Inhaled Nitric Oxide Inhibits Human Platelet Aggregation, P-Selectin Expression, and Fibrinogen Binding In Vitro and In Vivo

André Gries, MD; Christoph Bode, MD; Karlheinz Peter, MD; Axel Herr; Hubert Böhrer, MD; Johann Motsch, MD; Eike Martin, MD

Background—Recent data suggest that inhaled NO can inhibit platelet aggregation. This study investigates whether inhaled NO affects the expression level and avidity of platelet membrane receptors that mediate platelet adhesion and aggregation.

Methods and Results—In 30 healthy volunteers, platelet-rich plasma was incubated with an air/5% CO₂ mixture containing 0, 100, 450, and 884 ppm inhaled NO. ADP- and collagen-induced platelet aggregation, the membrane expression of P-selectin, and the binding of fibrinogen to the platelet glycoprotein (GP) IIb/IIIa receptor were determined before (t₀) and during the 240 minutes of incubation. In addition, eight patients suffering from severe adult respiratory distress syndrome (ARDS) were investigated before and 120 minutes after the beginning of administration of 10 ppm inhaled NO. In vitro, NO led to a dose-dependent inhibition of both ADP-induced (3±3% at 884 ppm versus 70±6% at 0 ppm after 240 minutes; P<.001) and collagen-induced (13±5% versus 62±5%; P<.01) platelet aggregation. Furthermore, P-selectin expression (36±7% of t₀ value; P<.01) and fibrinogen binding (33±11%; P<.01) were inhibited. In patients with ARDS, after two who did not respond to NO inhalation with an improvement in oxygenation had been excluded, an increase in plasma cGMP, prolongation of in vitro bleeding time, and inhibition of platelet aggregation and P-selectin expression were observed, and fibrinogen binding was also inhibited (19±7% versus 30±8%; P<.05).

Conclusions—NO-dependent inhibition of platelet aggregation may be caused by a decrease in fibrinogen binding to the platelet GP IIb/IIIa receptor. (Circulation. 1998;97:1481-1487.)

Key Words: platelets • P-selectin • fibrinogen • nitric oxide • respiration

Nitric oxide has been found to relax vascular smooth muscle and to inhibit platelet adhesion and platelet aggregation in vitro. Inhaled NO decreases elevated pulmonary arterial pressure and pulmonary vascular resistance and improves oxygenation. This principle has been used successfully to treat ARDS, persistent pulmonary hypertension of the newborn, and pulmonary hypertension in patients who have undergone cardiac surgery. Despite rapid inactivation of inhaled NO by hemoglobin and high selectivity for the pulmonary vascular system, recent data suggest that inhaled NO also has systemic effects on hemostasis by inhibiting platelet function. During NO inhalation, bleeding time was prolonged in animals and in healthy volunteers, and platelet aggregation was inhibited in patients with ARDS. In addition, inhibition of platelet aggregation was also observed during NO inhalation after acute massive pulmonary embolism in pigs.

Although several NO-releasing compounds have been investigated and the platelet inhibitory effect has been shown to be dose dependent in vitro and in vivo, it is currently unknown whether the inhibitory effect of inhaled NO on platelet function is also dose related. Furthermore, the mechanisms by which inhaled NO may affect platelet function are not completely understood. Inhibition of fibrinogen binding to the platelet membrane via an increase in intracellular cGMP concentration has been postulated to be responsible for the inhibition of platelet adhesion and aggregation. However, fibrinogen binding to human platelets during NO inhalation has not been investigated in detail. The present study was performed to investigate the dose dependency of platelet inhibition in vitro and in patients with ARDS by use of platelet aggregation studies, determination of in vitro bleeding time, and flow cytometry to elucidate the mechanism by which inhaled NO affects platelet function.

Methods

NO Gas In Vitro

Platelet Preparation and NO Administration

After approval from the ethics committee and informed consent from the study subjects had been obtained, venous blood was drawn from...
Determination of P-Selectin Expression and Fibrinogen Binding

To determine P-selectin expression and fibrinogen binding to the platelet GP IIb/IIIa receptor, additional PRP samples were carefully taken from the tube containing PRP at tₗ₀, t₁₂₀, t₂₄₀, and t₄₈₀ during administration of 0, 100, and 884 ppm NO. There were no centrifugation or vortexing steps, so that in vitro platelet activation was avoided. To determine P-selectin expression, PRP was diluted in HEPES solution, and to measure fibrinogen binding, in Tyrode’s buffer (Roth). P-selectin and fibrinogen were measured after stimulation with ADP (Mölab, FC, 2 μmol/L). The samples were incubated with the specific antibodies CD62P CloneAC1.2 (Becton Dickinson) to determine P-selectin expression and anti-human fibrinogen FITC-conjugated chicken antibody (Biopool) to determine fibrinogen binding for 20 minutes in the dark at 22°C. After fixation with Cellfix (Becton Dickinson), the samples were measured in a flow cytometer within 6 hours. The platelet population was analyzed at a low flow rate and identified on the basis of forward and sideways scatter characteristics. For each sample, 10,000 platelets were collected. Data were analyzed with LYSIS II software (FACScan, Becton Dickinson).α-21

NO Gas in Older Volunteers

Because of the age imbalance between the normal volunteers in the in vitro study and the ARDS patients, PRP from five additional volunteers (55 to 65 years old) was incubated with 0, 100, and 884 ppm NO. Platelet aggregation, P-selectin expression, and fibrinogen binding were investigated as described above.

50 ppm NO In Vitro

The NO concentrations used in the in vitro investigation were relatively high (100 to 884 ppm). To investigate a lower NO concentration, PRP from five additional volunteers was incubated with 50 ppm for 240 minutes. Platelet aggregation, P-selectin expression, and fibrinogen binding were investigated as described above.

Inhaled NO in Patients With ARDS

Patients and NO Administration

After approval by the ethics committee and informed consent from the relatives had been obtained, eight surgical intensive care patients (64±4 years old; seven men and one woman) diagnosed with ARDS who fulfilled the criteria of ARDS according to the consensus conference25 were investigated. Two patients had been admitted with multiple trauma, four had undergone coronary revascularization, major vascular surgery had been performed in one, and one had developed ARDS after major abdominal surgery. Exclusion criteria were suspected or confirmed intracranial hemorrhage, leukopenia, and a previous history of severe chronic kidney, liver, or lung disease.

All patients were sedated with intravenous midazolam (Dormicum; Hoffmann-LaRoche) and fentanyl (Janssen) and mechanically ventilated to ensure an arterial carbon dioxide tension (PaCO₂) between 40 and 50 mm Hg (EVITA 2, Dräger). In all patients, hemodynamic measurements were performed with a pulmonary artery catheter (93–631–5.5F, Baxter Healthcare Corp) and a radial arterial catheter (20 gauge; Abbo-cath-T, Abbott), and data were continuously displayed on a multichannel oscillograph (Vicom SMU-612, Hellige). After the patients had been selected, 10 ppm inhaled NO (Messier Griesheim) were administered with a commercially supplied administration unit connected to the ventilator (NÖdomo, Dräger). Inspiratory and expiratory concentrations of NO and NO₂ were analyzed continuously at the proximal end of the endotracheal tube with the NO/NO₂/NO₃ analyzer (Zellweger-EccO).

To analyze platelet function, the first 10 mL of arterial blood was discarded, and thereafter samples were carefully withdrawn into plastic tubes (Monovette) before (t₀) and 120 minutes after (t₁₂₀) NO administration was initiated.
**Determination of In Vitro Bleeding Time**
With a platelet function analyzer (PFA 100, Dade), the in vitro bleeding time was determined according to the following method, described by Kratzer and Born: At a constant negative pressure, samples of citrated whole blood (800 μL) are suctioned through a small capillary and a filter membrane with a diameter of 150 μm. The filter membrane is covered with collagen and soaked with epinephrine. During movement through the capillary, platelets adhere and aggregate at the filter membrane, diminishing blood flow until it stops. The total time in seconds of the blood flow is called in vitro bleeding time and is measured electronically.

**Determination of Platelet Aggregation**
As described above, whole blood (8 mL) was centrifuged at 500g for 5 minutes and subsequently at 2500g for 10 minutes to prepare PRP, and ADP-induced platelet aggregation (FC, 5 μmol/L) was measured. Because of the short half-life of NO and its rapid inactivation after contact with hemoglobin, all samples were centrifuged immediately and measured within 30 minutes after sampling.

**Determination of P-Selectin Expression and Fibrinogen Binding**
To determine P-selectin expression and fibrinogen binding to the platelet membrane in patients during NO inhalation, whole-blood samples (20 μL) were measured as described above. In these patients, P-selectin expression was determined without (basal P-selectin) and after activation with ADP (FC, 2 μmol/L).

**Measurement of Platelet Count, White Blood Cell Count, and Plasma cGMP**
Platelet count and white blood cell count were measured with a Coulter Counter STKS (Coulter Electronics). Arterial plasma samples that had been stored deep frozen (−80°C) until measurement were used to determine plasma cGMP levels with a commercially supplied enzyme immunoassay used according to the manufacturer’s guidelines (Immunodiagnostik).

**Statistical Analysis**
All results are given as mean±SEM. In addition, the values are also given as a percentage of the baseline value (t₀, given as 100%) to demonstrate the relative changes during the time course of P-selectin expression and fibrinogen binding. Statistical analysis was performed with one-way ANOVA for repeated measurements, followed by the Scheffé test to demonstrate changes in platelet aggregation, P-selectin expression, and fibrinogen binding and to analyze the differences between the two groups. For additional data analysis, the Wilcoxon signed rank test for paired samples was used in the ARDS patients. To analyze whether the effect of NO on platelet function was due to changes in fibrinogen binding, the changes in aggregation (aggregation ratio) and in fibrinogen binding (fibrinogen-binding ratio) during incubation with 884 ppm NO were correlated by the Pearson test. A value of P<.05 was considered to be statistically significant.

**Results**

**NO Gas In Vitro**
In the control group, ADP-induced platelet aggregation did not change during the study (72±5% at t₀, 73±7% at t₁₀, 67±7% at t₁₅₀, 68±6% at t₂₀, 65±5% at t₁₂₀, 67±7% at t₁₈₀, and 70±6% at t₂₄₀). During administration of 100 ppm NO, ADP-induced platelet aggregation was inhibited, reaching statistical significance at t₁₀ (46±7%, P<.05), t₁₅₀ (41±7%, P<.05), and t₂₀ (26±4%, P<.01). Administration of 450 ppm NO significantly inhibited ADP-induced platelet aggregation at t₀ (43±10%, P<.05), t₁₀ (32±7%, P<.01), t₁₅₀ (23±7%, P<.01), and t₂₀ (12±4%, P<.001). The highest NO concentration (884 ppm) resulted in the most pronounced inhibition of platelet aggregation: 37±5% at t₀ (P<.01), 38±6% at t₂₀ (P<.01), 29±5% at t₁₀ (P<.001), 15±5% at t₁₂₀ (P<.001), 9±5% at t₁₅₀ (P<.001), and 3±3% at t₁₈₀ (P<.001) (Fig 2). In accordance with these findings, collagen-induced platelet aggregation also was dose-dependently inhibited during NO administration, reaching statistical significance during administration of 450 and 884 ppm NO. During administration of 450 ppm (884 ppm) at t₁₂₀, t₁₅₀, and t₂₄₀, collagen-induced platelet aggregation was 49±8% (55±3%, P<.05), 45±6% (37±3%, P<.01), and 25±10% (13±5%, P<.001), respectively (Fig 3).

In vitro administration of 100 ppm NO led to a significant inhibition of P-selectin expression at t₁₀ (76±12% versus t₀ value, P<.05). During incubation with 884 ppm NO, P-selectin expression was inhibited at t₀, t₁₀, t₁₅₀, and t₂₀ (77±12%, P<.05; 73±13%, P<.05; 67±23%, P<.01; and 36±7%, P<.01, respectively) (Fig 4). Furthermore, in accordance with the other findings, NO administration to PRP resulted in a significant inhibition of platelet fibrinogen binding to the GP IIb/IIIa receptor. Compared with t₀ values, during administration of 450 ppm (884 ppm) NO, fibrinogen binding was 73±17% at t₁₀ (66±16%, P<.05), 57±30% at t₁₅₀ (51±15%, P<.01), and 48±28% at t₂₀ (33±11%, P<.01) (Fig 5). Moreover, a significant correlation between the aggregation ratio and the fibrinogen-binding ratio was found during NO incubation (Fig 6).

NO-induced dose-related inhibition of platelet aggregation, P-selectin expression, and fibrinogen binding was also observed in the older study population (Table I). In comparison
with the younger population, no statistically significant differences were observed.

Incubation with 50 ppm NO also led to a significant inhibition of ADP-induced platelet aggregation in vitro. In addition, a slight inhibition in P-selectin expression and a significant inhibition in fibrinogen binding were observed (Table 2).

**Inhaled NO in Patients With ARDS**

Two patients suffering from ARDS who had undergone cardiac surgery did not respond to NO inhalation with an improvement in oxygenation. NO was withdrawn in these patients, and they were excluded from the study. In the remaining six patients, the in vitro bleeding time was prolonged (99 ± 13 seconds at t₀ versus 71 ± 11 seconds at tₙ, P < .05) and ADP-induced platelet aggregation was inhibited (19 ± 3% versus 26 ± 2%, P < .05) during inhalation of 10 ppm NO. In addition, NO inhalation significantly inhibited both basal and ADP-stimulated P-selectin expression: 6.7 ± 1.7% versus 10.1 ± 2.2% (P < .05) and 43 ± 6% versus 57 ± 7% (P < .05), respectively. In accordance with these findings, the fibrinogen binding to the platelet GP IIb/IIIa receptor was also inhibited during NO inhalation in patients with ARDS (19 ± 7% versus 30 ± 8%, P < .05). In addition, NO therapy led to a significant increase in plasma cGMP levels (8.1 ± 1.6 versus 4.4 ± 1.2 ng/mL, P < .05) but had no influence on platelet and white blood cell count (Table 3).

**Discussion**

The present study demonstrates that inhaled NO inhibits platelet aggregation, P-selectin expression, and fibrinogen binding to the GP IIb/IIIa receptor of human platelets in vitro in a dose-dependent manner. These results could be substantiated in a clinical situation: besides prolonging the in vitro bleeding time, NO inhalation in patients with ARDS led to an inhibition of platelet aggregation, P-selectin expression, and fibrinogen binding.

In addition to their vasodilatory properties, systemically administered NO-releasing compounds have been shown to inhibit platelet function in vitro and in vivo, and their antithrombotic effect is proposed to be beneficial. The inhibition of platelet function may be explained by an activation of the soluble guanylate cyclase in the platelet cytosol, leading to an increase in cGMP levels. Increased cGMP levels in platelets can induce a decrease in intracellular calcium ion concentration, which may contribute to an inhibition of fibrinogen binding to the GP IIb/IIIa receptor on the surface of the platelet membrane, which mediates platelet aggregation. Furthermore, an increase in intracellular cGMP levels at the early stage of aggregation has been proposed, which could be further enhanced by relatively low concentrations of NO and finally lead to platelet disaggregation. In the present study, we were not able to determine intracellular cGMP levels. However, NO therapy in patients with ARDS led to a significant increase in plasma cGMP levels, which have also been demonstrated to reflect NO-induced guanylate cyclase activation in vivo.

The inhibition of platelet aggregation was reported to be dependent on the NO concentration in vitro and in healthy volunteers. Moreover, the antiplatelet effect was persistent despite a hemodynamic tolerance during nitroglycerin therapy in pigs. Conversely, bleeding time was shortened after systemic inhibition of NO production. The results of the present study are in accordance with these findings, demonstrating a dose-dependent inhibition of both ADP- and collagen-induced platelet aggregation during NO administration in vitro. In comparison, in another study, 200 and 400 ppm inhaled NO were found to significantly inhibit ADP-induced maximal platelet aggregation. When collagen was used to induce platelet aggregation, however, this inhibition was observed later and to a lesser extent, as also reported by other investigators. This may be explained by the relatively high collagen concentration that was used in the aggregation studies. Lower concentrations of collagen or the use of epinephrine, which led to good results in the in vitro studies, may also explain the differences in results.
Platelet aggregation (FC, 5 μmol/L), %

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<th>100 ppm</th>
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<tr>
<td>60</td>
<td>93±8</td>
<td>46±8†</td>
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<tr>
<td>120</td>
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<td>240</td>
<td>37±8†</td>
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Collagen-induced platelet aggregation (FC, 0.19 mg/mL), %

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<td>240</td>
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P-selectin expression (FC, 2 μmol/L), %

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<td>120</td>
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<tr>
<td>180</td>
<td>72±4</td>
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<td>240</td>
<td>47±5*†</td>
<td>39±10‡</td>
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Fibrinogen binding (FC, 2 μmol/L), %

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<td>240</td>
<td>39±4*†</td>
<td>30±9†</td>
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NO was administered in vitro at 100 and 884 ppm. Values are mean±SEM.

*P<.05, †P<.01, ‡P<.001 vs baseline.

bleeding time measurements, might have revealed a comparable platelet inhibitory effect.

In the present study, inhibition of ADP-induced platelet aggregation and prolongation of in vitro bleeding time were also observed during inhalation of 10 ppm NO in vivo. A prolongation of bleeding time during NO administration was observed in rabbits,8 whereas in rats, no change in bleeding time was observed at 80 ppm NO.15 In healthy volunteers, 30 ppm inhaled NO also prolonged bleeding time in a study population. In addition, platelet activation was abolished during NO administration in pigs after acute pulmonary embolism.11 In accordance with our findings, most of these studies demonstrated inhaled NO to have an inhibitory effect on platelet function. Differences in regard to bleeding time and platelet aggregation may be explained by the different methods and study populations that were used10,11,18,23,28 and are therefore not necessarily contradictory.

In contrast to the in vitro findings, we cannot say whether the inhibition of platelet function by inhaled NO is also dose dependent in vivo, because only one NO concentration was administered. The maximal inhibition of platelet aggregation was already achieved at 3 ppm NO during inhalation of 1 to 100 ppm NO in a comparable study population.10 In healthy volunteers inhaling 30 ppm NO, no further prolongation of bleeding time was observed at 80 ppm.24 In comparison with these studies, no definitive dose-related effect of inhaled NO on platelet function could be found in animals, and further studies are necessary to investigate a dose relation in a larger study population.

In activated platelets, P-selectin is translocated to the platelet surface from α-granules.20 Inhibition of NO synthesis led to an increase in P-selectin expression.22 Furthermore, the

TABLE 1. ADP- and Collagen-Induced Platelet Aggregation and ADP-Activated P-Selectin Expression and Fibrinogen Binding to the GP IIb/IIIa Receptor as a Percentage of Baseline Values at 60, 120, 180, and 240 Minutes in Older Volunteers (55 to 65 Years Old)

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<td>ADP-induced aggregation (FC, 5 μmol/L), %</td>
<td>87±10</td>
<td>63±11</td>
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<td>P-selectin expression (FC, 2 μmol/L), %</td>
<td>92±21</td>
<td>85±19</td>
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<td>Fibrinogen binding (FC, 2 μmol/L), %</td>
<td>73±21</td>
<td>64±9</td>
<td>&lt;.05</td>
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Values are mean±SEM.

*P<.05 vs baseline.

TABLE 2. ADP-Induced Maximal Platelet Aggregation (FC=5 μmol/L), ADP-Stimulated P-Selectin Expression and Fibrinogen Binding (FC=2 μmol/L) as a Percentage of Baseline Values 180 and 240 Minutes After the Beginning of NO Administration (50 ppm) In Vitro

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Values are mean±SEM.

TABLE 3. Platelet Count, White Blood Cell Count, Plasma cGMP, In Vitro Bleeding Time, ADP-Induced Platelet Aggregation, Basal and ADP-Stimulated P-Selectin Expression, and ADP-Stimulated Fibrinogen Binding in Patients With ARDS Before (0) and 120 Minutes After Start of Administration of 10 ppm Inhaled NO

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<tr>
<td>Platelet count, cells/mL</td>
<td>171±26</td>
<td>176±28</td>
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<tr>
<td>White blood cell count, cells/mL</td>
<td>15.4±1.3</td>
<td>15.2±1.6</td>
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<td>cGMP, ng/mL</td>
<td>4.7±1.1</td>
<td>7.7±1.4*</td>
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<tr>
<td>In vitro bleeding time, s</td>
<td>71±11</td>
<td>99±13*</td>
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<tr>
<td>Platelet aggregation (FC, 5 μmol/L), %</td>
<td>26±2</td>
<td>19±3*</td>
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<tr>
<td>Basal P-selectin expression (FC, 2 μmol/L), %</td>
<td>10.1±2.2</td>
<td>6.7±1.7*</td>
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<tr>
<td>ADP-stimulated P-selectin expression (FC, 2 μmol/L), %</td>
<td>57±7</td>
<td>43±6*</td>
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<tr>
<td>Fibrinogen binding (FC, 2 μmol/L), %</td>
<td>30±8</td>
<td>19±7*</td>
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Values are mean±SEM.

*P<.05 vs baseline.
administration of NO-releasing compounds inhibited P-selectin expression in vitro, and in several clinical conditions. In keeping with the data from the aggregation study, dose-dependent inhibition of P-selectin expression in ADP-activated platelets was observed in the present study during NO administration in vitro. In patients with ARDS, basal and ADP-activated P-selectin expression were also inhibited during NO inhalation, reflecting its platelet inhibitory effect. P-selectin also mediates leukocyte and endothelial interaction, which was significantly affected by NO in vivo. In the present study, the platelet and white blood cell counts remained stable during NO administration, most likely excluding a relevant leukocyte or platelet sequestration.

A decrease in platelet fibrinogen binding was observed after administration of various NO-releasing compounds in vitro and in vivo. Furthermore, the increase in GP IIb/IIIa receptor expression was abolished during administration of S-nitrosoglutathione and nitroglycerin in patients with unstable angina and acute myocardial infarction and during percutaneous transluminal coronary angioplasty. The results of the present study are comparable to these findings, because the binding of fibrinogen to the GP IIb/IIIa receptor was inhibited in vitro and during NO inhalation in patients with ARDS. As with the results of the aggregation studies, this inhibition was dose dependent in vitro. Moreover, in the present study, a significant correlation between aggregation ratio and fibrinogen-binding ratio was found, suggesting that the prolongation of the in vitro bleeding time and the inhibition of platelet aggregation are most likely due to the inhibition of fibrinogen binding to the platelet surface GP IIb/IIIa, which is required for platelet adhesion and aggregation. Because we were not able to measure the amounts of GP IIb/IIIa receptors on platelets, we cannot say whether inhaled NO influences the structure or the number of expressed GP IIb/IIIa receptors or both. Nevertheless, recent studies suggest that NO may inhibit the number of expressed GP IIb/IIIa receptors in vitro and in vivo. Thus, the expression of GP IIb/IIIa receptors may also be influenced by inhaled NO, which may contribute, at least in part, to the inhibition of fibrinogen binding, as demonstrated in the present study.

Although the in vitro model seems to be useful because it was possible to obtain mechanical platelet activation by means of gas bubbles and a filter membrane was used to imitate the alveolar membrane, the in vitro findings cannot be completely extrapolated to the clinical setting. In the present study, relatively high concentrations of NO were used in vitro. Nevertheless, our results seem to be relevant, because a significant inhibition of platelet aggregation and fibrinogen binding was already observed at 50 and 100 ppm, concentrations that have been used in patients. Furthermore, PRP was used, whereas in the lung, NO comes into contact with hemoglobin, which is known to inhibit its effects. On the other hand, the inhibitory effect on platelet function may be more pronounced in the pulmonary vessels, where inhaled NO may improve the microcirculation, because NO inhalation decreased platelet sequestration in the lungs during extracorporeal circulation in pigs. In the present study, however, the inhibition of platelet aggregation and fibrinogen binding in vivo supports the in vitro findings.

In conclusion, the results from the present study provide new evidence of an inhibitory effect of inhaled NO on platelet function. In fact, the inhibition of platelet adhesion and aggregation seems to be mediated via an inhibition of fibrinogen binding to the platelet membrane. The antiaggregatory effect could be beneficial: in a canine model of platelet-mediated reocclusion after thrombolysis, inhaled NO improved the coronary artery patency ratio, and platelet activation was inhibited after pulmonary embolism in pigs.

Because of its positive effect on hemodynamics and its antiplatelet action, the administration of inhaled NO in critically ill patients may be a beneficial and attractive adjunct to anticoagulation therapy. The present study provides a rational basis for investigating the efficacy of inhaled NO as an antiplatelet agent in further clinical studies.

References
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