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Platelet inhibitory effects of the nitric oxide donor drug MAHMA NONOate in vivo in rats

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Abstract

The platelet inhibitory effects of the nitric oxide (NO) donor drug MAHMA NONOate ((*Z*-1-{*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)amino]}diazen-1-ium-1,2-diolate) were examined in anaesthetised rats and compared with those of *S*-nitrosoglutathione (GSNO; an *S*-nitrosothiol). Bolus administration of the aggregating agent ADP dose-dependently reduced the number of circulating free platelets. Intravenous infusions of MAHMA NONOate (3–30 nmol/kg/min) dose-dependently inhibited the effect of 0.3 μmol/kg ADP. MAHMA NONOate was approximately 10-fold more potent than GSNO. MAHMA NONOate (0.3–10 nmol/kg/min) also reduced systemic artery pressure and was again 10-fold more potent than GSNO. Thus MAHMA NONOate has both platelet inhibitory and vasodepressor effects in vivo. The dose ranges for these two effects overlapped, although blood pressure was affected at slightly lower doses. The platelet inhibitory effects compared favourably with those of GSNO, even though NONOates generate free radical NO which, in theory, could have been scavenged by haemoglobin. Therefore platelet inhibition may be a useful therapeutic property of NONOates.

Keywords: Platelet inhibition, in vivo; MAHMA NONOate; Nitric oxide (NO) donors; *S*-nitrosoglutathione; Vasodepression systemic

1. Introduction

The term nitric oxide (NO) donor is used to describe any compound that can generate NO. The numerous different NO donors are classified according to their chemical structures and the manner in which they generate NO. All classes of NO donor drug have vasodilator properties. In addition, some, but not all, are effective inhibitors of platelet activation. For example, the NO donor drug glyceryl trinitrate (an organic nitrate) has been used as a vasodilator in the treatment of cardiovascular disease for the past 140 years. However this drug is a poor inhibitor of platelet activation in vitro (Sogo et al., 2000; Homer and Wanstall, 2002) and in vivo (Mehta and Mehta, 1980). In contrast *S*-nitrosothiols, a different class of NO donor drugs, are potent inhibitors of platelet activation. This has been demonstrated both in vitro (Radomski et al., 1992; Sogo et al., 2000; Homer and Wanstall, 2002) and in vivo (De Belder et al., 1994; Salas et al., 1998). In particular the *S*-nitrosothiol, *S*-nitrosoglutathione (GSNO), has been shown to inhibit ADP-induced platelet aggregation in vivo at concentrations that also decrease systemic artery pressure (Radomski et al., 1992).

Recently the pharmacological properties of another group of NO donor drugs, the diazeniumdiolates (NONOates), have been described. Like S-nitrosothiols, NONOates inhibit platelet aggregation in vitro (Raulli, 1998; Sogo et al., 2000; Homer and Wanstall, 2002). However their effects on platelets in vivo are not known. The in vivo pharmacological profile of NONOates may not necessarily be the same as their in vitro profile because, in vivo, the NO generated from these drugs may, like authentic NO, be readily scavenged by haemoglobin in red blood cells (Tsikas et al., 2001). The lack of in vivo information on the effects of this class of NO donor on platelets prompted the present study in which MAHMA NONOate ((*Z*-1-{*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)amino]}diazen-1-ium-1,2-diolate) has been examined in anaesthetised rats. The ability of MAHMA NONOate to inhibit ADP-induced aggregation/adhesion has been investigated, using a decrease in the number of circulating free platelets as a measure of ADP-induced aggregation/adhesion, as described by Radomski et al. (1992). The anti-platelet effects of MAHMA NONOate have been compared with those of GSNO and, in addition,

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the effects of both drugs on systemic artery pressure have been quantified.

2. Materials and methods

The experiments in this study comply with the "Code of Practice for Animal Experiments" of the National Health and Medical Research Council of Australia and have been approved by the University of Queensland Animal Experimentation Ethics Committee.

2.1. Surgical procedure

Male Wistar rats (mean body weight 343 ± 6.4 g, $n = 40$) were anaesthetised with pentobarbitone (60–90 mg/kg, ip). The animals were placed on a heat pad throughout the experiment to maintain body temperature at approximately 37 °C. The trachea was cannulated and the lungs artificially ventilated (60–90 strokes/min; 1.5 ml/stroke) with a Ugo Basile (Comerio, Varese, Italy) Rodent ventilator. In all experiments a jugular vein and a carotid artery were cannulated. In the experiments studying free platelet count the left femoral vein and right femoral artery were also cannulated. Heparin (approximately 15 IU/ml blood volume, bolus iv) was administered via either the jugular vein (pressure experiments, see Section 2.2) or the femoral vein (free platelet count experiments, see Section 2.3). Supplemental pentobarbitone (9–10 mg/kg, bolus iv) was administered as required throughout the experiments to maintain anaesthesia.

2.2. Measurement of systemic artery pressure

Systemic artery pressure (mm Hg) was measured via the carotid artery with a Bentley Trantec pressure transducer (American Edwards Laboratories, Santa Ana, CA, USA) and recorded with a Ugo Basile Gemini chart recorder. Once the pressure had stabilised following surgery an initial baseline pressure was obtained. Continuous infusions (infusion rate 0.01, 0.03 or 0.10 ml/min) of NO donor drug (MAHMA NONOate or GSNO) or saline were administered via the jugular vein until the changes in pressure in response to the drugs reached a plateau (≤ 5 min). In some experiments, two or three consecutive infusions of the same NO donor drug in ascending concentrations were administered with the second and third infusions commencing 40 min after the completion of the previous infusion. At this time the pressure had returned to pre-infusion values ($P > 0.05$, one-way ANOVA), i.e. there was no longer any effect of the preceding dose of the NO donor.

Systemic artery pressure was taken as the diastolic pressure + 1/3 (systolic–diastolic pressure) and determined (i) immediately prior to each NO donor infusion (pre-infusion), and (ii) when the response to the NO donor had reached a plateau (post-infusion). The responses to the NO donor drugs were expressed as the per cent change in the systemic artery pressure, calculated as:

$$\frac{\text{systemic artery pressure (pre infusion)} - \text{systemic artery pressure (post infusion)}}{\text{systemic artery pressure (pre infusion)}} \times 100\%$$

The ED_{25} (dose that induced 25% decrease of the pre-infusion systemic artery pressure) was interpolated from the mean dose–response curves.

2.3. Measurement of reduction in free platelets

Platelet aggregation and/or adhesion in vivo was measured as the disappearance of free platelets from whole blood (Radomski et al., 1992). To determine the free platelet count, blood samples were collected from the cannulated femoral artery using a Unopette Microcollection System (Becton Dickinson, Franklin Lakes, NJ, USA). Twenty microliters of blood were collected into a capillary pipette and immediately diluted (1:100) in a reservoir containing the red blood cell haemolysing agent ammonium oxalate (11.45 g/l). Diluted samples were transferred to a haemocytometer, allowed to settle for at least 10 min and the free platelets were counted using a light microscope. For each sample the average of two counts was obtained and the number of free platelets per microlitre in the sample determined. To obtain an initial platelet count samples were collected and counted 60 min after the completion of surgery, and then at 10 min intervals, until a stable count was reached. The experimental protocol was commenced only if the initial free platelet count was greater than 7×10^5 platelets/μl and there was no evidence of aggregated platelets in the diluted haemolysed blood sample.

ADP (0.1, 0.3 or 1 μmol/kg, bolus iv) was administered via the femoral vein and caused an immediate reduction in the free platelet count. In preliminary experiments the time to peak reduction was found to be 0.5 min. Therefore the free platelet count was measured in samples obtained 1 min before (control) and 0.5 min after (ADP present) the delivery of each dose of ADP. Responses to ADP were expressed as percent reduction of the control free platelet count and calculated as:

$$\frac{\text{free platelet count (control)} - \text{free platelet count (ADP present)}}{\text{free platelet count (control)}} \times 100\%$$

The effects of the NO donor drugs were examined on responses to 0.3 μmol/kg ADP (see Section 3.2.1 for selection of ADP dose). No more than three consecutive responses to ADP (0.3 μmol/kg) were obtained in each animal because, in preliminary experiments, three consecutive responses were shown to be reproducible whereas a fourth response was not. An interval of 60 min was

allowed between each ADP response at which time the free platelet count in the absence of ADP was not different from the initial (first control) count ($P>0.05$, one-way ANOVA).

In preliminary experiments 0.3 μmol/kg ADP was found to cause a very transient (< 1 min) but significant change in systemic artery pressure (mean systemic pressure (mm Hg); pre ADP, 80 ± 9.02; post ADP 34 ± 5.47; $n=7$; $P<0.0001$, paired *t*-test). Hence two separate groups of rats were used to examine the effects of the NO donor drugs on (i) systemic artery pressure (see Section 2.1) and (ii) ADP-induced platelet aggregation/adhesion.

The effects of continuous infusions (infusion rate 0.01, 0.03 or 0.10 ml/min) of first drug vehicle and then MAHMA NONOate or GSNO, administered via the jugular vein, were examined on consecutive responses to ADP (0.3 μmol/kg). The infusion of NO donor drug or vehicle was commenced 5 min before the administration of ADP. Blood samples for platelet counts were then obtained 1 min before (control) and 0.5 min after (ADP present) the administration of ADP and the response to ADP determined (see above). Times between (i) the commencement of the NO donor infusion and ADP administration (5 min) and (ii) consecutive NO donor infusions (40 min) were based on the results of the blood pressure experiments (see Section 2.2). The inhibition by the NO donor drugs of the ADP response was expressed as:

$$\frac{\text{ADP response (drug vehicle)} - \text{ADP response (NO donor)}}{\text{ADP response (drug vehicle)}} \times 100\%$$

The ED_{50} (dose that caused 50% inhibition of the ADP response) was interpolated from the mean dose–response curves.

2.4. Drugs and solutions

Sources of drugs were as follows: ADP (Sigma, Australia); GSNO (*S*-nitrosoglutathione, Sigma); heparin sodium (David Bull Laboratories, Australia; ampoules); MAHMA NONOate (*Z*-1-{*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)amino]}diazen-1-ium-1,2-diolate; Cayman, USA); sodium pentobarbitone (Merial, Australia). Stock solutions of drugs were prepared as follows: GSNO and ADP (5 μmol/ml) in 0.9% w/v saline; MAHMA NONOate in 0.01 M NaOH. The concentrations of stock solutions of NO donors varied depending on rat weight and infusion rate. All dilutions were prepared in 0.9% w/v saline apart from MAHMA NONOate that was diluted in 0.01 M NaOH except for the final dilution that was made in 0.9% w/v saline immediately before use. During the experiments the drug solutions were kept on ice and the NO donor drugs protected from light.

2.5. Statistical analysis

Mean values were calculated from data obtained from a number (n) of different animals and are quoted with their S.E.M. Differences between mean values have been assessed by paired *t*-test or unpaired *t*-test (comparison of two values) or by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test (comparison of more than two values).

3. Results

3.1. Systemic artery pressure

The initial mean systemic artery pressures in the series of experiments with MAHMA NONOate and GSNO were, respectively, 91 ± 7.9 and 79 ± 6.9 mm Hg ($n=6–7$). These values were not significantly different ($P>0.05$, unpaired *t*-test). Infusions of saline had no effect on systemic artery pressure. Infusions of MAHMA NONOate (0.3–10 nmol/kg/min) and GSNO (10–100 nmol/kg/min) caused dose-dependent decreases in mean systemic artery pressure (Fig. 1). MAHMA NONOate was more potent than GSNO, with a potency difference (based on ED_{25} values) of approximately one order of magnitude (Fig. 1).

3.2. Platelets

3.2.1. Selection of ADP dose

ADP caused a dose-dependent reduction in the free platelet count (Table 1). From the data in Table 1 the dose of ADP selected to examine the effects of the NO donor drugs was 0.3 μmol/kg.

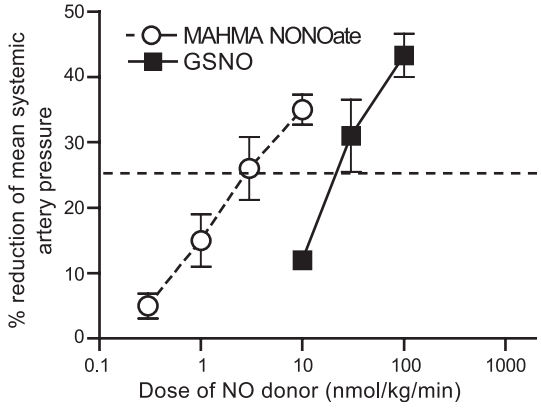

Fig. 1. Mean concentration–response curves to MAHMA NONOate and GSNO on mean systemic artery pressure. Responses are presented as changes in the systemic artery pressure expressed as a percentage of the pre-drug value. Points are mean values with S.E.M. shown by vertical bars except when smaller than the size of the symbols. Numbers of rats were: MAHMA NONOate, $n=5$; and GSNO, $n=3–5$. The broken line corresponds to 25% reduction in pressure. ED_{25} values were interpolated from the mean curves and were 3 and 24 nmol/kg/min for MAHMA NONOate and GSNO, respectively.

Table 1
Effects of ADP on free platelet count

ADP dose (μmol/kg)	Free platelet count (10^5 platelets/μl)		% Reduction[a]
	Control	ADP	
0.1	8.9 ± 0.6 (7)	7.4 ± 0.9 (7)	20 ± 7.7 (7)
0.3	10.7 ± 1.5 (3)	7.0 ± 1.5 (3)**	36 ± 7.1 (3)
1.0	10.3 ± 0.8 (4)	6.0 ± 1.8 (4)*	43 ± 15.7 (4)

Values are means ± S.E.M. Numbers of animals are in parentheses.
*0.05>*P*>0.01, **0.01>*P*>0.001 when compared with corresponding control values (paired *t*-test).

[a] % Reduction in free platelet count is calculated as the difference between the free platelet count obtained in the absence of ADP (control) and the count obtained in the presence of ADP (ADP) and expressed as a percentage of the free platelet count obtained in the absence of ADP (control).

3.2.2. Effect of NO donor infusion on platelet count

At the concentrations examined continuous infusions of the NO donor drugs, or their vehicles, had no effect on free platelet count except 30 nmol/kg/min MAHMA NONOate which caused a small but significant decrease (Table 2; $P < 0.05$, one-way ANOVA and Dunnett's post hoc test). The free platelet counts were measured 4 min after the commencement of the infusion, i.e. 1 min before the ADP was administered (see Section 2.3).

3.2.3. Effect of NO donor infusion on ADP response

The mean response to 0.3 μmol/kg ADP in the presence of vehicle in the series of experiments with MAHMA NONOate (% reduction in free platelets; 36 ± 3.5, $n = 18$) was not significantly different from that obtained in the series with GSNO (34 ± 4.7, $n = 14$: $P > 0.05$, unpaired *t*-test). MAHMA NONOate (3–30 nmol/kg/min) and GSNO (100–1000 nmol/kg/min) caused dose-dependent inhibition of the response to 0.3 μmol/kg ADP (Fig. 2). The response to ADP was abolished, or virtually abolished, by the highest doses of MAHMA NONOate and GSNO studied, i.e. 30 and 1000 nmol/kg/min, respectively. MAHMA NONOate was more potent than GSNO and the potency difference, based on values of ED_{50}, was approximately one order of magnitude.

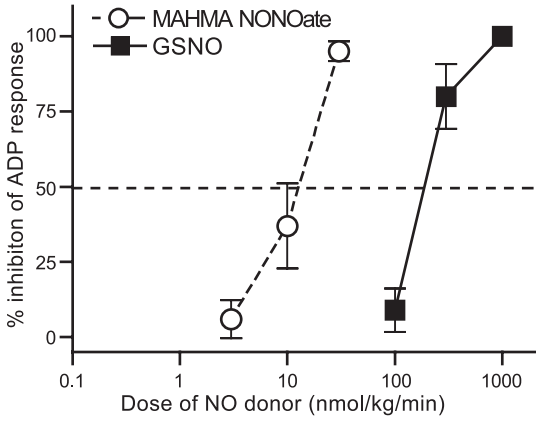

Fig. 2. Mean concentration–inhibition curves to MAHMA NONOate and GSNO on platelets. Responses to the NO donors are expressed as percentage inhibition of the response to the platelet activating agent ADP (0.3 μmol/kg) obtained in the presence of vehicle only. Points are mean values with S.E.M. shown by vertical bars except when smaller than the size of the symbols. For *n* values see Table 2. The broken line corresponds to 50% inhibition of the ADP response. ED_{50} values were interpolated from the mean curves and were 14 and 216 nmol/kg/min for MAHMA NONOate and GSNO, respectively.

Table 2
Effect of NO donor drugs on free platelet count

Free platelet count (10^5 platelets/μl)				
A				
Pre-infusion	Vehicle	MAHMA NONOate (nmol/kg/min)		
		3	10	30
11.1 ± 0.3 (12)	11.8 ± 0.3 (12)	9.8 ± 0.3 (4)	10.0 ± 0.5 (8)	9.3 ± 0.6 (6)*
B				
Pre-infusion	Vehicle	GSNO (nmol/kg/min)		
		100	300	1000
11.6 ± 0.3 (9)	11.6 ± 0.3 (9)	11.5 ± 0.5 (4)	11.0 ± 0.3 (7)	10.3 ± 0.3 (3)

Values are means ± S.E.M. Numbers of animals are in parentheses.
A: Values obtained from the series of experiments examining the effects of MAHMA NONOate.
B: Values obtained from the series of experiments examining the effects of GSNO.
* $P < 0.05$ when compared with corresponding pre-infusion value (one-way ANOVA and Dunnett's post hoc test).

4. Discussion

This study has demonstrated, for the first time, that MAHMA NONOate has platelet inhibitory effects in vivo. This NO donor drug inhibited platelets at doses that overlapped those producing systemic vasodepressor effects. Therefore, as with *S*-nitrosothiols but in contrast to organic nitrates, platelet inhibition may be a useful pharmacological/therapeutic property of NONOates in addition to vasodilatation.

Before attempting to quantify the effects of MAHMA NONOate and GSNO on ADP-induced aggregation/adhesion, it was important to establish whether either of the NO donors had any direct effect on the free platelet count. GSNO (at the doses studied) neither increased nor decreased the platelet count. MAHMA NONOate also had no effect on platelet number, except at the highest dose that caused a significant, though small, reduction in free platelets. However we are confident that this small change would not have caused erroneous interpretation of the results. This is because any decrease in free platelets by MAHMA NONOate would tend to exaggerate the response to ADP and hence, if anything, underestimate the inhibition of ADP by MAHMA NONOate. Yet, despite this, the highest dose of MAHMA NONOate virtually abolished the response to ADP.

The potency of GSNO as an inhibitor of the effect of ADP was consistent with that reported in other in vivo studies in rats (Radomski et al., 1992; Picunio et al., 1999). The potency of MAHMA NONOate was shown to be approximately 10-fold greater than that of GSNO. This potency difference between the two drugs was comparable to that seen in vitro in experiments in platelet rich plasma using either collagen or ADP as the aggregating agent (Homer and Wanstall, 2002). Of particular relevance are the negative log EC_{50} values that were obtained for inhibition of ADP-induced aggregation by MAHMA NONOate and GSNO in vitro; these values, derived from concentration–response data published by Homer and Wanstall (2002), were one log unit apart, viz. MAHMA NONOate 5.83 ± 0.19; GSNO 4.86 ± 0.25 ($n = 6$). An important difference between in vitro experiments in platelet-rich plasma and in vivo experiments like the ones carried out in the present study is the presence, in vivo, of red blood cells, and hence haemoglobin. Since haemoglobin can scavenge NO, including NO generated from MAHMA NONOate (Raulli, 1998), the presence of red blood cells has the potential to influence potency by affecting the availability of free NO. The fact that the relative potencies of MAHMA NONOate and GSNO were the same in vivo and in vitro suggests either that the presence of red blood cells influences both drugs equally or, alternatively, that it does not influence the anti-platelet effects of these NO donors at all.

The initial expectation was that the effects of GSNO (on both platelets and blood vessels) would not be compromised in vivo by the presence of red blood cells. This was based on evidence that GSNO administered intravenously to rats readily *S*-transnitrosates albumin in plasma to form *S*-nitrosoalbumin (Tsikas et al., 2001). In this form NO is protected from the scavenging effects of haemoglobin because *S*-nitrosoalbumin cannot readily cross the membranes of red blood cells. *S*-nitrosoalbumin has been described as a carrier, store and biological source of NO (Stamler et al., 1992; Tsikas et al., 2001) and has been shown to inhibit platelet aggregation in its own right (Simon et al., 1993; Tsikas et al., 1999).

The initial expectation with respect to MAHMA NONOate was that it might be less effective than GSNO in vivo. This was because MAHMA NONOate, unlike *S*-nitrosothiols, generates predominantly NO free radical (Feelisch and Stamler, 1996), which is only a weak nitrosating agent (Tsikas et al., 2001). Hence, when compared with *S*-nitrosothiols, the NO from MAHMA NONOate may be less likely to form *S*-nitrosoalbumin, thus leaving the NO unprotected. In the circulation the majority of free radical NO is thought to diffuse into red blood cells, where it is largely oxidised by oxyhaemoglobin to form methaemoglobin and nitrate, i.e. the NO is scavenged (Tsikas et al., 2001). If free radical NO derived from MAHMA NONOate is rapidly scavenged by haemoglobin, it was perhaps surprising that MAHMA NONOate was more potent than GSNO at inhibiting platelets in vivo.

There are several possible explanations for this apparent anomaly. Firstly, although NO is considered to be a weak nitrosating agent, it has been suggested by Crane et al. (2002) that there are plasma components which may facilitate the formation of higher oxides of NO, including N_2O_3 which is a potent nitrosating agent (Espey et al., 2001). These nitrosating agents may subsequently nitrosate thiols (both low molecular weight thiols and albumin) leading to the formation of the corresponding *S*-nitrosothiols (Crane et al., 2002). The finding that DEA NONOate, a short-acting NONOate with a half life similar to that of MAHMA NONOate, i.e. approximately 2 min, generates *S*-nitrosothiols in vitro when incubated in platelet rich plasma (Crane et al., 2002) supports this. Furthermore, experiments in washed platelets, instead of platelet rich plasma, demonstrated the central importance of *S*-nitrosoalbumin (Crane et al., 2002). A second consideration is that there is more than one way in which NO can interact with haemoglobin, i.e. NO can bind not only to haem iron but also to thiols of haemoglobin, depending on the oxidation state of the haem iron (McMahon et al., 2002). *S*-nitrosation of a cysteine residue of haemoglobin yields *S*-nitrosohaemoglobin. This compound has been described as part of a complex means of NO transportation in vivo (McMahon et al., 2002) although this remains controversial (Rassaf et al., 2003). Importantly, the *S*-nitrosothiols, *S*-nitrosoalbumin (Tsikas et al., 1999) and *S*-nitrosohaemoglobin (Pawloski et al., 1998), both retain NO-like bioactivity, i.e. they inhibit platelet aggregation.

Plasma streaming, a characteristic of blood flow in healthy blood vessels in vivo, is a third possible explanation as to why the anti-platelet effects of NO derived from MAHMA NONOate may not be affected by haemoglobin in vivo. Plasma streaming results in red blood cells flowing through the centre of blood vessels, surrounded by platelet rich plasma adjacent to the endothelium (Aarts et al., 1988). Megson et al. (2000) have proposed that the separation of red blood cells from platelets could preserve any NO that is in the vicinity of the platelets and, thus, could facilitate NO-mediated platelet actions of both endothelium-derived NO and exogenous NO under normal conditions in vivo.

Precise comparison of the potency of MAHMA NONOate on blood pressure (assumed to reflect the vasodilator effect of the drug) with its potency on platelets was not possible because of the different definitions of potency for the two effects. However the ‘threshold’ dose for an effect of MAHMA NONOate on platelets (3 nmol/kg/min) was 10-fold higher than the ‘‘threshold’’ dose for vasodepression (0.3 nmol/kg/min). This difference is, if anything, less than that seen in vitro, where there was a 45-fold difference in potency between blood vessels and platelets (Homer and Wanstall, 2002). Although MAHMA NONOate was slightly less potent on platelets than on blood vessels in this in vivo study, the dose ranges for the platelet and vascular effects overlapped. It is therefore possible, though yet to be demonstrated, that these two pharmacological properties

could be achieved concurrently if MAHMA NONOate were used therapeutically.

Like MAHMA NONOate, GSNO was more potent in reducing blood pressure than inhibiting platelet aggregation/adhesion. These results are in contrast to previous reports that GSNO is a platelet-selective NO donor in vivo both in rats (Radomski et al., 1992) and in humans (De Belder et al., 1994; Ramsay et al., 1995). In rats 300 nmol/kg/min GSNO effectively inhibited platelet aggregation/adhesion but had only a small effect on systemic pressure (Radomski et al., 1992). In the present study 100 nmol/kg/min GSNO, the highest dose of GSNO examined on blood pressure, caused a large reduction (approximately 40%) in pressure but had only a very minimal effect on platelets. The reason for this discrepancy is not known.

In conclusion, NONOates are considered to be potentially useful therapeutic agents because of their predictable release of NO. Although their vasodilator properties are well established in vitro and in vivo, this is the first demonstration of their anti-platelet effects in vivo. The data showed not only that MAHMA NONOate is effective in inhibiting platelets in vivo but also that it compared favorably with GSNO. The provision of antiplatelet data and vasodepression data within a single study enabled us to show that, although the effect on blood pressure occurred at slightly lower doses than the effect on platelets, the dose range for these two therapeutically useful properties overlapped.

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