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Characterization of the vasodilator properties of peroxynitrite on rat pulmonary artery: role of poly (adenosine 5′-diphosphoribose) synthase

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1 The pulmonary vasculature is constantly exposed to oxygen and reactive oxygen species such as nitric oxide (NO) and superoxide anions which can combine at a near diffusion limited rate, to form the powerful oxidant, peroxynitrite ($ONOO^-$). When formed in large amounts, $ONOO^-$ is thought to contribute to tissue injury and vascular dysfunction seen in diseases such as the acute respiratory distress syndrome (ARDS) and septic shock. Recent studies have shown that $ONOO^-$ can cause vasodilatation and at higher concentrations can activate poly (adenosine 5′-diphosphoribose) synthase (PARS) leading to consumption of nicotinamide adenine dinucleotide (NAD^+) and adenosine 5′-triphosphate (ATP). As the lung represents a prime site for $ONOO^-$ formation, we characterized its effects on pulmonary vascular tone and on endothelial function. In addition, we have assessed the role of PARS in producing the vasoactive properties of $ONOO^-$ on pulmonary artery rings.

2 Isolated pulmonary artery rings from rats were mounted in organ baths containing warmed and gassed (95% O_2: 5% CO_2) Krebs buffer. Force was measured with isometric force transducers. After equilibration, $ONOO^-$ (10 nM – 100 μM) was added in a cumulative manner. In separate experiments designed to assess any vasodilator properties of $ONOO^-$, tissues were pre-contracted with the thromboxane mimetic U46619 (1 μM). Once a stable base-line was achieved, $ONOO^-$ was added in a cumulative fashion. $ONOO^-$ had no significant effect on resting pulmonary artery tone but caused concentration-dependent relaxations of pre-contracted vessels in the range 1 μM to 100 μM. In some experiments the effects of freshly prepared $ONOO^-$ solutions were compared with those allowed to decay at 4°C for 2 days.

3 In some experiments either vehicle or $ONOO^-$ (1, 10 or 100 μM) was added for 15 min before U46619 (1 μM). Concentration-response curves to the endothelium-dependent vasodilator, acetylcholine (10 nM – 100 μM) were then constructed. In these experiments, $ONOO^-$ (1 μM or 10 μM) had no effect on the actions of acetylcholine. However, at the highest concentration tested (100 μM), $ONOO^-$ increased acetylcholine-induced relaxations.

4 The vasodilator actions of $ONOO^-$ were unaffected by the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM) or by removal of superoxide anions with superoxide dismutase (SOD) (30 units ml^{-1}). However, the relaxations induced by $ONOO^-$ were significantly inhibited by the PARS inhibitor, 3-aminobenzamide (10 μM). In contrast to its effects on $ONOO^-$, 3-aminobenzamide had no effect on the relaxation caused by acetylcholine or sodium nitrite, but actually increased that induced by sodium nitroprusside.

5 These data show that $ONOO^-$ causes vasodilatation of rat pulmonary arteries, probably via activation of PARS. Moreover, at concentrations where relaxation was achieved, $ONOO^-$ did not affect the ability of pulmonary artery rings to relax to acetylcholine. We propose that $ONOO^-$, but not endothelially derived NO, activates PARS resulting in the rapid depletion of ATP and a consequent reduction in contraction as well as other active processes of vascular smooth muscle. The finding that 3-aminobenzamide inhibited the actions of $ONOO^-$ but not acetylcholine, suggests that NO and $ONOO^-$ cause relaxation by independent mechanisms. It has been suggested that $ONOO^-$ is responsible for the vascular hyporesponsiveness to constrictor agents seen in experimental sepsis. This observation together with our current finding, that 3-aminobenzamide inhibits the relaxation induced by $ONOO^-$ but not by acetylcholine, suggests that inhibitors of PARS may reduce the persistent hypotension seen in sepsis without affecting the actions of endothelium-derived NO. Thus, the use of PARS inhibitors may represent a novel therapeutic approach to the treatment of septic shock.

Keywords: Rat pulmonary arteries; peroxynitrite; poly-ADP ribosyltransferase; 3-animobenzamide; superoxide dismutase (SOD); NO; N^G-nitro-L-arginine methyl ester (L-NAME)

Introduction

Under physiological conditions, a powerful vasodilator, nitric oxide (NO) is continuously released by endothelial cells to regulate organ blood flow and perfusion pressure (Moncada *et al*., 1991). Endothelial-derived NO formation is catalysed by endothelial NO synthase (eNOS; Pollock *et al*., 1991), which by virtue of its calcium dependency, forms discreet quanta of NO. Under inflammatory conditions, such as occur in septic shock (Salter *et al*., 1991; Mitchell *et al*., 1993) a calcium-independent isoform of NOS is induced (iNOS; Busse & Mulsch, 1990) that produced copious amounts of NO, a process which is thought to contribute to the fall in blood pressure seen in clinical sepsis (Petros *et al*., 1991). Production of large

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amounts of NO by iNOS in the vascular smooth muscle has been associated with suppression of mitochondrial respiration (Gent *et al*., 1992) and a reduction of arterial contractility.

During inflammatory events, such as sepsis, the presence of large amounts of activated leukocytes results in elevated levels of superoxide anions, formed by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system. The coupled production of excess NO and superoxide leads to the formation of an unstable intermediate termed peroxinitrite ($ONOO^-$) (Beckman *et al*., 1990). $ONOO^-$ formation is kept to a minimum under normal conditions by endogenous superoxide dismutase (SOD), which removes superoxide, and by the limited capacity of eNOS to form NO. However, when iNOS is expressed in conditions where superoxide formation is increased, or SOD activity is decreased, $ONOO^-$ is formed in excess. $ONOO^-$ is commonly described as a toxic oxidant that inhibits cellular respiration (Radi *et al*., 1994; Salgo *et al*., 1995; Szabó *et al*., 1996) and may contribute to endothelial dysfunction in diseases such as septic shock (Liu *et al*., 1994; Villa *et al*., 1994; Kooy *et al*., 1995). $ONOO^-$ inhibits cellular respiration directly by inhibiting mitochondrial electron transport (Radi *et al*., 1994) and indirectly by activating the nuclear enzyme, poly (adenosine 5′-diphosphoribose) synthase (PARS; Salgo *et al*., 1995). $ONOO^-$ causes DNA breaks which consequently triggers a futile cycle by the PARS pathway, resulting in depletion of nicotinamide adenine dinucleotide (NAD^+) and adenosine 5′-triphosphate (ATP) (Zhang *et al*., 1994).

$ONOO^-$ is a vasodilator of some vascular preparations *in vitro* including rat isolated perfused hearts (Moro *et al*., 1994), bovine pulmonary artery (Wu *et al*., 1994) and canine coronary artery (Liu *et al*., 1994). Moreover, in the rat aorta, activation of PARS by endogenous $ONOO^-$ accounts for the vascular hyporeactivity seen in this tissue after experimental sepsis (Szabó *et al*., 1996). However, the role of PARS activation in $ONOO^-$-induced vasodilatation of pulmonary vessels has not been addressed and the potential role of PARS activation in smooth muscle relaxation induced by endothelium-derived NO has not been studied.

Since the pulmonary circulation represents a prime site for oxidant formation, we have characterized the effects of $ONOO^-$ on vascular tone in rat isolated pulmonary arteries. Secondly, we have assessed the role of PARS, by use of the specific inhibitor, 3-aminobenzamide (Banasik *et al*., 1992), in modulating vascular responses induced by $ONOO^-$, the endothelium-dependent vasodilator acetylcholine, and the direct smooth muscle relaxant, sodium nitroprusside. Finally, we have assessed the effects of $ONOO^-$ on the ability of vessels to relax to endothelium-dependent and -independent dilator drugs.

Methods

Preparation of $ONOO^-$

$ONOO^-$ was prepared as described by Beckman *et al*. (1990). Briefly, equal volumes (6 ml) of freshly prepared sodium nitrate (2 M; in distilled water) and hydrogen peroxide (2.5 M in 2.9 M nitric acid) were combined in a reaction vessel, and the resultant intense yellow coloured solution stabilized by the immediate addition of 6.6 ml of 4.2 M sodium hydroxide. All solutions used in the process were kept chilled over ice. Excess hydrogen peroxide was removed from the $ONOO^-$ by passing it through a manganese dioxide column. The solution was then divided into aliquots and stored at -20°C for no longer than two weeks before use. Once frozen, $ONOO^-$ accumulates at the top of the frozen solution and can, on partial defrosting, be easily removed in a more concentrated form. The concentration of $ONOO^-$ was determined by use of its molar extinction coefficient (1670 M^{-1} cm^{-1} at $A_{302\,nm}$). The concentration of $ONOO^-$ was measured daily. The mean concentration of all solutions used in this study was 25.5 mM (range: 16.2 to 55.1 mM). The pH of the stock solution of $ONOO^-$ was 11.8. $ONOO^-$ was diluted in distilled water to obtain adequate concentrations, just before application to the vascular rings. Using rat isolated pulmonary artery, we have found that drugs have to be added at 100 fold dilution (maximum of 20 μl into 2 ml for each concentration) to avoid disruptions in tension due to volume changes in the bath. Thus, the highest concentration of $ONOO^-$ that could be consistently added was 100 μM. For this reason, we were unable to complete the concentration-response curve for $ONOO^-$. Control solutions (vehicle) were prepared by combining the three solutions described above in the absence of sodium nitrite or hydrogen peroxide prepared to similar pH (11.9). Distilled water was used to dilute carrier solutions in the same manner as that used to dilute the $ONOO^-$ solution.

Tissue preparation and organ bath experiments

The investigation was performed in accordance with the Home Office Animals (Scientific Procedure) Act 1986, published by Her Majesty's Stationery Office, London. Male Wistar rats (weight 275–300 g) were killed by cervical dislocation. Pulmonary arteries were dissected and cut into rings 2-mm wide. From each rat, 4 rings were usually obtained. The rings were mounted over a pair of rigid wires, and suspended in 2 ml organ baths containing oxygenated (95% O_2–5% CO_2) Krebs-Henseleit solution at 37°C with the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25.5 and glucose 5.6. One of the wires was fixed, and one attached to a force transducer (FT. 03 Grass Instruments, Quincy, MA). Changes in isometric force were recorded on a polygraph multichannel recorder (Grass Model 7). A uniform baseline tension of 500 mg was applied. After 20 min of equilibration, the rings were constricted with KCl (40 mM) and relaxed to baseline by repeated washing with Krebs-Henseleit solution. Rings were left to equilibrate in the bath for a total of 45 min and washed every 20 min. $ONOO^-$ or carrier solution was then added in a cumulative fashion (10 nM–100 μM) to pulmonary artery rings that were either pre-contracted by approximately 500 mg with the thromboxane mimetic, U46619 (1 μM) or left under basal tension. In separate experiments, the effects of freshly prepared solutions of $ONOO^-$ were compared with those allowed to decay for 48 h, which had lost 65% of their original $ONOO^-$. In some experiments the PARS inhibitor, 3-aminobenzamide (1 or 10 mM; Banasik *et al*., 1992), the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM) or SOD (30 units ml^{-1}) were added 15 min before U46619.

In experiments designed to assess the effects of $ONOO^-$ on endothelium-dependent responses, $ONOO^-$, carrier solution or Krebs-Henseleit buffer was added at different concentrations, before the addition of U46619 (1 μM). Dilator responses were then recorded in each ring to increasing concentrations of acetylcholine (10 nM–100 μM).

Drugs

Acetylcholine chloride, sodium nitroprusside, L-NAME, SOD, 3-aminobenzamide and U46619 (9,11-dideoxy-11α,9α epoxymethanoprostaglandin $F_{2\alpha}$ were obtained from Sigma (Poole, Dorset, U.K.). Drugs were dissolved in distilled water except for L-NAME and 3-aminobenzamide, which were dissolved in Krebs-Henseleit solution. All solutions were prepared on the day of the experiment.

Statistical analysis

Results are expressed as mean$\pm$s.e.mean for n (separate animals) experiments. Relaxations in response to $ONOO^-$, carrier solution, acetylcholine, sodium nitroprusside or sodium nitrite are expressed as the percentage of induced tone. Data were analysed by either one- or two-way analysis of variance as suggested by GraphPAD INSTAT, GraphPAD Software, San Diego, CA and GraphPAD PRISM,

version 2. A P value less than 0.05 was considered to indicate statistical significance.

Results

Effects of $ONOO^-$ on spontaneously or U46619 contracted vessels

In quiescent rings, $ONOO^-$ (10 nM to 100 μM) had no significant relaxant effect and at the highest concentration used, caused a slight but not significant (one-way ANOVA) contraction in rat pulmonary arteries ($n=8$; data not shown). In order to demonstrate a vasorelaxant effect of $ONOO^-$, the study was continued in pre-constricted vessels. The thromboxane mimetic U46619 (1 μM) caused a consistent (534 ± 17 mg) and long-lasting (at least 1 h) increase in tone. In rings pre-contracted with U46619, $ONOO^-$ induced concentration-dependent relaxations, with an apparent E_{max} of $65\pm7.3\%$ at 100 μM. The carrier solution for $ONOO^-$ caused a slight relaxation of pre-contracted rings, although its effects were significantly less than those observed with $ONOO^-$ (Figure 1). $ONOO^-$ which had been allowed to decay for 48 h was significantly less potent as a vasodilator than the freshly prepared solution (Figure 2). Sodium nitrite also caused concentration-dependent relaxations of pre-contracted rat pulmonary artery.

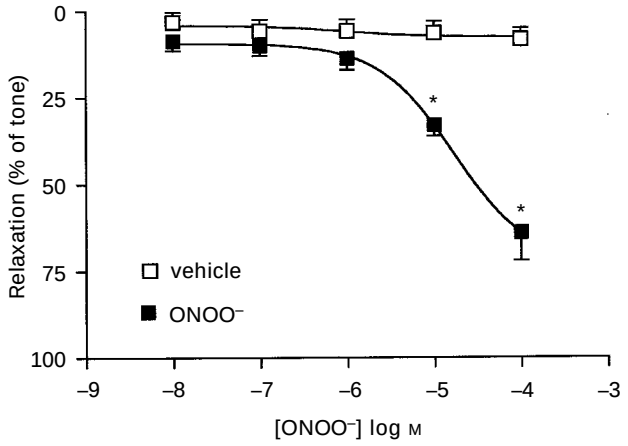

Figure 1 Vasodilator effects of $ONOO^-$ on rat pulmonary artery. Tissues were pre-contracted with U46619 as described in the Methods section. Concentration-response curves for peroxynitrite (■) were compared with volume matched additions of carrier solution (□). Results are the mean of 6 (carrier solution) or 18 ($ONOO^-$) separate experiments; vertical lines show s.e.mean. A statistically significant difference (denoted by $*P<0.05$) was found by upaired t test at the concentrations illustrated between the responses to $ONOO^-$ and the responses to the carrier solution.

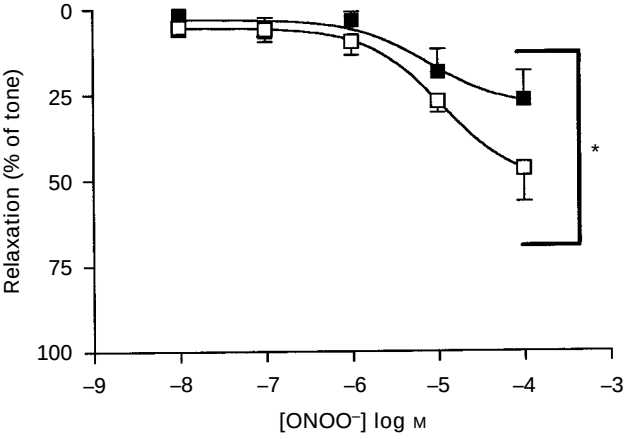

Figure 2 Comparison of the potency of freshly prepared $ONOO^-$ (□) with solutions allowed to decay for 2 days at 4°C (■). Tissues were pre-contracted with U46619 according to the Methods section. Results are the mean of 5–8 separate experiments; vertical lines show s.e.mean. A statistically significant difference (denoted by $*P<0.05$) was found by 2-way ANOVA between the responses to freshly prepared $ONOO^-$ solutions and those allowed to decay for 2 days.

Characterization of the effects of pre-exposure to $ONOO^-$ on endothelium-dependent vasodilatation

After pre-contraction with U46619 (1 μM), acetylcholine (10 nM – 100 μM; Figure 3) caused concentration-dependent

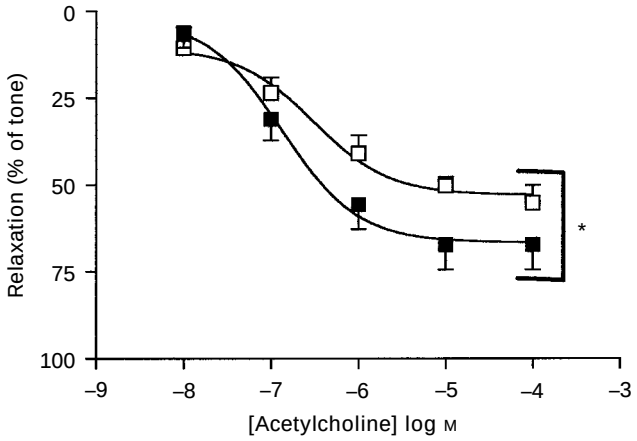

Figure 3 Effect of pre-exposure of pulmonary artery to $ONOO^-$ (100 μM) on the vasodilator properties of acetylcholine. Tissues were pre-contracted with U46619 according to the Methods section. The response of pulmonary arteries to acetylcholine in vessels pretreated with carrier solution (□) and the responses after exposure to $ONOO^-$ (■) are shown. Results are the mean of 5 separate experiments; vertical lines show s.e.mean. A statistically significant difference (denoted by $*P<0.05$) was found by 2-way ANOVA between the responses of vessels to acetylcholine with or without pre-exposure to $ONOO^{-1}$. In the same experiments $ONOO^{-1}$ at 1 μM or 10 μM did not significantly affect the vasodilator properties of acetycholine ($n=5$).

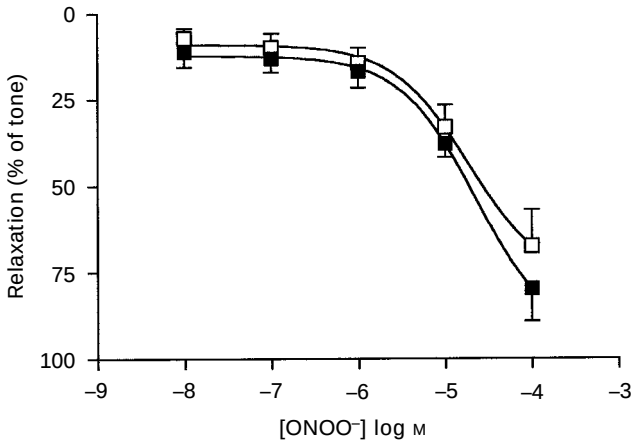

Figure 4 Effect of L-NAME (100 μM) on the vasodilator actions of $ONOO^-$. Tissues were pre-contracted with U46619 according to the Methods section. Control response to $ONOO^-$ (■) and in the presence of L-NAME (□) are shown. Results are the mean of 5 separate experiments; vertical lines show s.e.mean. No difference was seen in the ability of $ONOO^-$ to relax tissues in the presence or absence of L-NAME. In similar experiments, L-NAME (100 μM) inhibited the vasodilatation caused by acetylcholine by more than 60%.

vasodilatation. The vasodilator response to acetylcholine was increased after pretreatment with $ONOO^-$ at 100 μM (Figure 3), but was unaffected (not significant by two-way ANOVA) by $ONOO^-$ at 1 μM or 10 μM (data not shown; $n=6$).

Effects of pretreatment with L-NAME, SOD and 3-aminobenzamide on $ONOO^-$ concentration-response curves

The vasodilator properties of $ONOO^-$ were unaffected by pretreatment with L-NAME (100 μM; Figure 4) or SOD (30 units ml^{-1}), (data not shown; $n=7$). However, 3-aminobenzamide at 10 mM but not at 1 mM (Figure 5) significantly inhibited the relaxations induced by $ONOO^-$. By contrast, 3-aminobenzamide (10 mM) had no effect on the vasodilator properties of either acetylcholine (Figure 6) or sodium nitrite (Figure 7). However, 3-aminobenzamide (10 mM) increased the relaxations induced by sodium nitroprusside (Figure 8).

Discussion

Here we show that $ONOO^-$ relaxes pre-contracted rat pulmonary arteries at concentrations that do not cause endothelial dysfunction. Moreover, the relaxations induced by $ONOO^-$ were inhibited by the PARS inhibitor 3-aminobenzamide. By contrast, 3-aminobenzamide had no effect on the relaxations caused by endothelium-derived NO.

In the present study, the vasodilator effect of $ONOO^-$ on rat pulmonary arteries occurred over a concentration range of 1 μM to 100 μM, which is in agreement with observations with canine coronary artery (Liu *et al*., 1994), rat coronary artery (Villa *et al*., 1994) and bovine pulmonary artery (Wu *et al*., 1994). $ONOO^-$ is unstable at neutral pH and as such its bio-

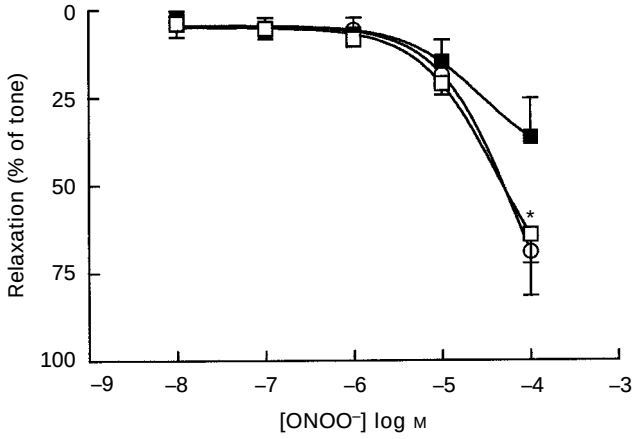

Figure 5 Effect of 3-aminobenzamide on the vasodilator actions of $ONOO^-$. Tissues were pre-contracted with U46619 according to the Methods section. Control response to $ONOO^-$ (□), in the presence of 1 mM 3-aminobenzamide (○) and in the presence of 10 mM 3-aminobenzimide (■) are shown. Results are the mean of 8–9 separate experiments; vertical lines show s.e.mean. A statistically significant difference (denoted by $*P<0.05$) was found, by unpaired t test at the concentration shown between the responses of vessels to $ONOO^-$ in the absence with those in the presence of 3-aminobenzamide at 10 mM but not at 1 mM.

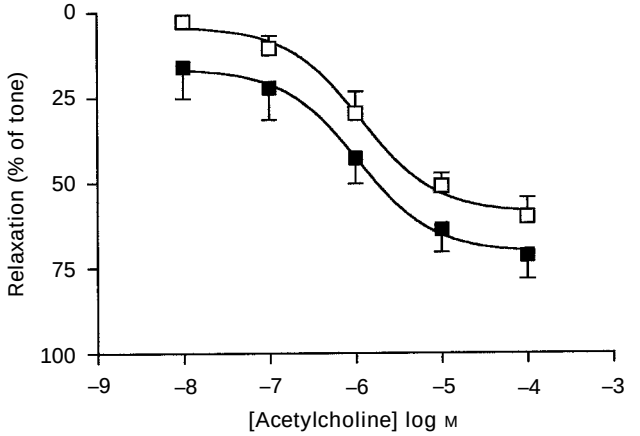

Figure 6 Effect of 3-aminobenzamide on the vasodilator actions of acetylcholine: (□) control responses; (■) responses in the presence of 3-aminobenzamide. Tissues were pre-contracted with U46619 according to the Methods section. Results are the mean of 5–6 separate experiments; vertical lines show s.e.mean.

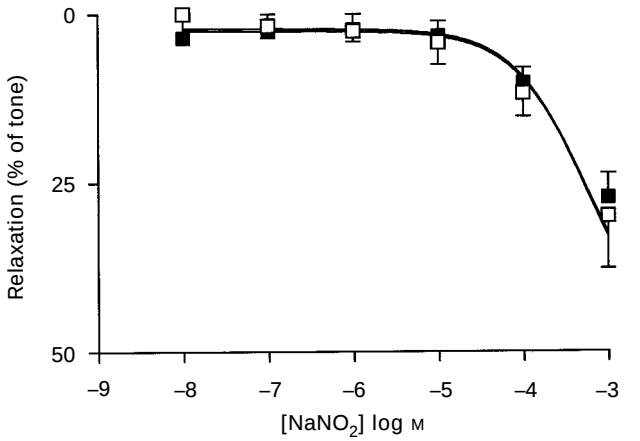

Figure 7 Effect of 3-aminobenzamide on the vasodilator actions of sodium nitrite: (□) control responses; (■) responses in presence of 3-aminobenzamide 10 mM. Tissues were pre-contracted with U46619 according to the Methods section. Results are the mean of 4 separate experiments; vertical lines show s.e.mean.

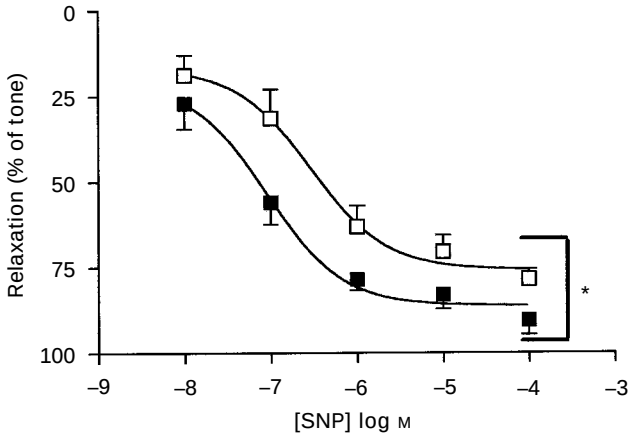

Figure 8 Effect of 3-aminobenzamide on the vasodilator actions of sodium nitroprusside (SNP): (□) control responses; (■) responses in presence of 3-aminobenzamide (10 mM). Tissues were pre-contracted with U46619 according to the Methods section. Results are the mean of 6 separate experiments; vertical lines show s.e.mean. A statistically significant difference (denoted by $*P<0.05$) was found, by 2-way ANOVA, between control responses and those after pre-exposure to 3-aminobenzamide.

logical activity should decay with time. We found a significant loss in potency and efficacy of $ONOO^-$ stored for 2 days at 4°C when compared to freshly prepared solutions, consistent with $ONOO^-$ being the active component. Our solutions were produced according to the method of Beckman and co-workers (1990) by use of a starting solution of sodium nitrite. In addition, $ONOO^-$ can break down to form nitrite. Thus, we felt that it was important to ensure that the effects of $ONOO^-$ on pulmonary artery tone were not caused by nitrite contamination. Sodium nitrite did relax rat pulmonary artery, but, was less potent and less efficacious than $ONOO^-$. Moreover, the vasodilator properties of $ONOO^-$ but not sodium nitrite were inhibited by 3-aminobenzamide. Taken together, these observations establish that $ONOO^-$ and not a contaminant or breakdown product was responsible for the relaxations observed in our experiments.

In the canine coronary artery, the vasodilator properties of $ONOO^-$ were potentiated by SOD (Liu *et al*., 1994), suggesting that superoxide anions were present either in the carrier solution of were released by the vessel. By contrast, we found no effect of SOD on the actions of $ONOO^-$ on rat pulmonary artery. In addition, we found no effect of L-NAME on $ONOO^-$, suggesting that it does not act by stimulating the endothelium to release NO.

The relaxant effects of $ONOO^-$ described in this study were greatly reduced by the PARS inhibitor, 3-aminobenzamide. PARS is a nuclear enzyme that is activated after DNA damage resulting in the utilization of NAD^+ and consequent depletion of cellular ATP (Schraufstatter *et al*., 1986). $ONOO^-$ at relatively high concentrations (1 mM) has been shown to activate the PARS pathway in vascular smooth muscle and to contribute to the hyporesponsiveness seen in rat aorta in experimental sepsis (Szabó *et al*., 1996). Our data support this concept and show, for the first time, that the vasodilator effects of relatively low concentrations of $ONOO^-$ are mediated by PARS activation. In addition, we show that 3-aminobenzamide has no effect on acetylcholine-induced relaxation, suggesting that NO *per se* does not relax pulmonary arteries via PARS. By contrast, 3-aminobenzamide actually increased the vasodilator properties of sodium nitroprusside. This suggests that sodium nitroprusside activates PARS in such a way as to reduce its biologial activity.

$ONOO^-$ has been described as a toxic oxidant that can cause endothelium dysfunction resulting in the inhibition of acetylcholine-induced relaxation of rat coronary vessels (Villa *et al*., 1994). However, this effect of $ONOO^-$ was not concentration-dependent since it inhibited acetylcholine responses at 3 μM and at 1000 μM, but not at 30 μM or 100 μM (Villa *et al*., 1994). By contrast, in bovine pulmonary arteries, $ONOO^-$ had no effect on acetylcholine-induced endothelium-dependent relaxations (Wu *et al*., 1994). We found that pretreatment with $ONOO^-$ actually increased the responses of pulmonary arteries to acetylcholine. Thus, in our studies $ONOO^-$ did not compromise the ability of the endothelium to release and/or respond to NO. The reason why $ONOO^-$ increased the response to acetylcholine is not clear. However, our study suggests that NO and $ONOO^-$ relax pulmonary arteries by different mechanisms, which may be additive. Studies in other vascular preparations show that the vasodilator properties of $ONOO^-$ are inhibited by oxyhaemoglobin and/or inhibitors of soluble guanylate cyclase (Liu *et al*., 1994; Villa *et al*., 1994; Wu *et al*., 1994), leading the authors to suggest that $ONOO^-$ relaxes vessels by spontaneous or metabolic release of NO. However, oxyhaemoglobin may be able to bind $ONOO^-$ directly, in the same way that it binds NO and thereby inhibit its actions. In addition, $ONOO^-$ may, like other oxidants (see Waldman & Murad, 1987), activate soluble guanylate cyclase directly, without the involvement of NO. Thus, these observations do not necessary contradict our hypothesis that $ONOO^-$ and NO relax vascular tissue by independent mechanisms.

In conclusion, peroxynitrite relaxes rat pulmonary artery directly, at concentrations that do not cause endothelial dysfunction and without stimulating the release of NO or superoxide. Moreover, unlike endogenously-released NO (by acetylcholine), or the nitrovasodilator sodium nitroprusside; $ONOO^-$ appears to cause relaxation by activation of PARS. We propose that $ONOO^-$ activates PARS resulting in the depletion of cellular ATP and reducing all active processes of vascular smooth muscle, including vasoconstriction. It has been suggested that $ONOO^-$ is responsible for the vascular hyporeactivity seen in experimental sepsis (Szabó *et al*., 1996). This observation together with our finding that 3-aminobenzamide inhibits relaxations induced by $ONOO^-$ but not by acetylcholine, suggests that inhibitors of PARS may reduce the persistent hypotension seen in sepsis without affecting the actions of endothelium-derived NO. This would be of particular importance to the pulmonary circulation, where septic shock is often accompanied by pulmonary hypertension. Thus, inhibitors of PARS may represent a novel therapeutic strategy in the treatment of diseases such as septic shock.

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