Endogenous Nitric Oxide Acts as a Natural Antithrombotic Agent In Vivo by Inhibiting Platelet Aggregation in the Pulmonary Vasculature

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Summary

Nitric oxide (NO) is a powerful vasodilator and an inhibitor of platelet aggregation in vitro. While the ability of NO to modulate vascular tone in vivo has been proven, only a few studies have assessed its platelet inhibitory activity in vivo.

We have employed two complementary animal models of pulmonary platelet thromboembolism to assess the antithrombotic activity of endogenous NO in vivo.

The inhibition of nitric oxide synthase (NOS) by L-NAME significantly potentiated while the administration of the NOS substrate L-arginine significantly reduced the accumulation of 111In-labelled platelets in the pulmonary vasculature of rabbits induced by intravenous collagen plus epinephrine. L-NAME or L-arginine did not, however, modify 111In-labelled erythrocyte distribution in lungs and phenylephrine had no effect on platelet accumulation following collagen + adrenaline, suggesting that the effects of L-NAME were not due to vasoconstriction but rather to a direct modification of platelet function. In mice, L-NAME significantly reduced the release of collagen + adrenaline required to induce thromboembolic mortality, increased the fall in circulating platelets and increased the % of pulmonary vessels occluded by platelet thrombi. The effects of L-NAME were reversed by L-arginine but not by a dose of nicardipine exerting maximal vasodilatation. Phenylephrine did not potentiate collagen + adrenaline-induced mortality.

In the pulmonary vasculature in vivo, endogenous NO inhibits collagen + adrenaline-induced aggregation and enhances platelet disaggregation. This natural modulator function of NO is exerted via a direct effect on platelets and not as a result of haemodynamic changes.

Introduction

Nitric Oxide (NO) is released from the endothelium, platelets and leukocytes in response to a number of circulating agonists (1) and haemodynamic factors (2). NO is an important local factor in the regulation of vascular tone (3) and exhibits antiaggregatory activity in vitro (4, 5) and ex vivo (6). The role of endogenous NO in modulating platelet function and coagulation in vivo is, however, unclear.

NO has been shown previously to reduce cyclic flow variations in stenosed and endothelium-injured arteries of dogs and rabbits (7). NO causes vasodilatation and changes in vessel diameter can be associated with changes in cyclic flow (8, 9); thus, it is not clear whether the effects observed following inhibition of NO were entirely the result of alterations in platelet function or whether changes in vessel tone played a role. In addition, NO synthase inhibition has been shown to shorten bleeding time in humans (10); vasoconstriction, however, is a determinant of bleeding time and no attempt was made to evaluate the contribution of systemic vasoconstriction in this study. It is possible, therefore, that some of the antithrombotic and antihaemostatic effects of NO in vivo, previously attributed to an action on platelets (7, 10-13), may well be partly explained by an action on the vessel wall as there is no evidence of a direct modulation of physiological platelet function by endogenous nitric oxide.

Some of the effects of endogenous NO upon thrombin-induced platelet accumulation in the pulmonary vasculature of the rabbit have been examined previously (14). The mechanism by which endogenous NO affects thrombin-platelet accumulation is unclear since thrombin, as well as stimulating platelet aggregation, induces a multitude of effects, including fibrin deposition, vascular damage and vessel constriction, all of which may be influenced by endogenous NO (14-17). Moreover, NO has been suggested to enhance endogenous fibrinolysis (15, 16) and this may contribute to the regulation of thrombin-induced platelet deposition in lungs (18, 19).

Under normal physiological conditions, platelets are exposed to several aggregating agents and synergism between these agonists may be an important phenomenon in optimising platelet activation in thrombosis and haemostasis (20). In the present study, therefore, we have investigated the role of endogenous NO upon platelet accumulation induced by a low dose of a mixture of collagen and adrenaline in animal models where thrombus formation is primarily platelet mediated. Experiments were designed to differentiate between effects on platelets and those on blood vessels. The automated isotope monitoring system (21) allows monitoring of radio-labelled platelet aggregation in different organs and is a non-invasive method that, unlike many other in vivo models of thrombosis, uses animals with an intact endothelium at all times. In addition, a mouse model of pulmonary embolism (22) has been employed to explore the effects of NO inhibition upon pulmonary vessel occlusion histologically as well as the effects of NO inhibition on mortality. The effects of the NOS inhibitors, L-N(G)-nitro arginine methyl ester (L-NAME) and nitro-L-arginine (16), as well as L-arginine (L-arg), the precursor of NO, were investigated.
Materials and Methods

Drugs

The sources of the drugs used were as follows: ADP, D-arginine, L-arginine, D-NNAME, L-NNAME, nitro-L-arginine and phenylephrine hydrochloride (Sigma); adrenaline (Mascia Brunelli, Milan, Italy); Diazepam (Valium, Roche); soluble equine tendon collagen (Hornom Chemie, Munich, Germany); Hypnorm (lentanyl citrate 0.315 mg ml−1 and fluanisone 10 mg ml−1; Janssen Pharmaceuticals Ltd., Oxford); 111Indium oxine (Amersham International) and nicardipine (Novartis, Milan, Italy). All drugs were dissolved in 0.9% saline.

111In-Labelled Platelet and Erythrocyte Monitoring in Rabbits

Animals

The study was carried out on New Zealand White, male rabbits weighing 2.0-3.0 kg (Foxfield, Petersfield, Hampshire UK and Charles River, Calco, Italy). Animals were fed a normal diet and received water ad libitum.

111In-labeling of platelets

Full details of the protocol for the isolation and radiolabelling of rabbit platelets have been described elsewhere (21). 9 ml blood was collected from the right ear artery into 3.8% (w/v) trisodium citrate and centrifuged (225 g for 15 min) to obtain platelet rich plasma (PRP). PRP was buffered in Ca++-free Tyrode solution containing 300 mg ml−1 prostaglandin E1 (CFTP) and centrifuged at 675 g for 15 min. After removal of the supernatant, the surface of the platelet pellet was washed with CFTP. The platelets were gently resuspended in 1 ml of CFTP and incubated for 2 min at 37°C with 1.8 MBq 111Indium oxine. After a further centrifugation (640 g for 15 min) the supernatant containing free 111In oxine was removed and the platelets resuspended in 2 ml CFTP.

111In labelling of erythrocytes

Blood was taken and PRP obtained as before and discarded. A 1 ml sample of packed erythrocytes was washed by centrifugation (225 g for 15 min) in 10 ml CFTP. An erythrocyte pellet was formed by centrifugation (640 g for 15 min) and the supernatant discarded. The pellet was washed with CFTP and resuspended before incubation with 111In oxine as described for platelet labelling. The erythrocytes were pelleted once again (640 g for 15 min) and washed and resuspended in 2 ml CFTP.

Experimental procedure

Animals were anaesthetised with diazepam (4 mg kg−1, i.p.) followed 10 min later by Hypnorm (0.4 ml kg−1, i.m.). Neuroleptanalgesia was maintained by further i.m. injections of Hypnorm (0.1-0.2 ml) as necessary (approximately 30 min intervals). 111In-labelled platelets (or erythrocytes) were administered via a butterfly cannula in a left marginal ear vein and allowed to equilibrate in the circulation for 40 min before challenge with platelet agonists. Circulating 111In oxine was removed and the platelets resuspended in 2 ml CFTP.

Administration of agonists and drugs

Intravenous bolus injections were made into a left marginal ear vein via a butterfly cannula. Agonists were flushed into the circulation with 0.5 ml of 0.9% saline. It has been suggested that the first challenge with an aggregating agent may produce an abnormally large response with baseline values resetting at a slightly different level (23, 24). For this reason, all animals were given an initial dose of ADP (10 µg kg−1 i.v.) before the main experiments were initiated (21).

L-NNAME (10 mg kg−1), L-arg (500 mg kg−1) and D-arg (500 mg kg−1) were administered 5 min prior to platelet agonists. When given in combination, L-arg or D-arg was administered 5 min prior to L-NNAME at a dose (900 mg kg−1) previously shown to reverse the effects of this dose of L-NNAME (14). Phenylephrine (20 µg kg−1 min−1) was infused for 20 min prior to injection of collagen + adrenaline and then for a further 10 min.

Collagen (20 µg kg−1) and adrenaline (1.2 µg kg−1) were administered either separately or in combination as bolus injections via a marginal ear vein, allowing 60 min between responses.

Blood pressure measurement in rabbits

Rabbits were anaesthetised as described above and the left common carotid artery was cannulated. Systemic blood pressure was monitored via a pressure transducer (Druck Ltd.) attached to the cannula.

Pulmonary Thromboembolism in Mice

Animals

Male CD1 mice, body weight 20-25 g, (Charles River, Calco, Italy) were caged and fed a regular diet for at least 1 week before use. Mice were accustomed to handling by skilled investigators who carried out injections with minimal disturbance to the animals. The total duration of each experiment was 15 min and all surviving animals were immediately killed by exposure to ether vapours. No anaesthetic was used during the experiments because of the short duration and because it has been shown previously that anaesthesia interferes with pulmonary thromboembolism in mice (25).

Experimental procedure

The method has been described elsewhere (22, 26). Drugs were administered in a fixed volume of 100 µl via a tail vein 2 min prior to administration of thrombogenic agonists. Pulmonary thromboembolism was induced by i.v. injection of a mixture of collagen (250 µg ml−1) and adrenaline (15 µg ml−1). In each experimental session, at least 5 animals per treatment group were tested; control groups were run at the beginning and end of every session. The majority of non-surviving animals injected with collagen + adrenaline died within 3 min. Any animals which did not die within this time were killed after 15 min (or immediately if obviously distressed) and recorded as survivors. In some experiments, nicardipine (100 mg kg−1 i.p.) was administered 1 h prior to collagen + adrenaline. This study was approved by the Committee on Ethics of animal experiments of the University of Perugia.

Tissue histology in mice

This methodology has been described elsewhere (26). Briefly, mice were rapidly killed with ether, 2 min after injection of 40 µl collagen + adrenaline, and the lungs removed, rinsed with cold saline and immediately fixed in 10% formalin. Tissues were blocked and paraffin sections cut at 5-6 µm and then stained with haematoxylin and eosin. At least 4 fields, at a magnification of 400×, were observed for every specimen. The specimens were examined under light microscopy by a pathologist unaware of the treatment administered to the animals. The total number of identifiable vessels per field was counted and the percentage of them occluded by thrombotic material was recorded.

Platelet counts in mice

Blood was collected by cardiac puncture from mice under ether anaesthesia and anticoagulated with 1/8th vol. of tripotassium EDTA (14.5 mg ml−1 of blood). After thorough mixing, platelets were counted optically by the Brecher-Cronkite (27) method. Platelet counts were carried out 2 min after i.v. thrombotic challenge in saline pre-treated controls or drug pre-treated animals.

Statistics

All values from rabbits are expressed as mean ± S.E. Mean. Responses to platelet agonists are expressed as maximum percentage increase in 111In counts above values recorded immediately prior to injection of the agonist (max. %
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Additionally, the trapezoidal area under the curve (AUC) of the plot of the % increase in radioactive counts against time was calculated for each response. Control and experimental values were compared using one-way ANOVA followed by a multiple comparison (Tukey) test. Responses in mice are presented as % mortality at a particular dose of collagen and adrenaline, percentage of occluded vessels in histological sections and number of circulating platelets/L of blood. Chi square was used to analyse mortality and one-way ANOVA followed by multiple comparison test (Scheffe’s test) was used for all other mouse data. For all data, a P value < 0.05 was considered significant.

Results

**111 In Labelled Platelet and Erythrocyte Monitoring in Rabbits**

Adrenaline (1.2 μg kg⁻¹) induced a small degree of platelet accumulation in the pulmonary vasculature (4.8 ± 0.6%, AUC = 17.5 ± 2.6, n = 8). Collagen (20 μg kg⁻¹) also induced platelet accumulation in the lung (6.2 ± 1.4%, AUC = 57.7 ± 24.7, n = 4). When administered together with adrenaline, collagen produced an additive increase in platelet accumulation in the pulmonary vasculature measured as peak response (Fig. 1a) and a greater than additive increase measured as AUC (95.0 ± 13.4, n = 8) (Fig. 1b). Neither agonist induced significant changes in erythrocyte associated counts in the pulmonary vasculature when given separately (n = 4, data not shown) but, following collagen + adrenaline, a slight fall was observed (-4.7 ± 0.7%, n = 4).

L-NAME (10 mg kg⁻¹) induced a modest increase in both platelet (4.8 ± 0.3%, n = 6) and erythrocyte (3.8 ± 0.5%, n = 5) associated basal counts in the pulmonary vasculature and a non-significant change in mean systemic blood pressure from 70 ± 4 mm Hg to 75 ± 8 mm Hg (n = 4) 5 min after i.v. injection.

Adrenaline-induced platelet accumulation (4.8 ± 0.6%) was not potentiated by prior treatment with L-NAME when measured as max. % increase in counts (5.0 ± 0.4%, n = 6; Fig. 1a), although the AUC (50.0 ± 7.9) was significantly (P < 0.05) higher than control values (17.5 ± 2.6; Fig 1b) due to a prolonged disaggregation period (Fig. 2). Collagen-induced platelet accumulation was significantly potentiated by L-NAME, both in terms of max. % increase (16.4 ± 3.1, n = 5; Fig. 1a) and AUC (122.8 ± 27.8; Fig. 1b). Collagen + adrenaline-induced platelet accumulation (10.3 ± 1.7%, n = 8) was significantly increased following L-NAME (19.6 ± 3.4%, n = 6; Fig. 1a) as was the AUC (408.6 ± 92.6; Fig. 1b). Pre-treatment with L-NAME did not, however, significantly affect the slight drop in erythrocyte associated counts in the pulmonary vasculature following collagen + adrenaline (-4.7 ± 0.7% and -5.7 ± 0.8% without and with L-NAME respectively, n = 4). D-NAME (10 mg kg⁻¹) had no significant effect on collagen + adrenaline-induced platelet accumulation (8.9 ± 2.2%, n = 5).

Phenylephrine (20 μg kg⁻¹ min⁻¹) increased basal platelet associated ¹¹¹ In-counts by 5.2 ± 0.7%, and erythrocyte associated ¹¹¹ In-counts by a similar level (5.1 ± 0.9%). Mean systemic blood pressure changed non-significantly from 72 ± 5 mm Hg to 75 ± 9 mm Hg (n = 4) after 20 min infusion of phenylephrine (20 μg kg⁻¹ min⁻¹). Pretreatment with...
this dose of phenylephrine had no effect on platelet accumulation induced by collagen + adrenaline measured as either max. % increase (6.5 ± 1.5, n = 5) or AUC (116 ± 67, n = 5) compared to saline controls (7.3 ± 1.9, AUC = 118 ± 56, n = 5).

L-arginine (500 mg kg⁻¹) reduced platelet (-4.6 ± 0.2%, n = 5) and erythrocyte (-5.8 ± 0.8%, n = 4) associated basal radioactivity in the pulmonary vasculature. Pre-treatment with L-NAME (500 mg kg⁻¹) significantly reduced platelet + adrenaline-induced platelet accumulation, (2.6 ± 1.0%, n = 6) compared to saline pre-treated controls (8.4 ± 0.9%, n = 5), whereas D-arginine (500 mg kg⁻¹) had no effect (Fig. 3). Pre-treatment with L-arginine (900 mg kg⁻¹) prevented L-NAME potentiation of collagen + adrenaline-induced platelet accumulation so that values (9.4 ± 1.8%, n = 5) were not significantly different to control levels (Fig. 3), whereas D-arginine (900 mg kg⁻¹) had no significant effect on this response (22.2 ± 3.7%, with D-arginine, n = 4; 16.4 ± 3.1, without).

**Discussion**

We have shown here that, in two different animal models of platelet pulmonary thromboembolism, endogenous NO appears to reduce collagen plus adrenaline-induced platelet accumulation in lungs since pre-treatment with competitive inhibitors of NO synthase (NOS) provoked a striking decrease in circulating platelets produced by collagen + adrenaline and increased significantly the percentage of occluded vessels in histological sections of lungs from animals injected with 40 μl of the mixture of collagen (coll.) and adrenaline (adr.).

**Table 1** Effects of the suppression or potentiation of the synthesis of endogenous NO on circulating platelets and number of occluded lung vessels in a mouse model of pulmonary thromboembolism

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Circulating Platelets (x10⁶/μl)</th>
<th>Occluded Vessels (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>105 ± 5</td>
<td>50 ± 0</td>
</tr>
<tr>
<td>Coll. + adr. + Saline</td>
<td>429 ± 65</td>
<td>43.1 ± 16.0</td>
</tr>
<tr>
<td>Coll. + adr. + L-NAME</td>
<td>126 ± 18*</td>
<td>76.6 ± 5.9*</td>
</tr>
<tr>
<td>Coll. + adr. + L-NAME + L-Arg.</td>
<td>342 ± 99</td>
<td></td>
</tr>
<tr>
<td>Coll. + adr. + Nic.</td>
<td>328 ± 89</td>
<td></td>
</tr>
<tr>
<td>Coll. + adr. + L-NAME + Nic.</td>
<td>161 ± 28*</td>
<td></td>
</tr>
<tr>
<td>Coll. + adr. + L-Arg.</td>
<td>26.9 ± 8.5*</td>
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</tr>
</tbody>
</table>

Levels of circulating platelets and % occluded pulmonary vessels in histological sections were measured following i.v. injection of 40 μl of the mixture of collagen (coll.) and adrenaline (adr.). Animals were pretreated with saline, L-NAME (50 mg kg⁻¹), L-arginine (L-arg.) or nicardipine (nic.l.) * Significantly (P < 0.05) different from saline pre-treated animals injected with collagen + adrenaline.
potentiation of agonist-induced pulmonary embolism. This was evidenced in rabbits by enhanced $^{111}$In-labelled platelet accumulation in the pulmonary vasculature and in mice by increased mortality and by an increased number of platelet pulmonary emboli as detected by lung histology. D-NAME, the inactive enantiomer of L-NAME, did not modify pulmonary embolism induced by collagen plus adrenaline in either model, suggesting that the effects of L-NAME are the consequence of NOS inhibition and not of some noxious effect of this substance. In addition, the simultaneous administration of L-NAME and a large excess of the natural NOS substrate, L-arg, but not of the inactive enantiomer D-arg, completely prevented the prothrombotic effects of L-NAME in both rabbits and mice.

As further proof of the role of endogenous NO in preventing platelet pulmonary thromboembolism, pre-treatment of animals with a large dose of L-arg, the metabolic precursor of NO, significantly reduced the accumulation of $^{111}$In-labelled platelets in rabbit lungs and reduced mortality and number of pulmonary emboli in mice.

We selected two animal models in which the thrombogenic stimulus acts primarily on platelets and blood coagulation is only minimally involved (21, 22, 24). Our results demonstrate that, under these experimental conditions, the action of endogenous NO is exerted at the level of platelets and not through an action on the vessel wall or on other components of the haemostatic system. This suggestion comes from a series of observations: 1) L-NAME, at doses producing striking potentiation of $^{111}$In-labelled platelet accumulation in the pulmonary vasculature, did not modify collagen + adrenaline-induced erythrocyte distribution in rabbit lungs; 2) L-NAME and nitro L-arginine potentiated, and L-arg decreased, the number of platelet emboli observed in lung histological sections in mice following collagen + adrenaline; 3) L-NAME potentiated and L-arg decreased the drop in the number of circulating platelets provoked by collagen + adrenaline in mice; 4) pre-treatment of mice with nicardipine, at a dose producing maximal vasodilatation (26, 28), did not affect the changes in platelet number produced by L-NAME pre-treatment, suggesting that the protective effect of arginine is due to a platelet dependent mechanism. This high dose of nicardipine was chosen because lower doses (30 and 10 mg kg$^{-1}$) produced submaximal vasodilatation (26, 28). 5) Vasoconstriction induced by the $\alpha_2$-adrenoceptor agonist, phenylephrine, did not affect collagen + adrenaline-induced platelet accumulation in rabbit lungs. A dose of phenylephrine was selected which produced an elevation in baseline $^{111}$In-counts in the pulmonary vasculature similar to that produced by L-NAME using either platelets or erythrocytes as markers. Such increases in basal counts most likely occur as a result of blood redistribution and are not due to effects on platelets (14). Phenylephrine has been shown to elicit effective vasoconstriction when administered to rabbits by intravenous infusion at doses similar to that used here (29). Moreover, the effects of the chosen doses of L-NAME and phenylephrine on systemic blood pressure were very similar and relatively small. The lack of effect of phenylephrine on collagen + adrenaline-induced platelet accumulation in rabbits and mortality in mice further indicates that the potentiation of platelet accumulation following L-NAME does not occur due to the haemodynamic effects of NOS inhibition but rather from a reduction of the direct action of NO upon platelets.

Our data suggest that endogenous NO regulates platelet function differentially depending on the agonist used. When the stimulus used was a weak platelet agonist, such as adrenaline (present paper), ADP or PAF (14), block of NO production affected mainly platelet disaggregation whilst, with stronger stimuli such as collagen, collagen plus adrenaline (present paper) or thrombin (14) platelet aggregation was also inhibited. The influence of NO upon platelet accumulation depends upon the anatomical site in which platelet aggregation is induced since L-NAME does not potentiate thrombin-induced platelet accumulation in the cerebral vasculature (14). The prolonged disaggregation in the pulmonary vasculature following PAF, ADP, adrenaline, collagen and thrombin, in the presence of L-NAME, also demonstrates the physiological role of NO in the lung in regulating platelet disaggregation in contrast with the cerebral vasculature where the primary function of the haemostatic system is the rapid formation of a platelet plug in the event of a rupture. Moreover, in the mouse model histologic studies show that the injection of collagen plus epinephrine induces the formation of platelet microemboli only in lungs and not in other organs.

The central role played by NO in the pulmonary circulation suggests that the main source of endogenous NO in our models is the endothelium, although the platelets themselves may be an additional source in view of previous findings with L-Arg (30). Moreover, the fact that NO inhibition per se did not cause a significant platelet accumulation in the lungs suggests that the basal release of NO from endothelial cells (and/or platelets) is probably not critically involved in modulating platelet function, whereas the stimulation of NO synthesis that takes place during platelet activation, possibly via released vasoactive substances (e.g. 5-HT from platelets) (31), allows attainment of local levels of NO which are sufficient to regulate the extent of platelet activation. Data obtained in humans and very recently published, showing that the inhibition of endogenous basal NO production does not increase platelet aggregation in vivo but that the enhanced endogenous biosynthesis of NO obtained by the i.v. administration of L-Arg does inhibit it (30), further support the suggestion that the platelet modulatory role of endogenous NO only becomes apparent after appropriate stimulation.

In conclusion, the experiments reported here indicate that endogenous NO is an important regulator of agonist stimulated platelet function in vivo. NO appears to play a crucial role in reducing platelet activation and in accelerating platelet disaggregation in the pulmonary vasculature. Given the ability of NO to reduce fibrinogen levels (16), increase t-PA activity (15) and the dramatic effects of L-NAME on thrombin-induced platelet aggregation and disaggregation (14), it cannot be excluded that NO may also be important as a modulator of endogenous fibrinolysis.

References


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