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The effects of peroxynitrite on rat aorta: interaction with glucose and related substances

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Abstract

Peroxynitrite (1–100 μM) induced a concentration-dependent relaxation of rat aortic rings; the $\log EC_{50}$ and maximum relaxation on endothelium-denuded rings were -5.31 ± 0.03 and $105 \pm 5\%$, $n = 6$, respectively. The presence of the endothelium significantly impaired this relaxation ($\log EC_{50}$, -4.41 ± 0.04; maximum relaxation, $71 \pm 4\%$; $n = 6$); an effect which was reversed by the inhibitor of nitric oxide synthase, N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM). Incubation with a high concentration of peroxynitrite (1 mM, 10 min followed by washout) had no effect on subsequent relaxation to acetylcholine (0.01–1 μM). It did, however, significantly depress subsequent contraction to phenylephrine (1–300 nM). This depression was dependent upon the presence of D-glucose in the Krebs solution, could be reversed by the inhibitor of soluble guanylate cyclase, methylene blue (10 μM) and reversed spontaneously after 2 h. When peroxynitrite (1 mM) was mixed with D-glucose (11 mM) and subsequently neutralised to remove unreacted peroxynitrite, a new more potent relaxant was formed. Despite this, the ability of peroxynitrite (1–100 μM) to produce relaxation of endothelium-denuded rings was similar in normal and glucose-free Krebs. Glycerol (22 mM), which like D-glucose is membrane permeant, also reacted with peroxynitrite (1 mM) to form a new more potent relaxant. L-cysteine (1 mM) had no effect by itself on the tone of aortic rings and when present in the tissue bath had no effect on the ability of peroxynitrite or neutralised peroxynitrite (1–100 μM) to produce relaxation. It did, however, potentiate the relaxant actions of the products formed from the reaction of peroxynitrite with D-glucose or glycerol. The membrane impermeant sugars, mannitol and sorbitol (each 11 mM) also reacted with peroxynitrite (1 mM), but expression of the vasorelaxant properties of their respective derivatives was seen only in the presence of L-cysteine (1 mM). Membrane permeance cannot, however, explain why peroxynitrite reacts with D-glucose and glycerol, but not mannitol or sorbitol to form products with intrinsic relaxant activity, as the product formed from the impermeant sugar, L-glucose (11 mM), also has intrinsic activity. The relaxant potency of this product was equipotent to that formed from D-glucose and was also potentiated by L-cysteine (1 mM). These result confirm that peroxynitrite can react with glucose and other compounds with alcohol functional groups to form vasorelaxant species. The relaxation induced when peroxynitrite is added to rat aortic rings is not, however, dependent upon this reaction since it occurs in glucose-free Krebs.

Keywords: Nitric oxide (NO); Peroxynitrite; Sugar; Vasodilatation; L-cysteine; Sodium nitrite; Methylene blue; Haemoglobin

1. Introduction

Several studies have now shown that superoxide anion (O_2^-) can influence the biological activity of nitric oxide (NO) (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986; Martin and Mian, 1995). The reason for this is that O_2^- can react with NO thus destroying its biological activity. The product of this reaction is peroxynitrite (Beckman et al., 1994; Pryor and Squadrito, 1995):

$$O_2^- + NO \rightarrow ONOO^-$$

Peroxynitrite is a highly reactive species which attacks a range of biological targets. For example, peroxynitrite has been shown to cause DNA strand breakage (Inoue and Kawanishi, 1995; Szabo et al., 1996), promote oxidation of protein (Gatti et al., 1994), ascorbic acid, uric acid and plasma sulfydryl groups (Van Der Vliet et al., 1994), disrupt mitochondrial function (Szabo and Salzman, 1995; Lizasoain et al., 1996) and induce lipid peroxidation (Radi et al., 1991). In the form shown in the above equation,

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peroxynitrite is relatively stable at alkaline pH. However, under physiological pH conditions the anion is converted to peroxynitrous acid which rapidly (half-life ≅ 1 s) decays, ultimately to form nitrate (Beckman et al., 1994; Crow and Beckman, 1995; Pryor and Squadrito, 1995):

$$ONOO^- + H^+ \leftrightarrow ONOOH \rightarrow HNO_3 \leftrightarrow NO_3^- + H^+$$

Recently, several studies have shown that under physiological conditions peroxynitrite exerts a prolonged vasorelaxant action. This relaxation has been observed in a range of isolated tissues, including dog coronary artery (Liu et al., 1994), rat heart (Villa et al., 1994), bovine pulmonary artery (Wu et al., 1994) and rabbit aorta (Furchgott and Jothianandan, 1996; Moro et al., 1995) as well as in the anaesthetised rat (Kooy et al., 1995). The relaxant action of peroxynitrite has been shown to have many characteristics in common with NO; it is blocked by the scavenger of NO, haemoglobin (Liu et al., 1994; Villa et al., 1994; Moro et al., 1995) and by the inhibitor of soluble guanylate cyclase, methylene blue (Liu et al., 1994; Wu et al., 1994). These relaxant actions cannot be due to the direct action of peroxynitrite itself because of its inherent instability at physiological pH values. It therefore seems reasonable to assume that the long acting vasorelaxation observed is due to the formation of a secondary, more stable species. Indeed, a recent study by Moro et al. (1995) investigating the action of peroxynitrite in rabbit aortic strips has provided evidence that it may react with glucose to form a stable relaxant.

The aim of this investigation was firstly to characterise the effects of peroxynitrite on the rat aorta; to date little information is available on its actions on this tissue and secondly to investigate the reaction of peroxynitrite with D-glucose and related substances containing the alcohol functional group. A preliminary account of these findings has already been published (Dowell and Martin, 1996).

2. Materials and methods

2.1. Preparation of aortic rings and tension recording

The preparation of aortic rings for tension recording was essentially similar to that described by Martin et al. (1985). Briefly, male Wistar rats weighing 250–300 g were killed by stunning and exsanguination. The aorta was removed, cleared of adhering fat and connective tissue and cut into 2.5 mm wide transverse rings with a razor blade slicing device. Endothelial cells were removed from some rings by gently rubbing the intimal surface (30–60 s) with a moist stick. Successful removal of the endothelium was later confirmed by the inability of acetylcholine (1 μM) to elicit relaxation. The aortic rings were mounted under 1 g resting tension on stainless steel hooks in 10 ml organ baths and bathed at 37°C in Krebs solution containing (mM): NaCl, 118; KCl, 4.8; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 24; D-glucose, 11 and gassed with 95% O_2 and 5% CO_2. Tension was recorded isometrically with Grass FT03 transducers and displayed on a MacLab (E Series, AD Instruments). Tissues were allowed to equilibrate for 60 min before experiments were begun, during which time the resting tension was re-adjusted to 1 g if required.

2.2. Experimental protocols

Vasodilator activity of peroxynitrite and its derivatives was assessed on endothelium-denuded and endothelium-containing rings of rat aorta. In these experiments the rings were contracted with a sub-maximal (50% of maximum) concentration of phenylephrine (100–200 nM for endothelium-containing rings and 10–20 nM for endothelium-denuded rings) and cumulative concentration–response curves to peroxynitrite and its derivatives (see below) were constructed. In preliminary experiments it was confirmed that none of the residual components of the peroxynitrite solution affected vascular tone, with the exception of sodium nitrite as detailed below. It was also confirmed that addition of peroxynitrite did not affect bath pH. The volume of peroxynitrite added to the bath did not exceed 100 μl for each concentration–response curve. In experiments where the effects of the scavenger of NO, haemoglobin, the inhibitor of soluble guanylate cyclase, methylene blue, or the inhibitor of NO synthase, N^G-nitro-L-arginine methyl ester (L-NAME), were to be investigated, they were added to the tissue bath 10 min before the addition of peroxynitrite or its derivatives. In experiments involving the use of L-NAME in endothelium-containing preparations the concentration of phenylephrine was adjusted to compensate for the elevation of tone due to the inhibition of basal nitric oxide.

The effects of exposure to high concentrations of peroxynitrite on vascular reactivity were also studied. In these experiments control responses were obtained to phenylephrine (1–300 nM, endothelium-denuded rings) or to acetylcholine (10–1000 nM, endothelium-containing rings). Following washout and re-equilibration, peroxynitrite, neutralised peroxynitrite (0.1 or 1 mM) or Krebs solution (100 μl) was added to endothelium-containing or endothelium-denuded rings for 10 min. The tissue baths were then repeatedly washed for a period of 10 min in order to remove any traces of contaminating nitrite and decomposed peroxynitrite, after which time cumulative concentration–response curves were immediately constructed either to phenylephrine (1–300 nM, endothelium-denuded rings) in the presence and absence of methylene blue (10 μM), or to acetylcholine (10–1000 nM, endothelium-containing rings) following constriction with a sub-maximal concentration of phenylephrine. Where indicated, some of the experiments on phenylephrine-induced contraction of endothelium-denuded rings were performed in glucose-free Krebs. For these tissues, all stages of preparation and

experimentation were performed in Krebs solution from which the glucose had been omitted.

2.3. Synthesis of peroxynitrite and its derivatives

Peroxynitrite was synthesised according to the method of Beckman et al. (1994). Briefly, sodium nitrite (0.6 M, 10 ml) was rapidly mixed with acidified hydrogen peroxide (H_2O_2, 0.7 M; HCl, 0.7 M, 10 ml). The reaction was immediately stopped and the products stabilised by the addition of sodium hydroxide (1.5 M, 10 ml). Excess hydrogen peroxide was removed by passing the solution down a column of manganese dioxide (1.5 g). Removal of hydrogen peroxide was confirmed to be more than 98% efficient by measuring absorbance at 220 nm, thus eliminating any direct vasoactive actions of hydrogen peroxide. The resulting concentration of peroxynitrite was determined by measuring the absorbance at 302 nm in 1 M sodium hydroxide (ε_{302} nm = 1670 M^{-1} cm^{-1}). The concentration of peroxynitrite synthesised ranged from 50–130 mM. All dilutions of peroxynitrite solutions were made in 0.5 M NaOH. Peroxynitrite was stored for up to 1 week at −20°C. The concentration of peroxynitrite was determined spectrophotometrically immediately prior to use. Neutralised peroxynitrite was prepared by adjusting the pH of the solution to 8.7 with HCl (0.05 M); the complete decomposition of peroxynitrite was confirmed by measuring the absorbance at 302 nm. Residual nitrite in these solutions was measured by the formation of a diazo product by a variant of the method of Green et al. (1982). Briefly, 60 μl of the sample was mixed with equal volumes of the Greiss reagents (60 μl of 1% sulphanilic acid in 2 M HCl, followed by 60 μl of 1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in H_2O). The absorbance was read at 550 nm using a Dynatech (Billinghurst, UK) microplate reader and the nitrite concentration in samples was assessed using a range of standards consisting of sodium nitrite in 0.5 M NaOH. In order to confirm that the effects of neutralised peroxynitrite were solely due to residual nitrite in the solution, acidified ammonium sulphamate was used to remove it (personal communication from Furchgott). Briefly, ammonium sulphamate (1 M) was added to the neutralised peroxynitrite solution using a volume sufficient to give 10 times the estimated nitrite concentration. The mixture was then acidified with HCl to a pH of 2. This process results in the destruction of nitrite and formation of nitrogen gas.

In order to investigate its potential to react with a range of sugars and related alcohols, peroxynitrite (1 mM) was rapidly mixed at room temperature for 30 s with solutions of either D- or L-glucose, mannitol, sorbitol (each 11 mM) or glycerol (22 mM). The pH values of the resultant solutions were subsequently adjusted to 8.7 with HCl (0.05 M) to ensure all peroxynitrite had decayed before assay of relaxant activity in normal glucose-containing Krebs solution. As controls, the same procedure was carried out for each agent using neutralised peroxynitrite.

2.4. Materials

Acetylcholine chloride, ammonium sulphamate, L-cysteine (free base), haemoglobin (bovine), N^G-nitro-L-arginine methyl ester (L-NAME), methylene blue, phenylephrine hydrochloride and sodium nitrite were obtained from Sigma (Poole, UK). All drugs were dissolved in distilled water and dilutions made in Krebs solution. Solutions of haemoglobin were prepared as previously described (Martin et al., 1985).

2.5. Statistical analysis

Results are expressed as the mean ± S.E.M. for *n* separate experiments. EC_{50} and $\log EC_{50}$ values were calcu-

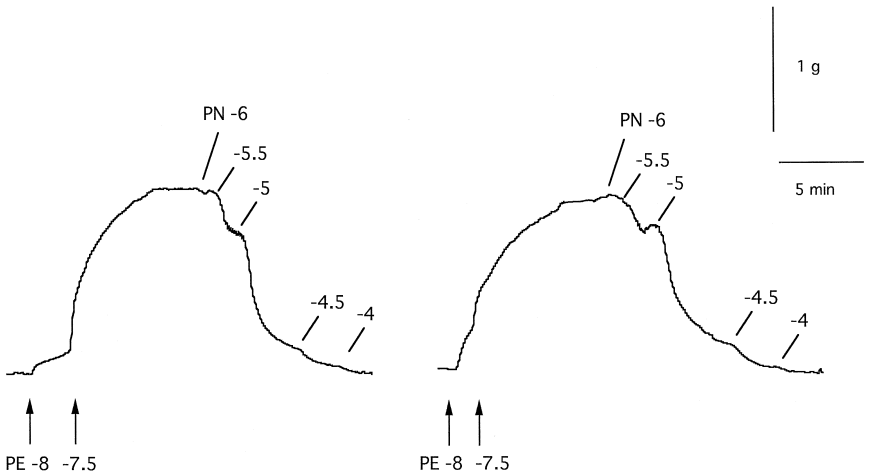

Fig. 1. Experimental traces showing peroxynitrite (PN)-induced relaxation in a phenylephrine (PE)-contracted endothelium-denuded ring of rat aorta. The second trace was obtained on the same tissue 2 h after the first, demonstrating reproducibility. The trace is representative of 6 separate experiments. Drug concentrations are expressed in log molar units.

lated by a computer-based curve fitting program (Prism, GraphPad). Multiple comparisons were made by one-way analysis of variance (ANOVA). If $P < 0.05$, individual pairs of means were then compared by the Bonferroni multiple comparisons test.

3. Results

3.1. Relaxant effects of peroxynitrite on rat aortic rings

Following induction of sub-maximal tone with phenylephrine, peroxynitrite (1–100 μM) induced a concentration-dependent relaxation on both endothelium-containing and endothelium-denuded rings (Fig. 1 and Fig. 2a and b). Endothelium-containing rings were significantly less sensitive to peroxynitrite ($\log EC_{50}$ value, -4.41 ± 0.04, $n = 6$) than those which had the endothelium mechanically removed ($\log EC_{50}$ value, -5.31 ± 0.03, $n = 6$, $P < 0.001$). Treatment with the NO synthase inhibitor L-NAME (100 μM) augmented peroxynitrite-induced relaxation on endothelium-containing rings ($\log EC_{50}$ value, -5.19 ± 0.07, $n = 6$, $P < 0.001$ compared to control endothelium-containing rings) but was without effect on endothelium-denuded rings ($\log EC_{50}$ value, -5.22 ± 0.04, $n = 4$) (Fig. 2c). When relaxation to peroxynitrite was repeatedly examined over a period up to 6 h it was found to be reproducible (Fig. 1).

On endothelium-denuded rings, relaxation to peroxynitrite (1–100 μM) was inhibited by haemoglobin (10 μM), a scavenger of NO; the $\log EC_{50}$ value for peroxynitrite was increased from -5.47 ± 0.03, $n = 16$ to -4.81 ± 0.13, $n = 11$, $P < 0.001$ and the maximum response was reduced from $101 \pm 3\%$, $n = 16$ to $73 \pm 11\%$, $n = 11$, $P < 0.05$. Relaxation was also attenuated by methylene blue (10 μM), an inhibitor of soluble guanylate cyclase; the $\log EC_{50}$ value for peroxynitrite was increased to -4.97 ± 0.06, $n = 16$, $P < 0.001$, however the maximum response was not significantly reduced $89 \pm 7\%$.

As previously reported (Liu et al., 1994), when the solution of peroxynitrite was neutralised before addition to the tissue, the relaxation observed at each concentration was slower in onset and lower in magnitude on both endothelium-containing ($\log EC_{50}$ value, -4.13 ± 0.05, $n = 6$, $P < 0.01$) and endothelium-denuded rings ($\log EC_{50}$ value, -4.87 ± 0.05, $n = 5$, $P < 0.001$) (Fig. 2a and b). Experiments with authentic sodium nitrite indicated that the residual relaxant activity of solutions of neutralised peroxynitrite was consistent with the presence of unreacted nitrite (126 ± 17 mM, $n = 4$) (data not shown). Furthermore, when neutralised peroxynitrite was reacted with acidified ammonium sulphamate, the nitrite content fell below the level of detection, and all relaxant activity was lost (Fig. 2b). When an equivalent concentration of ammonium sulphamate was added to phenylephrine-contracted aortic rings no change in tone was observed.

3.2. Effects of peroxynitrite on acetylcholine-induced relaxation and phenylephrine-induced contraction

Incubation of aortic rings with peroxynitrite or neutralised peroxynitrite (0.1 mM, 10 min followed by re-

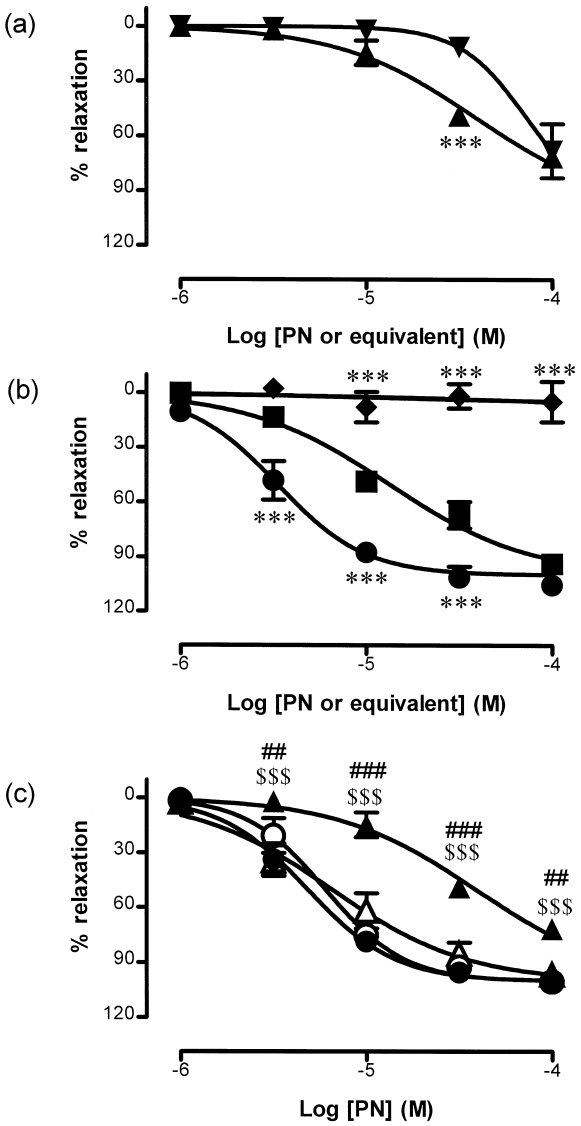

Fig. 2. Concentration–response curves showing relaxation to peroxynitrite and neutralised peroxynitrite on phenylephrine-contracted (a) endothelium-containing (▲, peroxynitrite; ▼, neutralised peroxynitrite) and (b) endothelium-denuded (●, peroxynitrite; ■, neutralised peroxynitrite; ♦, neutralised peroxynitrite treated with acidified ammonium sulphamate) rings of rat aorta and (c) the ability of L-NAME (100 μM; △, endothelium-containing; ○, endothelium-denuded) to increase the sensitivity to peroxynitrite in endothelium-containing but not endothelium-denuded rings (▲, control endothelium-containing; ●, control endothelium-denuded). The concentration axis indicates the concentration of peroxynitrite (PN) or equivalent. Each point is the mean ± S.E.M. of 4–6 observations. Where the S.E.M. cannot be seen they lie within the symbols. $^{***}P < 0.001$ compared to neutralised peroxynitrite. $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ compared to endothelium-containing rings in the presence of L-NAME. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to peroxynitrite in endothelium-denuded rings.

peated washing) had no significant effect on subsequent endothelium-dependent relaxation to acetylcholine (10–1000 nM) or contraction to phenylephrine (1–300 nM; endothelium-denuded rings) (data not shown). Furthermore, in endothelium-containing rings incubation with 1 mM peroxynitrite or neutralised peroxynitrite (10 min

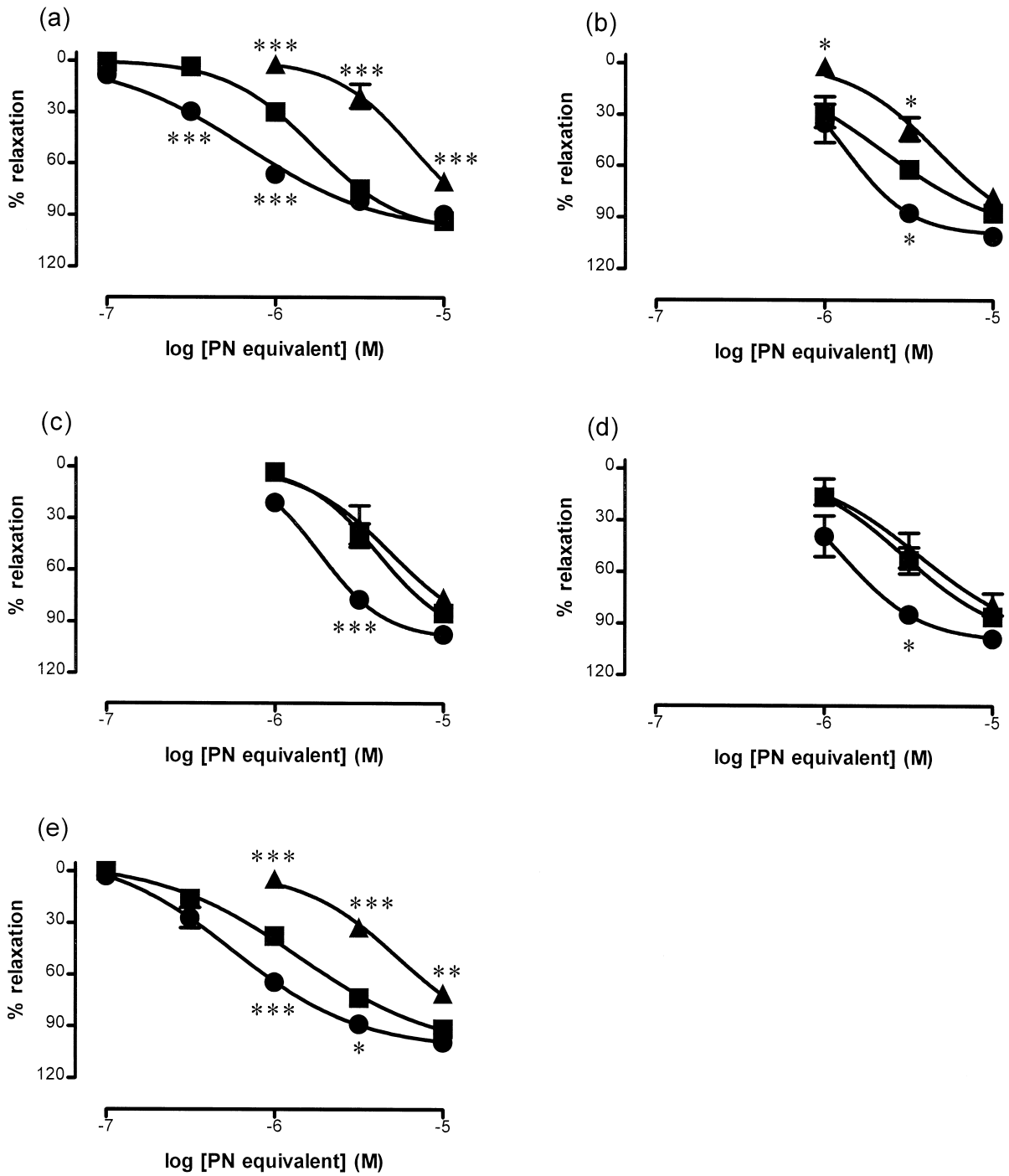

Fig. 3. Concentration–response curves showing contraction to phenylephrine in time-matched control (▲) endothelium-denuded rings of rat aorta and contraction following exposure to peroxynitrite (1 mM, 10 min followed by washout, ●) or neutralised peroxynitrite (1 mM, 10 min followed by washout, ■), in (a) normal glucose-containing Krebs and (c) glucose-free Krebs. Also shown (b and d) are contractions to phenylephrine in tissues following exposure to sodium nitrite (2 mM, 10 min followed by washout, ♦), in (b) normal glucose-containing Krebs and in (d) glucose-free Krebs together with their respective time-matched controls (▲). Panel (e) shows time-matched control (s) concentration–response curves to acetylcholine in endothelium-containing rings contracted with phenylephrine and relaxation to acetylcholine following exposure to peroxynitrite (1 mM, 10 min followed by washout, ●) or neutralised peroxynitrite (1 mM, 10 min followed by washout, ■) constructed 10 min after washout of peroxynitrite or neutralised peroxynitrite. Each point is the mean ± S.E.M. of 5–7 observations. Where the S.E.M. cannot be seen they lie within the symbols. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control. $^{\$\$\$}P < 0.001$ compared to neutralised peroxynitrite.

followed by washout) had no effect on subsequent relaxation to acetylcholine (10–1000 nM) when examined after 10 min (Fig. 3e), 1 or 3 h (data not shown). In contrast, treatment of endothelium-denuded rings with 1 mM peroxynitrite (10 min followed by washout) resulted in a profound depression of subsequent (10 min later) phenylephrine-induced contraction (1–300 nM; Fig. 3a). Exposure to neutralised peroxynitrite (1 mM, 10 min followed by washout) had only a slight depressant effect which was mimicked by incubating the rings with a concentration of nitrite (2 mM, 10 min followed by washout) equivalent to that found in solutions of neutralised peroxynitrite (Fig. 3b). The ability of peroxynitrite to depress phenylephrine-induced contraction was completely blocked by the inhibitor of soluble guanylate cyclase, methylene blue (10 μM; data not shown). Furthermore, the depression of contraction induced by peroxynitrite was reversible; when contractile responses were examined 2 h following exposure to peroxynitrite, they had recovered to control levels (data not shown).

In experiments performed in glucose-free Krebs exposure to peroxynitrite or neutralised peroxynitrite (1 mM, 10 min followed by washout) depressed phenylephrine-induced contraction to a similar extent (Fig. 3c). It was clear that neutralised peroxynitrite had caused a significantly greater depression of phenylephrine-induced contraction in the absence of glucose (Fig. 3c) and this was mimicked if endothelium-denuded rings were incubated with authentic sodium nitrite (2 mM, 10 min followed by washout) in glucose-free Krebs (Fig. 3d).

3.3. Reaction of peroxynitrite with D-glucose

When peroxynitrite (1 mM) was mixed with D-glucose (11 mM) and subsequently neutralised to remove any unreacted peroxynitrite prior to assay on phenylephrine-contracted endothelium-denuded rings, a new more potent relaxant was formed (Fig. 4a). In contrast, when neutralised peroxynitrite (1 mM) was mixed with D-glucose (11 mM) no new relaxant was formed; the relaxant response observed was similar to that seen with neutralised peroxynitrite alone (data not shown).

The ability of peroxynitrite (1–100 μM) to induce relaxation in endothelium-denuded rings was compared in normal and in glucose-free Krebs. Fig. 4b shows that peroxynitrite-induced relaxation was similar in the presence and absence of glucose in the Krebs. The weaker relaxant activity of neutralised peroxynitrite was also unaffected by the presence or absence of glucose in the Krebs (data not shown).

3.4. Effect of L-cysteine

Since thiols are known to enhance the release of NO from a wide variety of compounds (Feelisch, 1991), the actions of the thiol-containing amino acid, L-cysteine, were examined on relaxation produced by peroxynitrite and the product of its reaction with D-glucose. Addition of L-cysteine (1 mM) to endothelium-denuded rings had no relaxant action by itself and had no effect on relaxation to either peroxynitrite ($\log EC_{50}$ values: control, -5.32 ± 0.04, $n = 5$; in the presence of L-cysteine, -5.31 ± 0.04, $n = 5$) or neutralised peroxynitrite ($\log EC_{50}$ values: control, -4.79 ± 0.07, $n = 4$; in the presence of L-cysteine, -4.88 ± 0.08, $n = 4$). In contrast, the presence of L-cysteine (1 mM) in the tissue bath significantly potentiated the relaxation produced by the product of the reaction of peroxynitrite with D-glucose (Fig. 5a). L-cysteine (1 mM) had no effect, however, on relaxation induced by neutralised peroxynitrite mixed with D-glucose (data not shown).

3.5. Reaction of peroxynitrite with membrane permeant and impermeant sugars and related substances

As with D-glucose (Fig. 5a), when peroxynitrite was mixed with another membrane permeant agent, glycerol (22 mM), and subsequently neutralised to remove any unreacted peroxynitrite, a new, more potent vasodilator

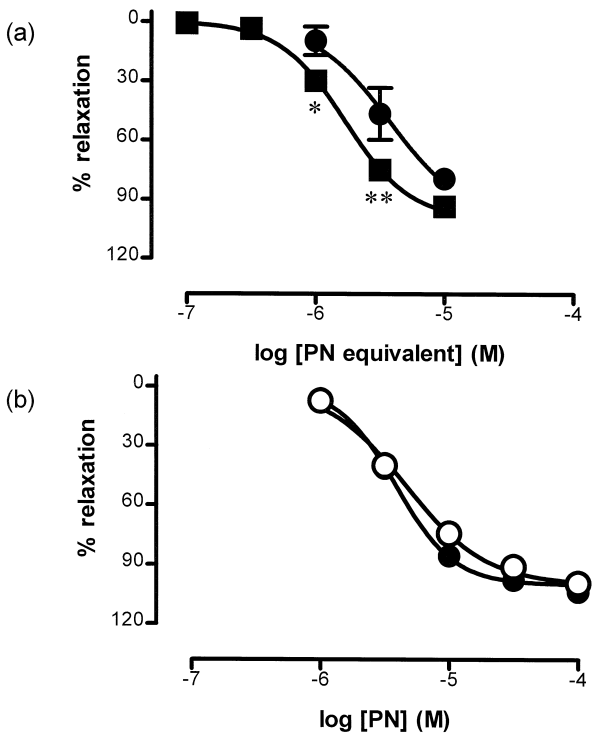

Fig. 4. Concentration–response curves for relaxation on phenylephrine-contracted, endothelium-denuded rings of rat aorta in response to (a) peroxynitrite (●) and the reaction product of D-glucose (11 mM) and peroxynitrite (1 mM) following neutralisation to remove any unreacted peroxynitrite (■). In (b) the relaxant actions of peroxynitrite are shown in experiments using normal glucose-containing (11 mM) Krebs (●) and glucose-free Krebs solution (○). The concentration axis indicates the concentration of peroxynitrite (PN) or equivalent. Each point is the mean ± S.E.M. of 6–10 observations. Where the S.E.M. cannot be seen they lie within the symbols. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with peroxynitrite.

substance was formed (Fig. 5b). Furthermore, the relaxation produced by this novel relaxant was potentiated by the presence of L-cysteine (1 mM) in the tissue bath. In contrast, mixing neutralised peroxynitrite with glycerol failed to generate any new relaxant activity (Fig. 5b), even in the presence of L-cysteine (1 mM) in the tissue bath (data not shown).

When peroxynitrite was mixed with the membrane-impermeant sugars, mannitol and sorbitol (each 11 mM) and subsequently neutralised to remove any unreacted peroxynitrite, no new relaxant activity was evident (Fig. 5c and d). A reaction had occurred with both sugars, however, since with each, relaxation was potentiated by the presence of L-cysteine (1 mM) in the tissue bath. Mixing neutralised

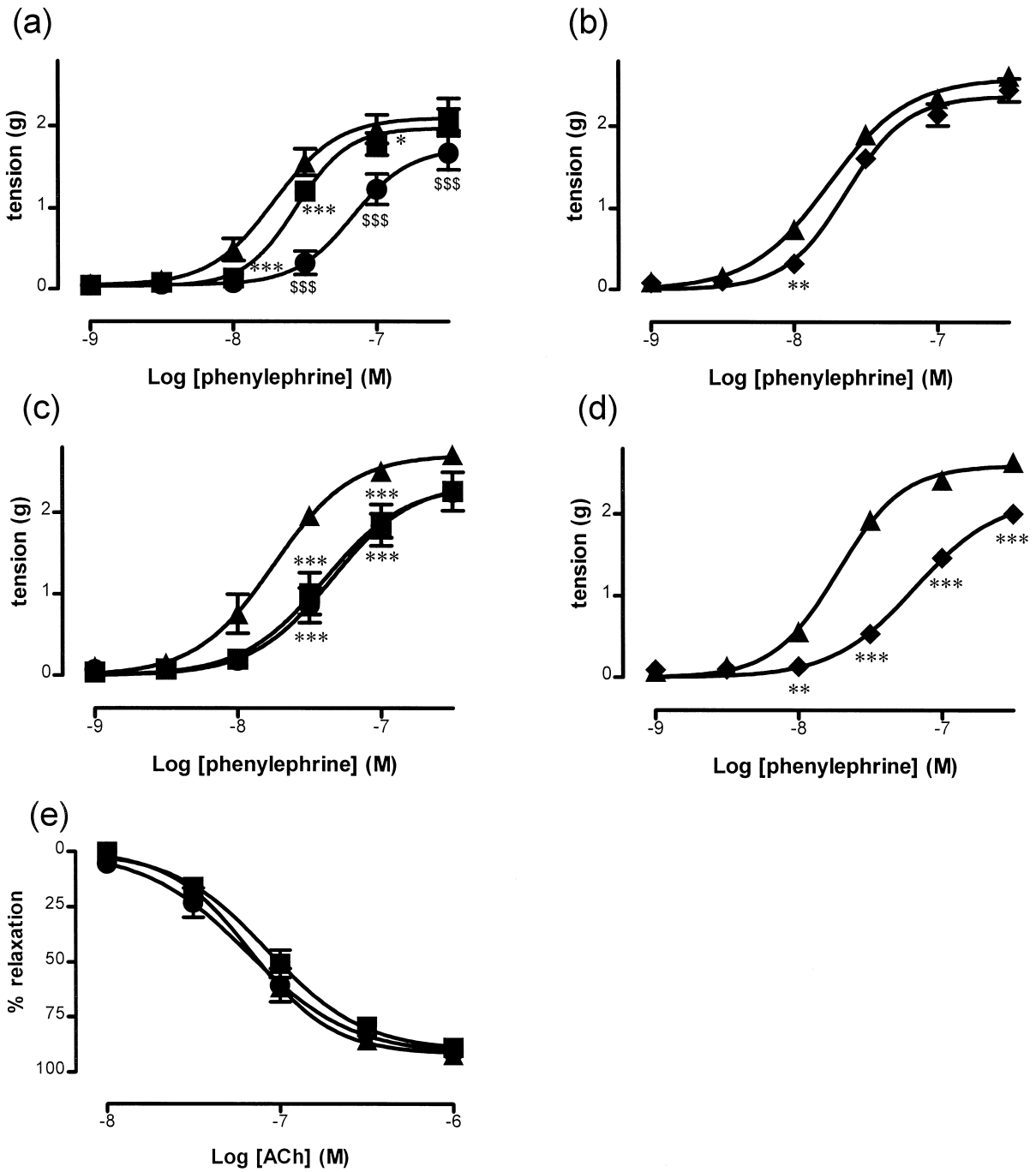

Fig. 5. Concentration–response curves for relaxation on phenylephrine-contracted, endothelium-denuded rings of rat aorta in response to the products formed from the reaction of (a) D-glucose (11 mM), (b) glycerol (22 mM), (c) mannitol (11 mM), (d) sorbitol (11 mM) and (e) L-glucose (1 mM) with peroxynitrite (1 mM; ■), following neutralisation to remove any unreacted peroxynitrite, or with neutralised peroxynitrite (1 mM, ▲). In each case the effect of adding L-cysteine (1 mM, ●) to the tissue bath on subsequent relaxation to the product of the reaction of peroxynitrite with each of the sugars is also shown. The concentration axis indicates the concentration of peroxynitrite (PN) equivalent. Each point is the mean ± S.E.M. of 6–10 observations. Where the S.E.M. cannot be seen they lie within the symbols. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to the sugar reacted with peroxynitrite.

peroxynitrite with mannitol or sorbitol failed to generate any new relaxant activity (Fig. 5c and d), even in the presence of L-cysteine (1 mM) in the tissue bath (data not shown). In contrast, when peroxynitrite was mixed with L-glucose (11 mM), which is also membrane impermeant, and subsequently neutralised to remove any unreacted peroxynitrite, a new more potent relaxant was formed (Fig. 5e). The relaxation produced by this novel product was potentiated by the presence of L-cysteine (1 mM) in the tissue bath. As with the other sugars, mixing neutralised peroxynitrite with L-glucose failed to generate any new relaxant activity (Fig. 5e), even in the presence of L-cysteine (1 mM) in the tissue bath (data not shown).

4. Discussion

We have shown that peroxynitrite induced powerful, concentration-dependent vasodilatation of rat aortic rings, thus, confirming previous findings obtained on bovine pulmonary artery rings (Wu et al., 1994), canine coronary artery rings (Liu et al., 1994), rabbit aortic strips (Moro et al., 1995) and the isolated perfused rat heart (Villa et al., 1994). As had been reported previously, the relaxant potency of peroxynitrite was greater than could be accounted for by the presence of unreacted nitrite contaminating the solution. The relaxant activity of this contaminating nitrite was seen in our study in both endothelium-containing and endothelium-denuded tissues, where neutralised peroxynitrite induced a weak relaxant response which was equal in magnitude to that produced by equivalent concentrations of authentic sodium nitrite. Confirmation that this relaxant activity was entirely due to contaminating nitrite was obtained with the use of ammonium sulphamate; this reactant led to complete loss of both nitrite and vasodilator activity. Consistent with previous reports, the relaxant response to peroxynitrite was blocked by haemoglobin, an inhibitor of NO (Liu et al., 1994; Villa et al., 1994; Moro et al., 1995) and by methylene blue, an inhibitor of soluble guanylate cyclase (Liu et al., 1994; Wu et al., 1994). It is therefore likely that the relaxant actions of peroxynitrite proceed through the donation of NO.

In contrast to findings published on canine coronary arteries, where the presence of the endothelium had no effect on relaxation to peroxynitrite (Liu et al., 1994), our data showed that endothelium-containing rings of rat aorta were significantly less sensitive to peroxynitrite than endothelium-denuded rings. Such an ability of the endothelium to depress the relaxant actions of NO donors has previously been reported in rat aorta (Shirasaki and Su, 1985). It appears that high basal activity of NO desensitises soluble guanylate cyclase to stimulants of this enzyme (Moncada et al., 1991). Consistent with this explanation was our finding that following treatment with L-NAME, a nitric oxide synthase inhibitor, the sensitivity to the NO donor peroxynitrite, in endothelium-containing but not endothelium-denuded rings was increased. Thus, in rat aorta where basal activity of NO is high, assays of relaxant activity are best conducted on endothelium-denuded rings.

Peroxynitrite is highly reactive and it has been proposed that under certain inflammatory conditions it may be responsible for damaging vascular tissue, thereby impairing vascular function (Buttery et al., 1996; Myatt et al., 1996). Indeed, a recent study by Villa et al. (1994) demonstrated that when peroxynitrite (3–1000 μM) was perfused into an isolated rat heart, rapid tachyphylaxis to its relaxant effects occurred and responses to a range of other vasodilators, including acetylcholine, were also significantly impaired. Consequently, we wished to investigate if a similar impairment of function could be observed in rat aorta. Firstly, no tachyphylaxis was observed; the concentration–response curve to peroxynitrite remained unchanged when repeated over a 6 h period, consistent with studies on rabbit aorta (Moro et al., 1995). The tachyphylaxis observed in the rat heart (Villa et al., 1994) may therefore result from the effects of direct infusion into a whole organ, which may trigger events not seen in an isolated blood vessel. Furthermore, in our experiments, when rings were exposed to a high level of peroxynitrite (1 mM, 10 min) the endothelium-dependent relaxant response to acetylcholine remained unaffected for up to three hours after exposure. Thus, no functional damage to the endothelium was evident. The subsequent ability of phenylephrine to cause contraction was, however, significantly reduced, but this was not the result of damage since it was completely reversed by the inhibitor of soluble guanylate cyclase, methylene blue. Furthermore, the depression reversed spontaneously with time and had disappeared by 2 h. These findings suggest that the depression of phenylephrine-induced contraction, like the relaxation produced by peroxynitrite, results from an NO-dependent mechanism.

It is clear that the powerful, long-acting vasorelaxation and the subsequent depression of phenylephrine-induced contraction observed when peroxynitrite is added to aortic rings cannot be mediated directly. Peroxynitrite is inherently unstable, so it seems likely that its ability to promote long lasting relaxation by an NO-dependent mechanism results from a reaction with either the tissue or a component of the bathing Krebs solution. It has recently been suggested that peroxynitrite reacts with glucose to form an NO-donor with the characteristics of an organic nitrate/nitrite (Moro et al., 1995; Furchgott and Jothianandan, 1996). The results of our study confirm that if peroxynitrite is pre-reacted with D-glucose and subsequently neutralised to remove any unreacted peroxynitrite, then indeed, a stable, more potent vasodilator substance is formed. In contrast, if neutralised peroxynitrite is mixed with D-glucose some dilator activity is seen, however this is similar to that observed with neutralised peroxynitrite alone and is due to contaminating nitrite. Furthermore, the depression of phenylephrine-induced contraction following

exposure to a high concentration of peroxynitrite (1 mM, 10 min) is dependent upon the presence of glucose in the Krebs. When glucose is absent no peroxynitrite-dependent component of depression of contraction is seen; it was noted that neutralised peroxynitrite had a more pronounced depressant action in the absence of glucose, however this was directly mimicked by authentic nitrite. The reason why nitrite should cause a more pronounced depression of contraction in the absence of glucose is unknown. Despite the glucose dependence of the ability of peroxynitrite to depress phenylephrine-induced contraction and the ability of peroxynitrite to react with glucose to form a relaxant, it appears that the relaxation we observe when peroxynitrite is added directly to the tissue bath is not dependent upon this reaction. This was clearly illustrated when the relaxant activity of peroxynitrite was found to be identical in normal and in glucose-free Krebs.

These findings contrast with the results obtained by Moro et al. (1995), who reported that the relaxation to peroxynitrite in a cascade bioassay on rabbit aortic strips was strictly dependent upon the concentration of glucose in the perfusion medium and those of Furchgott and Jothianandan (1996), who found that the absence of glucose reduced the relaxant potency of peroxynitrite by a factor of 1.5 in rabbit aortic rings. Such striking differences may result from the nature of the experimental protocols used in each case. In the cascade bioassay system (Moro et al., 1995) a 3 s delay occurred between injection of peroxynitrite into the buffer and its arrival at the first assay tissue. Most if not all the peroxynitrite would have decayed by this time, a fact pointed out by the authors. In the study on rabbit aortic rings (Furchgott and Jothianandan, 1996) the tissue baths used had a volume twice that used in our study, thus, again, possibly delaying the arrival of the peroxynitrite at the tissue. Another marked difference between our study and those on rabbit aorta (Moro et al., 1995; Furchgott and Jothianandan, 1996) is the concentration of peroxynitrite required. The EC_{50} for peroxynitrite-induced relaxation in our study in endothelium-denuded rings of rat aorta was approximately 2–5 μM, whereas in the rabbit aorta the EC_{50} for peroxynitrite was found to be in the order of 60–100 μM (Furchgott and Jothianandan, 1996; Moro et al., 1995). Perhaps, at high concentrations of peroxynitrite the reaction with glucose is important; indeed we ourselves found that at a concentration of 1 mM, peroxynitrite significantly depressed the generation of phenylephrine tone via a glucose-dependent mechanism. However, at 0.1 mM, a concentration 3-fold higher than that required to induce maximal relaxation in rat aortic rings, peroxynitrite had no effect on subsequent contraction to phenylephrine. Thus, it seems likely that peroxynitrite can induce vasorelaxation through two mechanisms: firstly, as the result of an interaction with glucose, which in our experiments appears only to be important at high (1 mM) concentrations of peroxynitrite. In rabbit aorta, however, where high concentrations of peroxynitrite are required (Furchgott and Jothianandan, 1996) this mechanism seems entirely responsible for peroxynitrite-induced relaxation. Secondly, peroxynitrite must form a potent dilator substance by reacting either with another component of the Krebs or by reacting directly with the tissue itself. A possible candidate is the bicarbonate present in the Krebs. Recently several reports have shown that CO_2/HCO_3^- reacts with peroxynitrite to form an $ONOOCO_2^-$ adduct, which in turn reacts with other biological molecules in a manner distinct from that of peroxynitrite itself (Fukuyama et al., 1996; Lymar et al., 1996; Uppu et al., 1996). The feasibility of studying this in an organ bath is limited as CO_2/HCO_3^- is the basis of the buffer in Krebs and thus cannot be omitted. Whilst other buffers, such as HEPES, are available, these are known to react with peroxynitrite, again producing NO donors (Liu et al., 1994). A more likely biological target is tissue thiols. Peroxynitrite has been shown to oxidise plasma thiols (Van Der Vliet et al., 1994), including the single L-cysteine of albumin (Radi et al., 1991), as well as glutathione in erythrocytes (Soszynski and Bartosz, 1996). Furthermore, the anti-platelet actions of peroxynitrite are only seen in the presence of glutathione or albumin (Moro et al., 1994) and glutathione enhances the ability of peroxynitrite to release nitric oxide (Wu et al., 1994; Mayer et al., 1995) and stimulate guanylate cyclase (Mayer et al., 1995). These effects are believed to reflect formation of NO-releasing *S*-nitrosothiols (Moro et al., 1994; Mayer et al., 1995)

As discussed previously (Moro et al., 1995) it is likely that the product of the reaction between peroxynitrite and glucose is an organic nitrate/nitrite. It is well established that some such compounds, for example glyceryl trinitrate and amyl nitrite, release NO in the presence of a thiol such as the amino acid L-cysteine (Feelisch, 1991). Thus, if peroxynitrite does form an organic nitrate/nitrite when reacted with glucose, the relaxant response observed may be potentiated by the presence of a thiol. We found that L-cysteine did indeed increase the relaxant potency of the product of pre-reacting peroxynitrite with D-glucose, but was without effect on the relaxation to peroxynitrite itself or to neutralised peroxynitrite which had been mixed with D-glucose. These results are supported by a previous study showing that L-cysteine releases NO from the product formed from the reaction of peroxynitrite with glucose (Moro et al., 1995). The effects of L-cysteine provide further evidence that peroxynitrite (1–100 μM)-induced relaxation is not dependent upon the presence of glucose in the Krebs. Specifically, if the reaction of peroxynitrite with glucose in the Krebs had played a role in the relaxation then the response should have been potentiated by L-cysteine, but this was not the case.

The ability of glucose to react with peroxynitrite is shared with other sugars and molecules containing an alcohol functional group, including fructose, glycerol and glyceraldehyde (Moro et al., 1995). We wished to extend

these observations by comparing the ability of peroxynitrite to react with membrane-permeant and membrane-impermeant alcohols and to examine the relaxant potency of the products of these reactions. We confirmed that a new stable relaxant was also formed from the reaction of peroxynitrite with the membrane-permeant alcohol, glycerol, and its ability to produce relaxation too was potentiated by L-cysteine. When the membrane-impermeant sugars, mannitol and sorbitol, were mixed with peroxynitrite, however, no new relaxant activity was initially evident; the relaxation observed was similar to that seen when each sugar was mixed with neutralised peroxynitrite and resulted from contaminating nitrite. Reactions had occurred, however, since with each sugar a more potent vasodilator action was seen in the presence of L-cysteine in the tissue bath. Thus, the membrane impermeant sugars, mannitol and sorbitol, do react with peroxynitrite, but expression of the vasodilator properties of their derivatives requires the presence of a thiol (L-cysteine). Although L-cysteine also potentiates the relaxant actions of the derivatives formed from the reaction of peroxynitrite with the membrane permeant molecules, D-glucose and glycerol, these derivatives have intrinsic vasodilator activity. Membrane permeance cannot explain the difference in activity, however, as the product of the reaction of peroxynitrite with the impermeant sugar, L-glucose, also has intrinsic activity. The difference in potency of the derivatives may simply reflect a difference in the reaction efficiencies between the different sugars and peroxynitrite.

In summary, these results confirm that peroxynitrite can react with glucose and other sugar molecules to form novel vasorelaxant species whose ability to release NO is augmented by L-cysteine. The relaxation produced when peroxynitrite is added to rat aortic rings is not, however, dependent upon this reaction since it is identical in glucose-free and glucose-containing Krebs and is not potentiated by L-cysteine.

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