Exogenous nitric oxide inhibits in vivo platelet adhesion following balloon angioplasty

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Objectives: The aim was to investigate the effects of an exogenous source of nitric oxide on in vivo platelet adhesion at the site of endothelial denudation after balloon angioplasty. Methods: The study group consisted of 12 anaesthetised Large White pigs. Pigs were randomised to receive SIN-1 (3-morpholino-sydnonimine), an exogenous donor of nitric oxide, or placebo before and during balloon induced vessel wall injury. Platelet deposition was quantified using the injection of autologous $^{111}$indium labelled platelets. Platelet function was also monitored by the measurement of bleeding time and ex vivo whole blood aggregometry. Results: Superficial vessel wall injury was confirmed histologically and platelet monolayer formation was demonstrated by scanning electron microscopy. Platelet deposition at the site of endothelial denudation was markedly reduced following SIN-1 administration compared to placebo: 1.266(SEM 0.063) v 1.732(0.060) log platelets $\times 10^6$ cm$^{-2}$, p=0.001. SIN-1 raised platelet cyclic GMP concentration, from 4.47(2.48) to 6.14(2.44) pg-platelet$^{-1}$ (p<0.01) and prolonged the bleeding time, from 135(5) to 202(6) s (p=0.001), but had non-significant effects on ex vivo whole blood aggregometry. Conclusions: Exogenous nitric oxide, through the activation of platelet soluble guanylate cyclase, inhibits platelet adhesion in vivo following balloon angioplasty.

Endothelium derived nitric oxide plays an important role in the regulation of vascular smooth muscle tone. More recently it has been shown also to modulate platelet function. The stimulated release of nitric oxide reduces platelet aggregation in the intact animal, while the competitive inhibition of nitric oxide synthesis leads to an enhancement of platelet aggregation in vivo. From in vitro studies, it has been shown that nitric oxide can also reduce the adhesion of platelets to cultured endothelial cells and this implies that it has an additional role in determining the "non-adhesive" properties of vascular endothelium.

Nitrovasodilators serve as an exogenous source of nitric oxide and therefore possess important potential antiplatelet properties. Nitric oxide is released from the organic nitrates through their interaction with tissue dependent thiol containing compounds, but is liberated spontaneously from SIN-1 (3-morpholino-sydnonimine) and sodium nitroprusside, the antiplatelet effects of which are demonstrable in vitro. The organic nitrates have been observed in some studies to inhibit platelet aggregation ex vivo but in others the findings have been contradictory. Molsidomine, the active metabolite of which is SIN-1, and sodium nitroprusside have both been shown to inhibit ex vivo platelet aggregation. Glyceryl trinitrate prolonged the bleeding time in healthy males and isosorbide dinitrate was synergistic with prostaglandin E1 in reducing platelet accumulation on peripheral atheromatous plaques. However, direct evidence in vivo to support the antiadhesive effects of nitric oxide is lacking, either through its endogenous production or through its exogenous administration.

When endothelium is damaged or denuded, local production of nitric oxide is lost and exposure of the subendothelium acts as a stimulus to platelet activation. If the internal elastic lamina remains intact, platelet deposition is restricted to that of an adherent monolayer. Under these circumstances, inhibition of platelet deposition can only be achieved through the modification of platelet-vessel wall interaction. In this study we have used a model of in vivo platelet adhesion to investigate the effects of SIN-1 on platelet deposition following endothelial denudation in the pig carotid artery.

Methods

The study was performed using local farmyard pigs of a Large White variety. The pigs were aged 3-4 months and their average weight was 30 kg. The following compounds were used in this study: $^{111}$indium chloride, cyclic guanosine monophosphate (GMP) and cyclic adenosine monophosphate (AMP) assay kits (Amersham International plc); SIN-1 (Casella AG, Frankfurt, Germany); fentanyl, ADP, Evans blue dye, tropolone, trichloracetic acid (Sigma, Poole, United Kingdom); halothane (May and Baker Pharmaceuticals, Dagenham, United Kingdom); ketamine (Parke-Davis, Pontypool, United Kingdom); heparin (CP Pharmaceuticals, Wrexham, United Kingdom); glutaraldehyde (Bio-Rad, Hemel Hempstead, United Kingdom); paraformaldehyde (Merek Ltd, Eastleigh, United Kingdom); pentobarbitone (RMB Animal Health, Dagenham, United Kingdom).

Pigs were sedated with intramuscular ketamine (750 mg). Anaesthesia was induced by inhalation of 0.5% halothane and was maintained by the continuous intravenous infusion of fentanyl (0.01 mg·ml$^{-1}$), etomidate (0.04 mg·ml$^{-1}$) and...
ketamine (1 mg·ml⁻¹). The pigs were mechanically ventilated and continuous electrocardiographic monitoring was performed throughout the procedure. Following surgical exposure, a 9F sheath was inserted into the right femoral artery and arterial blood pressure was monitored thereafter through its sidearm. All experiments were performed according to the Home Office guidelines on animal experimentation.

Experimental protocol

Pigs were randomised to receive an intravenous infusion of either SIN-1 (10 μg·kg⁻¹·min⁻¹, n=6) or placebo (normal saline; n=6), which was started 1 h before angioplasty and was continued until the end of the experiment. This dose of SIN-1 was found in preliminary experiments to lead to a significant prolongation in the bleeding time and to a twofold rise in platelet cyclic GMP concentration. In addition, all animals received a bolus of heparin (50 U·kg⁻¹) at the time of starting treatment with either SIN-1 or placebo, and this was followed by an intravenous infusion at a dose (50 U·kg⁻¹·h⁻¹) that has been shown not to influence platelet deposition following superficial arterial wall injury. An 8F 8 mm Meditech balloon catheter was advanced under fluoroscopic control into the left carotid artery and was positioned at a level between the first and third cervical vertebrae. The balloon was inflated to six atmospheres for 30 s on five occasions, with a 60 s interval between each inflation. A similar protocol was repeated in the right carotid artery. After an interval of 15 min following the last inflation, 100 ml of 0.5% Evans blue dye in 0.9% saline was injected into the ascending aorta in order to demarcate areas of endothelial loss. The animal was then given an overdose of pentobarbitone. After desanguination using physiological saline (Kreb’s solution), perfusion fixation of the vessels was performed in situ at physiological pressure (100 mm Hg) for 15 min, using 5 litres of 2% glutaraldehyde and 1% paraformaldehyde solution in sodium phosphate buffer (pH 7.4). The vessels were carefully excised and immersed in fixative for a further 12 h.

Tissue analysis

Each artery was cleaned and stripped of adventitia. The area of balloon injury was easily identifiable by the localised intensity of Evans blue staining. Each dilated area was divided into two segments. In addition, a segment of the distal uninjured vessel was taken from each carotid artery to act as control. The segments were placed in a gamma well counter for quantitation of platelet deposition (see below) and were later opened longitudinally to inspect for uniformity of Evans blue staining. Two sections were obtained from each segment for histological analysis and were stained with haematoxylin-eosin and with Van Giesen elastic stains. Histological sections were viewed by two observers who were blinded to the randomisation procedure. In order to limit our observations to the effects of SIN-1 on platelet adhesion, segments with deep arterial injury were excluded from analysis. Previous studies have shown that with rupture of the internal elastic lamina and deep arterial injury, platelet aggregation at the site of vessel wall injury leads to platelet-thrombus formation. Two or three specimens were taken from each segment and following coating with carbon and gold-palladium alloy using standard techniques were examined by scanning electron microscopy.

Quantitation of platelet deposition

Autologous pig platelets were labelled with ¹¹¹indium tropolone using a method that has been previously described and were reinfected 24 h prior to the procedure. In order to ensure that the ¹¹¹indium activity in the samples of platelet rich plasma and platelet poor plasma was counted separately in a gamma well counter. The proportion of the total counts that were contained within the platelet poor plasma was less than 4% in all experiments, implying that minimal extravasation of ¹¹¹indium had occurred into the plasma. Indium activity on each arterial segment and in three samples of blood drawn immediately prior to death was also counted in a gamma well counter. Each sample of blood was weighed using a microbalance and the volume of each was calculated after weighing 1 ml of blood from the same animal. Having measured the platelet count of each sample (Technicon H-1 system, Bayer Diagnostics, Basingstoke, United Kingdom) the number of platelets per cent per minute was calculated and the platelet deposition on each arterial segment was thereby determined. The surface area of arterial segments was derived from their length and diameter and the platelet deposition on each was expressed per square centimetre. The mean platelet deposition on all superficially injured arterial surface was then calculated for each animal.

Platelet studies

Arterial blood was drawn into a syringe that contained anticoagulant (1:9, 3.8% trisodium citrate). Samples were taken for ex vivo whole blood aggregometry and for measurement of platelet cyclic GMP and cyclic AMP concentrations at baseline (before SIN-1, placebo or heparin therapy) and again 60 min after treatment was begun (see below).

Ex vivo whole blood aggregometry – Impedance whole blood aggregometry was initiated immediately after withdrawal of the blood samples. Arterial blood (0.5 ml) and normal saline (0.5 ml) were placed in a plastic cuvette and incubated at 37°C. Aggregometry was measured on pretreatment samples following the addition of a threshold concentration of the agonist ADP (1-5 × 10⁻⁷M) and was repeated on the post-treatment sample using the same predetermined concentration of agonist. On each occasion the extent of aggregation was calculated as the increase in electrical impedance 5 min after the addition of the aggregating agent.

Platelet cyclic GMP and cyclic AMP concentrations – Platelet rich plasma was obtained by centrifugation of arterial blood at 140 g for 10 min. The platelet count in each plasma sample was determined, and 1 ml samples were then centrifuged at 2500 g for a further 1 min to obtain a platelet pellet. One millilitre of 6% trichloracetic acid was added to each platelet pellet and vortexed for 1 min. Samples were then centrifuged at 9000 g for 15 min and the aqueous phase was stored at −20°C. The cyclic GMP and cyclic AMP contents within the aqueous phase were later assayed using commercially available radioimmunoassay kits and the concentration of each was then expressed per 10⁷ platelets.

Bleeding time – The ear bleeding time was measured at baseline and again immediately prior to angioplasty, using a standard technique. The ear was cleaned and an incision was made on the posterior surface using a Medipoint blood lancet, to a depth of 3 mm, taking care to avoid superficial veins. The ear was then placed in a beaker of isotonic saline that had been warmed to 37°C and the interval of time was measured between puncture and the cessation of bleeding.
Statistics
The mean platelet deposition on superficially injured and on distal uninjured arterial segments was calculated in each animal. Logarithmic transformation was then performed since these data are not of a normal distribution. The log mean platelet deposition for the two groups of pigs was compared using an unpaired Student t test. Baseline and post-treatment values were compared using a paired Student t test for those variables that were measured before and after SIN-1 or placebo administration. Results are expressed as mean(SEM). A difference was considered statistically significant when p<0.05.

Results
Platelet deposition
The results are summarised in the figure. After superficial vessel wall injury and endothelial denudation, quantitative platelet deposition was significantly reduced following administration of SIN-1, when compared with placebo. Deposition of platelets in the distal uninjured segments, however, was similar in both treatment and control groups. Platelet deposition was significantly greater on balloon injured than on distal uninjured segments in both groups of animals treated with either SIN-1 (p=0.0002) or placebo (p=0.0001). Histology confirmed the loss of endothelium at the site of balloon injury in all cases and scanning electron microscopy revealed that platelet deposition in the presence of endothelial denudation was universally limited to a monolayer. Qualitative assessment of the electron micrographs also revealed that although platelets were closely opposed and profuse in number following placebo treatment, they were widely separated and scant in number in the presence of SIN-1.

Platelet studies
The results are summarised in the table. Treatment with SIN-1, but not placebo, led to a significant prolongation in bleeding time. Although there was a tendency towards inhibition of ex vivo platelet aggregation following administration of both SIN-1 and placebo, these changes were relatively minor and were statistically non-significant. Platelet cyclic GMP concentration altered little following placebo treatment but rose significantly after SIN-1 administration. There was no significant change, however, in platelet cyclic AMP concentration following either SIN-1 or placebo treatment.

Discussion
The results of this study provide the first evidence that nitric oxide inhibits platelet adhesion in vivo. Treatment with SIN-1 led to a marked reduction in quantitative platelet deposition following endothelial denudation which could only be explained by an attenuation of platelet-vessel wall interaction. These effects of SIN-1 on platelet function were accompanied by a significant rise in platelet cyclic GMP concentration, implying that they were mediated through the activation of platelet soluble guanylate cyclase. Prostaglandin synthesis was not blocked with a cyclo-oxygenase inhibitor in this study although the absence of a significant rise in platelet cyclic AMP concentration following treatment with SIN-1 means that it is unlikely that the activation of platelet adenylate cyclase contributed to the observed changes in platelet function.

SIN-1, as well as reducing platelet deposition following endothelial denudation, also led to a marked prolongation in bleeding time. Previous investigators have shown that glyceryl trinitrate prolongs the bleeding time in patients undergoing cardiac surgery as well as in healthy males. These observations are consistent with the putative actions of exogenous nitric oxide on in vivo platelet adhesion. An impairment of platelet-vessel wall interaction would certainly account for a prolongation in the bleeding time, although an additional effect of SIN-1 on endogenous fibrinolysis cannot be excluded. SIN-1 inhibits the release by stimulated platelets of plasminogen activator inhibitor type 1 and sodium nitroprusside induces fibrinolysis in vivo, at lower concentrations than those required for inhibition of platelet aggregation. It has therefore been suggested that the fibrinolytic activity of nitric oxide may be related to its antiadhesive effects on platelets, with both being stimulated by a relatively small increase in platelet cyclic GMP concentration.

We can only speculate as to why SIN-1 failed to inhibit ex vivo platelet aggregation in the present study. It is possible that the predominant antiplatelet effects of nitric oxide in vivo are to inhibit platelet adhesion and that the dose of SIN-1 that was used in this study led to a rise in cyclic GMP concentration that was insufficient to alter platelet aggregation. In addition, the coadministration of heparin, albeit at a low dose, may have been proaggregatory and may have partially reversed the antiaggregatory effects of SIN-1. Under these circumstances, however, we would expect to have observed an increase in
ex vivo platelet aggregation in placebo treated animals. A relatively short delay in analysis may lead to an underestimate of the true effects of glyceryl trinitrate on ex vivo platelet aggregation, since the antplatelet effects of this compound decay rapidly as nitric oxide is consumed after sampling. Such a factor, however, is unlikely to be important in the presence of SIN-1, from which nitric oxide continues to be released spontaneously ex vivo. Finally, it remains possible that a discrepancy exists between events occurring in vivo and ex vivo and, therefore, that ex vivo platelet aggregation is a poor reflection of the true effects of antplatelet agents in the whole animal.

Previous studies have used a similar model of arterial wall injury to investigate the effects of platelet inhibitor and thrombin inhibitor therapy on platelet deposition following balloon angioplasty in the pig. While a reduction in platelet aggregation and thrombus formation has been demonstrated in the presence of deep arterial injury, none of the agents studied has previously proved effective in the inhibition of platelet adhesion following superficial endothelial disruption.

That exogenous nitric oxide may lead to the inhibition of platelet adhesion following endothelial denudation in vivo has important potential therapeutic implications. Endothelial damage, followed by platelet adhesion and then local platelet-thrombus formation, plays an important role in vascular pathophysiology. The development of atheroma reduces the activity of endothelium derived relaxing factor, thereby exposing the arterial wall to the unopposed actions of factors that mediate vasospasm and also increase the risk of thrombosis. Spontaneous arterial injury and local platelet thrombus formation are subsequently important in the pathogenesis of acute ischaemic syndromes such as unstable angina and acute myocardial infarction. Platelet-arterial wall interaction is also important in the early stages of thrombus formation following coronary angioplasty and has been implicated, through the release of platelet derived growth factors, in the subsequent pathophysiology of intimal hyperplasia. Whether exogenous nitric oxide, through its ability to inhibit platelet adhesion in vivo, could influence such examples of vascular pathophysiology remains speculative, although the results of this study suggest that this is an area of potentially fruitful research for the future.

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Key terms: nitric oxide; platelet adhesion; balloon angioplasty


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