should have included such a component.

In summary, we found no significant effects of either l-NMMA infusion or NO inhalation on platelet function or thrombin generation in healthy volunteers. There are both supporting and conflicting data for these findings from earlier studies. The present results were obtained using several different methods that have previously been sensitive enough to detect changes in platelet function in response to physiological and pharmacological interventions. Our results do not contradict the idea that NO may act locally to influence platelet–vessel-wall interactions, especially in patients with atherosclerotic lesions. However, platelets in circulating blood do not seem to be influenced by either inhibition of NO synthesis or exogenous NO administration by inhalation, presumably due to the rapid inactivation of NO in blood.
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