A novel S-nitrosothiol causes prolonged and selective inhibition of platelet adhesion at sites of vascular injury


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Abstract

Objective: Platelet adhesion to areas of endothelial denudation following angioplasty is an important factor contributing to the limitations of this technique. Lipophilic S-nitrosothiols like S-nitroso-N-valerylpenicillamine (SNVP) are novel nitric oxide (NO) donor drugs with anti-platelet and vasodilator properties that are selective for areas of endothelial denudation. Here we assess the inhibitory effect of SNVP on platelet adhesion to angioplastied rabbit carotid arteries. Methods: A rabbit model was used to measure adhesion of radiolabelled platelets to carotid arteries following balloon angioplasty. The effects of SNVP were compared to the conventional NO donor, nitroglycerin (NTG). Electron microscopy was used to visualize adhering platelets. Results: Angioplasty resulted in endothelial denudation with only a modest reduction in vessel contractility. In vivo administration of NTG and SNVP (both 200 nmol) prevented the hyper-aggregability (~20%) of circulating platelets caused by angioplasty. However, bolus NTG failed to inhibit adhesion of radiolabelled platelets 30 min after angioplasty, despite inducing a transient 30% reduction in systemic blood pressure. In contrast, equimolar SNVP had little effect on blood pressure but markedly inhibited platelet adhesion (62% compared to control; P < 0.003). Platelet adhesion was confirmed with electron microscopy. Conclusion: The prolonged effects of SNVP at sites of endothelial damage suggest that novel S-nitrosothiols might offer a means of targeted delivery of an antiplatelet agent to areas of vascular injury.

Keywords: Angioplasty; Arteries; Nitric oxide; Platelets

1. Introduction

Percutaneous transluminal coronary angioplasty is a common clinical intervention used to improve blood flow through stenosed coronary arteries. Thrombosis is a feature of angioplasty that limits the success of the procedure in the short term and is believed to contribute to vascular remodelling and, ultimately, restenosis [1–5]. Current anti-thrombotic therapies offer benefits in angioplasty, however, neointimal hyperplasia remains a major problem leading to a 15–30% incidence of restenosis [6–8].

Loss of the protective effects of endothelium-derived nitric oxide (NO) is critical to increased platelet adhesion. NO has powerful anti-platelet actions and NO donors can reduce platelet activation [9] and adhesion [10,11] following angioplasty. However, existing NO donor drugs are not selective for areas of endothelial damage and dosing is limited by systemic hypotension. S-Nitrosothiols are generally recognised to be platelet-selective NO donor drugs [12] and we have recently described several novel S-

Abbreviations: ACh, acetylcholine; ADP, adenosine 5'-diphosphate; Hep-Sal, heparinised saline; l-NAME, N^3-nitro-l-arginine methyl ester; NO, nitric oxide; NTG, nitroglycerin; PE, phenylephrine; PRP, platelet rich plasma; SNVP, S-nitroso-N-valerylpenicillamine
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nitrosothiols, including S-nitroso-N-valerylpenicillamine (SNVP), which have a prolonged vasodilator activity that is selective for blood vessels with experimentally denuded endothelium [13].

Using a rabbit model, we tested the hypothesis that platelet adhesion to the intimal surface of common carotid arteries following angioplasty is inhibited to a greater extent by SNVP than by the conventional nitrate, nitroglycerin (NTG).

2. Methods

All procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 (UK Home Office). All chemicals were obtained from Sigma, unless stated otherwise. SNVP was synthesized by a published method [14].

2.1. Surgical procedure

Adult male New Zealand white rabbits (2.5–3.5 kg; n = 60) were anaesthetised as previously described [15]. The left femoral artery was cannulated (3F) for the measurement of systemic blood pressure and heart rate (CAPTO SP844 transducer, AD Instruments) and 5 ml of blood was withdrawn into a Monovette™ tube containing 0.4 ml sodium citrate (3.8%). The external carotid artery was cannulated and a 2.5×20 mm angioplasty balloon (Boston Scientific SCIMED) was passed into the common carotid artery (Fig. 1). Angioplasty was performed on a 40-mm section of artery, using 2×30 s inflations (10 atm, with a 15-s interval). The balloon was withdrawn under 4 atm pressure. Sham operations involved cannulation of the common carotid artery without balloon inflation. The artery was recannulated to administer a 0.2-ml bolus of heparinised saline (Hep-Sal; 25 U/ml; Genus Express), NTG (Schwarz Pharma) or SNVP (both 200 nmol) immediately upstream of the angioplastied region (Fig. 1). Both SNVP [13] and NTG [16] release 1 molar equivalent of NO. Blood flow over the angioplastied region was restored and animals were killed 35 min after angioplasty with pentobarbitone (Genus Express). Both common carotid arteries were dissected free and placed in standard Krebs solution [13]. Arteries were then cleaned of connective tissue and divided into rings for further assessment (Fig. 1).

2.2. Preparation and measurement of radiolabelled platelets

Citrated blood was centrifuged with prostacyclin (300 ng/ml; Affiniti Research Products) to obtain pelleted platelets. Platelets were radiolabelled with $^{111}$InCl$_3$ (NEN™ Life Science Products) and re-suspended in prostacyclin-free solution [17]. The final suspension contained ~330×10$^6$ platelets/ml (determined with a Coulter AC.T8 Haematology Analyser, Coulter Electronics). Approximately 1 ml platelet suspension (50–300×10$^6$ platelets; radioactivity=50–800×10$^5$ decays per minute; dpm) was re-injected into the donor rabbit via a marginal ear vein ~10 min before angioplasty. Radioactivity of blood samples (100 μl) and segments of carotid artery (~5 mm) was assessed by a Packard 1900TR liquid scintillation analyser.

2.3. Platelet aggregation

Five ml blood samples were taken from the femoral artery before angioplasty and immediately before the animal was killed. Platelet-rich plasma (PRP; 0.5 ml) was pre-warmed to 37 °C for 5 min in a two-channel platelet aggregometer (Cronolog Ca560, Labmedics) [18]. Platelet aggregation in response to adenosine 5′-diphosphate (ADP; 8 μmol/l) was measured turbidometrically for a period of

Fig. 1. Schematic representation of the surgical technique and segmentation of the isolated rabbit carotid used for experimental protocols.
5 min following agonist addition. On account of the small volumes of PRP available, experiments were restricted to a single concentration of ADP. Previous experiments have shown that S-nitrosothiols are capable of inhibiting aggregation in vitro in response to this concentration of ADP [19].

2.4. Organ bath studies

Vessel rings (3 mm) were suspended in a 10-ml myograph (Multi Tissue Bath System 700MO, Danish Myo Technology) and bathed in oxygenated (95% O₂, 5% CO₂) Krebs solution at 37 °C. Tension was applied to vessels in stepwise increments to obtain a resting tension of 7 g and allowed to equilibrate for 30–40 min. Rings were contracted (×3) to obtain the maximum contraction to high K⁺ Krebs (NaCl: 4.7 mmol/l; KCl: 118 mmol/l). Rings were exposed to phenylephrine (PE; 0.1–10 μmol/l) to investigate the effect of in vivo NO donor administration on blood vessel contractility. Following precontraction with EC₅₀ PE (~3 μmol/l), endothelial cell function was assessed with acetylcholine (ACh; 0.01–30 μmol/l). Vessels were precontracted with EC₅₀ PE (~1 μmol/l) and the response to the NO scavenger oxyhemoglobin (10 μmol/l; to inhibit both endogenous and exogenous NO) and the NO synthase inhibitor N⁰-nitro-L-arginine methyl ester (L-NAME; 200 μmol/l; to inhibit only endogenous NO) was measured.

2.5. Electron microscopy

Segments of carotid artery (3 mm) were fixed in 3% glutaraldehyde and osmium tetroxide, dehydrated in graded acetone and critical point dried with CO₂. The intimal surface was examined using a Philips 505 scanning electron microscope following gold–palladium alloy sputter coating (SC500 Sputter coater, Emscope Laboratories). Following fixation and dehydration, several samples were imbedded in araldite and 60 nm sections cut (Reichert OMU4 Ultracut microtome). Sections were stained with uranyl acetate and lead citrate (LKB Ultrostainer) and examined using a Philips CM12 transmission electron microscope.

2.6. Data analysis

Data analysis was carried out using GraphPad Prism (Version 3.02, GraphPad Software Inc., San Diego, CA, USA). Radiolabelled-platelet adhesion was expressed as an index, standardised to vessel length (cm) and whole blood radioactivity (dpm/100 μl blood). Thus, an index of 1.0 indicates that all the radiolabelled platelets in 100 μl of blood adhered to a 1-cm segment of vessel. Values are expressed as mean±S.E.M. Statistically significance was defined as P<0.05 (ns=non significant). ANOVA, paired and unpaired Student’s t-tests, were used where appropriate.

3. Results

3.1. Effect of angioplasty on vessel function

Compared to contralateral control vessels, angioplastied rings showed a 29±8% decrease in vasoconstriction to high K⁺ Krebs (P=0.02; n=6; Fig. 2a) and decreased
maximum vasoconstriction to PE (0.1–10 μmol/l; \( P = 0.002; n = 6 \); Fig. 2b). Responses to high K\(^+\) Krebs and PE in sham operated vessels were not different from those in contralateral vessels (\( P = n s; n = 7 \)).

Following precontraction with PE (2.7±0.2 μmol/l; \( n = 12 \)), ACh (0.01–30 μmol/l) produced a similar degree of concentration-dependent vasodilatation in un-injured contralateral arteries and sham vessels, with almost complete loss of tone at 30 μmol/l ACh (Fig. 3a). There was marked attenuation of the maximum vasodilatation to ACh in angioplasted vessels, from 90±2 to 20±10% (\( P < 0.001; n = 6 \)).

In the presence of PE (1.3±0.1 μmol/l; \( n = 11 \)), oxyhemoglobin (10 μmol/l) increased tone from 54±3 to 92±5% (\( n = 7 \)) of the maximal K\(^+\) Krebs-induced contraction in the contralateral vessels (Fig. 3b). In the same precontracted vessels, L-NAME (200 μmol/l) increased vessel tone from 59±1 to 73±2% (\( n = 6 \); Fig. 3b). These responses to oxyhemoglobin and L-NAME were similar in sham-operated in comparison to contralateral vessels (\( P = n s; n = 6–7 \)). In angioplastied vessels, oxyhemoglobin and L-NAME had no effect on vessel tone (\( P = n s; n = 3–5 \)).

The removal of endothelial cells in the intima of angioplastied arteries was confirmed by immunohistochemistry (results not shown).

3.2. Effect of drug bolus on vessel function ex vivo

Bolus administration of either NO donor immediately after angioplasty in vivo had no significant effect on responses to high K\(^+\) Krebs, PE (EC\(_{50}\)=0.6–1.5 μmol/l), ACh (EC\(_{50}\)=47–120 nmol/l), oxyhemoglobin or L-NAME in either angioplasted or contralateral vessels (\( P = n s; n = 5–6 \) for all; results not shown).

3.3. Effect of drug bolus on blood pressure

Before angioplasty, baseline systolic and diastolic blood pressure was 61±4 and 44±3 mmHg (\( n = 21 \)), respectively, and heart rate 220±9 bpm (\( n = 17 \)). Hep-Sal bolus (0.2 ml) had no effect on blood pressure or heart rate (Table 1 and Fig. 4). NTG bolus (200 nmol) caused a significant transient reduction in both systolic and diastolic blood pressure, whereas SNVP (200 nmol) had no effect (Table 1 and Fig. 4).
Table 1

<table>
<thead>
<tr>
<th>% Change after treatment</th>
<th>Saline</th>
<th>NTG</th>
<th>SNVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic pressure</td>
<td>1±1⁰ (9)</td>
<td>-31±6* (6)</td>
<td>-7±1⁰ (6)</td>
</tr>
<tr>
<td>Diastolic pressure</td>
<td>0±2⁰ (9)</td>
<td>-30±6* (6)</td>
<td>-6±4⁰ (6)</td>
</tr>
<tr>
<td>Heart rate</td>
<td>-2±1⁰ (9)</td>
<td>0±4⁰ (6)</td>
<td>0±4⁰ (6)</td>
</tr>
<tr>
<td>Platelet aggregation</td>
<td>26±4 (4)</td>
<td>-11±10* (5)</td>
<td>-8±23⁰ (5)</td>
</tr>
</tbody>
</table>

* P<0.05, unpaired t-test compared to saline control; † P<0.05, paired t-test, aggregatory response to 8 µmol/l ADP in platelet rich plasma derived from blood taken before compared to that taken after angioplasty. ** P>0.05 (number of experiments in brackets).

3.4. Effect of drug bolus on platelet aggregation

Following angioplasty with Hep-Sal, there was a 9.3±2.6 mV increase (~25%) in platelet aggregation to ADP (8 µmol/l) compared to before angioplasty (Table 1). In contrast, there was no increase in aggregability of platelets in PRP from rabbits that received NTG or SNVP after angioplasty (Table 1).

3.5. Radiolabelled platelet adhesion to carotid arteries

In angioplastied vessels, there was an almost 20-fold increase in platelet adhesion (index=0.25±0.04; Fig. 5) compared to contralateral vessels (index=0.013±0.01 dpm). NTG did not reduce platelet adhesion in angioplastied vessels, in comparison to Hep-Sal (P=ns; n=6 and 7, respectively), whereas SNVP reduced it by 62±7% (P=0.003; n=7; Fig. 5): and to a greater extent than NTG (P=0.01; n=6).

3.6. Electron microscopy

Adhesion of platelets to the lumen of blood vessels was confirmed using scanning electron microscopy (Fig. 6). Following a Hep-Sal bolus, angioplastied vessels showed adhering and activated platelets covering the entire luminal surface (Fig. 6b). Transmission electron microscopy revealed platelets with pseudopodia extending over the intimal surface and with a loss of cytoplasmic granules, confirming both adhesion and activation (Fig. 6b inset). In angioplastied vessels treated with SNVP, the number of activated platelets adhering to the luminal surface was substantially reduced (Fig. 6c).

4. Discussion

We have demonstrated that a novel S-nitrosothiol, SNVP, reduces adhesion of radiolabelled platelets to rabbit carotid arteries following angioplasty in vivo, without significantly affecting systemic blood pressure. In contrast, equimolar NTG caused an undesirable decrease (30%) in systemic blood pressure, but failed to inhibit local platelet adhesion. Both NO donors prevented circulating platelet hyper-aggregability induced by angioplasty, suggesting that the added benefits of SNVP are due to a prolonged...
antiplatelet action selectively at sites of endothelial cell damage.

4.1. Platelet activation and angioplasty

The major limitations of angioplasty are thrombosis and restenosis of treated arteries. The technique inevitably damages the vascular endothelium, leading to platelet activation, that is a key event in the initiation of vascular remodelling [5,20]. Current anti-thrombotic therapies, including aspirin, clopidogrel and GPIIb/IIIa antagonists, have been shown to reduce platelet adhesion and intimal thickening following angioplasty [6–8]. However, maintaining sufficiently high local drug concentration over a
number of hours can be problematic [2,21,22]. Recently, sirolimus (rapamycin)-eluting stents have been shown to be particularly effective at inhibiting neointimal hyperplasia [8,23], but this approach does not have anti-platelet actions and the long-term effectiveness (>1 year) has yet to be established. Despite current advances in drug delivery catheters [2] and deployment of stents [8], endothelial cell damage and platelet activation still remain a significant problem, highlighting the need for additional therapies which counteract the multiple factors involved in the response to injury.

4.2. Role of conventional NO donors

Delivery of NO is an attractive alternative to conventional anti-thrombotic agents, because NO exhibits a range of beneficial actions, including vasodilatation, regulation of inflammatory cell function, inhibition of smooth muscle cell mitogenesis and inhibition of platelet activation [24]. Indeed, NO donors inhibit platelet activation following angioplasty [9–11,25]. The most common clinically used NO donors are the organic nitrates, such as NTG, although the selectivity profile of traditional nitrates (veins-> arteries->platelets) is unfavourable. Our results are in keeping with the recognised selectivity profile of NTG; it caused a profound systemic hypotension, demonstrating that vasoactive concentrations of NTG were being used that may induce unwanted complications in patients undergoing angioplasty.

NTG is a poor inhibitor of platelet aggregation in vitro [26], possibly because PRP lacks the factors necessary for the biotransformation to active NO [27]. In vivo, NTG infusion can inhibit platelet activation, presumably via vascular tissue-mediated biotransformation of NTG to NO [28]. However, NTG failed to prevent adhesion of radiolabelled platelets to the intimal surface of angioplastied carotid arteries. These results suggest that bolus NTG can influence activation of circulating platelets, but its short-term effects are not capable of preventing adhesion to the exposed subendothelial surface [11], presumably because NTG is not selectively retained in this region. Similarly, other drugs, such as aspirin, have also been shown to prevent platelet hyper-responsiveness without reducing platelet adhesion [3].

4.3. Potential of S-nitrosothiols

S-Nitrosothiols exhibit a number of properties that might prove advantageous in angioplasty. They show greater selectivity for arteries than veins [29] and, unlike organic nitrates, are also powerful inhibitors of platelet aggregation both in vitro [19] and in vivo [12]. S-Nitrosothiols can generate sufficient NO in PRP to inhibit platelet aggregation [19]. We have previously demonstrated that SNVP causes sustained NO-mediated vasodilatation selectively in blood vessels with damaged endothelium [13]. In addition, other novel lipophilic S-nitrosothiols produce sustained vasodilatation in human arteries and veins both ex vivo [30] and in vivo [31], which may potentially be of great benefit in preventing vasospasm in angioplastied arteries. This evidence strongly suggests that locally-delivered SNVP is able to specifically target areas of endothelial damage, a feature that is not shared by conventional NO donors.

Equimolar concentrations of SNVP were compared with NTG, because both compounds release 1 molar equivalent of NO [16]. In contrast to NTG, SNVP had minimal effects on systemic blood pressure, but caused a >60% reduction in platelet adhesion to angioplastied carotid arteries. Both SNVP and NTG inhibited in vitro platelet aggregation in response to ADP, however, only SNVP inhibited platelet adhesion following angioplasty. Here, SNVP was administered as a concentrated bolus immediately upstream of the angioplastied region (Fig. 1). Our previous results [13] are consistent with the hypothesis that SNVP is retained in the exposed subendothelial layers, where it decomposes slowly, generating NO in sufficiently high concentrations to inhibit platelet adhesion locally. Similar findings with radiolabelled S-nitrosoalbumin have been confirmed to be due to adhesion of this agent to the area of injury [25]. The inhibitory effect of SNVP on platelet adhesion suggests that this compound might have therapeutic potential in the prevention of acute thrombosis at the site of angioplasty, possibly from a single administration, although its impact on long-term complications of interventional procedures remains to be determined.

4.4. Study limitations

Damaged vessels lose their hyperactivity to platelets within 8 h after injury, despite incomplete endothelial regrowth [21,32]. However, presently it is unknown whether the antiplatelet effects of SNVP persist for >30 min after angioplasty. In endothelium-denuded rat femoral arteries, ~75% of the vasodilatation to novel S-nitrosothiols is still present at 4 h [33], emphasising the long-acting nature of these compounds. This complementary vasodilator effect would be beneficial in limiting vasoconstriction occurring after angioplasty. However, the results of functional experiments in the current study failed to confirm a prolonged vasodilator effect in rings from angioplastied carotid arteries treated with SNVP. There was no difference in the maximum contraction to high K+ Krebs or PE between vessels that received different NO donors and no evidence from oxyhemoglobin experiments of NO-mediated, sustained vasodilatation in SNVP-treated vessels. However, these results do not preclude the possibility that sufficient S-nitrosothiol remains in the vessel to inhibit platelet adhesion, but not to cause vasodilatation. Alternatively, the setup time and processing of the isolated carotid ring experiments may reduce the concentrations of drug retained in the tissue. Further experiments are needed.
to investigate the mechanism and duration of action of drugs like SNVP, and will help to establish their therapeutic potential in the prevention of restenosis, either alone or as an adjunct to current therapies.

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