Functional regulation of vascular and platelet activity during thrombosis by nitric oxide and endothelial nitric oxide synthase

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Summary

Nitric oxide (NO) regulates both vascular tone and platelet function. Since thrombotic diseases and their animal models consist of both vascular and platelet components, the functional mechanisms by which NO and endothelial nitric oxide synthase (eNOS) regulate thrombotic events are unclear.

Experiments were conducted by measuring collagen induced aggregation of freely circulating radiolabelled platelets in the pulmonary vasculature of anaesthetised mice *via* external detection probes. In addition, cardiac haemodynamic function was assessed by invasive catheterisation during thrombotic stimulation.

Platelet aggregation responses were shown to occur independently of changes in vessel tone induced by pharmacological vasoconstriction or vasodilatation. Acute NOS inhibition significantly potentiated the amplitude and duration of platelet aggregation and an NO donor had an inhibitory effect. In contrast, in eNOS^{-/-} mice, the amplitude of platelet aggregation was not affected although the response was protracted following moderate thrombotic stimulation. Thrombosis induced changes in haemodynamic performance were sensitive to vasomodulation and were potentiated by both NOS inhibition and in eNOS^{-/-} mice.

In conclusion, endogenous NO and exogenously applied NO donors exert an antithrombotic effect *in vivo* through a direct suppression of platelet aggregation. In contrast, eNOS exerts a powerful antithrombotic effect upon the vascular components of thrombosis but has a more subtle effect on the duration of thrombotic responses that are platelet mediated. Our data demonstrate the differential roles of eNOS and general NO bioavailability in regulating vascular and platelet activity during thrombosis.

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Key words

animal models, endothelium, nitric oxide, pharmacology, platelet

Introduction

Arterial thrombotic diseases such as myocardial infarction are driven by inappropriate activation of platelets but additionally include vascular elements, such as arterial dysfunction, which can contribute to both the onset and severity of the condition (1, 2). Similarly, animal models of thrombosis have both platelet and vascular components (3). The contribution of each component to the measured thrombotic response has generally not been defined (4) and is likely to vary between models. Thus, the functional mechanisms through which nitric oxide (NO), which modifies both platelet and vascular function, regulates thrombotic responses are unclear.

NO is a labile signalling molecule that relaxes vascular smooth muscle and hence regulates vascular tone (5). Additionally, NO negatively regulates platelet aggregation and adhesion (6, 7) and inhibits platelet aggregation *in vivo* (8). NO therefore has multiple antithrombotic properties (9). Although the vascular and platelet modifying effects of NO *in vitro* are well documented, the roles of NO and the various NOS enzymes in regulating the platelet and vascular components of thrombosis *in vivo* are poorly understood. This lack of understanding is largely due to the absence of experimental models in which the contributions of the vascular and platelet components of the thrombotic response are defined.

We have previously used rabbit (8) and mouse (10) models of platelet thromboembolism to demonstrate that platelet aggregation *in vivo* is critically regulated by endogenous NO but that loss of endothelial nitric oxide synthase (eNOS) does not potentiate the platelet aggregation response due to compensatory mechanisms that have not been fully defined or redundancy between eNOS and other negative regulators of

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platelet function (10). A lack of thrombotic phenotype in eNOS^{-/-} mice has been reported by a number of groups (11, 12), whereas others contradict these reports by describing modified thrombotic responses (13-15). We hypothesised that eNOS exerts a limited or negligible anti-platelet effect but modifies the vascular elements of thrombosis due to its vasoactive properties (16, 17). Such differential effects of eNOS would explain apparently conflicting data concerning the role of this enzyme in regulating thrombotic events and further characterise its functional role in the cardiovascular system. In the current paper we have addressed this hypothesis using an *in vivo* model of platelet thromboembolism in which parameters are defined that are platelet driven but contrast in their dependence on vessel tone.

Methods

Mice

eNOS knock-out mice (NOS-3^{-/-}, Strain: 0026847) were purchased from Jackson Laboratory, ME, USA along with control C57Bl/6J control mice. All animal procedures were conducted under our Home Office Project License PPL 70/6358, approved by the Ethical Review Panel at Imperial College London and refined in association with the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) (18).

Materials

All reagents were purchased from Sigma (Dorset, UK) with the exception of ¹¹¹Indium Oxine (GE Healthcare, Bucks, UK) and collagen (Nycomed Pharma, Germany). All drugs were dissolved in saline, apart from collagen which was dissolved in the manufacturers' buffering solution, and were administered as an intravenous bolus injection or infusion as described. Drug and molecular target nomenclature in this paper conforms to the *British Journal of Pharmacology's* Guide to Receptors and Channels (19).

Blood collection and platelet labelling

Blood was collected from terminally anaesthetised (10 ml kg⁻¹ urethane (25%) i.p.) donor mice by cardiac puncture, platelets were isolated by centrifugation and radiolabelled with 1.8 MBq ¹¹¹Indium Oxine as previously described (18, 20). Radiolabelled platelets were resuspended in 200 µl Ca²⁺-free Tyrode's solution per mouse and infused into anaesthetised recipient mice *via* a femoral vein. Circulating

platelets were allowed to equilibrate for 15 min. Platelet agonists and drugs were administered *via* an exposed femoral vein.

In vivo platelet aggregation

Platelet responses were measured as increases in platelet-associated counts in the pulmonary vascular bed following intravenous injection of collagen. Data was collected *via* a 1cm SPEAR (Single Point Extended Area Radiation) detector (eV Products, PA, US) fixed over the pulmonary vascular bed and recorded on a UCS-20 spectrometer (Spectrum Techniques, TN, US) using custom made software (Mumed Systems, London, UK). We have previously shown that increases in radioactive counts in this model represent the trapping of platelet aggregates formed in the systemic circulation in the pulmonary vasculature (18).

Haemodynamic assessment

Blood pressure and cardiac contractility were measured by surgically exposing and cannulating the right common carotid artery of anaesthetised (10 ml kg⁻¹ urethane (25%) i.p.) mice with a 1.4 Fr pressure catheter (Millar, Tx, US). The catheter was advanced to the aortic arch to measure aortic pressure and then to the left ventricle to measure cardiac contractility (dP/dt_{max}) and left ventricular pressure (LVP) as previously described (18, 21). These parameters were continuously measured before and following induction of thromboembolism by intravenous injection of collagen.

Experimental protocol

The experimental protocols were identical for the platelet aggregation and haemodynamic experiments. Each preparation was allowed to stabilise for 15 min before the administration of experimental or control compounds. Drugs were administered intravenously 10 min (5 min for SNP) before injection of collagen and the response recorded for 10 min.

Analysis of results

Stable baseline values were recorded 1 min before the addition of the drug or vehicle.

All results are expressed as percentage changes from baseline values. Changes in mean radioactive counts or haemodynamic parameters caused by the test drug were compared with time-matched vehicle controls using one-way analysis of variance with Benferroni post-analysis. All values are expressed as the mean $\pm$ s.e. mean; differences in the mean were interpreted as significant when $p < 0.05$.

Results

Thrombotic and haemodynamic response to collagen

Collagen induced thromboembolism *in vivo* that was characterised by a rapid increase in radioactive counts in the pulmonary detection probe representing trapping of platelet aggregates in the pulmonary vascular bed as previously described (18). Platelet responses peaked and returned slowly to baseline as platelets disaggregated. Collagen also impaired haemodynamic function measurable as a transient fall in cardiac contractility (dP/dt_{max}) and left ventricular pressure (LVP) due to occlusion of pulmonary vessels and subsequent impairment of cardiac haemodynamic function (18). Typical traces showing the time course of the platelet and haemodynamic response to $75 \mu g\ kg^{-1}$ collagen are shown in Fig 1 and demonstrate that the changes in both platelet and haemodynamic parameters occur over a similar time frame.

We were able to define dose-dependent changes in platelet aggregation measured as the mean maximal % increase in counts at collagen doses of 25-100 $\mu g\ kg^{-1}$ (Fig 2A). These doses of collagen also produced dose-dependent concomitant changes in dP/dt_{max} and LVP during haemodynamic assessment as previously reported (18).

Effect of NOS inhibition and vasomodulation on blood pressure

Administration of the NOS inhibitors L-NAME (N_{ω} -Nitro-L-arginine methyl ester hydrochloride, 50 mg kg^{-1}) or ADMA (*asym*-Dimethylarginine, 50 mg kg^{-1}) significantly elevated aortic pressure compared with saline, D-NAME (N^G -Nitro-D-arginine methyl ester hydrochloride) or SDMA (*sym*-Dimethylarginine) controls (Fig 3A). To distinguish the effects of vasoconstriction from platelet mediated effects, we established concentrations of the $\alpha 1$ -adrenergic agonist and vasoconstrictor phenylephrine that produced similar increases in aortic pressure to those observed with

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3 L-NAME. Phenylephrine ($80 \mu\text{g kg}^{-1} \text{ min}^{-1}$ for 5 min) produced a similar change in the
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6 aortic pressure profile as 50 mg kg^{-1} L-NAME (Fig 3B) such that the mean increases
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8 in aortic pressure were not different following administration of these two agents (Fig
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10 3A). The NO donor SNP (sodium nitroprusside, $10 \mu\text{g kg}^{-1}$) reduced blood pressure and
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12 we established that the dihydropyridine calcium-channel antagonist and hypotensive,
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14 nicardipine, reduced pressure with a similar profile to SNP at $100 \mu\text{g kg}^{-1}$ (Fig 3C) such
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16 that the peak changes in blood pressure obtained with these agents were not different
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18 (Fig 3A). We also confirmed that, under our experimental conditions, aortic pressure
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20 was significantly elevated in $\text{eNOS}^{-/-}$ mice compared with wild-type controls as
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22 previously reported (22), data not shown.
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30 *Effect of NO and eNOS on platelet aggregation in vivo*

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32 The NOS inhibitors L-NAME (50 mg kg^{-1}) and ADMA (50 mg kg^{-1}) significantly
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34 potentiated collagen induced platelet aggregation and delayed the return to baseline
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36 compared to D-NAME and SDMA controls respectively (Fig 4A-B). In contrast,
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38 phenylephrine, at doses shown to elevate aortic pressure to a similar extent as NOS
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40 inhibition, had no effect on collagen induced aggregation. In addition, continuous
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42 infusion of phenylephrine throughout the recording period failed to enhance the
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44 response to collagen (Fig 4A-B).
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49 The NO donor, SNP, significantly reduced collagen induced aggregation whereas
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51 nicardipine, at concentrations producing a comparable reduction in aortic pressure, had
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53 no effect (Fig 4A-B).
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56 To assess the role of eNOS in a thrombotic model shown to be driven by platelet
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58 aggregation but independent of changes in vascular tone, we carried out dose-responses
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60 to collagen in $\text{eNOS}^{-/-}$ and wild-type mice. Collagen ($12\text{-}75 \mu\text{g kg}^{-1} \text{ i.v.}$) induced dose-

dependent platelet aggregation responses *in vivo* in eNOS^{-/-} and WT mice (Fig 4C-D). In contrast to experiments with NOS inhibitors, the amplitude of response (measured as maximal increase in counts) was not significantly different between eNOS^{-/-} and WT controls at any of the doses tested (Fig 4D). The total response (measured as AUC), although not different at low levels of collagen (12 – 25 µg kg⁻¹), was significantly elevated in eNOS^{-/-} mice at higher collagen doses (50-75 µg kg⁻¹, Fig 4F) due to a protracted return to baseline at these concentrations (Fig 4C-D).

Effect of NO and eNOS on haemodynamic responses in vivo.

Having established that the NOS inhibitors L-NAME and ADMA enhanced platelet aggregation responses *in vivo*, we went on to look at their effect on the cardiac haemodynamic changes elicited by collagen induced thromboembolism. Both L-NAME and ADMA significantly enhanced the fall in dP/dt_{max} and LVP following injection of collagen (Fig 5A-B). In contrast to platelet aggregation responses, which were not modified by phenylephrine (Fig 4A-B), there was a significant potentiation of the fall in dP/dt_{max} and LVP during collagen induced thromboembolism in the presence of phenylephrine compared with saline controls (Fig 5A-B). Similarly to platelet responses, SNP significantly reduced the collagen induced haemodynamic response but in contrast to the platelet response, which was not modified by nicardipine (Fig 4A-B), this agent significantly reduced the fall in dP/dt_{max} and LVP following injection of collagen (Fig 5A-B).

In order to assess the role of eNOS in a model shown to be driven by platelet aggregation but influenced by changes in vascular tone, we carried out experiments in eNOS^{-/-} mice. The fall in both dP/dt_{max} and LVP following collagen (50 µg kg⁻¹) were

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3 significantly and dramatically enhanced in eNOS^{-/-} mice compared to wild-type controls
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Discussion

The animal model employed in this paper was developed to allow platelet aggregation responses to be assessed *in vivo* and thus investigate the regulation of platelet function and thrombus formation by mediators derived from tissues external to the platelet such as the vascular endothelium (18). Collagen induced dose-dependent platelet aggregation measurable as the trapping of radiolabelled platelet aggregates in the mouse pulmonary vasculature. It was unclear whether such responses were driven purely by the magnitude of the platelet response or whether changes in vascular tone could exert an effect. Thus, although we had previously reported an increase in platelet aggregation in this model following NOS inhibition (10), it was unclear whether this observation was entirely platelet mediated or was partially influenced by vascular changes. We therefore conducted a series of experiments to determine whether changes in blood pressure could affect the platelet response in our model. We found that vasoconstriction with phenylephrine and vasodilatation with nicardipine induced similar changes in blood pressure to those observed with NOS inhibitors and an NO donor respectively but had no effect on platelet aggregation. We therefore conclude that the observed ability of NOS inhibitors to enhance platelet aggregation is mediated through a direct effect on platelets such that endogenous NO exerts an antithrombotic effect by acting directly on platelets to negatively regulate their functional response. Similarly, NO donors are able to inhibit platelet aggregation *in vivo* through a direct action on platelets.

We also observed that platelet thromboembolic responses were accompanied by concomitant changes in cardiac haemodynamic performance. Occlusion of pulmonary vessels by platelet aggregates leads to overload of the right ventricle. Subsequent dilation and hypokinesis of the right ventricle reduces left ventricular pre-load and impairs left ventricular contractile function leading to a fall in cardiac output (23). The

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3 haemodynamic impact of pulmonary vessel occlusion during collagen induced
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5 thromboembolism was therefore assessed in the current study as reductions in left
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7 ventricular $dP/dt_{\max}$ and LVP. Haemodynamic changes due to pulmonary vessel
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9 occlusion, for example during pulmonary embolism in humans, are strongly influenced
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11 by vessel tone (24, 25). We therefore hypothesised that the observed changes in $dP/dt_{\max}$
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13 and LVP, in contrast to platelet responses, would also be influenced by vessel tone.
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15 We confirmed this by showing increases and decreases in $dP/dt_{\max}$ and LVP in the
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17 presence of phenylephrine and nicardipine respectively. Thus, we were able to define
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19 distinct parameters in our model (platelet aggregation and cardiac haemodynamic
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21 changes), both of which were dose-dependently determined by platelet aggregation but
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23 which contrasted in their sensitivity to changes in blood pressure. When we investigated
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25 the consequences of pharmacological NOS inhibition upon cardiac haemodynamic
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27 performance, we observed a potentiation of the response confirming the ability of
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29 endogenous NO to modify a multifaceted thrombotic response.
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33 Although inhibition of NOS with inhibitors without isoform specificity was shown to
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35 enhance both the magnitude and duration of the platelet aggregation response, a
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37 different effect was observed in $eNOS^{-/-}$ mice. We have previously reported a similar
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39 level of platelet aggregation *in vivo* in $eNOS^{-/-}$ and WT mice (10) and this was
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41 confirmed here for a range of collagen concentrations indicating that although
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43 endogenous NO represses platelet aggregation *in vivo* leading to responses of a smaller
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45 magnitude, this effect is not mediated through eNOS. We also assessed the full time-
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47 course of the platelet response in $eNOS^{-/-}$ and WT mice and found that during mild
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49 stimulation, there was no difference in the platelet response. This contrasts with data
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51 with NOS inhibitors in previous studies, where threshold platelet aggregation was
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53 enhanced by non-selective NOS inhibitors (8, 10). At higher concentrations of collagen,
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3 however, we found that although the magnitude of the response was not modified, there
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5 was an increase in the duration of the response (measured as a significantly increased
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7 AUC) demonstrating that eNOS plays a role in the resolution of the platelet thrombus
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9 during moderate to strong thrombotic stimulation.
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12 We also demonstrated that the magnitude and duration of the haemodynamic response
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14 to thrombotic stimulation were significantly and dramatically enhanced in eNOS^{-/-} mice.
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16 These data demonstrate that the thrombotic phenotype of eNOS^{-/-} mice is highly
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18 dependent upon the sensitivity of the assessed thrombotic parameter to changes in
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20 vessel tone. In models and diseases with a vascular component, loss of eNOS induces a
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22 powerful thrombotic effect. In contrast, the effect of eNOS upon platelet driven
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24 processes is more subtle and relates to the duration rather than the magnitude of the
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26 response.
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31 Our data provide an explanation for apparently conflicting data concerning the role of
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33 eNOS in different experimental models of thrombosis. eNOS plays an important role in
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35 determining the duration and hence severity of moderate thrombotic stimulation that is
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37 primarily platelet mediated. Thus, the role of eNOS may not be apparent in models of
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39 mild thrombosis or in those in which the dynamic progression of the thrombotic event is
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41 not monitored. In contrast, models with a vascular element are likely to consistently
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43 reveal a powerful antithrombotic effect of eNOS that is mediated through the vasoactive
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45 properties of this enzyme.
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52 Our data clarify apparently contradictory data from animal models by demonstrating the
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54 differential roles of NO derived from eNOS and general NO bioavailability in
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56 regulating vascular and platelet activity during thrombotic responses. We demonstrate
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58 that despite the failure of many experimental models of thrombosis to detect a
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3 thrombotic phenotype in the absence of eNOS, this enzyme is likely to play a role in
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6 determining the severity of platelet driven thrombotic events. eNOS is therefore a
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8 potential therapeutic target in the treatment of both platelet driven and vascular diseases.
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Conflict of interest

The authors have no conflicts to declare.

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Figure Legends

Figure 1

Time course of the platelet and haemodynamic responses during collagen induced platelet thromboembolism. The real-time radiolabelled response to collagen is shown as % change in counts against time and shows a rapid increase in counts as platelets aggregate in the pulmonary region followed by a slower resolution phase (top panel). The middle panel shows a real-time recording of ventricular pressure from a catheter situated in the left ventricle of an anaesthetised mouse plotted as pressure (mmHg) against time and shows a fall in systolic left ventricular pressure (LVP) upon injection of collagen and a slower return to baseline. The lower panel shows a real time recording of derived pressures (dP/dt) and shows and changes in dP/dt_{max} (contractile function) and dP/dt_{min} (speed of relaxation) during collagen injection. All traces are representative of n=5.

Figure 2

Dose-dependent platelet aggregation and haemodynamic responses *in vivo*. Dose-response following injection of collagen (25 – 100 µg kg⁻¹ i.v., n=6-7) plotted as time-course of % change in counts at each collagen dose (upper panel) and maximal % increase in platelet-associated counts against collagen concentration (lower panel). The graphs show increased platelet aggregation in response to increasing doses of collagen. Data are presented as mean ± S.E.M of maximal % change from stable baselines, n=5-7, **p<0.01, ***p<0.001.

Figure 3

Effect of modified NO bioavailability and vascular tone on basal blood pressure.

(A) Effect of NOS inhibitors L-NAME and ADMA (50 mg kg⁻¹), and inactive forms of these compounds (D-NAME and SDMA) at equimolar doses, phenylephrine (80 µg kg⁻¹ min⁻¹), the NO donor SNP (10 µg kg⁻¹) and nicardipine (100 µg kg⁻¹) on systolic arterial pressure (SP max). L-NAME and phenylephrine enhanced arterial pressure to a similar extent and ADMA also increased blood pressure whereas saline, D-NAME and SDMA had no effect. SNP and nicardipine reduced arterial pressure to a similar extent. Data are presented as mean maximal change in systolic arterial blood pressure (SP max % response) ± S.E.M., n=6-8. (B) Profile in real-time of response to L-NAME (50 mg kg⁻¹, broken line) and phenylephrine (80 µg kg⁻¹ x 5 mins, solid line) and (C) SNP (10 µg kg⁻¹, solid line) and nicardipine (100 µg kg⁻¹, broken line) showing similar response profile of the agents in each response. Data are presented as mean ± S.E.M. % change relative to stable baseline (%ΔSP) against time, n=8, **p<0.01.

Figure 4

Effect of modified NO bioavailabilty and vascular tone on collagen induced platelet

aggregation *in vivo*. (A) Mice were pre-treated with NOS inhibitors L-NAME (50 mg kg⁻¹) or ADMA (50 mg kg⁻¹) or the inactive forms of these compounds (D-NAME and SDMA) at equimolar doses. Alternatively mice were treated with the NO donor SNP (10 µg kg⁻¹), the hypertensive phenylephrine (80 µg kg⁻¹ min⁻¹) or the hypotensive nicardipine (100 µg kg⁻¹). Platelet aggregation was then induced with collagen (50 µg kg⁻¹ i.v.) and the mean maximal response recorded as maximal increase in platelet-associated counts (Max % Response). NOS inhibitors potentiated the response to collagen compared to saline pre-treated controls whereas D-NAME, SDMA and phenylephrine had no effect. SNP significantly reduced the platelet response to collagen

whereas nicardipine had no effect, $n=5-7$. (B) Real time traces showing the full time-course of the mean platelet response to collagen in the presence of the agents outlined above (broken lines) and control responses (solid lines), $n=5$. (C-D) Full time-course of the mean platelet response to collagen ($12-75 \mu\text{g kg}^{-1}$) in wild-type (WT, (C)) and $\text{eNOS}^{-/-}$ (D) mice, $n=5$. (E-F) Mean platelet response to collagen ($12-75 \mu\text{g kg}^{-1}$) in WT and $\text{eNOS}^{-/-}$ mice expressed as (E) maximal response and (F) trapezoidal area under curve (AUC). Data are shown as mean $\pm$ S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 5

Effect of modified NO bioavailability and vascular tone on collagen induced

haemodynamic changes *in vivo*. (A) Mice were pre-treated with NOS inhibitors L-NAME (50 mg kg^{-1}) or ADMA (50 mg kg^{-1}) or the inactive forms of these compounds (D-NAME and SDMA) at equimolar doses. Alternatively mice were treated with the NO donor SNP ($10 \mu\text{g kg}^{-1}$), the hypertensive phenylephrine ($80 \mu\text{g kg}^{-1}$) or the hypotensive nicardipine ($100 \mu\text{g kg}^{-1}$). Platelet aggregation was then induced with collagen ($50 \mu\text{g kg}^{-1}$ i.v.) and the accompanying changes in the haemodynamic parameters $\text{dP/dt}_{\text{max}}$ and systolic left ventricular pressure (LVP) recorded as maximal % changes. NOS inhibitors and phenylephrine potentiated the response to collagen compared to saline pre-treated controls whereas D-NAME and SDMA had no effect. SNP and nicardipine significantly reduced the haemodynamic response to collagen. Data are shown as mean $\pm$ S.E.M., $n=4-7$. (B) Typical traces of $n=4-7$ showing the full time-course of the haemodynamic response (as blood pressure (BP) and dP/dt) to collagen in the presence of the agents outlined above, (C) full time-course of the haemodynamic response (as blood pressure (BP) and dP/dt) to collagen in wild-type and $\text{eNOS}^{-/-}$ mice showing typical responses of $n=6-7$ and (D) mean maximal %

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haemodynamic response (dP/dt_{max} and systolic left ventricular pressure (LVP)) to
collagen in WT and $eNOS^{-/-}$ mice, $n=6-7$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig 1

Time course of the platelet and haemodynamic responses during collagen induced platelet thromboembolism. The real-time radiolabelled response to collagen is shown as % change in counts against time and shows a rapid increase in counts as platelets aggregate in the pulmonary region followed by a slower resolution phase (top panel). The middle panel shows a real-time recording of ventricular pressure from a catheter situated in the left ventricle of an anaesthetised mouse plotted as pressure (mmHg) against time and shows a fall in systolic left ventricular pressure (LVP) upon injection of collagen and a slower return to baseline. The lower panel shows a real time recording of derived pressures (dP/dt) and shows changes in dP/dt_{max} (contractile function) and dP/dt_{min} (speed of relaxation) during collagen injection. All traces are representative of n=5.

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Fig 2

Dose-dependent platelet aggregation and haemodynamic responses in vivo. Dose-response following injection of collagen (25 – 100 µg kg⁻¹ i.v., n=6-7) plotted as time-course of % change in counts at each collagen dose (upper panel) and maximal % increase in platelet-associated counts against collagen concentration (lower panel). The graphs show increased platelet aggregation in response to increasing doses of collagen. Data are presented as mean ± S.E.M of maximal % change from stable baselines, n=5-7, **p<0.01, ***p<0.001.

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Fig 3

Effect of modified NO bioavailability and vascular tone on basal blood pressure. (A) Effect of NOS inhibitors L-NAME and ADMA (50 mg kg⁻¹), and inactive forms of these compounds (D-NAME and SDMA) at equimolar doses, phenylephrine (80 μg kg⁻¹ min⁻¹), the NO donor SNP (10 μg kg⁻¹) and nicardipine (100 μg kg⁻¹) on systolic arterial pressure (SP max). L-NAME and phenylephrine enhanced arterial pressure to a similar extent and ADMA also increased blood pressure whereas saline, D-NAME and SDMA had no effect. SNP and nicardipine reduced arterial pressure to a similar extent. Data are presented as mean maximal change in systolic arterial blood pressure (SP max % response) ± S.E.M., n=6-8. (B) Profile in real-time of response to L-NAME (50 mg kg⁻¹, broken line) and phenylephrine (80 μg kg⁻¹ × 5 mins, solid line) and (C) SNP (10 μg kg⁻¹, solid line) and nicardipine (100 μg kg⁻¹, broken line) showing similar response profile of the agents in each response. Data are presented as mean ± S.E.M. % change relative to stable baseline (%ΔSP) against time, n=8, **p<0.01.

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Fig 4

Effect of modified NO bioavailability and vascular tone on collagen induced platelet aggregation in vivo. (A) Mice were pre-treated with NOS inhibitors L-NAME (50 mg kg⁻¹) or ADMA (50 mg kg⁻¹) or the inactive forms of these compounds (D-NAME and SDMA) at equimolar doses. Alternatively mice were treated with the NO donor SNP (10 $\mu\text{g kg}^{-1}$), the hypertensive phenylephrine (80 $\mu\text{g kg}^{-1}$ min⁻¹) or the hypotensive nicardipine (100 $\mu\text{g kg}^{-1}$). Platelet aggregation was then induced with collagen (50 $\mu\text{g kg}^{-1}$ i.v.) and the mean maximal response recorded as maximal increase in platelet-associated counts (Max % Response). NOS inhibitors potentiated the response to collagen compared to saline pre-treated controls whereas D-NAME, SDMA and phenylephrine had no effect. SNP significantly reduced the platelet response to collagen whereas nicardipine had no effect, n=5-7. (B) Real time traces showing the full time-course of the mean platelet response to collagen in the presence of the agents outlined above (broken lines) and control responses (solid lines), n=5. (C-D) Full time-course of the mean platelet response to collagen (12-75 $\mu\text{g kg}^{-1}$) in wild-type (WT, (C))

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3 and eNOS^{-/-} (D) mice, n=5. (E-F) Mean platelet response to collagen (12-75 $\mu\text{g kg}^{-1}$) in WT and
4 eNOS^{-/-} mice expressed as (E) maximal response and (F) trapezoidal area under curve (AUC). Data
5 are shown as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
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Fig 5

Effect of modified NO bioavailability and vascular tone on collagen induced haemodynamic changes in vivo. (A) Mice were pre-treated with NOS inhibitors L-NAME (50 mg kg⁻¹) or ADMA (50 mg kg⁻¹) or the inactive forms of these compounds (D-NAME and SDMA) at equimolar doses. Alternatively mice were treated with the NO donor SNP (10 µg kg⁻¹), the hypertensive phenylephrine (80 µg kg⁻¹) or the hypotensive nicardipine (100 µg kg⁻¹). Platelet aggregation was then induced with collagen (50 µg kg⁻¹ i.v.) and the accompanying changes in the haemodynamic parameters dP/dtmax and systolic left ventricular pressure (LVP) recorded as maximal % changes. NOS inhibitors and phenylephrine potentiated the response to collagen compared to saline pre-treated controls whereas D-NAME and SDMA had no effect. SNP and nicardipine significantly reduced the haemodynamic response to collagen. Data are shown as mean ± S.E.M., n=4-7. (B) Typical traces of n=4-7 showing the full time-course of the haemodynamic response (as blood pressure (BP) and dP/dt) to collagen in the presence of the agents outlined above, (C) full time-course of the haemodynamic response (as blood pressure (BP) and dP/dt) to collagen in wild-type and eNOS^{-/-} mice showing typical responses of n=6-7 and (D) mean maximal % haemodynamic response (dP/dtmax and systolic left ventricular pressure (LVP)) to collagen in WT and eNOS^{-/-} mice, n=6-7. *p<0.05, **p<0.01, ***p<0.001.

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What is known on this topic	What this paper adds
Nitric oxide regulates platelet and vascular function	Nitric oxide exerts a direct anti-thrombotic effect on platelets <i>in vivo</i>
Thrombosis is driven by platelet and vascular dysfunction	eNOS modifies the vascular component of thrombotic responses
The role of eNOS in thrombosis is unclear	eNOS potentiates the duration of moderate platelet driven thrombotic events