Anesthesiology
1997; 86:387–93

Inhaled Nitric Oxide Inhibits Platelet Aggregation after Pulmonary Embolism in Pigs

André Gries, M.D., Bernd W. Böttiger, M.D.,† Joachim Dörsam, M.D.,‡ Harry Bauer, M.D.,† Jörg Weimann, M.D.,* Christoph Bode, M.D.,§ Eike Martin, M.D.,‖ Johann Motsch, M.D.#*

Background: **Inhaled nitric oxide (NO) is reported to prolong bleeding time in animals and humans and to inhibit platelet aggregation in persons with acute respiratory distress syndrome. In pulmonary embolism (PE), inhibition of platelet aggregation appears useful because further thrombus formation may lead to right ventricular dysfunction that results in circulatory failure. In the present study, the effect of inhaled NO on platelet aggregation after acute massive PE was investigated.**

Methods: **After acute massive PE was induced in 25 anesthetized pigs by injecting microspheres, 5, 20, 40, and 80 parts per million inhaled NO were administered stepwise for 10 min each in 11 animals (NO group). In the control group (n = 14), NO was not administered. Adenosine diphosphate–induced initial and maximal platelet aggregation were measured before PE (t0), immediately after induction of PE (PE), at the end of each 10-min NO inhalation interval (t10–t40), and 15 min after cessation of NO inhalation (t55) in the NO group, and at corresponding times in the control group, respectively.**

Results: **Two animals in the control group and one in the NO group died within 10 min after PE induction and were excluded from analysis. Peaking at t40 and t55, respectively, initial (+13 ± 6%; $P < 0.05$) and maximal (+44 ± 17%; $P < 0.05$) platelet aggregation increased significantly after PE in the control group. In contrast, NO administration after PE led to a significant decrease in initial (maximum decrease, −9 ± 3% at t40; $P < 0.05$) and maximal (maximum decrease, −15 ± 7% at t30; $P < 0.05$) platelet aggregation. In the NO group, platelet aggregation had returned to baseline levels again at t55. In addition, NO administration significantly decreased mean pulmonary artery pressure and significantly increased end-tidal carbon dioxide concentration and mean systemic blood pressure.**

Conclusions: **Inhaled NO has a systemic and rapidly reversible inhibitory effect on platelet aggregation after acute massive PE in pigs. This may be beneficial in treating acute massive PE. (Key words: Animal model: pig. Blood: platelet aggregation. Gases: inhaled nitric oxide. Lung: pulmonary embolism.)**

ACUTE pulmonary embolism (PE) increases mean pulmonary artery pressure (MPAP) and may lead to right ventricular decompensation.[1] Mechanical obstruction of the pulmonary artery is aggravated when circulating platelets are activated. Platelet aggregation accompanied by the release of vasoactive substances such as serotonin and thromboxane A_2 may cause vasoconstriction of the pulmonary vessels, thus increase pulmonary hypertension and hypoxemia, and finally lead to circulatory failure.[2–4] Inhibition of platelet aggregation thus may be useful to treat massive PE.[5,6]

Nitric oxide (NO) relaxes vascular smooth muscle and inhibits platelet function.[7–9] Because inhaled NO decreases elevated pulmonary artery pressure and pulmonary vascular resistance and improves oxygenation, it has been used successfully to treat acute respiratory distress syndrome, newborns with persistent pulmonary hypertension, and patients with pulmonary hypertension after cardiac surgery.[10–12] Recently we observed that inhaled NO also can decrease MPAP and pulmonary vascular resistance after acute massive PE in piglets.[13]

Despite the rapid inactivation of inhaled NO after it contacts hemoglobin, and a high selectivity for the pulmonary vascular system,[11,14] recent data suggest that inhaled NO has systemic effects on hemostasis by inhibiting platelet function.[15–17] Although no data exist regarding the effect of inhaled NO on platelet function in acute massive PE, inhibition of platelet aggregation could be hypothesized as a further useful action. Thus we performed this study to investigate the effect of inhaled NO on platelet aggregation after acute massive PE.

*Resident in Anesthesiology, Department of Anesthesiology.

†Staff Anesthesiologist, Department of Anesthesiology.

‡Staff Urologist, Department of Urology.

§Associate Professor of Cardiology, Department of Cardiology.

‖Professor of Anesthesiology, Chairman, Department of Anesthesiology.

#Professor of Anesthesiology, Department of Anesthesiology.

Received from the Departments of Anesthesiology, Urology, and Cardiology, University of Heidelberg, Heidelberg, Germany. Submitted for publication March 29, 1996. Accepted for publication October 6, 1996. Presented in part at the Third Congress of the European Society of Anaesthesiology, Paris, France, May 1–3, 1995.

Address reprint requests to Dr. Gries: Department of Anesthesiology, University of Heidelberg, Im Neuenheimer Feld 110, D-69120 Heidelberg, GERMANY.

Materials and Methods

Animal Model of Pulmonary Embolism

After approval was obtained from the Governmental Animal Care Committee, 25 German Landrace piglets (weighing 15 ± 3 kg) were studied. The animals were handled according to the guiding principles published by the National Institutes of Health and the Council of the American Physiologic Society.‡‡ After premedication with 1 mg/kg azaperone given intramuscularly (Stressnil; Janssen, Neuss, Germany), the animals were anesthetized with 1 mg/kg midazolam (Dormicum; Hoffmann-LaRoche, Grenzach-Wyhlen, Germany) and 0.8 mg/kg piritramide given intravenously (Dipidolor; Janssen). For muscle relaxation, 0.3 mg/kg pancuronium bromide was administered intravenously (Pancuronium "Organon"; Organon Teknika, Eppelheim, Germany). Ringer's solution was administered at an infusion rate of 3 $ml \cdot kg^{-1} \cdot h^{-1}$. After tracheal intubation, the animals' lungs were mechanically ventilated with an air and oxygen mixture (inspiratory oxygen fraction = 0.3) to ensure arterial carbon dioxide tension between 35 and 40 mmHg. During the entire study, rectal body temperature was maintained between 35.5°C and 37°C using a warming pad. Using local cut-down procedures, an arterial catheter (Leader Cath 20G; Laboratories Pharmaceutique Vygon, Ecouen, France) was inserted into the right femoral artery and a central venous catheter (Arrow central vein catheterization set 14G; Arrow International, Reading, PA) was placed in the right femoral vein. A pulmonary artery catheter (93-631-5.5F; Baxter Healthcare, Irvine, CA) was inserted into the right internal jugular vein. After connecting each catheter line to a pressure transducer (Uniflow Pressure Monitoring Kit; Baxter Healthcare), the transducer output was displayed continuously on a multichannel oscillograph (Vicom SMU-612; Hellige, Freiburg, Germany).

After the end of the preparation period, PE was induced by stepwise injection of 300 μm microspheres (Sephadex, G50 coarse; Pharmacia Biotech, Freiburg, Germany) until MPAP increased to 45 mmHg. Fifteen minutes after injection of microspheres had begun when only slight fluctuations in MPAP and in mean systemic artery pressure were present, NO inhalation was started in the NO group (n = 11). For an overall inhalation time of 40 min, 5, 20, 40, and 80 parts per million (ppm) inhaled NO were administered in a stepwise manner, with each concentration maintained for 10 min. In the control group (n = 14), inhaled NO was not administered after PE was induced. During the study, all parameters were determined at defined and corresponding times in both groups.

Nitric Oxide Administration

Nitric oxide was commercially supplied by Messer Griesheim (Ludwigshafen, Germany) at an original gas concentration of 884 ppm NO in N_2. Using a special flowmeter, any desired mixture of NO, oxygen, and air could be administered. Inspiratory and expiratory concentrations of NO and nitrogen dioxide (NO_2) were analyzed continuously at the proximal end of the endotracheal tube by cheminluminescence ($NO/NO_2/NO_x$ analyzer CLD 700 AL; Zellweger-Ecco, Munich, Germany).[18]

Determination of Platelet Aggregation

To determine platelet aggregation, after the first 10 ml had been discarded, samples of arterial blood were carefully withdrawn into plastic tubes containing 3.8% sodium citrate (Monovette; Sarstedt, Nümbrecht, Germany) at time points before PE (t0), immediately after induction of PE (PE), at the end of each 10-min NO inhalation interval (t10–t40), and 15 min after cessation of NO inhalation (t55). The samples were centrifugated immediately at 500*g* for 5 min to obtain platelet-rich plasma, and subsequently at 2500*g* for 10 min to obtain platelet-poor plasma. Platelet concentration was fixed at 300 per nanoliter by adding platelet-poor plasma to platelet-rich plasma, and platelet aggregation was measured in a four-channel platelet amplifier (PAP-4; Biodata Corp., Hartboro, PA) at 37°C according to the method described by Born.[19] Platelet aggregation was induced by adenosine diphosphate (Mölab, Neuss, Germany; final concentration = 4 μM). Each test was done twice, and the mean value of both measurements was recorded. Because of the short half-life of NO and its rapid inactivation after contact with hemoglobin, all samples were centrifugated immediately and measured within 30 min after sampling. As shown in figure 1, aggregation was quantified by measuring the maximal slope of the aggregation curve (initial aggregation, angle α, measured in degrees) and the maximal extent of light transmission (maximal aggregation, A_{max}, measured as a percentage).

‡‡ National Institutes of Health: Guide for the care and use of laboratory animals (publication no. 85-23). Washington, DC, National Institutes of Health, 1985.

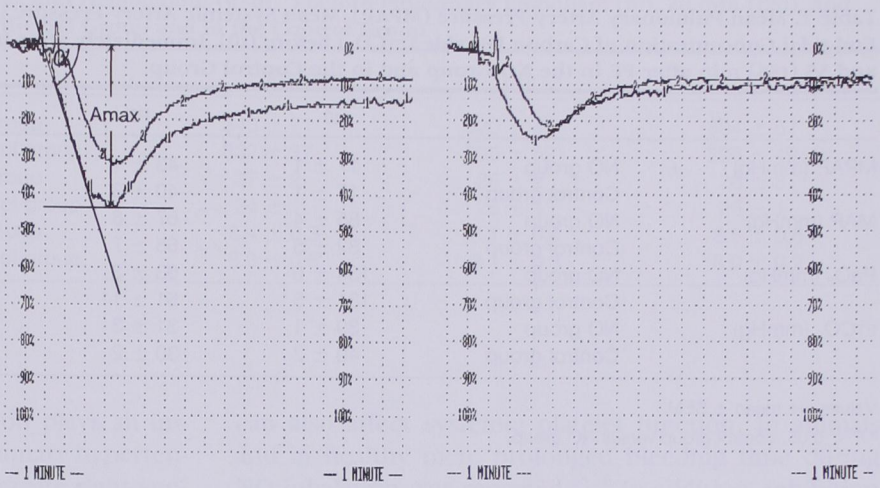

Fig. 1. Original printout of a double measurement of adenosine diphosphate (4 μM final concentration) induced platelet aggregation before pulmonary embolism (PE) (t0, left) and 40 min after induction of PE (t40, right) in pig 3 of the nitric oxide (NO) group during inhalation of 80 ppm NO. Platelet aggregation was quantified by measuring initial platelet aggregation (angle α; measured in degrees) and maximal platelet aggregation (A_{max}; measured as a percentage). For demonstration purposes, angle α (°) and A_{max} (%) are marked in one curve of the t0 printout.

Determination of Hematocrit Concentration and Platelet Count

In addition, samples were taken to determine hematocrit concentration and hemoglobin and platelet count at t0, t20, and t55 and measured using a Coulter counter (T 660; Coulter Electronic, Krefeld, Germany).

Hemodynamic and Respiratory Monitoring

To ensure a comparable degree of PE in all animals, the following hemodynamic and respiratory parameters were determined at t0, PE, t20, t40, and t55: MPAP, mean systemic artery pressure, arterial oxygen tension, and end-tidal concentration of carbon dioxide.

Pigs without Pulmonary Embolism

In five additional micropigs (weighing 5.5–7 kg), the effect of NO inhalation on platelet aggregation in pigs without PE was investigated. Anesthesia, animal preparation, NO administration, and the determination of platelet aggregation were performed as previously described. In these animals, 5, 20, and 80 ppm NO were administered in a stepwise manner, with each concentration maintained for 10 min. Thus the overall inhalation time was 30 min. Platelet aggregation was determined before, at the end of each 10-min inhalation period, and 15 min after cessation of NO administration. In addition, one German Landrace pig (weighing 14.5 kg) was used to study the effect of long-term NO administration. In this animal, 5 ppm NO were administered for an overall time of 240 min, and platelet aggregation was studied every 15 min in the first hour, and every 30 min during the ensuing hours.

Statistical Analysis

All results are given as means ± SEM. In addition, the values are also given as a percentage of baseline value (t0, fixed at 100%) to demonstrate the extent of change in the time course of platelet aggregation. Statistical analysis was performed using one-way analysis of variance for repeated measurements, followed by the Scheffé test to show changes in platelet aggregation and in hemodynamic and respiratory parameters in each group, and to analyze the differences between the two groups. A probability value less than 0.05 was considered significant.

Results

Pulmonary Embolism

The amount of microspheres needed to induce acute massive PE was comparable in both groups (NO group, 241 ± 73 mg; control group, 212 ± 28 mg; P = NS). Three animals, one in the NO group and two in the control group, died within 10 min after PE was induced and were excluded from data analysis. The severity of PE was comparable in both groups, and the injection of microspheres significantly increased MPAP and significantly decreased mean systemic artery pressure, arterial oxygen tension, and end-tidal concentration of carbon dioxide. Compared with the control group, NO inhalation after PE led to a significant decrease in MPAP and a significant increase in mean systemic artery pressure and end-tidal concentration of carbon dioxide (table 1).

Table 1. Mean Pulmonary Artery Pressure (MPAP), Mean Systemic Artery Pressure (MAP), Arterial Oxygen Tension (PaO_2), and End-tidal Concentration of Carbon Dioxide ($ETCO_2$) before (t0), Immediately after Induction of PE (PE), and 20 (t20), 40 (t40), and 55 (t55) min after PE in the NO Group and in the Control Group

		t0	PE	t20	t40	t55
MPAP (mmHg)	NO group	17 ± 1	43 ± 1	31 ± 1*	29 ± 2*	32 ± 1
	Control group	17 ± 1	40 ± 3	34 ± 1	34 ± 1	32 ± 1
MAP (mmHg)	NO group	85 ± 4	67 ± 8	74 ± 4*	75 ± 4	78 ± 5
	Control group	82 ± 5	65 ± 7	56 ± 5	61 ± 6	61 ± 7
PaO_2 (mmHg)	NO group	134 ± 6	63 ± 3	65 ± 2	63 ± 4	66 ± 3
	Control group	131 ± 5	61 ± 11	59 ± 4	61 ± 5	61 ± 5
$ETCO_2$ (mmHg)	NO group	39 ± 1	31 ± 3	40 ± 1*	40 ± 3	40 ± 3
	Control group	40 ± 2	30 ± 2	33 ± 2	33 ± 3	32 ± 3

Values are mean ± SEM.

* $P < 0.05$, control group *versus* NO group.

Platelet Aggregation in Animals with Pulmonary Embolism

In the control group, acute massive PE led to a significant increase in angle α and A_{max}. In contrast, we found a significant decrease in angle α and A_{max} in the NO group, in which 15 min after cessation of NO inhalation (t55), angle α and A_{max} returned to the t0 level (figs. 2 and 3).

The hematocrit and the hemoglobin concentrations were comparable and remained stable in both groups. The platelet count decreased slightly in both groups during the study period (table 2).

Platelet Aggregation in Animals without Pulmonary Embolism

In animals without PE, inhalation of 5 ppm NO significantly decreased angle α and A_{max} only after 10 min. The effect was transient, and we observed no further decrease in platelet aggregation during continued NO inhalation, although higher concentrations of NO were administered (table 3). Comparable to these findings, during inhalation of 5 ppm during 240 min, platelet aggregation also decreased after 15 min (−22%) and reached baseline levels again 1 h after the beginning of NO inhalation.

Discussion

This study shows that inhaled NO inhibits the increase of *ex vivo* adenosine diphosphate-induced platelet aggregation after acute massive PE in pigs. Furthermore, NO inhalation significantly improved hemodynamic and respiratory conditions.

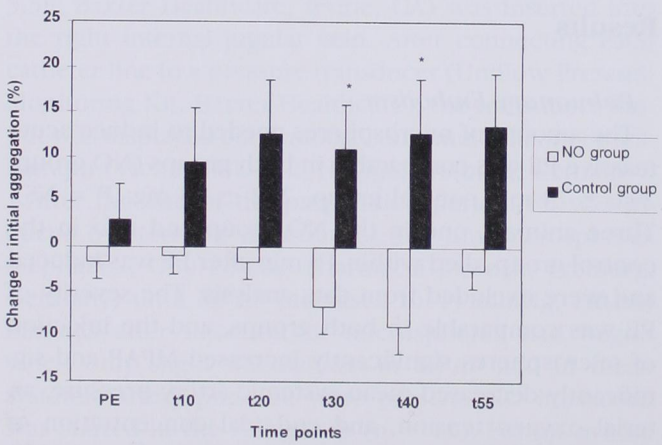

Fig. 2. Change in initial platelet aggregation in percentage of t0 value (before pulmonary embolism [PE]), immediately (PE), and 10 (t10), 20 (t20), 30 (t30), 40 (t40), and 55 (t55) min after induction of PE in the nitric oxide (NO) group and in the control group, respectively (mean ± SEM; *$P < 0.05$ for the control group compared with the NO group).

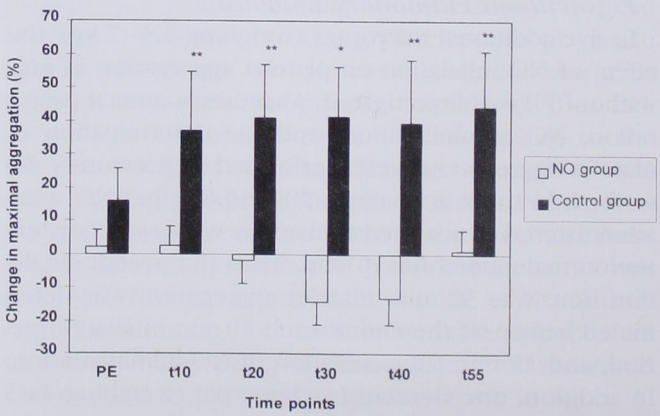

Fig. 3. Change in maximal platelet aggregation in percentage of t0 value (before pulmonary embolism [PE]), immediately (PE), and 10 (t10), 20 (t20), 30 (t30), 40 (t40), and 55 (t55) min after induction of PE in the nitric oxide (NO) group and in the control group, respectively (mean ± SEM; *$P < 0.05$, **$P < 0.01$ for the control group compared with the NO group).

Table 2. Platelet Count (nl^{-1}), Hematocrit (%), and Hemoglobin ($g \cdot dl^{-1}$) before Pulmonary Embolism (t0), 20 min (t20), and 55 min (t55) after Pulmonary Embolism in the NO Group and in the Control Group

		t0	t20	t55
Platelet count (nl^{-1})	NO group	340 ± 71	320 ± 45	304 ± 67
	Control group	332 ± 27	303 ± 52	296 ± 49
Hematocrit (%)	NO group	27 ± 2	26 ± 2	28 ± 3
	Control group	24 ± 1	26 ± 1	26 ± 1
Hemoglobin ($g \cdot dl^{-1}$)	NO group	8.4 ± 0.3	8.1 ± 0.4	8.5 ± 0.5
	Control group	8.0 ± 0.6	8.1 ± 0.3	8.0 ± 0.2

Values are mean ± SEM.

In acute massive PE, platelet activation plays an important role in the pathogenesis of pulmonary hypertension and hypoxemia[2,3,5,6,20] and can aggravate right ventricular afterload and cardiac ischemia, ultimately contributing to right ventricular failure.[1,21,22] Thus treatment with anti-platelet agents can help prevent death from acute PE.[2,6,23-26]

In contrast to intravenously administered NO-releasing compounds, inhaled NO can selectively decrease MPAP and right ventricular afterload and can improve arterial oxygenation in various conditions associated with acute pulmonary hypertension.[10,14]

In addition to its vasodilatory properties, NO inhibits platelet function *in vitro* and *in vivo*.[7,9,27] Inhibition of platelet adhesion and platelet aggregation is explained by an activation of the soluble guanylate cyclase in the platelet cytosol, leading to an increase in cyclic guanosine monophosphate levels.[28] When these levels are increased in platelets, the intracellular calcium-ion concentration decreases, which in turn inhibits fibrinogen binding to the glycoprotein IIb/IIIa receptor on the surface of the platelet membrane, which is required for platelet aggregation.[8,9,28]

Our findings support recent data that suggest that, in contrast to its local vasodilating properties, inhaled NO can also affect systemic platelet function. In animals and in healthy men, prolonged bleeding time during NO inhalation was reported.[15,16] In addition, inhibition of platelet aggregation was observed during the administration of inhaled NO in patients with acute respiratory distress syndrome.[17] In our study, the increase in platelet aggregation after PE that occurred in the control group was eliminated during NO inhalation. Furthermore, we found an additional modest inhibition in platelet aggregation during inhalation of 40 and 80 ppm in the NO group. We could speculate that the inhibitory effect on platelet function may be more pronounced locally in the pulmonary microcirculation, where inhaled NO may diminish additional clot formation. This is supported by experimental studies in pigs that reported that NO inhalation decreased platelet sequestration in the lungs during extracorporeal circulation.[29] Therefore, besides the pulmonary vasodilatory effect of inhaled NO,[13] additional inhibition of platelet activation may contribute to the decrease in MPAP and to the improvement in oxygenation, which may be beneficial in patients with PE.

Table 3. Initial (Angle α) and Maximal Platelet Aggregation (A_{max}) before and during Inhalation of 5, 20, and 80 ppm NO, Subsequently Administered, and 15 min after the End of NO Inhalation (End) in Pigs without PE

	Before	5 ppm	20 ppm	80 ppm	End
Angle α (°)	64 ± 2	56 ± 4*	64 ± 2	64 ± 3	61 ± 2
A_{max} (%)	37 ± 2	25 ± 2*	39 ± 3	36 ± 3	34 ± 4

Values are mean ± SEM.

* $P < 0.05$, versus baseline.

We are not certain whether the decrease in platelet aggregation after PE in the NO group was a dose- or a time-related effect, because we used a sequential mode of NO administration in our study. *In vitro*, inhibition of adenosine diphosphate-induced platelet aggregation was reported to depend on the NO concentration.[28,30] In contrast, in patients with acute respiratory distress syndrome who inhaled 1-100 ppm NO, the effect of inhaled NO on platelet aggregation was not dose dependent, and the maximal inhibition of platelet aggregation was observed at 3 ppm.[17] To our knowledge, the time-related effect of NO inhalation on platelet aggregation has not yet been investigated by others. In our study, investigation of platelet aggregation in one pig without PE during inhalation of 5 ppm NO for 240 min revealed

no time relation, because the decrease in platelet aggregation was maximal 15 min after the beginning of NO inhalation. In addition, a comparable time course in platelet aggregation was also observed in pigs without PE, when 5–80 ppm inhaled NO were administered in a sequential mode during a period of 30 min. These findings suggest that the inhibitory effect of inhaled NO on platelet aggregation is transient and *not* related to time. However, further studies are required to investigate a time and dose relation as well as possible adaptative mechanisms.

In the NO group, platelet aggregation returned to baseline 15 min after NO inhalation was stopped. In comparison, platelet aggregation has been reported to recover within 8–12 min after cessation of an infusion of sodium nitroprusside.[31] Thus inhaled NO seems to be a rapidly reversible platelet inhibitor, an attribute that may be desirable, particularly in patients in whom pulmonary hemorrhage may supervene.

In our study, we injected microspheres to induce acute massive PE, a method used commonly to induce acute pulmonary hypertension.[13,32–34] The increase in pulmonary vascular resistance is caused by the combination of mechanical obstruction of the vascular bed and active pulmonary vasoconstriction induced by vasoactive mediators.[3] As in other investigations, the injection of microspheres in our study caused hypoxia and led to an increase in MPAP and a decrease in mean systemic artery pressure in both groups.[1,3,13] In addition, although we did not investigate the release of thromboxane and serotonin, the increase in systemic platelet aggregation reflects the involvement of circulating platelets. Thus this experimental model seems to be useful for investigating the effect of inhaled NO in acute PE and relevant because results of autopsy studies have shown that more than 40% of PE in the clinical setting are microembolisms.[35]

A decrease in platelet count has been reported with PE.[2,5] In our study, we observed only a slight decrease in platelet count, whereas hematocrit and hemoglobin concentrations were in the normal range during the entire study.[36] In clot-induced PE, platelet consumption may be more pronounced than in our model, which might explain the lack of significant decrease in platelet count. Therefore care must be taken when extrapolating our findings to the situation of clot-induced PE.

In conclusion, we found a significant inhibitory effect of inhaled NO on platelet aggregation in an animal model of acute massive PE. Because of its positive effect on hemodynamics and its anti-platelet action after acute massive PE, administration of inhaled NO may be an attractive adjunct to anticoagulation therapy. Our data may provide a foundation for future clinical studies that evaluate the efficacy of NO either alone or combined with other antithrombotic agents to treat patients with PE.

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