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Interaction between peroxynitrite and L-cysteine: Effects on rat aorta

Fiona J. Dowell *, William Martin

Clinical Research Initiative, West Medical Building, University of Glasgow, Glasgow G12 8QQ, UK

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Abstract

In rings of rat aorta previously exposed to peroxynitrite (1 mM), L-cysteine and its analogues containing, but not those lacking, a thiol group produced a powerful transient relaxation. This relaxation is likely to result from the release of nitric oxide from a nitrated/nitrosated compound formed following reaction of peroxynitrite with a component of the tissue or bathing medium. Furthermore, when peroxynitrite was pre-mixed with L-cysteine a new relaxant species was formed. Analogues of L-cysteine with a free thiol reacted with peroxynitrite to form species with similar relaxant potencies. Analogues lacking a thiol formed products with relaxant activity, but less than with L-cysteine. Analogues with a free amino but no thiol or carboxylic functions formed products with potencies similar to those lacking only the thiol. If the amino is substituted and the thiol removed, no relaxant activity was generated. Thus, peroxynitrite reacts with L-cysteine to form a novel relaxant whose activity derives mainly from formation of its *S*-nitrosothiol, with a lesser component perhaps from an *N*-nitroso derivative. © 1998 Elsevier Science B.V.

Keywords: Peroxynitrite; Nitric oxide (NO); L-Cysteine; *S*-Nitrosothiol; Vasodilatation; Aorta, rat

1. Introduction

Peroxynitrite, the product of nitric oxide and superoxide anion, is a highly reactive species which has been implicated in the tissue damage associated with atherosclerosis (Beckman et al., 1994), arthritis (Kaur and Halliwell, 1994) and ischaemia-reperfusion injury (Wang and Zweier, 1996). Despite this, peroxynitrite has also been observed to produce effects which may be considered to be beneficial, such as prolonged vasodilatation (Liu et al., 1994; Villa et al., 1994; Wu et al., 1994; Dowell and Martin, 1997) and inhibition of platelet aggregation (Moro et al., 1994). These long lasting beneficial actions are difficult to reconcile with the extremely short half-life of peroxynitrite (~ 2 s; Beckman et al., 1990), but it seems that the oxidant reacts with a number of biological targets to form stable nitric oxide-releasing compounds.

There is strong evidence that tissue thiols represent one such biological target. Specifically, 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 and stimulate soluble guanylate cyclase (Wu et al., 1994; Mayer et al., 1995). The proposed role of thiols is supported further by the ability of thiol depleting agents to block peroxynitrite-induced elevation of endothelial cyclic GMP content (Mayer et al., 1995), and vasodilatation and release of nitric oxide by bovine pulmonary artery (Wu et al., 1994). *S*-nitrosothiols are powerful nitric oxide-releasing agents and it is therefore possible that formation of these following nitrosation of tissue proteins or low molecular weight thiols by peroxynitrite may explain the above actions. In support of this, a compound with an high-performance liquid chromatography (HPLC) elution profile identical to that of authentic *S*-nitrosoglutathione has been reported by two groups following the reaction of peroxynitrite with glutathione (Moro et al., 1994; Mayer et al., 1995). In contrast, others reported the reaction of peroxynitrite and glutathione to yield a product with an absorbance maximum at 338 nm, characteristic of *S*-nitrosothiols, but its HPLC elution profile differed from that of authentic *S*-nitrosoglutathione (Wu et al., 1994). Thus, although it is clear that peroxynitrite does indeed react with thiols, some doubt still exists as to the precise nature of the product(s) formed.

* Corresponding author. Tel.: +44-141-3306614; fax: +44-141-3304100; e-mail: f.dowell@bio.gla.ac.uk

Sugars and related compounds with alcohol functions represent an additional chemical class known to react with peroxynitrite to form nitrated or nitrosated derivatives with nitric oxide-releasing properties, and which are believed to contribute to the vasodilator activity of the oxidant (Moro et al., 1995; Furchgott and Jothianandan, 1996; Dowell and Martin, 1997). Our recent study (Dowell and Martin, 1997) suggests that the depression of vasoconstriction induced by high concentrations (>100 μM) of peroxynitrite in rat aorta involves mainly a reaction with glucose in the bathing medium, but vasodilatation produced by lower concentrations is glucose-independent and may reflect the reaction with thiols (Wu et al., 1994). An additional action of thiols has been highlighted, since despite having no effects on the tone of control rabbit aortic rings, glutathione and L-cysteine each produced powerful relaxation in tissues which had previously been bathed in glucose-containing medium, exposed to peroxynitrite and thoroughly washed (Furchgott and Jothianandan, 1996). This relaxant effect was believed to have resulted from the reaction of peroxynitrite with the alcohol functional group(s) of glucose to form nitrated/nitrosated products which were retained by the tissue and from which release of nitric oxide could be enhanced by glutathione and L-cysteine. Such enhanced release may proceed by a process analogous to the well-established ability of thiols to release nitric oxide from a wide range of organic nitrates and nitrites (Feelisch, 1991).

The aim of this study was to investigate further the interactions of peroxynitrite with thiols. Specifically, we made use of a number of structural analogues of L-cysteine to determine if the thiol group was solely responsible for: (i) formation of a nitric oxide-releasing species following reaction with peroxynitrite and (ii) the relaxation induced by L-cysteine in tissues previously exposed to a high concentration (1 mM) of peroxynitrite.

2. 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. (1986). 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 all 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 (in mM): NaCl, 118; KCl, 4.8; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 24; 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 rings of rat aorta. In these experiments, the rings were contracted with a submaximal concentration of phenylephrine (10–20 nM) and cumulative concentration–response curves to peroxynitrite and its derivatives (see below) were constructed.

The effects of exposure to high concentrations of peroxynitrite on vascular reactivity were also studied. In these experiments peroxynitrite or neutralised peroxynitrite (1 mM) were added to endothelium-denuded rings for 10 min. The tissue baths were then repeatedly washed for a period of 10 min, after which time the rings were contracted with phenylephrine (30 or 300 nM). When contraction had stabilised, L-cysteine (1 mM) or one of its analogues (Section 2.4) was added and the effects on tone recorded.

2.3. Synthesis of peroxynitrite and its derivatives

Peroxynitrite was synthesised according to the method of Beckman et al. (1990). 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 peroxynitrite 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). The resulting concentration of peroxynitrite was determined by measuring the absorbance at 302 nm in 1 M sodium hydroxide ($\varepsilon_{302\ nm} = 1670\ M^{-1}\ cm^{-1}$). The concentration of peroxynitrite synthesised ranged from 40–70 mM. All dilutions of peroxynitrite solutions were made in 0.5 M NaOH. 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.

In order to investigate its potential to react with thiols, peroxynitrite (1 mM) was rapidly mixed at room temperature for 30 s with neutral (pH 7) solutions of L-cysteine (10 mM) or its analogues (see below). 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 on aortic rings. As controls, the same procedure was carried out for L-cysteine and each analogue using neutralised peroxynitrite.

2.4. Analogues of L-cysteine

The analogues of L-cysteine were chosen in order to elucidate which of the functional groups (i.e. thiol, amino and carboxylic acid) are involved in the actions observed.

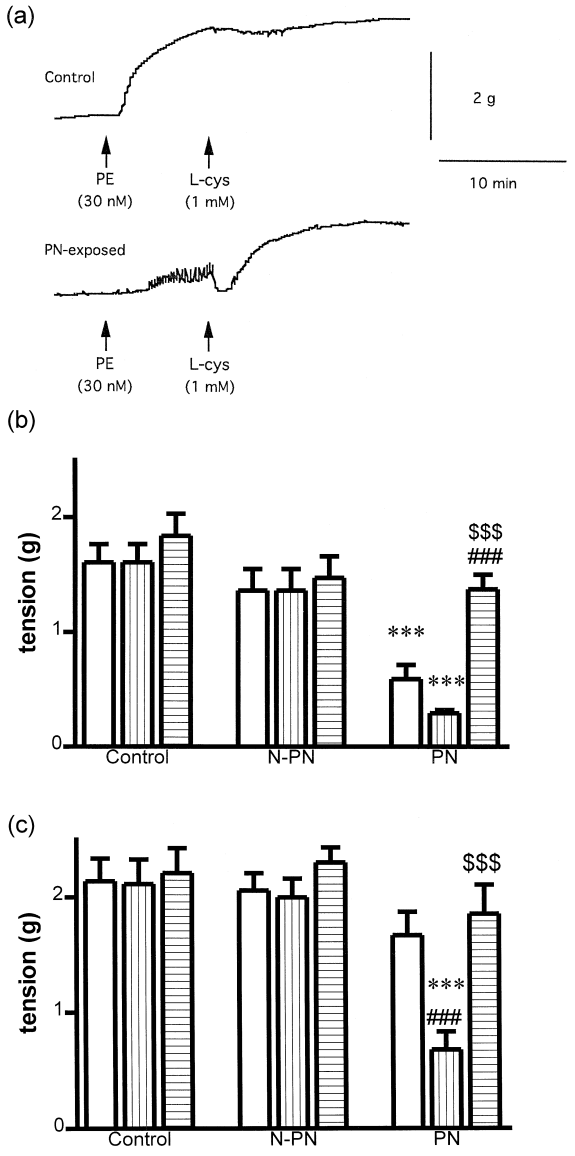

Fig. 1. Individual experimental traces (a) and mean data (b) and (c) showing contractile responses to phenylephrine (PE, 30 nM, (a) and (b); 300 nM, (c)) in control endothelium-denuded rings of rat aorta following exposure to peroxynitrite (PN, 1 mM, 10 min, followed by repeated washing for 10 min) or neutralised peroxynitrite (N-PN, 1 mM, 10 min, followed by repeated washing for 10 min). Open columns denote the stable level of tone achieved upon addition of phenylephrine, vertical lined columns the minimum level of tone after addition of L-cysteine (1 mM) and the horizontal lined columns represent the stable level of tone after addition of L-cysteine. Each data point is the mean ± S.E.M. of 6–8 observations. $^{***}P < 0.001$ compared to the corresponding control column, $^{###}P < 0.001$ compared to the level of tone prior to the addition of L-cysteine in each group, $^{\$\$\$}P < 0.001$ compared to the minimum level of tone after the addition of L-cysteine.

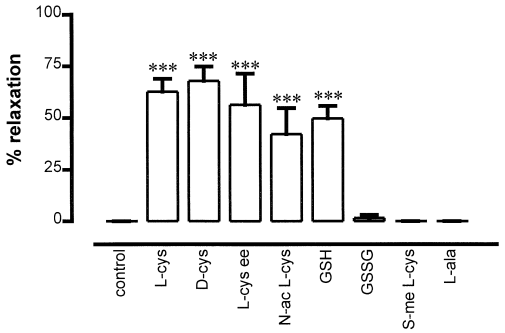

Fig. 2. Relaxant responses to analogues of L-cysteine (L-cys, 1 mM) following exposure to peroxynitrite (1 mM, 10 min, followed by repeated washing for 10 min) in endothelium-denuded rings of rat aorta contracted with phenylephrine (300 nM). The analogues used were D-cysteine (D-cys), L-cysteine ethyl ester (L-cys ee), *N*-acetyl L-cysteine (*N*-ac-L-cys), reduced glutathione (GSH), oxidised glutathione (GSSG), *S*-methyl L-cysteine (*S*-me-L-cys), L-alanine (L-ala). Responses are expressed as percentage relaxation of phenylephrine-induced tone. Each data point is the mean ± S.E.M. of 5–6 observations $^{***}P < 0.001$ compared to control.

The compounds used were divided into four main groups: analogues with a free thiol with or without a free amino or carboxylic acid group, i.e. D-cysteine (the optical isomer of L-cysteine), L-cysteine ethyl ester (esterified carboxylic acid group), *N*-acetyl-L-cysteine (acetylated amine group) and reduced glutathione (a tri-peptide containing L-cysteine); analogues lacking a free thiol but retaining a free amino and carboxylic acid group, i.e. *S*-methyl-L-cysteine (methylated thiol group), oxidised glutathione (two molecules of glutathione conjugated through a di-sulphide bridge) and L-alanine (another amino acid, similar in structure to L-cysteine but lacking the thiol functional group); analogues retaining an amino but lacking a free thiol or carboxylic acid group, i.e. L-cystine diethyl ester (two molecules of L-cysteine ethyl ester conjugated through a di-sulphide bridge) and L-alanine ethyl ester (L-alanine with an esterified carboxylic acid group) and finally, *N*-acetyl-L-alanine (L-alanine with an acetylated amine group), which lacks free thiol and amino groups but retains the carboxylic acid function.

2.5. Drugs

N-acetyl L-alanine, *N*-acetyl-L-cysteine, L-alanine, L-alanine ethyl ester dihydrochloride, D-cysteine, L-cysteine, L-cysteine ethyl ester hydrochloride, L-cystine diethyl ester dihydrochloride, glutathione (oxidised form, GSSG), glutathione (reduced form, GSH), phenylephrine hydrochloride and *S*-methyl-L-cysteine were obtained from Sigma (Poole, Dorset). All drugs were dissolved in distilled water and dilutions made in Krebs solution. All concentrations given are final bath concentrations.

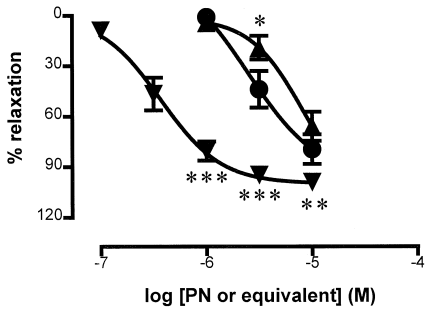

Fig. 3. Concentration–response curves showing relaxation of phenylephrine-contracted endothelium-denuded rings of rat aorta to peroxynitrite (●) alone, the product of peroxynitrite (1 mM) and L-cysteine (10 mM) following neutralisation to remove any unreacted peroxynitrite (▼) and the product of neutralised peroxynitrite (1 mM) and L-cysteine (10 mM, ▲). The concentration axis indicates the concentration of peroxynitrite (PN) or its equivalent. Responses are expressed as percentage relaxation of phenylephrine-induced tone. Each data point is the mean ± S.E.M. of 5–7 observations $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with peroxynitrite alone.

2.6. Statistical analysis

Results are expressed as the mean ± S.E.M. for *n* separate experiments. Comparisons were made by one-way analysis of variance (ANOVA). If $P < 0.05$, individual pairs of means were then compared using the Bonferroni multiple comparisons test.

3. Results

3.1. The effect of L-cysteine on peroxynitrite-induced depression of phenylephrine contraction

As previously reported (Dowell and Martin, 1997), incubation of aortic rings with peroxynitrite (1 mM, 10 min, followed by repeated washing) resulted in profound depression of subsequent (10 min later) phenylephrine-induced contraction (Fig. 1). This depression was marked at 30 nM phenylephrine (Fig. 1a and b), however, it was much less apparent at the near maximal concentration of 300 nM. In contrast, incubation with neutralised peroxynitrite (1 mM, 10 min followed by repeated washing) had no significant effect on phenylephrine-induced contraction (Fig. 1).

Since thiols are known to enhance release of NO from a wide variety of compounds (Moncada et al., 1991), the actions of L-cysteine were examined on the ability of peroxynitrite to depress phenylephrine-induced tone. In control rings, the addition of L-cysteine (1 mM) to phenylephrine (30 or 300 nM)-contracted tissues had no effect on the level of tone (Fig. 1). Similarly, in rings exposed to neutralised peroxynitrite (1 mM, 10 min, followed by repeated washing), L-cysteine (1 mM) did not affect the stable phenylephrine-induced contraction. However, in

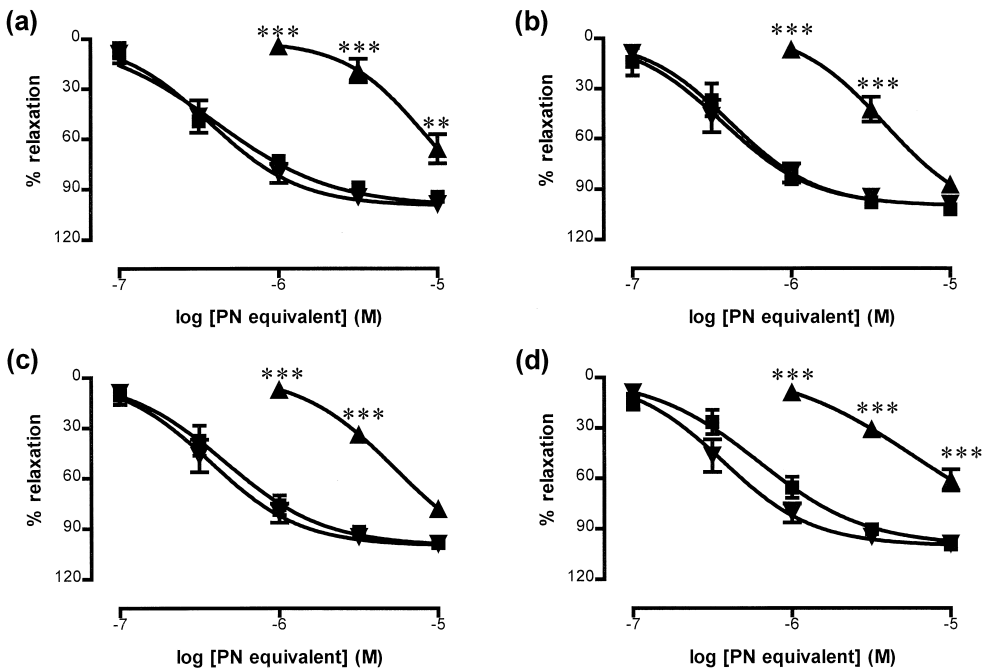

Fig. 4. Concentration–response curves for relaxation on phenylephrine-contracted, endothelium-denuded rings of rat aorta in response to the products formed from the reaction of (a) D-cysteine (10 mM), (b) *N*-acetyl L-cysteine (10 mM), (c) L-cysteine ethyl ester (10 mM) and (d) reduced glutathione (10 mM) with either peroxynitrite (1 mM; ■), following neutralisation to remove any unreacted peroxynitrite, or neutralised peroxynitrite (1 mM, ▲). In each case relaxation to the product formed from the reaction of L-cysteine (10 mM) with peroxynitrite (1 mM, ▼) is shown for comparison. The concentration axis indicates the concentration of peroxynitrite (PN) equivalent. Responses are expressed as percentage relaxation of phenylephrine-induced tone. Each point is the mean ± S.E.M. of 4–7 observations. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the analogue reacted with peroxynitrite.

rings exposed to peroxynitrite (1 mM, 10 min, followed by repeated washing), the addition of L-cysteine (1 mM) resulted in rapid relaxation (maximal after 1–2 min) followed by a contraction. This contraction was sufficient not only to reverse the initial L-cysteine-induced relaxation but also to restore tone to the control level seen in rings which had not been exposed to peroxynitrite.

3.2. The effect of analogues of L-cysteine

In order to investigate which functional group of the L-cysteine molecule was responsible for the observed vasorelaxation of phenylephrine-induced tone following exposure to peroxynitrite, a range of analogues was assayed as above. Specifically, rings were exposed to peroxynitrite (1 mM, 10 min, followed by repeated washing). Phenylephrine (300 nM) was then added and the tone allowed to stabilise before addition of the analogues. D-Cysteine, L-cysteine ethyl ester, *N*-acetyl-L-cysteine and reduced glutathione (all at 1 mM), each of which has a free thiol group, induced relaxation of similar magnitude to that of L-cysteine (Fig. 2). In contrast, analogues which lack a free thiol group, i.e. oxidised glutathione, *S*-methyl-L-cysteine and L-alanine (all at 1 mM), induced no relaxation of phenylephrine-induced tone following exposure to peroxynitrite (Fig. 2).

3.3. Reaction of peroxynitrite with L-cysteine

When peroxynitrite (1 mM) was mixed with L-cysteine (10 mM) and subsequently neutralised to remove any unreacted peroxynitrite prior to assay on phenylephrine-contracted rings of rat aorta, a new more potent relaxant was formed (Fig. 3). When neutralised peroxynitrite (1 mM) was mixed with L-cysteine (10 mM), however, no new relaxant was formed (Fig. 3); the relaxant response

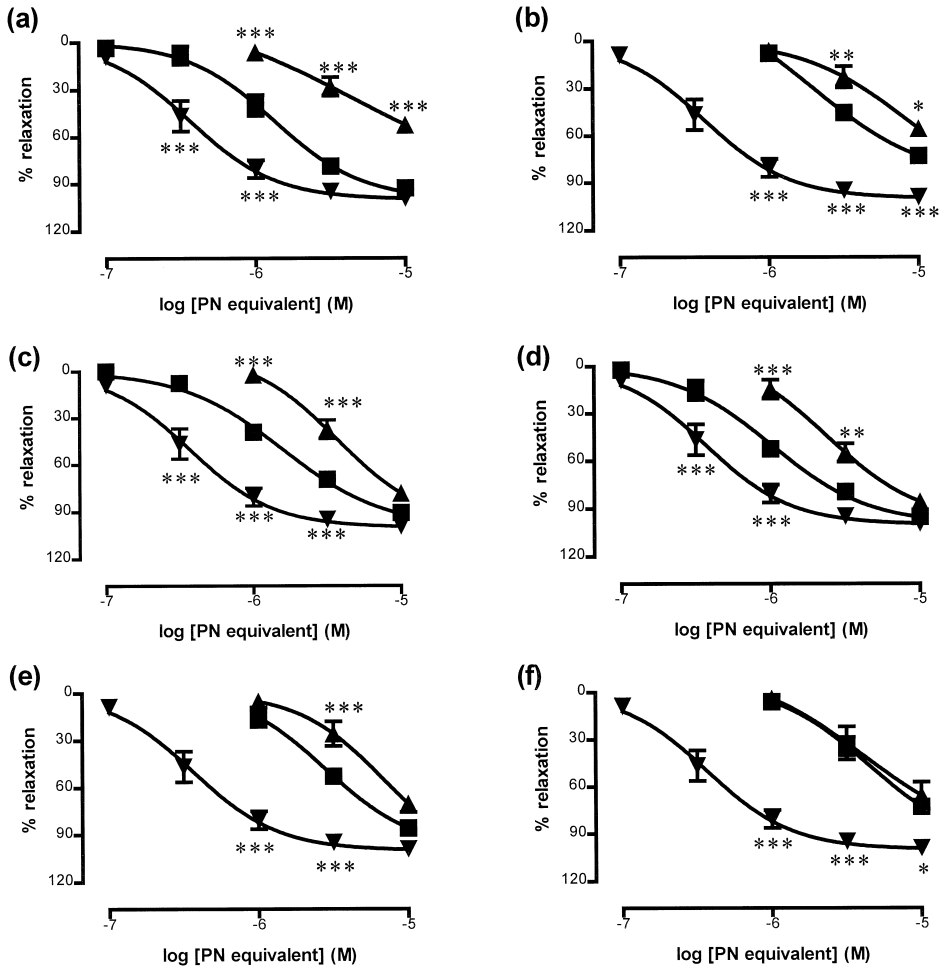

Fig. 5. Concentration–response curves for relaxation on phenylephrine-contracted, endothelium-denuded rings of rat aorta in response to the products formed from the reaction of (a) *S*-methyl L-cysteine (10 mM), (b) oxidised glutathione (10 mM), (c) L-alanine (10 mM), (d) L-cystine di-ethyl ester (10 mM), (e) L-alanine ethyl ester (10 mM) and (f) *N*-acetyl L-alanine (10 mM) with either peroxynitrite (1 mM; ■), following neutralisation to remove any unreacted peroxynitrite, or neutralised peroxynitrite (1 mM, ▲). In each case relaxation to the product formed from the reaction of L-cysteine (10 mM) with peroxynitrite (1 mM, ▼) is also shown for comparison. The concentration axis indicates the concentration of peroxynitrite (PN) equivalent. Responses are expressed as percentage relaxation of phenylephrine-induced tone. Each point is the mean ± S.E.M. of 7–10 observations. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the analogue reacted with peroxynitrite.

observed was similar to that seen with neutralised peroxynitrite alone (data not shown).

3.4. Reaction of peroxynitrite with analogues of L-cysteine

In order to investigate which functional group(s) of the L-cysteine molecule reacted with peroxynitrite to form the new relaxant species, a series of experiments was conducted in which peroxynitrite (1 mM) was reacted with analogues of L-cysteine (10 mM) and subsequently neutralised to remove any unreacted peroxynitrite. The products of these reactions were then assayed on phenylephrine-contracted rings of aorta and their relaxant potencies compared. Analogues with a free thiol group, namely D-cysteine, *N*-acetyl-L-cysteine, L-cysteine ethyl ester and reduced glutathione, reacted with peroxynitrite to form new relaxant species with potencies similar to that of the product of peroxynitrite and L-cysteine (Fig. 4). As with L-cysteine, when neutralised peroxynitrite (1 mM) was mixed with each of these analogues (10 mM) no new relaxant was formed (Fig. 4); the residual relaxant activity reflected that of neutralised peroxynitrite. When peroxynitrite was reacted with analogues lacking a free thiol group, i.e. with *S*-methyl-L-cysteine, oxidised glutathione or L-alanine (each at 10 mM) and subsequently neutralised to remove any unreacted peroxynitrite, the products formed, did exhibit new relaxant activity, but in each case this was significantly lower than with L-cysteine or the other thiols (Fig. 5a, b and c). When neutralised peroxynitrite (1 mM) was mixed with each of these analogues of L-cysteine (10 mM) no new relaxant was formed (Fig. 5a, b and c).

In order to elucidate the functional group of these molecules lacking a free thiol group which reacted with the peroxynitrite, a further series of analogues was investigated. Analogues retaining the amino but lacking the free thiol or carboxylic acid functions i.e. L-cystine diethyl ester and L-alanine ethyl ester (each at 10 mM) reacted with peroxynitrite (1 mM) to form products with a potency similar to that of analogues lacking only the thiol group (Fig. 5d and e). As with the other analogues, when neutralised peroxynitrite (1 mM) was mixed with L-cystine diethyl ester or L-alanine ethyl ester (each at 10 mM), no new relaxant was formed. In contrast, *N*-acetyl-L-alanine (10 mM), which has a substituted amino group and lacks the free thiol, failed to generate new relaxant activity when mixed with peroxynitrite (1 mM; Fig. 5f).

4. Discussion

As previously reported (Dowell and Martin, 1997), we found that prior exposure of endothelium-denuded rings of rat aorta to a high concentration (1 mM) of peroxynitrite followed by extensive washing, but not neutralised (decayed) peroxynitrite, resulted in profound depression of subsequent phenylephrine-induced contraction. Despite the short half-life of peroxynitrite ($t_{1/2} \sim 2$ s; Beckman et al., 1990), this depression is maintained for at least 1 h, following which the full contractile ability of phenylephrine slowly recovers (Dowell and Martin, 1997). The duration of the acute relaxant actions of peroxynitrite (Liu et al., 1994; Villa et al., 1994; Wu et al., 1994; Furchgott and Jothianandan, 1996) also greatly exceeds its expected lifetime, giving rise to the suggestion that this agent reacts to form nitric oxide-releasing products either with components of the tissue or with constituents of the bathing medium which are subsequently bound by the tissue. Indeed, the acute relaxation (Furchgott and Jothianandan, 1996) and subsequent depression of contraction (Dowell and Martin, 1997) observed following treatment with high concentrations of peroxynitrite are dependent upon the presence of glucose in the bathing medium. Thus, peroxynitrite is likely to have reacted with glucose to form a nitrated/nitrosated product which is bound by the tissue and which is able to release nitric oxide over a prolonged period (Moro et al., 1995; Furchgott and Jothianandan, 1996; Dowell and Martin, 1997).

Although without effect on control rings of rabbit aorta, reduced glutathione and L-cysteine each produce powerful relaxation in tissues previously incubated in glucose-containing medium and exposed to peroxynitrite followed by thorough washing (Furchgott and Jothianandan, 1996). This relaxation is believed to have occurred through enhanced release of nitric oxide from the nitrated/nitrosated product formed by the reaction of peroxynitrite with glucose. We wished to determine if this potentiation resulted from the well-characterised ability of thiols to promote release of nitric oxide from a wide range of nitrites and nitrates (Feelisch, 1991). To achieve this, we examined the ability of L-cysteine and certain of its thiol-containing and thiol-deficient analogues to enhance the ability of prior exposure to a high concentration of peroxynitrite (1 mM) to depress subsequent vasoconstriction to phenylephrine in rat aortic rings. In keeping with a previous observation (Furchgott and Jothianandan, 1996), we found that despite its lack of effect on phenylephrine-induced tone in control tissues or on tissues treated with neutralised peroxynitrite, L-cysteine had a powerful effect on tissues previously exposed to active peroxynitrite. This consisted of a profound but transient relaxation, followed by a rapid and sustained rise in tone which attained a magnitude equal to that seen with phenylephrine in tissues not treated with peroxynitrite. It is thus likely that the relaxation resulted from the ability of L-cysteine to accelerate greatly the rate of release of nitric oxide from the nitrated/nitrosated 'store' formed by the reaction of peroxynitrite with glucose. The reduced duration of the depression of phenylephrine-induced contraction would also be consistent with a more rapid depletion of nitric oxide from this 'store'.

Analogues of L-cysteine with a free thiol, regardless of the presence of a free amino or carboxylic acid function, i.e. D-cysteine, *N*-acetyl-L-cysteine, L-cysteine ethyl ester

and reduced glutathione, each produced relaxation of a similar magnitude and duration to that of L-cysteine when added to tissues previously exposed to peroxynitrite (1 mM). In contrast, analogues lacking a free thiol, i.e. *S*-methyl-L-cysteine, oxidised glutathione and L-alanine produced no relaxant effect. Thus, the relaxation produced by L-cysteine in tissues previously exposed to peroxynitrite is critically dependent upon the presence of its thiol group, suggesting it occurs by a process analogous to thiol-induced release of nitric oxide from organic nitrates/nitrites (Feelisch, 1991).

In addition to their capacity to promote release of nitric oxide from the reaction products of peroxynitrite and sugars, is the ability of thiols such as glutathione to directly react with peroxynitrite. Although there is agreement that the reaction results in the formation of an *S*-nitrosothiol, debate exists as to the precise nature of the product(s) formed. Two groups reported that the product co-eluted with authentic *S*-nitrosoglutathione in HPLC systems (Moro et al., 1994; Mayer et al., 1995), but another showed that despite having an absorbance peak at 338 nm characteristic of *S*-nitrosothiols, the product did not co-elute in their HPLC system with *S*-nitrosoglutathione (Wu et al., 1994). Consequently, with the aid of a number structural analogues we wished to establish which of the three functional groups, i.e. thiol, amino and carboxylic acid functions, of L-cysteine could react with peroxynitrite to produce novel relaxant activity. The results were clear. When analogues containing a free thiol group, i.e. D-cysteine, *N*-acetyl-L-cysteine, L-cysteine ethyl ester and reduced glutathione, were mixed with peroxynitrite and then neutralised to remove any unreacted peroxynitrite, powerful new relaxant activity equal in magnitude to that of the product with L-cysteine itself was found in each case. Neutralised (decayed) peroxynitrite was unable to form new relaxant activity with any of these analogues and the weak relaxant activity seen in each case simply reflected the presence of contaminating nitrite (Furchgott and Jothianandan, 1996; Dowell and Martin, 1997). The reaction, presumably producing the respective *S*-nitrosothiol, is therefore not stereospecific and occurs irrespective of the presence of a free amino or carboxylic acid group. Although the greater part of this new relaxant activity was lost when the reaction with peroxynitrite was conducted with analogues in which the thiol group was either substituted (*S*-methyl-L-cysteine), oxidised (oxidised glutathione) or absent (L-alanine), thereby precluding formation of *S*-nitrosothiols, some new activity was generated. Peroxynitrite was, however, unlikely to have reacted with the carboxylic acid groups to form this new relaxant activity because following reaction with analogues in which these were esterified and in which a free thiol was absent, i.e. L-cystine diethyl ester and L-alanine ethyl ester, relaxant activity similar in magnitude to that of the product with L-alanine was generated. The only functional group remaining, i.e. the amino group, seemed to be an obvious candidate for a the reaction with peroxynitrite to produce new relaxant activity and, indeed, when this was substituted (*N*-acetyl-L-alanine) no new relaxant activity was generated. Thus, when peroxynitrite reacts with L-cysteine, the major component of the new relaxant activity produced results from the formation of its *S*-nitrosothiol, and a lesser but still significant component appears to arise from the formation of an *N*-nitroso derivative. Such an *N*-nitroso derivative, resulting from the reaction of peroxynitrite with glutathione, may explain the formation of an *S*-nitrosothiol which failed to co-elute in an HPLC system with authentic *S*-nitrosoglutathione (Wu et al., 1994). However, it should also be noted that a further publication from this group has demonstrated the presence of a nitrated product ($GSNO_2$) following reaction of peroxynitrite with glutathione (Davidson et al., 1996).

In summary, our results show that L-cysteine stimulates powerful relaxation in rat aortic rings that have previously been exposed to a high (1 mM) concentration of peroxynitrite. This property is strictly limited to analogues possessing a free thiol group and is likely to result from enhanced release of nitric oxide from a nitrated/nitrosated compound formed by the reaction of peroxynitrite with glucose in the bathing medium, by a process analogous to the ability of thiols to release nitric oxide from nitrites and nitrates (Feelisch, 1991). In addition, peroxynitrite reacts directly with L-cysteine to form a powerful new relaxant species. Most of this new relaxant activity derives from formation of its *S*-nitrosothiol, with a lesser component perhaps from an *N*-nitroso derivative.

Acknowledgements

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