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In vitro comparison of two NONOates (novel nitric oxide donors) on rat pulmonary arteries

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Abstract

The pulmonary vasorelaxant properties of two NONOates (diazeniumdiolates) were examined because this novel group of nitric oxide (NO) donors may be useful in pulmonary hypertension. MAHMA NONOate ((*Z*)-1-{*N*-Methyl-*N*-[6-(*N*-methylammoniohexyl)amino]} diazen-1-ium-1,2-diolate) and spermine NONOate ((*Z*)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino}diazen-1-ium-1,2-diolate) decomposed at different rates (half-lives 1.3 min and 73 min, respectively; 37°C, pH 7.3) but generated the same total amount of NO. They fully relaxed submaximally contracted ring preparations of main and intralobar pulmonary arteries from rats. Responses were inhibited by the guanylate cyclase inhibitor, ODQ (1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one). Potency was not affected by choice of contractile spasmogen (phenylephrine, endothelin-1, thromboxane-mimetic) or endothelium removal, and tolerance did not develop; thus the drugs had properties important for use in pulmonary hypertension. MAHMA NONOate was 10–40-fold more potent than spermine NONOate but responses to spermine NONOate were more sustained (spermine NONOate > 60 min; MAHMA NONOate < 7 min). It is concluded that the differences in potency and time-course reflect the different rates of NO generation by these NONOates.

Keywords: MAHMA NONOate; Nitric oxide donor; NONOate; Pulmonary artery, rat; Pulmonary hypertension; Spermine NONOate

1. Introduction

The use of inhaled nitric oxide (NO) gas is one of the most significant, recent advances in the treatment of pulmonary hypertension (Zapol, 1996). However, NO has a very short half-life and therefore requires continuous administration. Moreover, specialised delivery equipment is required to avoid exposure of both patients and health care workers to toxic levels of NO and nitrogen dioxide, a toxic by-product of NO (Krebs et al., 1996). These factors may limit the use of inhaled NO gas, especially in the long-term treatment of chronic pulmonary hypertension, and have led to interest in NO donor drugs as possible alternatives to NO gas.

NONOates, otherwise known as diazeniumdiolates, are a novel group of NO donors. They are complexes of NO with nucleophiles (*X*) and have the general formula, $X\,N(O^-)N=O$ (Maragos et al., 1991; Hrabie et al., 1993). These compounds are generally stable as solids, but decompose in solution to generate NO (Keefer et al., 1996). Decomposition occurs at a predictable rate that depends on pH, temperature and the nature of the nucleophile (Maragos et al., 1991).

NONOates were described in the chemical literature over 30 years ago (Longhi et al., 1962; Ragsdale et al., 1965) but it is only recently that their potential as sources of NO, with biological properties, has been studied (Maragos et al., 1991; Morley et al., 1993). NONOates have been found to be effective vasodilators both in vitro (Maragos et al., 1991; Morley et al., 1993) and in vivo (Diodati et al., 1993; Vanderford et al., 1994). However, the in vitro effects of this novel group of NO donors have not previously been evaluated specifically on pulmonary blood vessels. The aim of this study was to examine the pulmonary vasorelaxant properties of two NONOates, MAHMA NONOate ((*Z*)-1-{*N*-Methyl-*N*-[6-(*N*-methylammoniohexyl)amino]}diazen-1-ium-1,2-diolate) and spermine NONOate ((*Z*)-1-{*N*-[3-Aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino}diazen-1-ium-1,2-diolate), that are reported to generate NO at different rates

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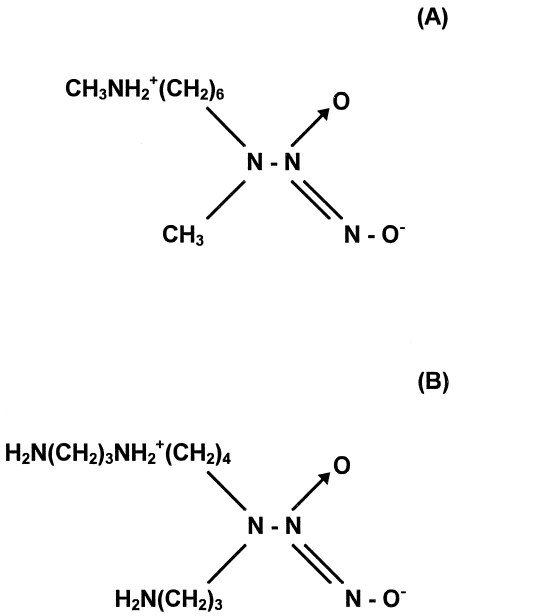

Fig. 1. Chemical structure of (A) MAHMA NONOate and (B) spermine NONOate.

(Fig. 1). In particular, the study was designed to (i) compare the potency of the two drugs and the time course of the relaxant responses, (ii) determine whether the drugs were equally effective in reversing contractions to a variety of physiologically important spasmogens and (iii) establish whether they induced tolerance, as occurs for some other types of NO donors. The rate of decomposition of each NONOate, under the experimental conditions used in the blood vessel experiments, and total NO production, have also been quantified.

A preliminary account of these data was presented to a meeting of the Australian Society of Clinical and Experimental Pharmacologists and Toxicologists, Canberra, December 1997 (Homer et al., 1997).

2. Materials and methods

2.1. Decomposition of NONOates

The rates of decomposition of MAHMA NONOate and spermine NONOate were determined spectrophotometrically using a Shimadzu UV spectrophotometer (Model No. UV-265, Shimadzu, Kyoto, Japan). Stock solutions of each NONOate (0.1 M) were prepared in sodium hydroxide (0.01 M) and added to 50 ml of physiological salt solution (PSS; composition in mM: NaCl 118; KCl 5.9; $CaCl_2$ 1.5; $MgSO_4$ 0.72; $NaHCO_3$ 25; glucose 11.7; Na_2EDTA 0.025; 95% O_2/5% CO_2) to produce a final NONOate concentration of 135 μM. The NONOate solution (pH 7.3) was maintained at 37°C throughout the experiment. Absorbance at wavelengths optimal for each NONOate (MAHMA NONOate 250 nm; spermine NONOate 252 nm) was measured, first within 10 s of the stock solution being added to the PSS (initial absorbance), and then intermittently for a total of either 10 min (MAHMA NONOate) or 4 h (spermine NONOate). In some experiments vascular rings, as used in isolated blood vessel experiments, were present in the solutions. The half-life of each NONOate was computed from plots of absorbance versus time for each individual experiment (GraphPad PRISM, version 2); mean half-lives from 4 experiments were expressed as geometric means with 95% confidence limits.

2.2. NO production from NONOates

Total NO production by each NONOate was determined as the concentration of its oxidation products, nitrite plus nitrate, in solution following decomposition of the parent nucleophile/NO complexes. Stock solutions (10 mM) of MAHMA NONOate and spermine NONOate were prepared in HCl (0.01 M and 1 M, respectively; pH ≤ 5) and allowed to decompose in sealed microfuge tubes until spectrophotometric measurements (at wavelengths shown in Section 2.1) indicated that decomposition of the nucleophile/NO complexes was complete. The concentrations of nitrite plus nitrate in dilutions of each stock solution were then determined using a commercial nitrite/nitrate colorimetric assay kit and a nitrate standard curve (Cayman Chemical, Ann Arbor, MI, USA). Plots of nitrite plus nitrate concentration vs. NONOate concentration were constructed from mean data obtained in 4 experiments.

2.3. Pulmonary artery preparations

On the day of the experiment, male Wistar rats (age 8–9 weeks; weight 303 ± 7.3 g, $n = 64$) were anaesthetised with pentobarbitone (120 mg/kg, i.p.), the thorax opened, and the main pulmonary artery or the lung removed.

2.3.1. Main pulmonary artery

Ring preparations (3 mm in length) of main pulmonary artery were cleared of connective tissue and mounted around two horizontal stainless steel wires in vertical organ baths containing PSS (37°C; 95% O_2/5% CO_2). In one series of experiments the endothelium was deliberately removed by rubbing the lumen of the preparation with forceps. Changes in force in the circular muscle were recorded isometrically with either a Statham Universal Transducer (UC3 + UL5) or a Grass FTO3 force displacement transducer attached to a micrometer (Mitutoyo, Tokyo, Japan). Preparations were set at a resting force of 10 mN to reflect in vivo pulmonary artery pressure, as in previous studies (Wanstall and O'Donnell, 1990; Wanstall et al., 1995). The preparations were then allowed to equilibrate for 1 h. During this time the PSS was changed every 15 min and the resting force readjusted if necessary.

2.3.2. Intralobar pulmonary artery

Intralobar pulmonary arteries (i.d. = 490–700 μm) were dissected from the left lung. Ring preparations (length = 1.3–2.0 mm), with the endothelium intact, were mounted on 40 μm diameter stainless steel wires in a small vessel myograph (Mulvany–Halpern type; Model 400A; AJP Trading, Aarhus, Denmark) containing PSS (37°C; 95% O_2/5% CO_2). The preparations were individually normalised to resting forces corresponding to 15 mm Hg (approximate in vivo transmural pressure). The mean resting force was 2.15 ± 0.16 mN ($n = 9$). The preparations were allowed to equilibrate for 30 min. Active force was recorded isometrically.

2.3.3. Protocols for pulmonary artery preparations

After equilibration, preparations were contracted submaximally with phenylephrine (0.1 μM; main pulmonary artery) or the thromboxane-mimetic, U46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$; 0.3 μM; intralobar pulmonary artery), and when the contraction reached equilibrium acetylcholine (0.3 μM) was added. A relaxant response to acetylcholine confirmed the presence of a functional endothelium. Preparations in which the endothelium was deliberately removed did not relax to acetylcholine. The preparations were then washed with PSS to restore baseline resting force. A reference contraction to K^+-depolarising PSS (in which 80 mM NaCl was replaced with 80 mM KCl) was obtained and the tissues were washed again. The preparations were then contracted submaximally with either phenylephrine (0.1 μM; main pulmonary artery only), U46619 (30 nM; main pulmonary artery only) or endothelin-1 (3 nM) and when the contraction was stable a cumulative concentration-response (relaxation) curve to MAHMA NONOate or spermine NONOate was obtained. When the spasmogen used to contract the preparation was endothelin-1, only one concentration-response curve was obtained per preparation, because endothelin-1 cannot be washed from the preparations. With the other spasmogens, the preparations were washed after the first concentration–response curve and, when resting baseline force was restored, the preparations were re-contracted. A concentration–response curve was then obtained to the other NONOate or, alternatively, one of the nucleophiles, drug solvents or nitrite were tested.

In some experiments on main pulmonary artery preparations two concentration–response curves were obtained to the same NONOate, first in the absence and then in the presence of 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ; 3 μM). The ODQ was added 30 min before the spasmogen contraction and remained in the bath during the concentration–response curve.

The time course of action of each NONOate was determined on main pulmonary artery preparations precontracted with phenylephrine (0.1 μM) by adding a single concentration of MAHMA NONOate (40 nM) or spermine NONOate (500 nM) and measuring the response at regular intervals for a total of 10 or 60 min, respectively.

The possible development of tolerance to glyceryl trinitrate and spermine NONOate (and cross tolerance between these drugs) was investigated on phenylephrine (0.1 μM)-contracted preparations of main pulmonary artery. A concentration–response curve to glyceryl trinitrate or spermine NONOate was obtained. The preparations were then incubated for 60 min in either PSS alone, or in the presence of 100 μM glyceryl trinitrate or 100 μM spermine NONOate (solution replaced every 15 min). After incubation, the preparations were washed 5 times over a 20 min period with PSS. Phenylephrine was immediately added to the bath and 10 min later a second concentration–response curve was obtained to either glyceryl trinitrate (on preparations pre-exposed to glyceryl trinitrate) or spermine NONOate (on preparations pre-exposed to either spermine NONOate or glyceryl trinitrate).

2.3.4. Analysis of data

Contractile responses to vasoconstrictor spasmogens were measured as increases in force (mN) from the resting baseline. Relaxant responses to the vasorelaxant drugs were measured as the difference between the peak relaxation for each concentration of drug and the steady state of the spasmogen precontraction. They were expressed as percentage reversal of the spasmogen-induced contraction and plotted against molar concentration of drug on a logarithmic scale. EC_{50} values (where EC_{50} is the concentration to give 50% of the maximum response to the particular drug) were interpolated from these plots. The potency of the NONOates was expressed as the negative log EC_{50}. In experiments to determine the time course of action of each drug, response (as % reversal) was plotted against time.

2.4. Drugs and solutions

The following drugs were used: acetylcholine chloride (Sigma), endothelin-1 (human, porcine; Auspep), glyceryl trinitrate (Pohl), MAHMA NONOate (Cayman), ODQ (1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one; Tocris Cookson), phenylephrine hydrochloride (Sigma), spermine NONOate (Cayman), spermine diphosphate (Sigma), sodium nitrite (BDH), and U46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$; Sigma). Stock solutions were prepared as follows: acetylcholine (10 mM), endothelin-1 (10 μM) and sodium nitrite (10 mM) in deionised water; ODQ (10 mM) in dimethyl sulfoxide; phenylephrine (10 mM) in 0.01 M HCl; U46619 (10 mM) in absolute ethanol; glyceryl trinitrate was purchased in ampoules (22 mM in ethanol); MAHMA and spermine NONOate (0.1 M) were prepared in 0.01 M NaOH unless stated otherwise. Dilutions were prepared in PSS, except the NONOate dilutions which were prepared in 0.01 M NaOH; all dilutions were kept on ice during the course of

an experiment. None of the solvents, at the concentrations used, affected blood vessel tone. In order to test the effects of the nucleophiles (see Section 2.3.3), a solution of spermine (100 mM in 1 M HCl) was prepared using spermine diphosphate, and MAHMA was prepared by allowing a solution of MAHMA NONOate (10 mM in 0.01 M HCl) to decompose for at least 16 h.

2.5. Statistical analysis

Mean values were calculated from data in preparations from a number (n) of different animals and are quoted together with S.E.M., with the exception of geometric mean half-life values which are quoted with 95% confidence limits. The statistical significance of differences between mean responses, expressed as % values (not necessarily normally distributed), was assessed by Mann–Whitney U-test (comparison of 2 values) or Kruskal–Wallis test (comparison of more than 2 values). For all other values, Student's t-test (comparison of 2 values) or One-way analysis of variance (ANOVA) followed, when appropriate, by Tukey–Kramer post hoc test (comparison of 3 values) was used.

2.6. Ethical approval

The experiments in this study were undertaken with the approval of The University of Queensland Animal Experimentation Ethics Committee.

3. Results

3.1. Decomposition of MAHMA NONOate and spermine NONOate

At pH 7.3 and 37°C, absorbance values of solutions of MAHMA NONOate and spermine NONOate in PSS decreased over time, indicating decomposition of the respective nucleophile/NO complexes under these experimental conditions. The half-lives for each NONOate, derived from the decomposition data, are shown in Table 1. The half-lives were the same whether or not vascular tissue was present (Table 1). MAHMA NONOate decomposed much more rapidly, i.e., had a shorter half-life, than spermine NONOate.

Table 1
Half-lives of MAHMA NONOate and spermine NONOate at 37°C and pH 7.3

	Half-life (min)	
	MAHMA NONOate	Spermine NONOate
No vascular tissue	1.3 (1.18–1.37) ($n = 4$)	73 (68.9–77.5) ($n = 4$)
Vascular tissue present	1.2 (1.05–1.37) ($n = 4$)	73 (70.6–75.9) ($n = 4$)

Values are geometric means with 95% confidence intervals in parentheses.
n = number of experiments.
Half lives were determined in physiological salt solution.
There was no significant difference between values in the presence and absence of vascular tissue (Student's t-test).

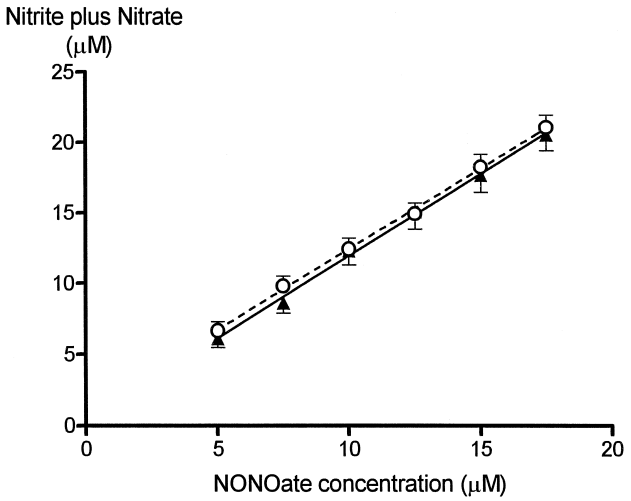

Fig. 2. Nitrite plus nitrate production from different concentrations of MAHMA NONOate (○) and spermine NONOate (▲). Values represent mean concentration ± S.E.M. (shown by vertical bars; $n = 4$). The lines represent the mean regression lines (MAHMA NONOate, broken line; spermine NONOate, solid line). The slopes of the regression lines were: MAHMA NONOate 1.14 ± 0.02, spermine NONOate 1.17 ± 0.03 ($n = 4$) ($P > 0.05$; Student's t-test).

3.2. NO production from MAHMA NONOate and spermine NONOate

Total nitrite plus nitrate production (representing NO production) following decomposition of each NONOate is shown in Fig. 2. The plots of nitrite plus nitrate vs. molar concentration of NONOate were linear (correlation coefficient MAHMA NONOate 0.999, spermine NONOate 0.998). The slopes of these plots indicated that, at the concentrations examined, between 1 and 2 mol of NO were produced per mol of NONOate; the values were the same for both drugs (Fig. 2).

3.3. Vasorelaxant effects of MAHMA NONOate and spermine NONOate on main and intralobar pulmonary arteries from rats

On main pulmonary artery preparations, MAHMA NONOate and spermine NONOate completely reversed the contractions induced by phenylephrine, U46619 and endothelin-1, i.e., maximum relaxation corresponded to 100% reversal. For each NONOate the potency (negative log EC_{50}) was the same irrespective of the spasmogen used to contract the preparations (Table 2). Spermine NONOate was significantly ($P < 0.01$) less potent (10–40 fold) than

Table 2
Potency (negative log EC_{50}) of MAHMA NONOate and spermine NONOate on rat main pulmonary artery and intralobar pulmonary artery contracted with different spasmogens

Tissue	Spasmogen[a]	Mean negative log $EC_{50} \pm$ S.E.M.	
		MAHMA NONOate	Spermine NONOate
Main pulmonary artery	Phenylephrine	7.37 ± 0.09 (8)	6.19 ± 0.07[b] (12)
	U46619	7.33 ± 0.10 (4)	6.36 ± 0.16[b] (4)
	Endothelin-1	7.61 ± 0.19 (4)	6.04 ± 0.14[b] (4)
Intralobar pulmonary artery	Endothelin-1	6.86 ± 0.15[c] (5)	5.86 ± 0.04[b] (4)

Values are means ± S.E.M. (numbers of preparations in parentheses).
[a]Concentrations of spasmogens were: phenylephrine, 0.1 μM; U46619, 30 nM; endothelin-1,3 nM. For each NONOate, potency values did not differ significantly on main pulmonary artery preparations contracted with different spasmogens (One-way ANOVA).
[b]Value significantly less than corresponding value for MAHMA NONOate $P < 0.01$ (Student's *t*-test).
[c]Value significantly less than on main pulmonary artery preparations contracted with endothelin-1 $P < 0.05$ (One-way ANOVA followed by Tukey–Kramer post-hoc test).

MAHMA NONOate (Table 2). The potencies in the absence of endothelium (phenylephrine contracted preparations, negative log EC_{50}: MAHMA NONOate 7.51 ± 0.06; spermine NONOate 6.35 ± 0.10; $n = 4$) were no different from those in the presence of endothelium (Table 2). The nucleophiles, viz. MAHMA and spermine, had no vasorelaxant effect on main pulmonary artery when tested at concentrations corresponding to the concentrations of NONOates that gave maximum relaxation. Nitrite, an oxidation product of NO, relaxed the preparations but only at concentrations ≥ 200 μM, i.e., greater than the highest amounts possibly produced from the concentrations of NONOates studied.

On intralobar pulmonary artery, MAHMA NONOate and spermine NONOate completely reversed contractions induced by endothelin-1. For MAHMA NONOate, but not spermine NONOate, the potency was significantly ($P < 0.05$) less than on endothelin-1 contracted main pulmonary artery (Table 2).

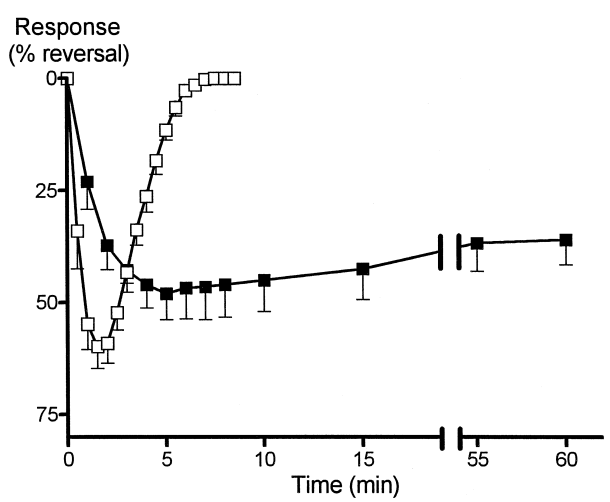

Fig. 3. Mean relaxant responses to MAHMA NONOate (40 nM, □) and spermine NONOate (500 nM, ■) over time. Values represent mean responses ± S.E.M. (shown by vertical bars; $n = 4$), expressed as percentage reversal of the contraction to phenylephrine (0.1 μM). Note that the time axis is interrupted between 15 and 55 min.

The time courses of responses on main pulmonary artery to each NONOate at concentrations approximately equal to the EC_{50} are shown in Fig. 3. The peak relaxation

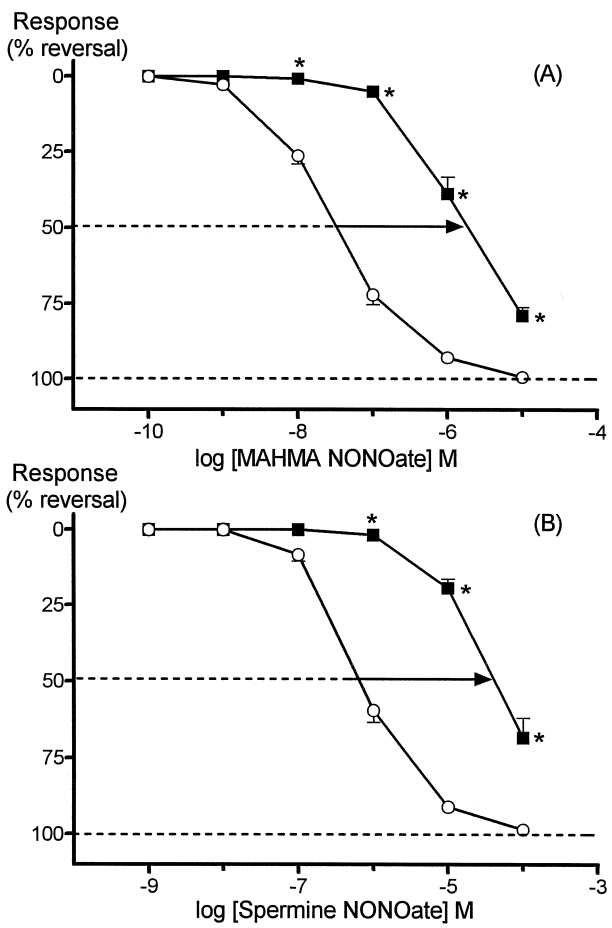

Fig. 4. Mean concentration–response curves to (A) MAHMA NONOate and (B) spermine NONOate in the absence (○) and presence (■) of ODQ (3 μM). Values represent mean responses ± S.E.M. (shown by vertical bars; $n = 4$) expressed as percentage reversal of the contraction to phenylephrine (0.1 μM). The stippled lines correspond to 100% reversal of the phenylephrine contraction. * Response significantly less than corresponding response in the absence of ODQ ($P < 0.05$; Mann–Whitney *U*-test).

to MAHMA NONOate (40 nM) was achieved in 1.5 min but relaxation was no longer present after 7 min (Fig. 3). In contrast, peak relaxation to spermine NONOate (500 nM) was achieved in 5 min and relaxation was maintained for at least 60 min (Fig. 3), i.e., the response after 60 min was not significantly different from the response after 5 min ($P > 0.05$; Kruskal–Wallis test).

3.4. The effects of the guanylate cyclase inhibitor, ODQ, on relaxant responses to MAHMA NONOate and spermine NONOate

Relaxant responses on main pulmonary artery to both MAHMA NONOate and spermine NONOate were significantly inhibited by 3 μM ODQ (Fig. 4). ODQ caused a parallel shift in the concentration-response curves to the NONOates. The shifts in the curves, measured at the level corresponding to 50% reversal of the pre-contraction (indicated by the arrows in Fig. 4), were 55- and 70-fold for MAHMA NONOate and spermine NONOate, respectively.

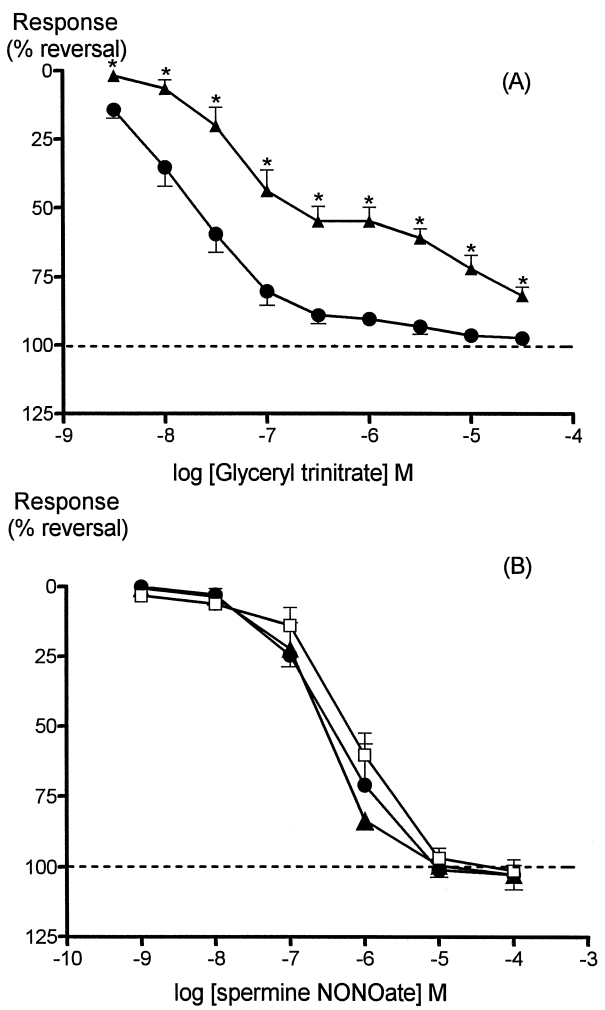

Fig. 5. Mean concentration–response curves to (A) glyceryl trinitrate or (B) spermine NONOate. Data obtained after 1 h incubation in either physiological salt solution (PSS) alone (●) or in PSS containing either 100 μM glyceryl trinitrate (▲) or 100 μM spermine NONOate (□). Values represent mean responses ± S.E.M. (shown by vertical bars; $n = 4$) expressed as percentage reversal of the contraction to phenylephrine (0.1 μM). The stippled lines correspond to 100% reversal of the phenylephrine contraction. ∗Responses significantly less than corresponding responses on preparations incubated in PSS alone $P < 0.05$ (Mann–Whitney *U*-test).

3.5. Tolerance and cross tolerance to glyceryl trinitrate and spermine NONOate

Pre-exposure of main pulmonary artery preparations to 100 μM glyceryl trinitrate induced tolerance to glyceryl trinitrate, as indicated by a significant decrease in responses at all concentrations examined (Fig. 5A). In contrast concentration–response curves to spermine NONOate were not affected by pre-exposure of arteries to either spermine NONOate (100 μM) or glyceryl trinitrate (100 μM) (Fig. 5B). No tolerance experiments were performed with MAHMA NONOate because of its short half life (see Section 3.1).

4. Discussion

In this study the in vitro pulmonary vascular effects of two drugs belonging to the novel class of NO donors known as NONOates have been systematically examined. MAHMA NONOate and spermine NONOate were effective vasorelaxants on main and intralobar pulmonary arteries precontracted with various physiological vasoconstrictors, but differed in both potency and time course of action. Vasorelaxant responses were neither potentiated nor diminished by removal of the endothelium.

The two particular NONOates examined were selected for study because reports in the literature indicated that the rates at which they decompose to generate NO are markedly different. This was demonstrated in the present study with the half-life of MAHMA NONOate being 56-fold less than that of spermine NONOate. For both drugs the half-lives (1.3 and 73 min, respectively) were within the range of values cited in the literature (Keefer et al., 1996). The values were unaltered by the presence of blood vessel rings, confirming that vascular tissue is not required for the decomposition of these compounds to generate NO.

The total amount of NO that a NONOate can generate varies for different compounds. The theoretical maximum value is 2 mol of NO per mol of parent compound (Maragos et al., 1991) but at neutral pH it is usually less than this (Feelisch and Stamler, 1996). In this study NO production from each compound was determined indirectly, as nitrite plus nitrate, following decomposition at pH ≤ 5. Even under these acid conditions the total amount of NO produced was less than 2 mol per mol of NONOate, but was the same for each of the two NONOates examined.

Our data suggest that, as anticipated, production of NO accounts for the pulmonary vasorelaxant responses produced by the NONOates. This conclusion is based on the following experimental observations. First, neither of the nucleophiles, MAHMA or spermine, caused relaxation at concentrations corresponding to the concentrations of the NONOates used. It was important to establish this, particularly for spermine, because high concentrations of spermine have previously been reported to have non-specific smooth muscle relaxant properties (De Meis, 1967). Second, the relaxant responses could not be attributed to nitrite, the oxidation product of NO. Finally, the responses were inhibited by ODQ, a selective inhibitor of soluble guanylate cyclase (Garthwaite et al., 1995), which is the enzyme involved in at least part of the relaxation induced by NO.

The inhibition of guanylate cyclase by ODQ is reported to be either non-competitive (Garthwaite et al., 1995) or irreversible, competitive (Schrammel et al., 1996). Hence, ODQ was expected to cause a non-parallel shift in the concentration–response curves to the NONOates, together with a depression in the maximum responses. The finding that ODQ caused parallel shifts in the NONOate concentration–response curves could indicate that part of the relaxation induced by the NO derived from these two drugs is independent of the guanylate cyclase/cyclic GMP pathway. A cGMP-independent component of vasorelaxation has recently been suggested for another NO donor, *S*-nitrosoglutathione (Brunner et al., 1996).

Our finding that the NONOates decomposed (and hence, by implication, generated NO) in the absence of vascular tissue confirms the findings of others (Morley et al., 1993). Furthermore it distinguishes the drugs from NO donors, such as the organic nitrates, which require the presence of tissue thiols and activation by tissue enzymes (Bauer et al., 1995). One of the clinical problems associated with organic nitrates is the development of tolerance. Tolerance is defined as an attenuation of responses to a NO donor in tissues that have been exposed to a high concentration of the same drug. It occurs in vivo (Van de Voorde et al., 1989) and can also be demonstrated in vitro (Henry et al., 1989). Tolerance is most evident with those NO donors that require tissue activation (Henry et al., 1989) but it is also seen, at least in vitro, with some NO donors that do not require enzymatic activation (e.g., 3-morpholinosydonimine (SIN-1); Henry et al., 1989; Kukovetz and Holzmann, 1989). Hence it was important to establish whether or not tolerance to the NONOates would develop. In the present study, using an experimental protocol that clearly illustrated tolerance to glyceryl trinitrate, no tolerance to spermine NONOate was observed. Furthermore responses to spermine NONOate were not diminished in preparations that had been made tolerant to glyceryl trinitrate, i.e., there was no cross-tolerance. It was not practical to investigate whether tolerance develops to MAHMA NONOate using this protocol. Due to its short half-life, it would have been necessary to replace MAHMA NONOate constantly during the incubation period, requiring the use of excessive quantities of this drug.

Studies with other NO donors, and also with NO itself, have indicated that these drugs are generally less potent on intralobar pulmonary arteries (resistance vessels) than on main pulmonary arteries (conduit vessels) (NO, Archer et al., 1996; sodium nitroprusside, Wanstall et al., 1997a; FK409, Wanstall et al., 1997b; SIN-1, Wanstall, unpublished). This potency difference may reflect (i) the involvement of calcium activated potassium channels in responses to NO and, (ii) a relative paucity of these channels in vascular smooth muscle cells from resistance compared with conduit pulmonary arteries (Archer et al., 1994, 1996). In keeping with this general trend, MAHMA NONOate was likewise less potent (6-fold) on the resistance vessels than on conduit pulmonary arteries. Surprisingly, spermine NONOate was virtually equipotent on both vessel types; the reason for this anomalous result is unknown at present.

Although both NONOates were effective pulmonary vasorelaxants, they differed in potency, with MAHMA NONOate being the more potent. They also differed in their onset and duration of action, with spermine NONOate having the slower time course, i.e., peak relaxation was achieved more slowly and the response was sustained for longer when compared with responses to MAHMA NONOate. Maragos et al. (1991) predicted that the potency of NONOates will depend on rate of NO release as well as total NO production. The overall NO production was the same for both NONOates. Hence, the difference in potency must be due to the differing rates of decomposition of each NONOate to produce NO (present study), together with the short half life of NO (Kelm and Yoshida, 1996). Initially, the amount of NO in solution produced by MAHMA NONOate would be greater than that produced by the same concentration of spermine NONOate, accounting for the observation that MAHMA NONOate was the more potent drug, i.e., had the lower EC_{50}. Spermine NONOate, with its longer half-life, would continue to release NO into solution long after most of the NO from MAHMA NONOate had been released and subsequently oxidised. This would account for the longer duration of action seen for spermine NONOate.

In summary, this study on the pulmonary vascular effects of NONOates was undertaken because this group of NO donors may have potential in the treatment of pulmonary hypertensive patients. This is the first detailed in vitro study of NONOates in pulmonary artery preparations. The results obtained with two different NONOates suggest that drugs in this group possess properties which could be considered useful in pulmonary hypertension. First, each drug effectively relaxed pulmonary artery preparations precontracted with a variety of physiological vasoconstrictors. This is important because elevated pulmonary vascular tone in pulmonary hypertension may involve a number of different vasoconstrictors (Barnes and Liu, 1995). Second,

the drugs remained effective in the absence of the endothelium. This is important because endothelial cells are reported to be functionally impaired in patients with pulmonary hypertension (Dinh-Xuan et al., 1991). Third, they were effective on small intralobar (resistance) arteries which contribute most to the elevated pulmonary vascular resistance in pulmonary hypertension. Finally, the NONOates, unlike some of the other NO donors, do not appear to induce tolerance; however this remains to be confirmed in vivo.

As potential therapeutic agents, NONOates have an advantage over other NO donors in that the choice of nucleophile can determine the duration of biological action, as illustrated in the present study. In pulmonary hypertension, a short-acting NONOate could be a useful alternative to prostacyclin for screening pulmonary hypertensive patients to identify those patients that are suitable candidates for long-term treatment with vasodilator drugs. A long-acting NONOate could be a suitable long-term therapeutic agent requiring intermittent, as opposed to continuous, administration. If the NONOates are to be used for pulmonary hypertension, selectivity for the pulmonary circulation would be required to avoid systemic vasodilatation. Since this study commenced, there have been three reports of attempts to achieve pulmonary selectivity by administering NONOates directly into the lungs of animals, i.e. to achieve selectivity by route of administration (Hampl et al., 1996; Brilli et al., 1997; Adrie et al., 1998). An alternative means of achieving pulmonary selectivity could conceivably be to develop a NONOate with a nucleophile that is, in itself, selective for the lung.

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