Oxidative release of nitric oxide accounts for guanylyl cyclase stimulating, vasodilator and anti-platelet activity of Piloty's acid: a comparison with Angeli's salt*

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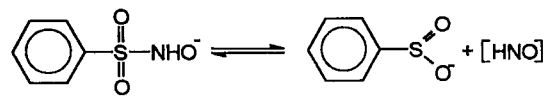
The decomposition of benzenesulphohydroxamic acid (Piloty's acid; PA) and some of its derivatives has been reported to yield nitroxyl ions (NO^-), a species with potent vasodilator properties. In a previous study we demonstrated that the oxidative breakdown of PA results in the formation of nitric oxide (NO) and suggested that NO rather than NO^- may account for its vasorelaxant properties. Using isolated aortic rings in organ baths, we now show that high concentrations of cysteine potentiate the vasorelaxant response to PA, whereas responses to Angeli's salt (AS), a known generator of NO^-, were almost completely inhibited. These different behaviours of PA and AS are mirrored by their distinct chemistries. By using HPLC it was shown that, at physiological pH and in the absence of oxidizing conditions, PA is a relatively stable compound. Direct chemical determination of NO, stimulation of soluble guanylyl cyclase, and measurement of platelet aggregation under various experimental conditions confirmed the requirement for oxidation to release NO from PA, and quite weak oxidants were found to be sufficient to promote this reaction. In contrast, at pH 7.4 AS decomposed rapidly to yield nitrite (NO_2^-) and NO^-, but did not produce NO on reaction with dioxygen (O_2) or hydrogen peroxide (H_2O_2). Thus sulphohydroxamic acids are a new class of thiol-independent NO-donors that generate NO rather than NO^- under physiological conditions.

INTRODUCTION

Since the recognition of nitric oxide (NO) as the ultimate mediator of the pharmacodynamic action of nitrovasodilators [1] there has been an increasing interest in the metabolism of compounds which are thought to produce activation of soluble guanylyl cyclase and subsequent vasorelaxation by releasing nitroxyl anion (NO^-) or its protonated form, hyponitrous acid (HNO), respectively, rather than the NO radical. Recently, the formation of NO^- has been postulated to account for the potent vasodilator activity of some derivatives of Piloty's acid (PA) [2]. This assumption was based largely on earlier observations showing that (i) in strongly alkaline aqueous solution PA undergoes hydrolytic decomposition to yield nitrous oxide (N_2O) and benzenesulphinic acid (BSI) [3] and (ii) the rate of decomposition of its sodium salt is determined by an equilibrium reaction [eqn. (1)], followed by conversion of the intermediate HNO to N_2O [4] [eqn. (2)]:



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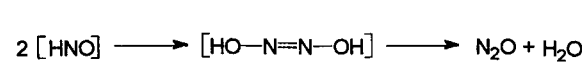

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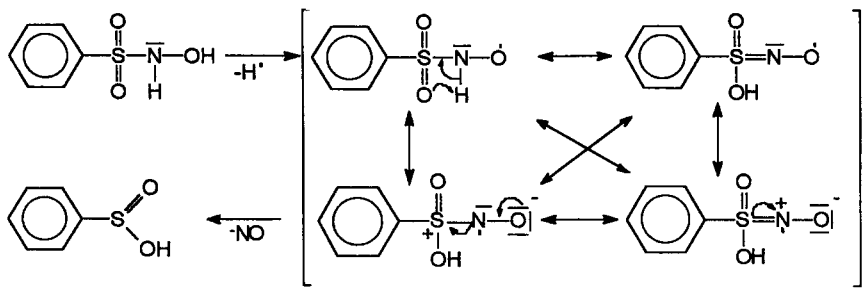
Recently, we proposed that at pH 7–8 oxidation of PA occurs on the intact molecule, resulting in the formation of a radical intermediate that decomposes to BSI and NO [5] [eqn. (3)]:



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Abbreviations used: AS, Angeli's salt; BSI, benzenesulphinic acid; CLD, chemiluminescence detector; CysNO, *S*-nitrosocysteine; HbO_2, oxyhaemoglobin; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; $ONOO^-$, peroxynitrite; PA, Piloty's acid; PE, phenylephrine hydrochloride; PRP, platelet-rich plasma.

* This paper is dedicated to Professor H. Möhrle on the occasion of his 65th birthday.

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Thus the release of NO rather than NO^- may account for the vasodilator activity of PA.

In a previous study we used L-cysteine as a tool for differentiating by bioassay between the action of NO and that of NO^- [6]. High concentrations of this thiol-containing amino acid were shown to cause almost complete inhibition of the vasorelaxant response to NO^- [generated from Angeli's salt (AS) and sodium nitroxyl], whereas responses to authentic NO and *S*-nitrosocysteine (CysNO) were largely enhanced. In the present study a similar approach was used in combination with direct and indirect determination of NO to investigate the possible relationship between non-enzymic decomposition and biological effect of PA in isolated vascular tissue and platelet-rich plasma (PRP). Our results demonstrate clearly that under physiological conditions PA directly releases NO upon oxidation [eqn. (3)] rather than NO^- via fragmentation reaction [eqn. (1)].

MATERIALS AND METHODS

Chemicals and solutions

AS (sodium trioxodinitrate, $Na_2N_2O_3$) was synthesized by a modified version of the procedure described by Smith and Hein [7] and stored under argon. Synthesis of the nitroxide 2-(4-methoxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (Compound **1**; see eqn. 5) was performed by a modified version [8] of the procedure described by Nadeu and Boocock [9]. **1** was obtained in 60% yield as blue needles from light petroleum (boiling range 60–80 °C) and revealed the following compound characteristics: melting point 101 °C; IR peaks (KBr, per cm) 2980, 2940 and 2850 (aliphatic C-H), 1610 (aromatic C=C); MS fragments (100 °C) 263 (17) [$M^{+\cdot}$], 174 (17), 135 (33), 134 (40), 133 (20), 84 (100), 69 (50); ESR (9.4 GHz, dichloromethane, 15 °C) $g = 2.00673$, $a(N) = 0.761$ [mT] 2 (N). Analytical $C_{14}H_{19}N_2O_3$ molecular mass, 263.32 Da; calc. C, 63.86; H, 7.27; N, 10.64; found C, 63.94; H, 7.39; N, 10.52. Stock solutions of **1** (10 mM) were made up in dioxane and further dilutions were prepared with saline solution. PA and potassium hexacyanoferrate(III) {$K_3[Fe(CN)_6]$} were purchased from Fluka (Buchs, Switzerland). Phenylephrine hydrochloride (PE) was purchased from Aldrich (Steinheim, Germany). N^G-Nitro-L-arginine methyl ester (L-NAME), L-cysteine and hydroxylamine (NH_2OH) were purchased as hydrochloride salts from Sigma (Deisenhofen, Germany). Sodium nitrite ($NaNO_2$) was from Riedel de Häen (Seelze, Germany). Solutions of AS were prepared in nitrite-free water (water for HPLC; Riedel de Häen) that had been deaerated and gassed with argon before use. Stock solutions (1 mM) of CysNO were prepared daily by acid catalysed *S*-nitrosation of L-cysteine with $NaNO_2$ in citrate/HCl buffer, pH 2.0. Solutions were kept on ice and protected from light, and appropriate dilutions were made up in citrate/HCl buffer immediately before use. Aqueous solutions of NO were prepared as described previously [10]. The Krebs–Henseleit buffer used consisted of (mM): NaCl 126.8, KCl 15.9, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 30.0, glucose 5.0, and was supplemented with 1 μM indomethacin to prevent formation of vasoactive prostanoids.

Measurement of vasorelaxing activity

Anaesthesized male Wistar rats (250–300 g) were killed, the thoracic aorta was carefully removed, cleaned of fat and connective tissue and cut into 4–5 mm endothelium-intact rings. The vascular rings were mounted in 20 ml organ baths containing oxygenated (O_2/CO_2, 95:5) Krebs–Henseleit buffer (pH 7.4) and allowed to equilibrate under a resting tension of 2 g at 37 °C with a complete exchange of the bathing solution every 15 min. After 90 min of equilibration, tissues were submaximally precontracted with PE (0.2 μM), and L-NAME (100 μM) was added to enhance long-term stability of the preparation. Complete concentration–response curves for the vasorelaxant action of various NO and nitroxyl-generating agents were constructed by continuous recording of changes in isometric tension after cumulative addition of test compounds to the organ bath. In some experiments either the newly synthesized nitroxide (**1**), hexacyanoferrate(III) or L-cysteine was added 5 min before addition of PA or AS. Relaxations were expressed as a percentage of initial contraction. Reported values represent the final bath concentration.

Detection of nitric oxide

NO generation on decomposition of PA was measured by chemiluminescence as described previously [11]. Briefly, a highly sensitive chemiluminescence detector (CLD 780 TR, eco Physics, Dürnten, Switzerland) was connected by stainless-steel tubing to the outlet of a water-jacketted, septum-sealed reaction chamber containing deaerated aqueous buffer solution (0.1 M phosphate or borate buffer, respectively, at 37 °C and pH values of 7.0, 7.4, 8.0 or 9.0). A stream of purified nitrogen was passed over the surface of the stirred sample (total volume 2.0 ml) at a rate of 100 ml/min, allowing the continuous monitoring of generated NO after introducing the test compounds by injection through the septum of the reaction chamber. The detection limit was 2 pmol of NO per ml per min under the conditions applied (integration time 5 s, signal-to-noise ratio > 3:1).

Stimulation of soluble guanylyl cyclase

The 105000 *g* supernatant of rat-liver homogenate was prepared as described by Ignarro et al. [12] and was used as a source of soluble guanylyl cyclase. The enzymic activity of this crude preparation was determined by radioimmunoassay as described by Kimura et al. [13] with a commercially available radioimmunoassay kit for cyclic GMP (Amersham, Braunschweig, Germany). Incubations were carried out in 50 mM phosphate buffer at pH 7.0, 7.4 or 8.0, at 37 °C in the presence of 4 mM $MgCl_2$ and 1 mM GTP with 30 μg of protein, and reactions were stopped after 15 min. In some experiments, either oxyhaemoglobin (10 μM) or potassium hexacyanoferrate(III) (50 μM) was added with PA.

Decomposition of PA

Products of the non-enzymic decomposition were analysed by reversed-phase HPLC on a Nucleosil C-18 column (250 mm × 5 mm) with a mobile phase consisting of an acetonitrile/water mixture (1:4, v/v; pH 2.5) running at 0.7 ml/min. The detection wavelength was set at 220 nm. Typical retention times for PA and BSI were 9.6 and 5.4 min, respectively. The HPLC system was calibrated daily with known concentrations of reference standards. Stock solutions (20 mM) of PA and the respective reference compounds were prepared in methanol and stored for up to 5 h at 4 °C until use. Dilutions were made up in 0.1 M phosphate or borate buffer, respectively, immediately before use. Incubation runs were performed in a total volume of 5.0 ml of either phosphate or borate buffer (0.1 M; pH 7.0–9.0) at 37 °C with stirring. The concentration of PA and its products of decomposition was determined at 30 min intervals after dilution of a 0.1 ml aliquot of the incubation mixture with the eluent (1:10). In some experiments incubations were performed in the presence of hexacyanoferrate(III) (1 mM). In another set of experiments incubations were performed in septum-sealed reaction vials under anaerobic conditions with

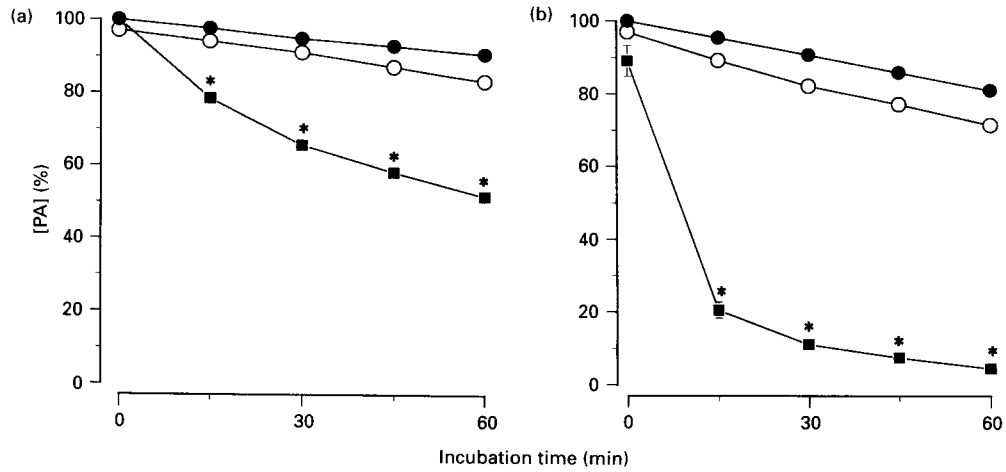


Figure 1 ***In vitro*** **decomposition of PA (100 μM) in 0.1 M phosphate buffer at 37 °C, at (a) pH 7.4 and (b) pH 8.0, as determined by HPLC**

Incubations were performed under three different conditions: under room air (○, control), under argon (●), and under room air in the presence of 100 μM hexacyanoferrate(III) (■). Data represent means ± S.E.M. for three individual experiments performed in duplicate. Asterisks denote statistically significant differences from control ($P < 0.05$).

buffer solutions that had been deaerated and bubbled for 30 min with argon before use.

Platelet aggregation

Venous blood was collected freshly from chronically catheterized Munich minipigs with sodium citrate/glucose solution (Biostabil; Biotrans GmbH, Dreieich, Germany). After centrifugation of the blood at 300 *g* for 10 min the supernatant, representing PRP, was carefully separated and immediately used for aggregation studies. Platelet-poor plasma was obtained by further centrifugation of the sediment at 3600 *g* for 10 min. Platelet aggregation was determined turbidimetrically at 37 °C under continuous stirring (1000 rev./min) using an APACT dual-channel aggregometer. Aggregation was induced by addition to the PRP of 1–5 μg/ml ADP (Boehringer Mannheim, Germany) after preincubation of the platelets for 1 min with increasing concentrations of PA (0.1–1 mM) or its vehicle (Tris/HCl buffer, 50 mM at pH 7.40). In some experiments incubations were carried out in the presence of hexacyanoferrate(III) (100 mM) or oxyhaemoglobin (10 μM). After addition of ADP the platelet response was recorded for 7 min by measuring the resulting changes in light transmission.

RESULTS AND DISCUSSION

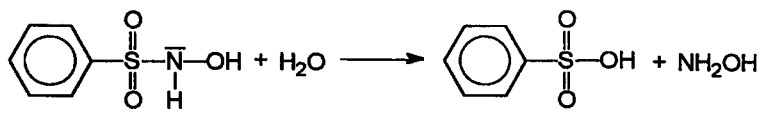
As previous investigations on the chemical decomposition of PA have been performed under anaerobic and strongly alkaline conditions only [14], we sought to study the compound's behaviour at physiologically more relevant pH values in the absence and presence of molecular oxygen and potassium hexacyanoferrate(III) as a one-electron oxidant. In the present investigation PA was found to undergo only slow decomposition at pH 7.4 and 8. Even under aerobic conditions at least 80 % of PA remained intact after 1 h at 37 °C (Figure 1). The only decomposition product detected by HPLC was BSI. Only minor kinetic differences were found between decomposition under an inert and decomposition under an oxygen-containing atmosphere. Decomposition of PA was, however, markedly enhanced on addition of hexacyanoferrate(III) (Figure 1). Apart from BSI as major product, minor traces of benzenesulphonic acid ($C_6H_5SO_2OH$) were detected under these conditions. The oxidative degradation of PA is strongly pH-dependent and, similarly to other OH-acidic compounds such as phenols and oximes, high pH favours decomposition.

Table 1 Concentrations of different nitric oxide and nitroxyl-generating agents eliciting half-maximal relaxation (EC_{50}) in isolated PE-precontracted rat aortic rings

Data are means ± S.E.M., where *n* indicates the total number of individual tissue rings from three to five animals.

Compound	EC_{50} (mol/l)	*n*
CysNO	$(2.91 \pm 1.33) \times 10^{-7}$	13
AS	$(3.16 \pm 0.80) \times 10^{-7}$	10
Nitric oxide	$(4.03 \pm 0.49) \times 10^{-7}$	11
Hydroxylamine	$(1.03 \pm 0.09) \times 10^{-6}$	18
PA	$(1.15 \pm 0.34) \times 10^{-6}$	11
Nitrite	$(1.63 \pm 0.40) \times 10^{-4}$	10

PA was found to have potent vasorelaxant activity in precontracted rat aortic rings in organ baths with an EC_{50} similar to that of NH_2OH and one-hundredth that of NO_2^-. PA was, however, a clearly weaker relaxant than either authentic NO, AS, or CysNO (Table 1). The tissue response to PA was abolished, in a concentration-dependent manner, in the presence of Methylene Blue ($n = 3$, results not shown), suggesting that relaxation was mediated by stimulation of soluble guanylyl cyclase and consequent increases in the level of cGMP. The similarity between the concentrations of PA and hydroxylamine to elicit half-maximal tissue relaxation might have been interpreted to indicate that the vasorelaxation produced by the former compound was due to its hydrolysis to NH_2OH according to eqn. (4):



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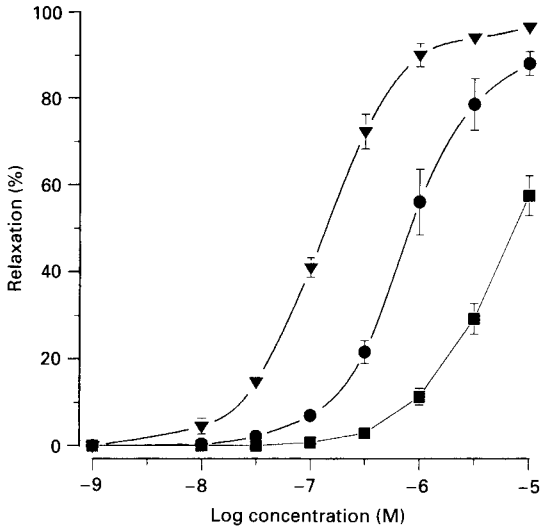


Figure 2 Concentration–response curve for the vasorelaxing action of PA in phenylephrine-precontracted endothelium-intact rat aortic rings in the absence (●) and presence of 100 mM of either potassium hexacyanoferrate(III) (▼) or the nitroxide, 1 (■)

Results are means ± S.E.M. for four or five individual experiments.

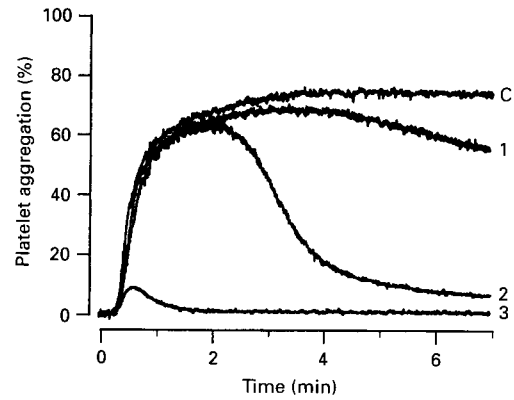
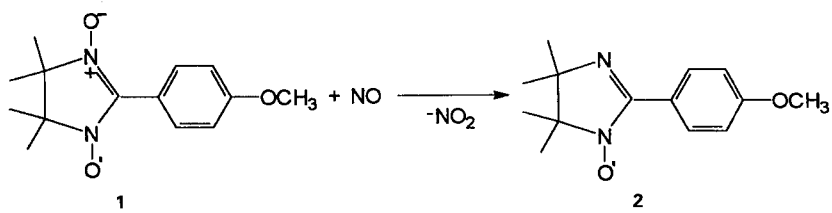


Figure 3 Superimposed original registrations showing the inhibitory effect of PA on ADP-induced platelet aggregation

Potentiation by potassium hexacyanoferrate(III) and reversal by oxyhaemoglobin (HbO_2). Trace C, Control aggregation; trace 1, PA 10 mM + $K_3[Fe(CN)_6]$ 100 mM + HbO_2 10 mM; trace 2, PA 10 mM; trace 3, PA 10 mM + $K_3[Fe(CN)_6]$ 100 mM. The tracings shown are representative of three separate experiments with similar results.

However, neither benzenesulphonic acid nor NH_2OH were formed during the decomposition of PA in phosphate buffer (pH 7.4, 37 °C) as evidenced by HPLC ($n = 3$, results not shown). Addition of the nitroxide (**1**) to the organ bath caused a considerable shift of the concentration–response curve for PA to the right (Figure 2), indicating that free NO was formed in the tissue, which reacted with **1** via a radical–radical reaction to form **2** and NO_2 [eqn. (5)] [15–17].



(5)

In contrast, addition to the organ bath of 10^{-4} M hexacyanoferrate(III), which itself did not affect vascular tone, induced a significant shift of the concentration–response curve for PA to the left (Figure 2), suggesting transformation to NO under oxidative conditions. This conclusion is supported by results obtained in PRP. PA proved to be a rather weak inhibitor of platelet aggregation, with an EC_{50} of approx. 3×10^{-4} M ($n = 3$). Its anti-platelet activity was, however, markedly potentiated, in a concentration-dependent manner, on addition of hexacyanoferrate(III) (see Figure 3). A leftward shift of the concentration–response curve by almost 2 log orders was observed at 10^{-4} M hexacyanoferrate(III), whereas the same concentration of the oxidant had no effect on the control response to ADP. This potentiation by hexacyanoferrate(III) was completely abolished in the presence of 10 μM oxyhaemoglobin, indicating the involvement of NO.

Formation of NO from PA was measured directly by chemiluminescence and indirectly by stimulation of soluble guanylyl cyclase. Virtually no formation of NO was detectable chemically under strictly anaerobic conditions. Addition of room-air-equilibrated buffer to an anaerobic solution of PA, however, induced the immediate release of NO (Figure 4). This is in agreement with results reported by other investigators earlier [18]. More efficiently than with O_2, hydrogen peroxide (H_2O_2) and hexacyanoferrate(III) induced NO generation from PA under these conditions. The accelerated decomposition of PA in the presence of hexacyanoferrate(III) observed upon alkalinization (see Figure 1) was associated with an increased rate of NO formation (Figure 5).

With rat-liver cytosol as an enzyme source, PA stimulated soluble guanylyl cyclase in a concentration- and pH-dependent manner with EC_{50} values of > 2000, 142 ± 13 and 17 ± 3 μM at pH 7.0, 7.4 and 8.0, respectively ($n = 3$). Addition of an equimolar concentration of hexacyanoferrate(III) increased enzyme stimulation by 50 μM PA from 189 ± 21 to 567 ± 42 pmol cGMP/min per mg of protein, whereas basal activity was only marginally influenced by hexacyanoferrate(III) alone (3.9 ± 0.3 compared with 3.0 ± 0.2 pmol cGMP/min per mg of protein) ($n = 3$). Enzyme activation by 50 μM PA was completely abolished in the presence of 10 μM oxyhaemoglobin ($n = 2$).

Although these results demonstrate unambiguously that NO can be formed from PA under oxidative conditions, they do not clarify whether NO was formed from the sulphohydroxamic acid directly or whether it originated from the oxidation of NO^- as proposed by Fukuto et al. [19], if the latter species were actually formed under these conditions. Because, at physiological pH, (i)

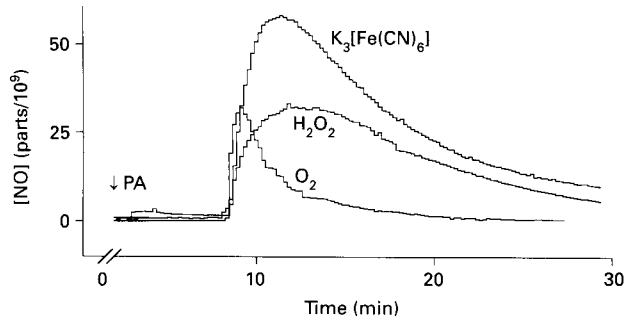


Figure 4 Effect of oxidation on the formation of NO from PA as measured by chemiluminescence

Conditions: 50 μM PA in 0.1 M phosphate buffer, pH 7.4, after 10 min addition of (1) room-air-equilibrated phosphate buffer (O_2), (2) hydrogen peroxide (H_2O_2), or (3) potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$). The final concentration of O_2, H_2O_2 or hexacyanoferrate(III) was 50 μM.

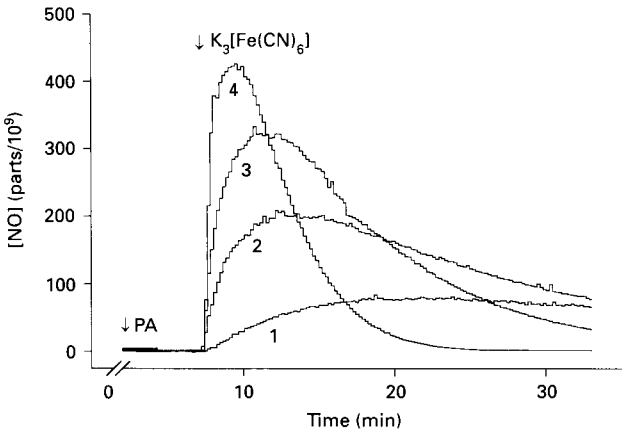


Figure 5 pH-dependence of PA oxidation to NO

Conditions: 50 μM PA in 0.1 M aqueous buffer; after 10 min addition of a 5-fold molar excess of $K_3[Fe(CN)_6]$: trace 1, pH 6.0; trace 2, pH 7.4; trace 3, pH 8.0; trace 4, pH 9.0. The tracings shown are representative of two or three similar experiments.

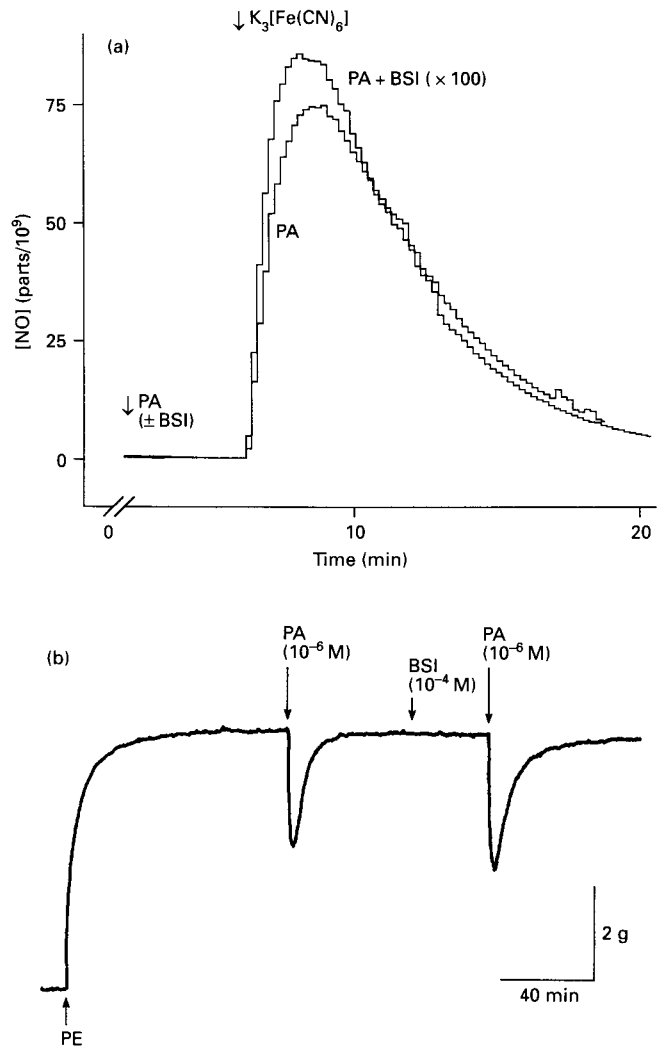


Figure 6 Lack of inhibitory effect of BSI on (a) NO formation from PA and (b) on vascular relaxation by PA

Experiments were performed at pH 7.4 and 37 °C in the absence and presence of a 100-fold molar excess of BSI over PA. For chemiluminescence measurements, NO generation was induced by addition of an equimolar (50 μM) amount of $K_3[Fe(CN)_6]$ to an aerobic solution of PA in 0.1 M phosphate buffer. PE, phenylephrine. The tracings shown are representative of two separate experiments with identical results.

PA, as a weak acid (pK_a 9.29 [4]), exists predominantly in protonated form, and (ii) PA is quite stable under non-oxidative conditions (see Figure 1), prior cleavage of HNO according to eqn. (1) cannot account for the dramatic increase in NO formation observed on oxidation. As in this case the former would represent the rate-limiting step, NO generation from PA could never exceed the rates of decomposition to NO^-. To corroborate this conclusion we investigated the oxidative decomposition of PA to NO and its relaxing effect in isolated vascular tissue in the presence of BSI which, according to eqn. (1), inhibits decomposition of PA to HNO [3]. A 100-fold molar excess of BSI over PA did not result in a decrease in NO generation (Figure 6a), nor did it inhibit vasorelaxation by PA (Figure 6b), demonstrating that HNO cleavage is not a prerequisite for NO formation to occur. This is furthermore substantiated by the lack of metal nitrosyl formation when attempting to detect NO^- by complexation with $[Ni(CN)_4]^{2-}$ as described by Nast et al. [20,21]. Even with very high concentrations of PA this test revealed only weak positive results at pH 10, confirming that in the pH range 7–8 the formation of NO from HNO is of little or no significance. Similarly, consecutive reaction products of NO^-/HNO, such as peroxynitrite ($ONOO^-$), for example, are likely to be quantitatively irrelevant for the observed effects during PA oxidation. We cannot exclude completely the possibility that small amounts of HNO derived from the non-oxidative decomposition of PA may, in part, have contributed either directly or indirectly (i.e. after oxidation to NO) to the biological effects observed. The extent of vasorelaxation and platelet inhibition under oxidative conditions, however, cannot be explained by this mechanism.

However, oxidation of PA to a nitroxide radical is more easily conceivable because corresponding N-substituted analogues have been detected by ESR spectroscopy [22,23]. Unlike the nitroxide derivative of PA, those radicals cannot stabilize by splitting off NO but undergo other reactions such as dimerization.

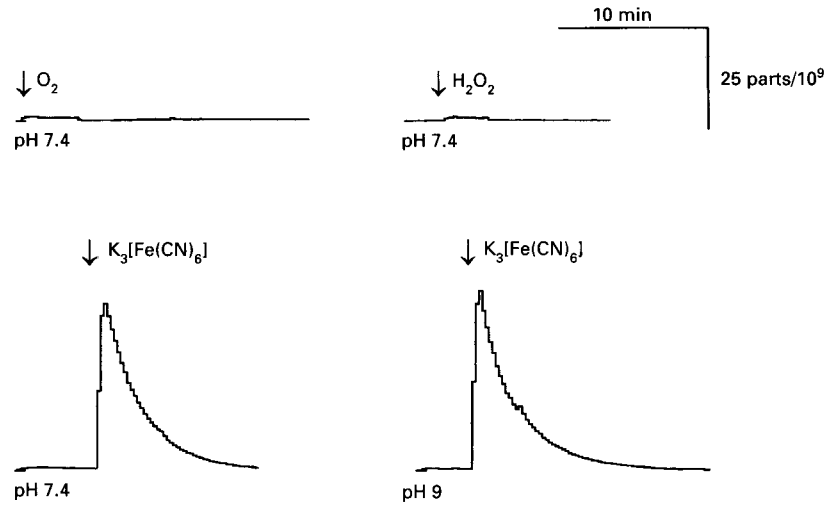


Figure 7 Effect of different oxidants and pH on the release of NO from Angeli's salt (AS)

Experiments were performed in 0.1 M phosphate buffer at 37 °C under nitrogen; 5 min after addition of AS, either O_2 (in the form of an appropriate volume of room-air-equilibrated buffer), hydrogen peroxide (H_2O_2) or $K_3[Fe(CN)_6]$ was introduced.The tracings shown are representative of 2 individual experiments with identical results.

Reactivity of AS

In contrast with PA, in the pH range 3–9 AS decomposes quite quickly to NO_2^- and HNO [24,25], whereas it is stable in more alkaline solution. On addition of hexacyanoferrate(III) the rates and absolute amounts of NO released from AS were comparable at pH 7.4 and 9 (Figure 7). Contrary to findings by Fukuto et al. [18] we were unable to detect any release of NO on addition of O_2 or H_2O_2 at physiological pH (Figure 7). The reason for this discrepancy is unknown. When aqueous solutions of AS were stored at room temperature under an inert atmosphere at pH 7.4, NO formation on addition of hexacyanoferrate(III) was considerably less than that obtained with freshly prepared solutions because part of the AS had undergone decomposition, via HNO, to N_2O ($n = 2$, results not shown).

It therefore seems that AS hydrolyses to HNO but does not generate NO at physiological pH. Whether the hexacyanoferrate(III)-induced release of NO is caused by direct oxidation of AS [eqn. (6)] or by the oxidation of intermediate HNO [eqns. (7 and 8)] remains to be investigated.

$$HN_2O_3^- \xrightarrow{-H^\bullet} N_2O_3^{\bullet-} \longrightarrow NO_2^- + NO \qquad (6)$$

$$HN_2O_3^- \longrightarrow NO_2^- + [HNO] \qquad (7)$$

$$[HNO] \xrightarrow{-H^\bullet} NO \qquad (8)$$

Effect of L-cysteine on PA- and AS-induced vasorelaxation

In isolated vascular tissue the relaxant responses to PA and AS revealed major differences when experiments were carried out in the presence of high concentrations of L-cysteine (Figure 8). As predicted from their respective EC_{50} values (see Table 1), AS was about 3 times more potent than PA when applied as a bolus to the organ bath. Relaxations elicited by either compound were generally short-lived. Addition of L-cysteine (3 mM) to the organ baths caused a transient relaxation of endothelium-intact rat aortic rings, perhaps due to the release of NO from storage forms in the tissue. After recovery of the tissues to their original contractile tone, the relaxant response to a second application of AS was almost completely blunted (see Figure 8). Almost the same degree of inhibition of AS action was found when cysteine was added to the organ bath first ($n = 3$). These findings are in agreement with previous results demonstrating that nitroxyl-mediated vasorelaxation is inhibited, in a concentration-dependent manner, by L-cysteine [6]. In contrast, relaxations induced by PA, similarly to those produced by authentic NO (not shown), were greatly enhanced in the presence of high concentrations of L-cysteine. These results thus confirm that the product of decomposition of PA in the tissue that accounts for its vasorelaxing activity is NO rather than NO^-.

Whereas PA can be oxidized physiologically to BSI and NO, even in the presence of high concentrations of L-cysteine, the

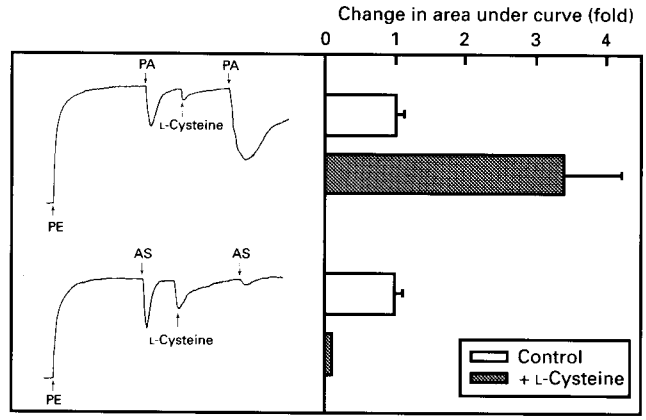


Figure 8 Differential effects of L-cysteine on vascular relaxation to equipotent concentrations of PA (1 μM) and AS (0.3 μM) in endothelium-intact rat aortic rings in organ baths

Representative tracings and means ± S.E.M. of three separate experiments.

situation is obviously different with AS. Here, the predominating reaction appears to be formation of NO^-, which by itself cannot activate soluble guanylyl cyclase directly but only after oxidative transformation into NO. If this oxidation is directly or indirectly inhibited by L-cysteine, target enzyme activation is precluded, and thus vasorelaxation does not occur. However, with all those compounds that can release NO even in the presence of thiols (as appears to hold true for PA), addition of the latter will actually lead to a prolongation of the tissue half-life of NO, thus potentiating its biological activity.

The finding that the rate of decomposition of PA at physiological pH and pO_2 is slow, and not markedly different from its decomposition rate in an inert atmosphere, suggests the involvement of an enzymic step in the oxidative release of NO from PA [5]. Although the identity of the enzyme(s) involved still remains to be elucidated, the differential action of L-cysteine indicates that the enzyme that accounts for the bioactivation of PA is not identical with that which mediates the vasorelaxing action of AS.

Collectively, the results of this study clearly demonstrate that under physiological conditions the decomposition product that accounts for the vasorelaxing and anti-platelet activity of PA is NO rather than NO^- as originally envisaged. Sulphohydroxamic acids with a free hydroxamic acid functional group can thus be considered prototypes of a new class of thiol-independent NO-donors capable of generating NO under oxidative conditions.

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