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RESEARCH PAPER

# Platelet aggregation responses are critically regulated *in vivo* by endogenous nitric oxide but not by endothelial nitric oxide synthase

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**Background and purpose:** Although exogenous nitric oxide (NO) clearly modifies platelet function, the role and the source of endogenous NO *in vivo* remain undefined. In addition, endothelial NO synthase (NOS-3) critically regulates vessel tone but its role in modulating platelet function is unclear. In this paper we have investigated the roles of endogenous NO and NOS-3 in regulating platelet function *in vivo* and determined the functional contribution made by platelet-derived NO.
**Experimental approach:** We used a mouse model for directly assessing platelet functional responses *in situ* in the presence of an intact vascular endothelium with supporting *in vitro* and molecular studies.
**Key results:** Acute NOS inhibition by $N_\omega$-nitro-L-arginine methyl ester hydrochloride (L-NAME) enhanced platelet aggregatory responses to thrombin and platelets were shown to be regulated primarily by NO sources external to the platelet. Elevation of endogenous NOS inhibitors to mimic effects reported in patients with cardiovascular diseases did not enhance platelet responses. Platelet responsiveness following agonist stimulation was not modified in male or female NOS-3$^{-/-}$ mice but responses in NOS-3$^{-/-}$ mice were enhanced by L-NAME.
**Conclusions and implications:** Platelets are regulated by endogenous NO *in vivo*, primarily by NO originating from the environment external to the platelet with a negligible or undetectable role of platelet-derived NO. Raised levels of endogenous NOS inhibitors, as reported in a range of diseases were not, in isolation, sufficient to enhance platelet activity and NOS-3 is not essential for normal platelet function *in vivo* due to the presence of bioactive NO following deletion of NOS-3.
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**Keywords:** animal model; endothelium; nitric oxide; platelet; thrombosis

**Abbreviations:** ADMA, *asym*-dimethylarginine; AUC, area under curve; CFT, $Ca^{2+}$-free Tyrode's solution; D-NAME, $N^G$-nitro-D-arginine methyl ester hydrochloride; eNOS, endothelial nitric oxide synthase; L-291, $N^G$–(2-methoxyethyl)arginine methyl ester; L-NAME, $N_\omega$-nitro-L-arginine methyl ester hydrochloride; L-NMMA, $N^G$-methyl-L-arginine acetate salt; NOS-3, nitric oxide synthase 3; PRP, platelet-rich plasma

## Introduction

Platelets synthesize nitric oxide (NO) from L-arginine by a constitutively expressed NO synthase (NOS), most likely NOS-3, also known as endothelial NOS (Radomski *et al.*, 1990; Sase and Michel, 1995) and platelet-derived NO has been shown to regulate thrombus formation *in vitro* and *in vivo* (Freedman *et al.*, 1998; Williams and Nollert, 2004). Recently, however, the presence of NOS-3 in platelets has been challenged (Ozuyaman *et al.*, 2005) and the consequences of genetic deletion of NOS-3 on functional responses of platelets *in vitro* are unclear (Marjanovic *et al.*, 2005; Ozuyaman *et al.*, 2005). The presence and role of NOS-3 in platelets therefore remains contentious. Nonetheless, platelets are negatively regulated *in vitro* by NO originating from exogenous sources (Mellion *et al.*, 1981) and the vascular endothelium (Radomski *et al.*, 1987). The contribution to platelet function *in vivo* made by platelet-derived NO, relative to NO from other sources, including the vascular endothelium, remains unresolved (Naseem and Riba, 2008). The issues concerning the relevance and roles of platelet-derived NO have recently been summarized by Naseem and Riba (2008) and Gkaliagkousi *et al.* (2007).

Impaired NO production by the vascular endothelium following deletion of NOS-3 in mice leads to hypertension (Huang *et al.*, 1995) and complete loss of NO-dependent vasodilatation (Harrington *et al.*, 2007). Thus, NOS-3 is a critical regulator of vessel tone and its absence would also be

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predicted to enhance platelet-mediated events such as haemostasis and thrombosis. Bleeding time is reduced in NOS-3$^{-/-}$ mice (Freedman *et al.*, 1999) although results from thrombosis models are less definitive. Models of carotid artery injury have shown both a lack of thrombotic phenotype in NOS-3$^{-/-}$ mice (Ozuyaman *et al.*, 2005; Dayal *et al.*, 2006) and an antithrombotic effect shown by a prolonged time to occlusion (Iafrati *et al.*, 2005; Marjanovic *et al.*, 2005) possibly due to up-regulated fibrinolysis (Iafrati *et al.*, 2005). There are also models in which loss of NOS-3 promotes thrombosis (Heeringa *et al.*, 2000). The role of NOS-3 in regulating the platelet response *in vivo* therefore remains undefined, partly due to conflicting data and partly because models of thrombosis involve a number of processes, such as platelet activity, vascular dysfunction, blood flow, tissue damage and coagulation, and do not functionally isolate the platelet.

In the present study we investigated the role of endogenous NO and NOS-3 in regulating *in vivo* platelet aggregatory responses to agonist stimulation using a mouse model recently developed in our laboratory (Tymvios *et al.*, 2008) based on validated protocols in larger mammals (May *et al.*, 1990; Emerson *et al.*, 1997; Emerson *et al.*, 1999b). Our data show that the aggregation of platelets was critically regulated *in vivo* by endogenous NO originating from sources external to the platelet but that normal platelet function was maintained in the absence of NOS-3.

## Methods

*Mice*

All animal care and experimental 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. Male, Balb/c mice (20–30 g) were purchased from Harlan (Bicester, UK) and had access to food and water *ad libitum*. NOS-3 knock-out mice (NOS-3$^{-/-}$, Strain: 0026847) were purchased from Jackson Laboratory, ME, USA along with control C57Bl/6J control mice.

*Blood collection and platelet labelling*

Blood collection and platelet labelling were conducted as previously published (Tymvios *et al.*, 2008). Briefly, blood was collected into acidified citrate-dextrose solution, from terminally anaesthetized (2 g·kg$^{-1}$ urethane, i.p.) donor mice by cardiac puncture. Platelet-rich plasma (PRP) was obtained by centrifugation, supplemented with $Ca^{2+}$-free Tyrode's solution (CFT) and centrifuged to produce a platelet pellet. The pellet was washed, resuspended with 1.8 MBq [$^{111}$In] indium oxine and incubated at room temperature for 5 min. Platelets were centrifuged again and finally resuspended in 50 µL CFT per mouse. The same numbers of donor and recipient mice were used so that each recipient received all platelets that could be collected from one donor mouse. All mice were given an initial dose of ADP (40 µg·kg$^{-1}$) to ensure that transfused platelets were functional, as previously reported (May *et al.*, 1990).

*Platelet monitoring*

We have recently published protocols for measuring platelet functional responses to agonist stimulation *in vivo* in mice (Tymvios *et al.*, 2008). Data was collected *via* 1 cm single-point extended area radiation detectors (eV Products, Saxonburg, PA, USA) fixed externally over the pulmonary vascular bed of anaesthetized (1.5 g·kg$^{-1}$ urethane i.p.) mice and recorded on a UCS-20 spectrometer (Spectrum Techniques, Oak Ridge, TN, US) using custom made software (Mumed Systems, London, UK). Radiolabelled platelets were infused *via* a tail vein and allowed to equilibrate for 20 min. Responses were measured as increases in platelet-associated counts in the pulmonary vascular bed following injection of platelet pro-aggregatory agonists *via* an exposed femoral vein.

In vivo *experimental design*

$N_\omega$-nitro-L-arginine methyl ester hydrochloride (L-NAME), $N^G$-nitro-D-arginine methyl ester hydrochloride (D-NAME), $N^G$-methyl-L-arginine acetate salt (L-NMMA) and *asym*-dimethylarginine (ADMA) were dissolved in saline and injected i.v. in 50 µL, 5 min prior to injection of platelet agonists. Where given, L-arginine was administered 5 min prior to NOS inhibitors, at a 10-fold higher concentration. L-291 [$N^G$–(2-methoxyethyl)arginine methyl ester] was administered at a dose of 30 mg·kg$^{-1}$ i.p. 1 h prior to injection of platelet agonists according to published protocols (Leiper *et al.*, 2007).

NOS-3$^{-/-}$ mice and wild-type controls were age and sex matched and compared under identical experimental conditions using the same batches of drugs, anaesthetic and platelet agonists.

In vitro *aggregometry*

Blood was collected from human volunteers by venepuncture. Informed consent was obtained from all blood donors and procedures were approved by the National Research Ethics Service (ref: 07/H0708/72). Platelets were isolated by centrifugation and resuspended in modified Tyrode–HEPES buffer. Platelets were incubated with L-NAME or D-NAME for 10 min and aggregation measured using an optical aggregometer (CHRONO-LOG, 500CA, Labmedics Ltd., Manchester, UK) at 37°C under constant stirring conditions.

*Western blotting*

Human and mouse washed platelets and hearts from NOS-3$^{-/-}$ and wild-type mice were lysed in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8.0) containing a mix of protease and phosphatase inhibitors (Sigma, Dorset, UK). Proteins were quantified using the BCA Protein Assay (bicinchoninic acid). The mouse endothelioma cell line sEnd (provided by Dr. Mary Cavanagh, Imperial College London) was used as a positive control for NOS-3 expression. Following dilution of 10 µg of protein in loading sample buffer (4×) (Invitrogen, Renfrew, UK), samples were heated at 95°C for 5 min and electrophoretically separated in a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride

membrane (BioRad, Hertfordshire, UK). Membranes were then blocked in 5% non-fat dry milk in PBS–Tween (0.1%) for 1 h, washed with PBS–Tween (0.1%) and incubated with the NOS-3 antibody (1:500 rabbit polyclonal; Sigma) overnight at 4°C. After washing with PBS-Tween (0.1%), the secondary HRP goat anti-rabbit antibody (1:8000; Dako, UK Ltd, Cambridgeshire, UK) was added to the membrane for 1 h at room temperature. Following washing, detection was carried out by enhanced chemiluminescence according to the manufacturer's protocol (Amersham).

*Data analysis and statistical procedures*

*In vivo* data was acquired as radioactive counts in consecutive 4 s monitoring periods and expressed as percentage changes in basal counts or maximum percentage increase above stable basal counts or trapezoidal area under curve of the percent change against time. All data are expressed as mean ± standard error of the mean. Where statistical comparisons were made a Student's *t*-test or one-way analysis of variance followed by a multiple comparison test was used to compare mean values and a $P$ value <0.05 denoted statistical significance.

*Materials*

All reagents were purchased from Sigma with the exception of [$^{111}$In]-indium oxine (GE Healthcare, Bucks, UK) and collagen (Nycomed Pharma, Berlin, Germany). L-291 was a kind gift of Dr James Leiper, University College London. Drugs were dissolved in saline and administered i.v. in 50 µL volumes. Drug and molecular target nomenclature in this paper conforms to the *British Journal of Pharmacology*'s Guide to Receptors and Channels (Alexander *et al.*, 2008).

## Results

*Platelet agonist responses are regulated by endogenous NO in vivo*

Injection of thrombin (250–1000 IU·kg$^{-1}$ i.v.) into mice induced a dose-dependent platelet response consisting of a rapid increase in platelet-associated counts followed by a return to baseline (Figure 1A). This response reflects the trapping of platelet aggregates, formed in the systemic circulation, in the pulmonary vascular bed followed by a resolution phase in which aggregates resolve (Tymvios *et al.*, 2008). The time taken to return to baseline was dose-dependent (Figure 1A) as was the magnitude of the response to thrombin, expressed as the maximal percent increase in counts (Figure 1B). Pretreatment with L-NAME (50 mg·kg$^{-1}$) and subsequent injection of thrombin required a 50-fold reduction in the dose of thrombin (5–20 IU·kg$^{-1}$ i.v.) needed to elicit responses that were similar to those following saline pretreatment (Figure 1B, blue line). Doses of thrombin greater than 20 IU·kg$^{-1}$ following L-NAME and 1000 IU·kg$^{-1}$ following saline induced thromboembolic mortality and so were not used. By measuring the full time course of the response to 10 IU·kg$^{-1}$ thrombin we were able to determine that L-NAME leads to a more rapid formation of platelet aggregates (shown as an increase in the positive gradient, Figure 1C) although platelet aggregates

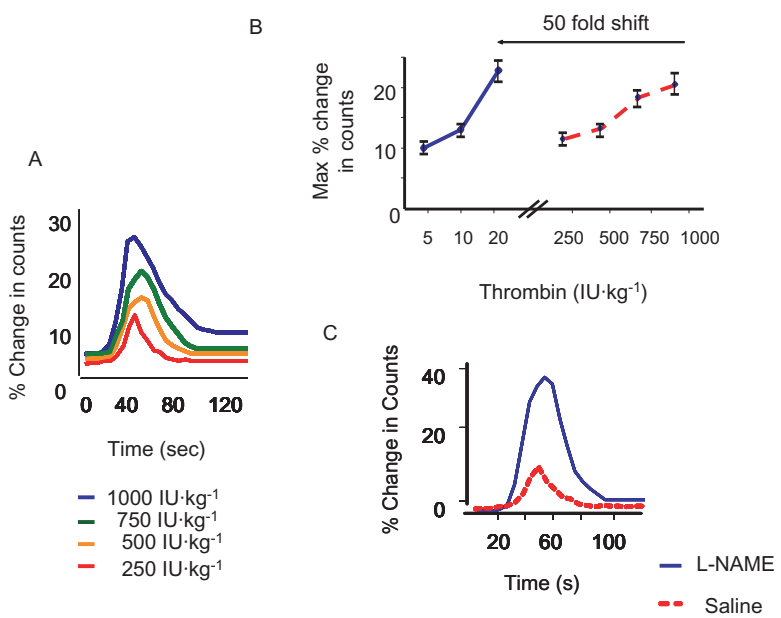


**Figure 1** Inhibition of systemic endogenous nitric oxide (NO) production by the NO synthase inhibitor $N_\omega$-nitro-L-arginine methyl ester hydrochloride (L-NAME) enhanced platelet reactivity *in vivo*. Circulating radiolabelled platelets were monitored in the pulmonary region of anaesthetized mice and aggregation responses measured as changes in platelet-associated radioactive counts following i.v. injection of thrombin. (A) Platelet responses to thrombin (250–1000 IU·kg$^{-1}$) in real time; typical traces are shown. (B) Thrombin (250–1000 IU·kg$^{-1}$) induced a dose-dependent platelet response indicated by an increasing maximal percent increase in counts ($n$ = 5). Pretreatment with L-NAME (50 mg·kg$^{-1}$) potentiated the response to thrombin such that a 50-fold reduction in thrombin (5–20 IU·kg$^{-1}$) was required to elicit a similar dose response, ($n$ = 6). (C) Time course of response to thrombin (10 IU·kg$^{-1}$) following pretreatment with saline or L-NAME showing the potentiated response in L-NAME treated mice; typical traces are shown ($n$ = 6).

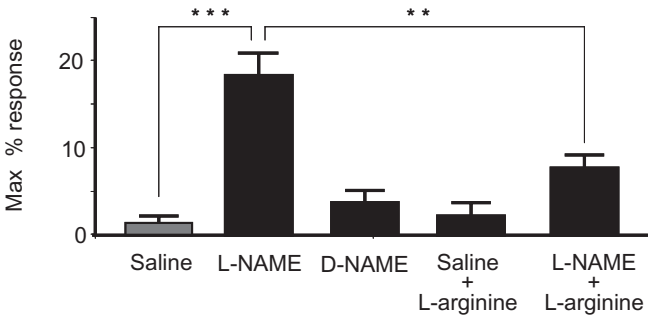


**Figure 2** L-NAME potentiated the platelet response to thrombin by inhibition of an L-arginine pathway. Pretreatment of mice with $N_{\omega}$-nitro-L-arginine methyl ester hydrochloride (L-NAME; 50 mg·kg$^{-1}$) significantly (***$P < 0.001$) enhanced the response to thrombin (10 IU·kg$^{-1}$) whereas $N^{G}$-nitro-D-arginine methyl ester hydrochloride (D-NAME; 50 mg·kg$^{-1}$) pretreatment had no effect. An excess of L-arginine (500 mg·kg$^{-1}$) had no effect on the response to thrombin alone but partially and significantly (**$P < 0.01$) reversed the effect of L-NAME. Data are show as the mean maximal percent increase in counts above stable baseline values, $n = 5$.

resolved in a time frame similar to that in saline treated controls (shown as a return to baseline within 100 s of stimulation, Figure 1C).

We then carried out a series of control experiments to demonstrate that the observed effect of L-NAME was due to inhibition of an L-arginine/NO pathway rather than a non-specific or noxious effect of this compound. When mice were treated with 10 IU·kg$^{-1}$ thrombin, there was a significant potentiation of the maximal response following pretreatment with L-NAME compared with pretreatment with saline ($P < 0.001$) or the inactive enantiomer of L-NAME, D-NAME ($P < 0.01$, Figure 2). We were also able to partially and significantly ($P < 0.01$) reverse the effect of L-NAME by pretreatment with an excess of L-arginine (500 mg·kg$^{-1}$, Figure 2) in line with previous observations on blood pressure in rats (Rees *et al.*, 1990).

*Platelet function* in vivo *is mediated by NO originating from the environment external to the platelet*

We carried out a series of *in vitro* and *in vivo* experiments to differentially treat platelets and the extra-platelet environment with L-NAME to determine the functionally relevant source of NO *in vivo*. We pretreated recipient mice with L-NAME and transfused platelets from donor mice pretreated with saline to create a model in which NO production was inhibited in the environment external to the platelet but monitored platelets were untreated. There was a significant potentiation of the response to thrombin in this model compared with recipient mice pretreated with saline (Figure 3A).

In order to confirm the lack of pro-aggregatory outcomes following selective treatment of platelets with L-NAME, we compared human platelet functional responses with an $EC_{50}$ concentration of thrombin (0.1 U·mL$^{-1}$) by conventional *in vitro* aggregometry. L-NAME (1 μM–1 mM) pretreatment *in vivo* had no effect on human platelet aggregation induced by thrombin (Figure 3B) or collagen (data not shown) compared with D-NAME. At high concentrations, L-NAME exerted a non-specific toxic or noxious inhibitory effect similar to that exerted by D-NAME at these concentrations (Figure 3B).

We were unable to detect NOS-3 protein in human or mouse platelet lysates. The validity of this finding was supported by Western blots showing NOS-3 expression in endothelial cells and heart tissue from wild-type but not from NOS-3$^{-/-}$ mice (Figure 3C).

*Effect of pathological levels of endogenous NOS inhibitors*

Having shown that NOS inhibition with L-NAME dramatically potentiated the platelet response *in vivo*, we hypothesized that raised levels of endogenously produced methylated NOS inhibitors such as ADMA and L-NMMA reported in a variety of cardiovascular conditions (Boger *et al.*, 1998; Miyazaki *et al.*, 1999), might enhance platelet responsiveness. Pretreatment with ADMA at doses producing blood concentrations up to 1 mM did not significantly affect the subsequent response to thrombin (100 IU·kg$^{-1}$) compared with saline pretreated controls (Figure 4A). At higher concentrations (10 mM), ADMA significantly ($P < 0.01$) potentiated the thrombin response (Figure 4A). Similarly, L-NMMA at concentrations up to 100 μM did not affect thrombin responses although higher concentrations (1 mM) significantly ($P < 0.01$) enhanced the response to thrombin. In order to enhance ADMA and L-NMMA concentrations intracellularly, that is, in the vicinity of NOS-3, we pretreated mice with the dimethylarginine dimethylaminohydrolase (DDAH) inhibitor L-291 at doses previously shown to inhibit the breakdown of endogenous NOS inhibitors and elevate blood pressure (Leiper *et al.*, 2007). L-291 pretreatment had no effect on thrombin induced platelet aggregation *in vivo* (Figure 4C).

*NOS-3 does not regulate platelet agonist responses* in vivo

The platelet response to thrombin was not significantly different in NOS-3$^{-/-}$ mice compared with age- and sex-matched C57Bl/6J control mice (Figure 5). We examined all the components of the development and resolution of the response and found no effect of the deletion of NOS-3 over the full time course of the thrombin response (Figure 5A), so that neither the mean peak response (Figure 5B) nor the AUC (Figure 5C) was affected by NOS-3 ablation. There was no difference in platelet responsiveness *in vivo* between male and female NOS-3$^{-/-}$ mice (Figure 5D). As NOS-3 activation has been linked with collagen activation in platelets (Riba *et al.*, 2005), we also compared responses to collagen in NOS-3$^{-/-}$ mice and C57Bl/6J controls and found no significant difference in platelet responsiveness (Figure 5E). When NOS-3$^{-/-}$ mice were pretreated with L-NAME (50 mg·kg$^{-1}$) there was a significant ($P < 0.01$) increase in thrombin induced platelet aggregation *in vivo* compared with D-NAME treated controls indicating sensitivity to acute NOS inhibition in the absence of NOS-3 (Figure 5F).

## Discussion and conclusions

The rationale for this study was based on contradictory data reporting both the presence (Ji *et al.*, 2007) and absence

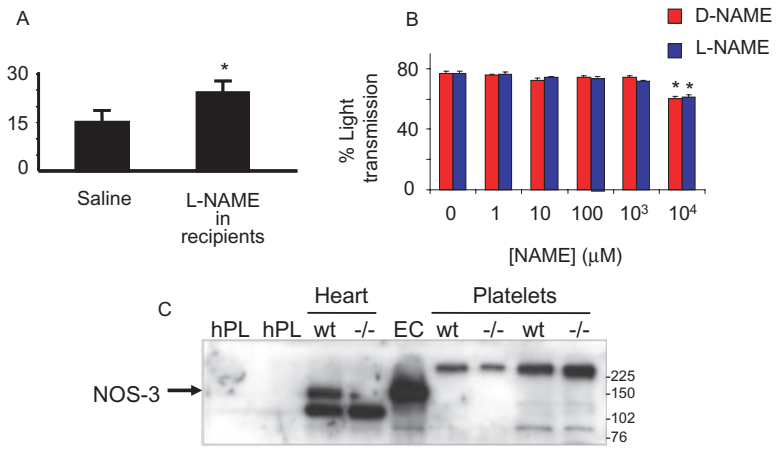


**Figure 3** Platelets are regulated *in vivo* by nitric oxide (NO) originating from the environment external to the platelet. (A) Transfusion of platelets from saline pretreated donors into $N_{\omega}$-nitro-L-arginine methyl ester hydrochloride (L-NAME; 50 mg·kg$^{-1}$) treated recipient mice significantly (*$P < 0.01$) potentiated the response to thrombin. (B) A range of concentrations of L-NAME or N$^{G}$-nitro-D-arginine methyl ester hydrochloride (D-NAME; 1 μM–1 mM) had no effect on *in vitro* platelet aggregation induced by thrombin. At higher concentrations both L-NAME and D-NAME significantly (*$P < 0.05$) inhibited the response. (C) A representative Western blot analysis of endothelial NO synthase (NOS-3). A band of 135 kDa was observed in endothelial cells (EC) but not human platelets (hPL). NOS-3 was detected in cardiac tissue from wild-type (wt) but not NOS-3$^{-/-}$ mice. Note that there was no band in platelets lysates from either wild-type (wt) or NOS-3$^{-/-}$ mice.

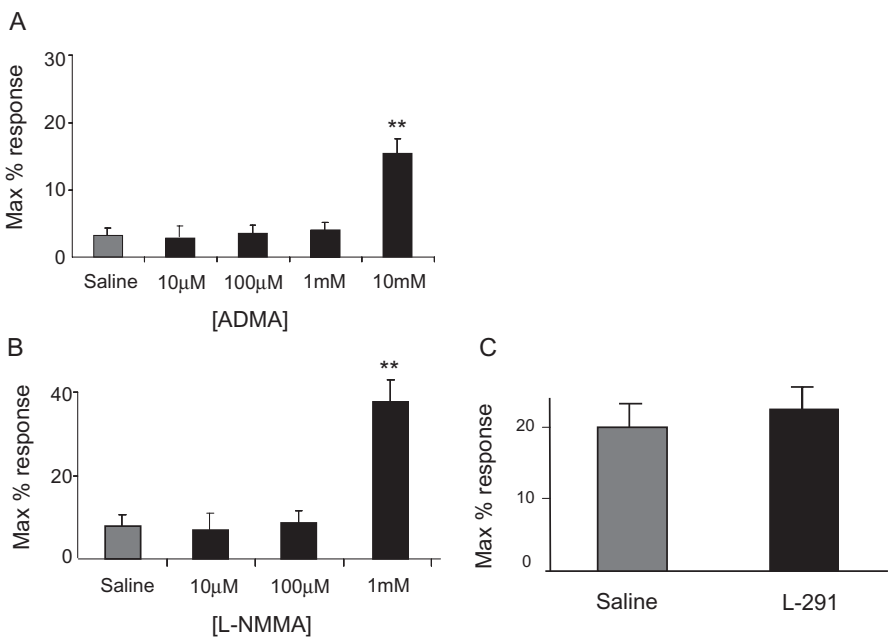


**Figure 4** The enhancement of platelet responses by endogenous nitric oxide syntase inhibitors *in vivo* required concentrations in excess of those found pathologically. Pretreatment with (A) *asym*-dimethylarginine (ADMA; 10 mM) or (B) N$^{G}$-methyl-L-arginine acetate salt (L-NMMA; 1 mM) significantly (**$P < 0.01$) increased the response to thrombin (100 IU·kg$^{-1}$) compared with saline pretreatment. Lower concentrations of ADMA (10 μM–1 mM) or L-NMMA (10–100 μM) had no effect. (C) Pretreatment with the dimethylarginine dimethylaminohydrolase inhibitor L-291 (30 mg·kg$^{-1}$) had no effect ($P > 0.05$) on subsequent thrombin (100 IU·kg$^{-1}$) induced platelet aggregation *in vivo*. Data are expressed as mean maximal percent increase in counts, $n = 5$.

(Gambaryan *et al.*, 2008) of functional NOS-3 in platelets and subsequent confusion concerning the role of platelet-derived NO, relative to other sources. In addition, the role of NOS-3 in regulating systemic platelet responsiveness *in vivo* was not known. We adopted an *in vivo* functional approach to address these issues and developed methodology for assessing platelet responses *in vivo* in the mouse (Tymvios *et al.*, 2008).

Although it is clear that both pharmacological inhibition of NOS (Rees *et al.*, 1990) and deletion of NOS-3 elevate blood pressure (Huang *et al.*, 1995), most clearly in males (Scotland *et al.*, 2005), the consequences of these interventions to thrombotic predisposition are less clear and conflicting data have been published (Iafrati *et al.*, 2005; Marjanovic *et al.*, 2005; Ozuyaman *et al.*, 2005; Dayal *et al.*, 2006). These contradictions may partly stem from the use of models that induce a range of vascular and haemostatic responses and do not functionally isolate the platelet. For example, Iafrati *et al.* (2005) described compensatory mechanisms including

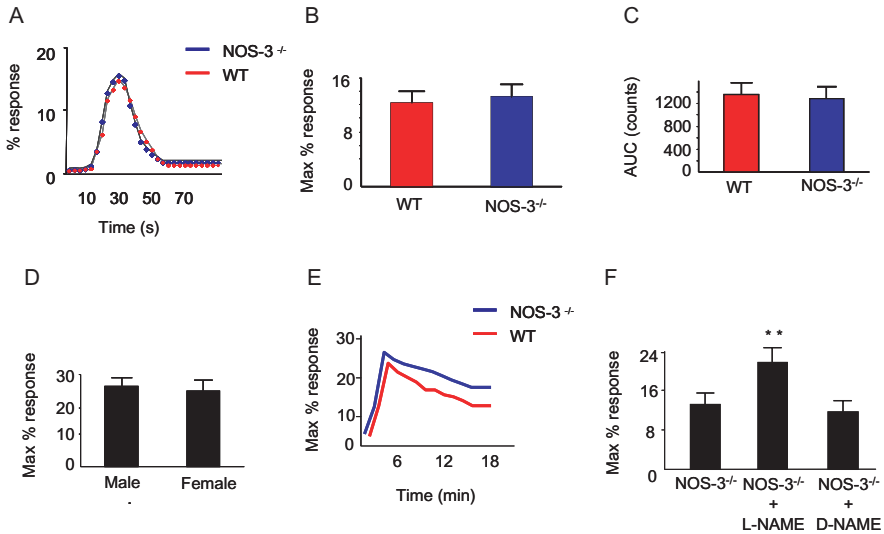


**Figure 5** Endothelial nitric oxide synthase (NOS-3) was not essential for normal platelet responsiveness $N^G$-methyl-L-arginine acetate salt (L-NMMA) potentiated platelet responses in the absence of NOS-3. No significant differences were observed in platelet counts in the pulmonary vasculature upon injection of thrombin into NOS-$3^{-/-}$ mice compared with wild-type (WT) controls. (A) Full time course of the response to thrombin (100 IU·$kg^{-1}$) in WT and NOS-$3^{-/-}$ mice; a typical response is shown ($n$ = 5). (B) Comparison of the mean maximal response to thrombin and (C) the mean area under curve (AUC) following injection of thrombin into WT and NOS-$3^{-/-}$ mice ($n$ = 5). (D) Comparison of the response to thrombin, expressed as maximal percent increase in male and female mice ($n$ = 5). (E) Full time course of the response to collagen in WT and NOS-$3^{-/-}$ mice; typical responses are shown ($n$ = 5). (F) L-NAME (50 mg·$kg^{-1}$) significantly ($P$ < 0.01) enhanced the maximal increase in platelet counts in NOS-$3^{-/-}$ mice compared with $N^G$-nitro-D-arginine methyl ester hydrochloride (D-NAME; 50 mg·$kg^{-1}$) pretreated controls; ($n$ = 4–6).

up-regulation of t-PA that counteracted the effects of NOS-3 deletion in a thrombosis model. In the present study, we investigated the consequences of NOS-3 ablation upon platelet responses *in vivo* and compared this with pharmacological inhibition of NO production. We hypothesized that measuring platelet function non-invasively in the mouse in the presence of an intact endothelium would maximize the measurable consequences of NOS inhibition. This was borne out by our data showing a 50-fold increase in platelet sensitivity to thrombin following NOS inhibition. Indeed, injection of thrombin at doses that elicited responses in control mice was fatal in mice pretreated with L-NAME. Platelets are therefore highly sensitive to NOS inhibition *in vivo* and endogenous NO is critical to normal platelet aggregation. Loss of all endogenous NOS activity, i.e. inhibition of NOS with L-NAME, led to fatal thromboembolism at even moderate does of thrombin.

We carried out a series of experiments to determine the relevance of the platelet as a source of NO by creating an animal model in which platelets were untreated but NOS was inhibited in the environment external to the platelet. Under these conditions we observed a potentiation of the response to thrombin that was similar to that observed following systemic NOS inhibition. Thus, platelets are regulated *in vivo* primarily by NO originating from outside the platelet. The most important source of extra-platelet NO is likely to be the vascular endothelium although there may also be a contribution from erythrocytes and other blood elements (Kleinbongard *et al.*, 2006). The lack of a functional role of platelet-derived NO was confirmed by conventional *in vitro* aggregometry and protein analysis suggesting that NOS-3 was either not expressed or was present at undetectable levels in both human and mouse platelets.

NO bioavailability *in vivo* depends on many factors including the presence of endogenous NOS inhibitors. These inhibitors take the form of methylated arginine derivatives such as ADMA and L-NMMA and their plasma concentration changes under varying physiological and pathological conditions and is regulated by the enzyme DDAH that breaks down these compounds to inactive forms (Vallance *et al.*, 1992; Boger *et al.*, 1998). There has been a recent surge of interest in the role of endogenous NOS inhibitors such as ADMA in cardiovascular diseases with debate raging over their causative roles (Vallance and Leiper, 2004). What is clear is that normal plasma concentrations of the endogenous inhibitors become elevated up to ten-fold in renal disease (MacAllister *et al.*, 1996), heart failure (Saitoh *et al.*, 2003), pulmonary hypertension (Gorenflo *et al.*, 2001) and diabetes (Paiva *et al.*, 2003). The consequences of these elevations on platelet function *in vivo* are not known but given the sensitivity of platelets to NOS inhibition that we have shown here, we hypothesized that pathologically raised concentrations of endogenous NOS inhibitors might promote thrombosis by enhancing platelet reactivity *in vivo*. Concentrations of ADMA and L-NMMA far in excess of those occurring pathologically (maximum 10 μM) had no measurable effect on the platelet response *in vivo*. These data suggest that the reported pathological elevations of ADMA and L-NMMA may not themselves enhance platelet responses and so cannot necessarily be used to predict increased risk of thrombosis. Our data and interpretation outlined above by infusing L-NMMA and ADMA assume that excessive exogenous infusion leads to an intracellular concentration that approaches that occurring pathologically. This is reasonable given that concentrations approximately 1000-fold higher than those reported in patients failed to promote platelet aggregation. Nonetheless we carried out additional

experiments with a DDAH inhibitor at concentrations shown to enhance intracellular NOS inhibitor concentrations (Leiper *et al.*, 2007), and found that this also failed to enhance platelet responsiveness. Our observations with ADMA and L-291 are important in the light of current initiatives to manipulate endogenous ADMA levels as a treatment for septic shock (Leiper *et al.*, 2007) and indicate there may be a window in which these compounds may be used to elevate blood pressure without increased risk of thrombosis.

We also present data showing that platelet reactivity is not modified following deletion of NOS-3 *in vivo*. It is essential that this data is viewed alongside our pharmacological data in which we showed that our model was sensitive to NOS inhibition. Thus, we conclude that although NO is a critical regulator of the platelet response *in vivo*, NOS-3 is not critical for normal platelet responsiveness following pro-aggregatory agonist stimulation.

The pharmacological agents used in this report are not specific to NOS-3 and also inhibit NOS-1 and NOS-2 at the concentrations used. We may therefore have identified a level of functional redundancy between NOS isoforms or else the reported enhanced fibrinolysis following NOS-3 deletion (Iafrati *et al.*, 2005) may be important although this not likely here given that we have shown a lack of phenotype in a model that is driven primarily by platelet aggregation (Emerson *et al.*, 1999a; Tymvios *et al.*, 2008). Additionally, there may be compensatory regulation of platelet activity by endothelium-derived prostanoids and EDHF. There are likely therefore to be unidentified mechanisms regulating the platelet response in NOS-3-deficient mice. It is important to identify these mechanisms and to determine whether they accompany impaired NOS-3 expression and activity in cardiovascular disease as such mechanisms may constitute disease pathways and may provide targets for treatment. As a first step in identifying these mechanisms we conducted experiments to determine if platelets were sensitive to NOS inhibition in the absence of NOS-3. L-NAME potentiated the platelet aggregation response in NOS-3$^{-/-}$ mice compared with D-NAME controls, indicating the persistence of the biosynthesis of NO in NOS-3$^{-/-}$ mice. Thus we conclude that platelets are regulated by NO originating from sources other than NOS-3, such as NOS-1 and NOS-2, and are likely to be regulated by endogenous NO from sources other than the vascular endothelium. Identifying the molecular and anatomical location of platelet regulatory NO will require further investigations involving various NOS knock-out lines and selective treatment or ablation of blood elements and tissue types together with comprehensive integrated molecular analysis.

In summary, we have shown that platelets are regulated by endogenous NO *in vivo* and acute NOS inhibition leads to enhanced platelet responsiveness although the pathological elevations of endogenous inhibitors of NOS reported in a range of diseases, were not sufficient by themselves to confer platelet hyperactivity. Platelets were regulated *in vivo* primarily by NO originating from the environment external to the platelet with a negligible or undetectable role of platelet-derived NO. Finally, NOS-3 was not a prerequisite for normal platelet function in male and female mice and this was at least partly due to the continuing presence of endogenous NO, in the absence of NOS-3. Fully identifying the mechanisms permitting normal platelet function in the absence of NOS-3 is an essential step in understanding the regulation of platelet function *in vivo* and in unravelling cardiovascular disease processes.

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## Conflict of interest

The authors have no conflicts to declare.

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