Inhaled NO inhibits platelet aggregation and elevates plasma but not intraplatelet cGMP in healthy human volunteers

BEGHETTI, Maurice, et al.

Abstract

Effects of inhaled nitric oxide (NO) on human platelet function are controversial. It is uncertain whether intraplatelet cGMP mediates the effect of inhaled NO on platelet function. We investigated the effect of 30 ppm inhaled NO on platelet aggregation and plasma and intraplatelet cGMP in 12 subjects. We performed platelet aggregation studies by using a photooptical aggregometer and five agonists (ADP, collagen, epinephrine, arachidonic acid, and ristocetin). During inhalation, the maximal extent of platelet aggregation decreased by 75% with epinephrine (P < 0.05) and ristocetin (5% P > 0.05) were unaffected. Platelet aggregation velocity decreased by 64% with collagen (P < 0.05). Plasma cGMP levels increased from 2.58 +/- 0.43 to 9.99 +/- 5.57 pmol/ml (P < 0.05). Inhaled NO inhibits platelet [...]
Inhaled NO inhibits platelet aggregation and elevates plasma but not intraplatelet cGMP in healthy human volunteers

Maurice Beghetti,1,4 Catherine Sparling,2 Peter N. Cox,3 Derek Stephens,5 and Ian Adatia1,3,4

Divisions of 1Cardiology and 2Haematology and Departments of 3Critical Care Medicine, 4Pediatrics, and 5Population Health Sciences, The Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada M5G 1X8

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Beghetti, Maurice, Catherine Sparling, Peter N. Cox, Derek Stephens, and Ian Adatia. Inhaled NO inhibits platelet aggregation and elevates plasma but not intraplatelet cGMP in healthy human volunteers. Am J Physiol Heart Circ Physiol 285: H637–H642, 2003. First published May 15, 2003; 10.1152/ajpheart.00622.2002.—Effects of inhaled nitric oxide (NO) on human platelet function are controversial. It is uncertain whether intraplatelet cGMP mediates the effect of inhaled NO on platelet function. We investigated the effect of 30 ppm inhaled NO on platelet aggregation and plasma and intraplatelet cGMP in 12 subjects. We performed platelet aggregation studies by using a photooptical aggregometer and five agonists (ADP, collagen, epinephrine, arachidonic acid, and ristocetin). During inhalation, the maximal extent of platelet aggregation decreased by 75% with epinephrine (P < 0.005), 56% with collagen (P < 0.005), and 20% with arachidonic acid (P < 0.05). Responses to ADP (8% P > 0.05) and ristocetin (5% P > 0.05) were unaffected. Platelet aggregation velocity decreased by 64% with collagen (P < 0.005), 60% with epinephrine (P < 0.05), 33% with arachidonic acid (P < 0.05), and 14% with ADP (P > 0.05). Plasma cGMP levels increased from 2.58 ± 0.43 to 9.99 ± 5.57 pmol/ml (P < 0.005), intraplatelet cGMP levels were unchanged (means ± SD: 1.96 ± 0.58 vs. 2.71 ± 1.67 pmol/10^9 platelets; P > 0.05). Inhaled NO inhibits platelet aggregation via a cGMP independent mechanism.

ENDOGENOUS NITRIC OXIDE (NO) is produced by the endothelial cell and plays an essential role in the maintenance of vascular homeostasis through relaxation of vascular smooth muscle and inhibition of platelet aggregation (20, 26, 27). The vascular effects of NO are thought to be limited and generally localized to the site of production through inactivation by hemoglobin (29). These properties have been exploited clinically, and inhaled NO is used as a specific pulmonary vasodilator in the assessment and treatment of pulmonary vascular disorders (1). In contrast, despite unequivocal evidence demonstrating inhibition of platelet aggregation by endogenous NO, the infusion of nitrovasodilators and gaseous NO in vitro, it remains controversial whether inhaled NO inhibits platelets during transit through the pulmonary circulation (3, 14). Furthermore, intraplatelet cGMP levels in response to inhaled NO have not been documented. Therefore, we investigated the effect of inhaled NO on platelet aggregation and cGMP production in normal human volunteers.

MATERIALS AND METHODS

Subjects and study design. Institutional ethics committee approval and informed consent were obtained before recruiting 12 healthy, nonsmoking subjects (3 female, 9 male, median age 35, range 30–50 yr) to participate in the study. None of the subjects took aspirin or other drugs known to affect platelet function in the 2 wk preceding the study.

Subjects were studied in the semirecumbent position. A 20-gauge angiocatheter (Becton Dickinson Vascular Access; Sandy, UT) was placed in an antecubital vein, permitting easy and rapid removal of samples for analysis. Blood samples were drawn immediately before and after 16–22 min of inhaling NO, as soon as the aggregometer was ready after completion of the baseline platelet analysis. Inhalation of NO was discontinued after the second blood sample was drawn. We analyzed venous blood samples for hemoglobin concentration, platelet count and function, and plasma and platelet cGMP levels. The centrifuge and platelet aggregometer were located in the same room as the subjects to minimize the delay between blood sampling and platelet aggregation studies. Blood pressure, heart and respiratory rate, and transcutaneous oxygen saturation were recorded every 5 min.

NO delivery. We provided a gas flow rate of 2–3 times the calculated minute volume to compensate for alterations in respiratory patterns. A nonrebreathing mask (disposable simple facemask; Baxter Medical Products) was fitted to each subject, and leaks were minimized. A gas analysis port, situated directly under the subjects’ noses, permitted continuous monitoring of the inspired NO concentration. Once the subjects were breathing comfortably at a rate of 14–18 breaths/min with the mask in place, we titrated NO (500 pm NO tank; Matheson Specialty Gases, Toronto, Ontario, Canada) to a concentration of 30–35 ppm into the flow of inspired piped air. The NO and NO2 concentrations were analyzed by chemiluminescence (Ecophysics CLD 700AL chemiluminescence NO/NO2/NOx gas analyzer; Amko Systems, Toronto, Ontario, Canada). Delivered NO concentrations were maintained between 30 and 35 ppm for each patient.

Address for reprint requests and other correspondence: I. Adatia, Dept. of Critical Care Medicine, Hospital for Sick Children, 555 University Ave., Toronto, Ontario, Canada M5J 1X8 (E-mail: ian.adatia@sickkids.ca).

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Blood sampling. After the first 5 ml were discarded, 19 ml of blood were collected gently and quickly distributed to appropriate tubes. The 9-ml aliquot of blood designated for platelet aggregation was transferred into plastic tubes and anticoagulated with sodium citrate (3.8%, 1:9 vol/vol) and processed immediately. The 10-ml aliquot was transferred to EDTA vacutainers (vacutainer system; Becton Dickinson, Rutherford, NJ) for whole blood platelet count, hemoglobin concentration, and cGMP levels. Platelet count and hemoglobin concentration were measured by using an automatic counter (Beckman Coulter, Canada).

Preparation of platelets and plasma samples for cGMP assays. Plasma and platelets for measurement of cGMP were prepared from the 10-ml aliquot of blood collected in EDTA tubes by centrifugation at 3,000 rpm for 90 s to obtain platelet-rich plasma (PRP) and platelet-poor plasma (PPP). PPP was stored at −70°C until analysis. PRP was centrifuged for a further 10 min at 3,000 rpm, and the supernatant was discarded. The remaining pellet was suspended in 2 ml modified Tyrode buffer solution (g/l: 8 NaCl, 0.2 KCl, 1 NaHCO3, and 0.05 NaH2PO4) containing BSA (2.5 g/l) with a pH of 7.4. HEPES (5 mM) was added to this solution. Samples were frozen at −70°C until analysis. Immediately before assay, cGMP was extracted by centrifuging with 6% trichloroacetic acid at 2,000 g for 15 min at 4°C. The supernatant was recovered and washed with water-saturated diethyl ether four times. The aqueous suspension extract was lyophilized, and the dried extract was dissolved in assay buffer (32). Plasma and platelet cGMP levels were determined in batches by using a commercially available radioimmunoassay (Amersham International). Results were expressed as picomoles per 10−9 platelets.

Platelet aggregation studies. PRP was obtained by centrifugation of fresh blood in the citrated tube at 1,500 rpm for 90 s at 20°C immediately after the sampling and collecting of supernatant. Platelets were counted to ensure a count within 200 and 250 × 109/l.

Platelet aggregation was assessed by using a four-channel platelet aggregometer (model PAP-4; Biodata, Horsham, PA) at 37°C. The platelet aggregometer records platelet aggregation photooptically on the basis of an increase in light transmission in platelet suspensions, if challenged by different agonists. Aggregation was induced by using 5 μM ADP (Sigma, St. Louis, MO), 7 mg/ml collagen (Sigma), 2 μM epinephrine (Abbott Laboratories; St. Laurent, Quebec, Canada), 0.5 mg/ml arachidonic acid (Bio/Data, Johns Scientific VWR, Canada Lab), and 1.5 mg/ml ristocetin (Helena Laboratories; Beaumont, TX). The aggregometer was calibrated with nonstimulated PPP and PRP. Maximal aggregation was expressed as the percent increase in light transmission after 5 min. The maximal slope of the aggregation curve reflected platelet aggregation over time or velocity of platelet aggregation (expressed as the angle between the horizontal and the curve in degrees). All studies were completed within 30 min of blood sampling and in the same order for each subject.

Statistical analysis. We determined that the data were distributed normally with the Kolgorov-Smirnov test. We analyzed the results of inhaled NO on platelet aggregation

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**Fig. 1. Platelet aggregometer tracing.** Platelet aggregation is inhibited in response to the agonists collagen, epinephrine, and arachidonic acid during inhalation of nitric oxide (NO) at 30 ppm. The response to ADP is unchanged from baseline. 1, ADP; 2, collagen; 3, epinephrine; 4, arachidonic acid.
(maximal intensity and velocity of aggregation) by paired *t*-test for each agonist. Blood pressure, heart rate, and transcutaneous oxygen saturation before and during inhalation were compared by using ANOVA for repeated measures. Plasma and platelet cGMP levels, before and during NO inhalation, were compared by paired *t*-test for each agonist. Blood pressure, heart rate, and transcutaneous oxygen saturation before and during inhalation were compared by paired *t*-test.

**RESULTS**

During NO inhalation the maximal extent of platelet aggregation decreased by 75% with epinephrine (*P* < 0.005), 60% with collagen (*P* < 0.005) and 20% with arachidonic acid (*P* < 0.05) (Fig. 1). There was no significant change with ADP (8%, *P* > 0.05) or ristocetin (5% *P* > 0.05) (Table 1). Similarly, the velocity of platelet aggregation decreased by 60% with collagen (*P* < 0.005), 60% with epinephrine (*P* < 0.05), and 33% with arachidonic acid (*P* < 0.05). There was no significant change with ADP (14%, *P* > 0.05) or ristocetin (0%) (Table 2).

Plasma cGMP levels increased from (means ± SD) 2.59 ± 0.43 to 9.99 ± 5.57 pmol/ml (*P* < 0.005). Intraplatelet cGMP at baseline was (means ± SD) 1.96 ± 0.58 compared with 2.71 ± 1.67 pmol/10^9 platelets during NO inhalation (*P* > 0.05) (Table 3). There was no change in blood pressure, heart rate, or transcutaneous oximetry (Table 4).

**DISCUSSION**

This study shows that, in healthy human volunteers, inhaled NO inhibits platelet aggregation during passage through the pulmonary circulation. The effect on platelets persists and is detectable in systemic venous blood. Furthermore, plasma cGMP levels increase during the inhalation but intraplatelet cGMP levels are unchanged.

The inhibition of platelet aggregation during inhalation of NO has been reported in patients with adult respiratory distress syndrome and healthy human volunteers (13, 14, 30). However, others have reported a
prolonged bleeding time but no change in platelet function during inhalation of NO (4, 5, 7, 10, 16). Indeed, lack of effect on bleeding time and platelet function also has been reported (3). In contrast, there is compelling evidence to suggest that NO mediates inhibition of platelet aggregation in vitro by using washed human platelets (19, 25–27). It seems likely that the apparently contradictory results in humans are related to the rapidity with which platelet aggregation studies are performed after bloodletting. We performed platelet aggregation responses to agonists as quickly as possible after blood sampling with the aggregometer and centrifuge located in the same room as the subject. Indeed, Samama et al. (30) was able to account for the apparent discordance between prolonged bleeding time and platelet function by repeating the study with minimal delay between bloodletting and platelet function testing.

Plasma cGMP increased during NO inhalation in the present study and confirms that sufficient NO was inhaled to activate guanylate cyclase, as reported previously (4, 5, 13, 14, 33). However, in the present study, the increase in plasma cGMP was not accompanied by an increase in intraplatelet cGMP despite inhibition of platelet aggregation. Although Albert et al. (5) reported discordance between plasma and intraplatelet cGMP responses to inhaled NO, there was no effect on the platelet variables they studied. Intraplatelet cGMP levels we report are similar to baseline intraplatelet cGMP levels and contrast with the 5- to 10-fold increases demonstrated when washed human platelets are exposed to NO in vitro (19, 21, 25–27). This suggests that the inhibition of platelet activation by inhaled NO is mediated neither by diffusion of cGMP liberated in plasma into the platelets or through activation of intraplatelet soluble guanylate cyclase. This finding contrasts with in vitro studies that suggest that NO and L-arginine mediate platelet inhibition through elevation of intraplatelet cGMP (19, 21, 25–27). However, NO-mediated cGMP independent mechanisms have been described in platelets (11, 23, 31) including inhibition of thromboxane A2. The formation of NO adducts with hemoglobin, which decreases platelet aggregation and adhesion without elevating intraplatelet cGMP, may explain also the effects of inhaled NO on platelet function in blood sampled from a peripheral vein, as well as activation during pulmonary transit (11, 23, 31). We used the method described by Watanabe et al. (32) who were able to detect changes in intraplatelet cGMP despite storage of the platelet-rich pellet in Tyrode’s buffer at −70°C. Therefore, it is unlikely that our results were due to cGMP degradation by phosphodiesterase during storage. However, because we did not measure intraplatelet cGMP levels before and after storage, we cannot exclude the possibility that platelet cGMP was degraded before assay.

Kermarrec et al. (17) reported decreased platelet aggregation using ADP as an agonist and increased intraplatelet cGMP levels in rats injected with lipopolysaccharide and exposed to NO for 10 h. The differences between our study and that of Kermarrec et al. include the model and length of time NO was inhaled. It is possible that prolonged NO inhalation and persistently elevated plasma cGMP levels permit diffusion of cGMP into circulating platelets from plasma or that prolonged inhalation of NO affects platelets and agonist responses differently from a short 20-min exposure.

Inhibition of human platelet aggregation by inhaled NO with an elevation in plasma cGMP has been reported in two previous studies (13, 14). Additionally, Gries et al. (13, 14) demonstrated prolongation of bleeding time and inhibition of P-selectin expression with decreased binding of fibrinogen to the platelet glycoprotein IIb/IIIa receptor. However, these authors did not report intraplatelet cGMP levels. Inhaled NO has been reported to prolong bleeding time in rabbits and rats (16), decrease neutrophil and platelet sequestration in pigs (18), and increase coronary artery patency after thrombolysis in dogs (2). Despite accumulation of a large clinical experience with inhaled NO, there has been little evidence to suggest an increased bleeding diathesis (20a).

Table 3. *cGMP levels in platelets and plasma at baseline and during NO inhalation*

<table>
<thead>
<tr>
<th></th>
<th>Baseline Platelet cGMP</th>
<th>NO Platelet cGMP</th>
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<td>2.73</td>
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<td>2.36</td>
<td>2.06</td>
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<td>1.92</td>
<td>4.24</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.96 ± 0.58</td>
<td>2.71 ± 1.67</td>
<td>2.58 ± 0.43</td>
<td>9.99 ± 5.57*</td>
</tr>
</tbody>
</table>

Mean values are ± SD. Platelet values are pmol/10⁹ platelets and plasma values are pmol/ml. *P < 0.005.

Table 4. *Blood pressure, oxygen saturation, and heart rate before, during, and after NO inhalation*

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>5 min NO</th>
<th>10 min NO</th>
<th>15 min NO</th>
<th>15 min after NO</th>
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</thead>
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<td>SBP, mmHg</td>
<td>122.6 ± 4</td>
<td>119.7 ± 3.9</td>
<td>118.5 ± 3.7</td>
<td>117.9 ± 3.8</td>
<td>119.9 ± 3.6</td>
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<tr>
<td>DBP, mmHg</td>
<td>73.2 ± 3.5</td>
<td>70.1 ± 2.2</td>
<td>70.2 ± 2.1</td>
<td>71.2 ± 2.6</td>
<td>70.8 ± 3.2</td>
</tr>
<tr>
<td>O₂ saturation, %</td>
<td>98.3 ± 0.38</td>
<td>97.9 ± 0.37</td>
<td>97.6 ± 0.36</td>
<td>97.5 ± 0.43</td>
<td>97.8 ± 0.32</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>76.8 ± 1.6</td>
<td>73.1 ± 1.6</td>
<td>73.8 ± 1.8</td>
<td>74 ± 2.2</td>
<td>75.8 ± 1.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. DBP, diastolic blood pressure, SBP, systolic blood pressure.
We found no change in platelet agglutination, in contrast to Samama et al. (30) who found that platelet agglutination was decreased, as detected by the response to ristocetin during NO inhalation, in patients with acute respiratory distress syndrome (ARDS). The difference in response to ristocetin may reflect a difference in endothelial cell von Willebrand expression in ARDS and pulmonary hypertension (24) compared with normal volunteers. Gries et al. (13) reported inhibition of ADP-induced platelet aggregation in patients with ARDS, suggesting that therapeutic responses to inhaled NO in disease may differ from the pharmacological actions of inhaled NO in healthy volunteers. In addition Gries et al. (13) described inhibition of ADP-induced platelet aggregation in PRP drawn from human volunteers exposed to high doses of NO over a prolonged period. We suggest that the mechanisms involved in inhibition of platelet aggregation may differ depending on whether NO is inhaled or added to platelets ex vivo.

We performed the platelet studies in citrated plasma. Citrate chelates external calcium and may modify platelet responses to agonists, such as ADP, that are dependent on thromboxane A2 (6, 22). Thus it is possible that the differential effects of inhaled NO on platelet aggregation were secondary to the change in plasma calcium. In addition, there may be an agonist dependence of the effects of inhaled NO on platelet function as suggested by Albert et al. (4). However, Gries et al. (13) described inhibition of ADP-induced platelet aggregation despite the use of citrated plasma.

We performed the study by using an indwelling catheter for sampling rather than repeated venipuncture. The results of our study on platelet aggregation are in agreement with some studies that used indwelling vascular lines for sampling (13, 14) and in disagreement with others (7, 10). However, it seems likely, as discussed above, that the variability is due to the delay between blood sampling and platelet function testing rather than the use of an indwelling catheter instead of repeated venipuncture. However, because we did not study a control group to exclude time-dependent changes in platelet aggregation, we cannot exclude the unlikely possibility that changes in platelet function are independent of the inhalation of NO.

We used a single dose of inhaled NO and, therefore, we cannot exclude the possibility that another NO dose would have a different effect on platelet aggregation. However, in general, the response to NO, as far as oxygenation and pulmonary vascular resistance is concerned, appears to be maximal between 5 and 20 ppm (8, 9, 12). It seems unlikely that the dose of 30 ppm was insufficient to elicit responses to the agonists ristocetin and ADP, especially because the dose was sufficient to elevate plasma cGMP.

We were unable to differentiate between the effect of inhaled NO and the effect of an elevated plasma cGMP level on platelet function. However, phosphodiesterase inhibitors such as dipyridamole may have a marked anti-aggregatory effect on platelets (28).

In summary, we have found that 30 ppm inhaled NO inhibits platelet aggregation in response to stimulation with collagen, arachidonic acid, and epinephrine in human volunteers. This is accompanied by increases in plasma but not intraplatelet cGMP levels, suggesting that, unlike the vascular effects of inhaled NO and in contrast to in vitro platelet effects of NO, the inhibition of platelet aggregation is mediated by a cGMP-independent mechanism. Inhaled NO may offer a novel method to administer a rapidly reversible inhibitor of platelet aggregation with effects in both the pulmonary and systemic circulation.

DISCLOSURES

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