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BIOASSAY DISCRIMINATION BETWEEN NITRIC OXIDE (NO·) AND NITROXYL (NO^-) USING L-CYSTEINE

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Nitroxyl (NO^-) is the one-electron reduction product of nitric oxide (NO·). Recently, NO^- generating compounds were shown to possess potent vasorelaxant activity and this was attributed to the ready conversion of NO^- to NO·. Because of its metastable character, direct chemical detection of NO^- or its conjugated acid, HNO, has not been accomplished yet. In order to gain further insight into the cellular mode of action of NO^- generating compounds we aimed at finding a means to discriminate NO^- from NO· by bioassay. Using isolated rat aortic rings in organ baths, we here show that high concentrations of L-cysteine cause complete inhibition of the vasorelaxant response to NO^- (generated from Angeli's salt and sodium nitroxyl) whereas responses to authentic NO· and S-nitrosocysteine are largely enhanced. Preliminary results indicate that the inhibition by L-cysteine of NO^- activity may be mediated in part by enzymatic and non-enzymatic mechanisms. Whether or not NO^- generating compounds will have promising therapeutic potential as a new classs of NO·-donors will not least depend on their interference with enzymatic routes susceptible to inhibition by NO^-.

The chemical and pharmacological identification of EDRF as nitric oxide (NO·) [1] and its involvement in diverse physiological processes in the cardiovascular, nervous and immune system, has not only opened up a new area of biological research [2], but has also directed the attention of investigators towards the chemistry and biochemistry of NO· and its redox-related forms, nitrosonium ion (NO^+) and nitroxyl anion (NO^-) [3]. Other compounds rather than NO· have been proposed to account for the biological activity of EDRF, among them NO^- [4].

The protonated form of NO^-, HNO, is a weak acid with a pK_a value of 4.7 [5], indicating that, at physiological pH, NO^- is the predominant form in aqueous solution. After protonation NO^- decomposes rapidly to yield nitrous oxide (N_2O) following dimerization and dehydration [6]:

$$NO^- + H^+ \rightleftharpoons HNO \quad (1)$$

$$2\,HNO \longrightarrow [\,HO\text{-}N{=}N\text{-}OH\,] \longrightarrow N_2O + H_2O \quad (2)$$

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NO^- also reacts with Fe(III) heme proteins such as methemoglobin, metmyoglobin [7] and Fe(III) cytochrome c [8] to form NO-adducts (reductive nitrosylation). Because of its metastable character, direct detection of HNO has not been accomplished yet, but generation of N_2O is generally considered to be indicative of the formation of NO^-. N_2O production has been observed upon decomposition of Angeli's salt ($Na_2N_2O_3$) in aqueous solution [6,9], upon hydrolysis of N-hydroxybenzenesulfonamide ($C_6H_5SO_2NHOH$, Piloty's acid) in alkaline aqueous solution [10] and upon non-enzymatic disproportionation of its N,O-diacylated derivatives [11]. The enzymatic oxidation of cyanamide (NH_2CN) by catalase/H_2O_2 and the chemical oxidation of N-hydroxyguanidine compounds have also been reported to be accompanied by formation of N_2O [12,13].

After the identification of N^G-hydroxy-L-arginine as an intermediate in the enzymatic conversion of L-arginine to $NO^\cdot$ and the realization that under certain conditions not only $NO^\cdot$ but also HNO may be formed [13], studies were performed in order to explore the biological activity, if any, of NO^-. NO^- has been described to be a potent vasorelaxant of both rabbit thoracic aorta and bovine intrapulmonary artery [4], but its actual relationship to the action of EDRF remains to be elucidated. Recently, we provided experimental evidence against the assumption that NO^- may be identical to EDRF. A comparison of the relaxing actions in a cascade superfusion bioassay of various EDRF candidates revealed major differences between $NO^\cdot$ and sodium nitroxyl (NaNO), the latter being more stable and less susceptible to inhibition by oxyhaemoglobin [14]. The present study was performed in order to compare directly the vasorelaxant activity of NO^- generated from Angeli's salt and NaNO with that of authentic $NO^\cdot$ and the $NO^\cdot$-donor compound S-nitrosocysteine (CysNO) in isolated aortic rings in organ baths. Whereas moderate concentrations of L-cysteine have been shown to be a useful tool for the discrimination of $NO^\cdot$ from S-nitrosothiols [14], we now show that, using the same tool at higher concentrations, $NO^\cdot$ can be clearly differentiated from NO^-.

MATERIALS AND METHODS

Chemicals and solutions: Sodium trioxodinitrate ($Na_2N_2O_3$, Angeli's salt) was synthesized by a modified version of the procedure described by Smith and Hein [15]. Sodium nitroxyl (NaNO) was synthesized according to the method of Zintl and Harder [16]. Both compounds were stored under argon until use. Phenylephrine hydrochloride (PE) and sodium diethyldithiocarbamate (Na-DETC) were purchased from Aldrich (Steinheim, FRG). L-N^G-nitro-arginine (L-NNA), ethylenediaminetetraacetic acid (EDTA), L-cysteine hydrochloride, hydroxylammonium chloride (NH_2OH, hydroxylamine) and superoxide dismutase (Cu-Zn isoform from bovine erythrocytes, SOD) were purchased from Sigma (Deisenhofen, FRG). Sodium nitrite ($NaNO_2$) was from Riedel de Häen (Seelze, FRG). Solutions of Angeli's salt and NaNO were prepared in nitrite-free water (water for HPLC, Riedel de Häen) bubbled with argon for 15-20 min prior to use. S-nitrosocysteine (CysNO) was synthesized by acid catalysed S-nitrosation of L-cysteine with $NaNO_2$ in 1 mM citrate buffer (pH=2). All dilutions were made in citrate buffer immediately before use and stored at 4°C and protected from light. Aqueous solutions of $NO^\cdot$ were prepared as described previously [17]. The Krebs-Henseleit buffer used consisted of: (in 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.

Bioassay: Anesthesized male Wistar rats (250-350 g) were sacrified, the thoracic aorta was carefully removed, cleaned up of fat and connective tissue and cut into 4-5 mm rings. The rings were then mounted in 20 ml organ baths containing oxygenated (95% O_2 / 5 % CO_2) Krebs solution (pH=7.4) and allowed to equilibrate under a resting tension of 2 g at 37°C. After equilibration tissues were submaximally precontracted with PE (0.2 µM). L-NNA (100 µM) was added in order to enhance the stability of the preparation. Complete concentration-response curves were obtained by continuous recording of changes in isometric tension following cumulative addition of test compounds.

Detection of nitric oxide: The generation of NO· upon the decomposition of Angeli's salt was measured by chemiluminescence as previously described [18]. Briefly, a highly sensitive chemiluminescence detector (CLD 780 TR, eco Physics) was connected to the outlet of a jacketed, septum-sealed micro-reaction chamber via stainless-steel tubings. A stream of purified nitrogen gas was passed over the surface of the sample at a rate of 100 ml/min, allowing for the continuous monitoring of generated NO· after introduction of test compounds into the buffer solution (0.1 M sodium phosphate, pH=7.4, 37°C) via the septum of the reaction chamber. The detection limit was in the ppt range.

RESULTS AND DISCUSSION

Angeli's salt was used as a source of NO^-, since the decomposition of Angeli's salt in aqueous solution has been shown to be essentially pH independent between pH 4 and 8. At physiological pH it exists predominantly in the form of the monoanion $HN_2O_3^-$ (pK_1=2.5, pK_2=9.5), which reversibly decomposes to form NO^- and nitrite (NO_2^-) [6,9]:

$$HN_2O_3^- \rightleftharpoons NO^- + NO_2^- + H^+ \qquad (3)$$

NO^- generated from Angeli's salt was found to have potent dilator activity in precontracted rat aortic rings with an EC_{50} value similar to that of CysNO and 1000 times more potent than its decomposition product, NO_2^- (Tab. 1). In contrast, NaNO was found to have considerably weaker activity than Angeli's salt. It should be noted, however, that the exact chemical nature of the compound described as NaNO in the literature is not completely clear at

Table 1. EC_{50} values for the relaxation response in isolated rat aortic rings. Data are presented as mean values ± S.E.M., where n indicates the total number of individual tissue rings from 3-5 animals.

Compound	EC_{50} [M]	n
CysNO	$2.91 \pm 1.33 \times 10^{-7}$	13
Angeli's salt	$3.16 \pm 0.80 \times 10^{-7}$	10
NO·	$4.03 \pm 0.49 \times 10^{-7}$	11
NH_2OH	$1.03 \pm 0.09 \times 10^{-6}$	18
NaNO	$6.31 \pm 1.63 \times 10^{-6}$	5
$NaNO_2$	$1.63 \pm 0.40 \times 10^{-4}$	10

present. Since we could not determine with a proper degree of accuracy the chemical composition of the solid obtained, it is possible that it was actually a mixture of monomeric and dimeric forms and thus not a sole source of NO^-.

Addition of increasing concentrations of L-cysteine to the organ baths caused a transient relaxation of endothelium-intact rat aortic rings (not shown). L-cysteine effectively reduced the relaxant response to NO^- from Angeli's salt in a concentration-dependent manner, with almost complete inhibition at 3 mM, whereas the response to an equipotent concentration of CysNO was greatly enhanced (Fig. 1). This enhancement is likely to be due to chemical stabilisation of CysNO by L-cysteine, which can complex transition metal ions that catalyse the decomposition of S-nitrosothiols [14]. The relaxation response to NaNO was inhibited by higher concentrations of L-cysteine to a similar extent as observed with Angeli's salt (n=3, not shown). In contrast, the action of $NO^\cdot$ was partially reduced at concentrations of L-cysteine up to 0.1 mM but was greatly enhanced at higher thiol concentrations, an effect which has been attributed to partial S-nitrosation of L-cysteine by an oxidised form of $NO^\cdot$ [14].

The electroaffinity of $NO^\cdot$ (EA= +0.024 eV) is one of the smallest positive electroaffinity values known [19]. Thus, any molecule with a higher EA value would be able to detach an electron from NO^- to yield $NO^\cdot$ in a one electron-transfer process. Recent observations indicate a marked increase in $NO^\cdot$ formation during the decomposition of Angeli's salt in phophate buffer (pH=7.4) after exposure to air [20], but the mechanism of this oxidation still requires elucidation. We have now confirmed, using chemiluminescence, that in oxygenated aqueous buffer Angeli's salt decomposes with the formation of small amounts of $NO^\cdot$ with a conversion rate in the order of 0.3 - 1.0 % (n=3, data not shown). However, if in the organ baths NO^- had been readily converted to $NO^\cdot$ by oxygen, we should have observed an augmented tissue response to Angeli's salt in the presence of high concentrations of L-cysteine (via formation of CysNO) whereas, in fact, the opposite effect was observed (Fig. 1).

There are several ways in which L-cysteine may have inhibited the action of NO^-: First, if NO^- were readily converted to $NO^\cdot$ in the tissue, the generation of superoxide radicals ($O_2^{\cdot-}$) by L-cysteine [21] may have counteracted the dilator action of the $NO^\cdot$ formed. Although superoxide dismutase (SOD) has been shown to increase the vasorelaxant activity of Angeli's salt [19] and to almost completely reverse the inhibitory effect of L-cysteine on $NO^\cdot$-mediated relaxation [14], in the present study addition of SOD (10 U/ml) did not reverse the inhibition by L-cysteine of the dilator action of NO^- (n=2, not shown). This suggests that generation of $O_2^{\cdot-}$ by L-cysteine is unlikely to account for the observed inhibition in the tissue response to NO^-.

The inhibitory action of L-cysteine may rather be due to interference with conversion of NO^- to $NO^\cdot$. In order to test whether NO^- may be converted to $NO^\cdot$ by traces of redox active metal ions present in the buffer solution, a process which may have been affected by L-cysteine due to its complexing action [14, 22], we compared the relaxation responses to equipotent concentrations of CysNO and Angeli's salt in the presence and absence of EDTA. As shown in

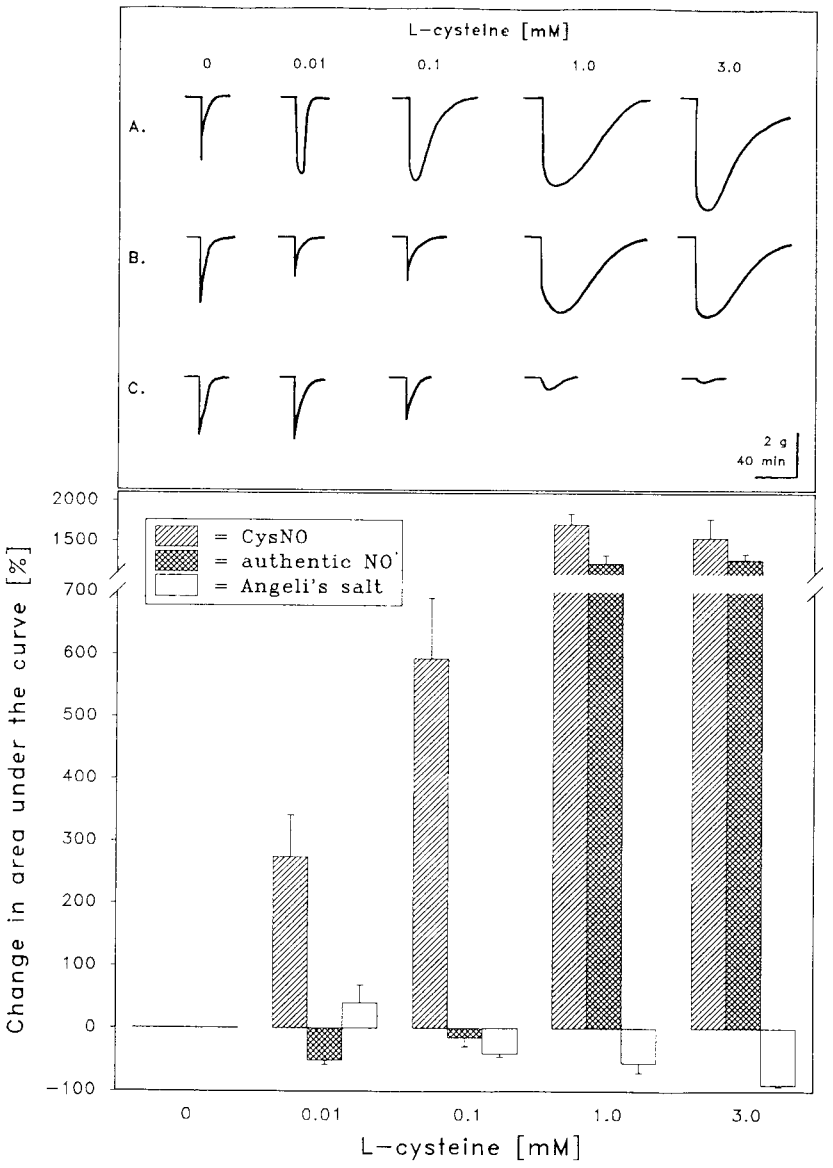

Fig. 1. Effect of different concentrations of L-cysteine on the relaxation to CysNO (0.3 μM), authentic NO· (3 μM) and Angeli's salt (0.3 μM) in endothelium-intact rat aortic rings. Results shown in the upper panel are representative tracings of 3 - 5 separate experiments and in the lower panel means ± S.E.M. of these experiments. A) CysNO, B) authentic NO·, C) Angeli's salt.

Fig. 2, EDTA had no effect on either the magnitude or the duration of the response to NO^-, ruling out the involvement of metal ion driven conversion of NO^- to NO·.

Alternatively, L-cysteine may inhibit an enzyme such as SOD which converts NO^- to NO· [23], since 0.1 mM sodium diethyldithiocarbamate (Na-DETC), a known chelator of copper-containing enzymes [24], partially mimicked the inhibitory effect of L-cysteine on the action of Angeli's salt and NaNO in this bioassay, leaving the action of NO· and CysNO unaffected (Fig. 3). Moreover, inhibition of the activity of SOD by L-cysteine has been reported previously

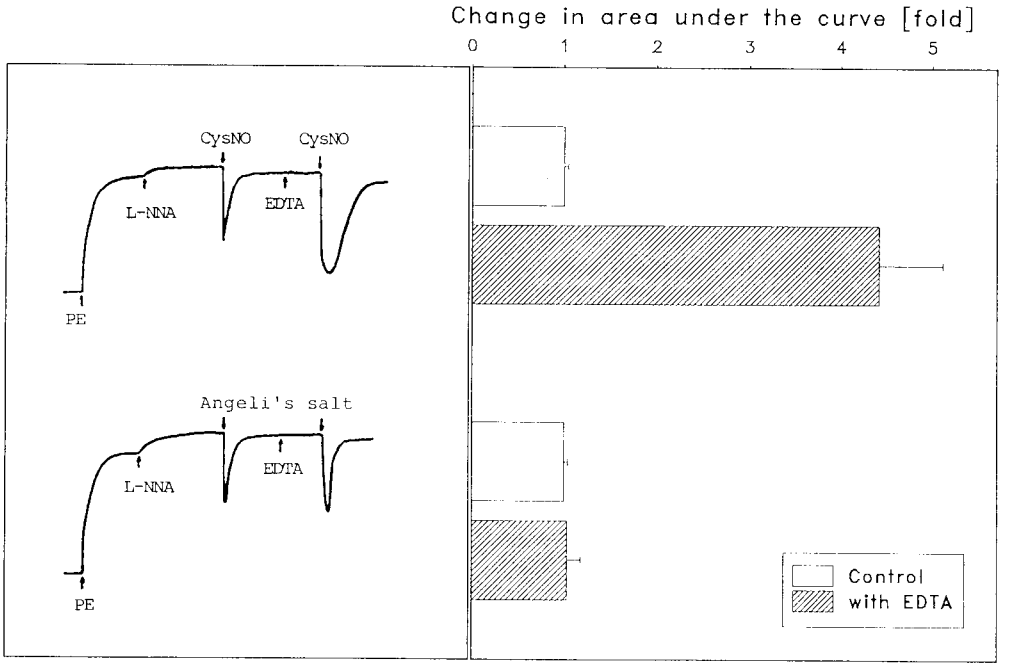

Fig. 2. Effect of EDTA (0.1 mM) on the relaxation to equipotent concentrations of CysNO (0.3 μM) and Angeli's salt (0.3 μM) in endothelium-intact rat aortic rings. Left panel shows representative tracings of 3 separate experiments. The results in the right panel are expressed as the means ± S.E.M. of these experiments.

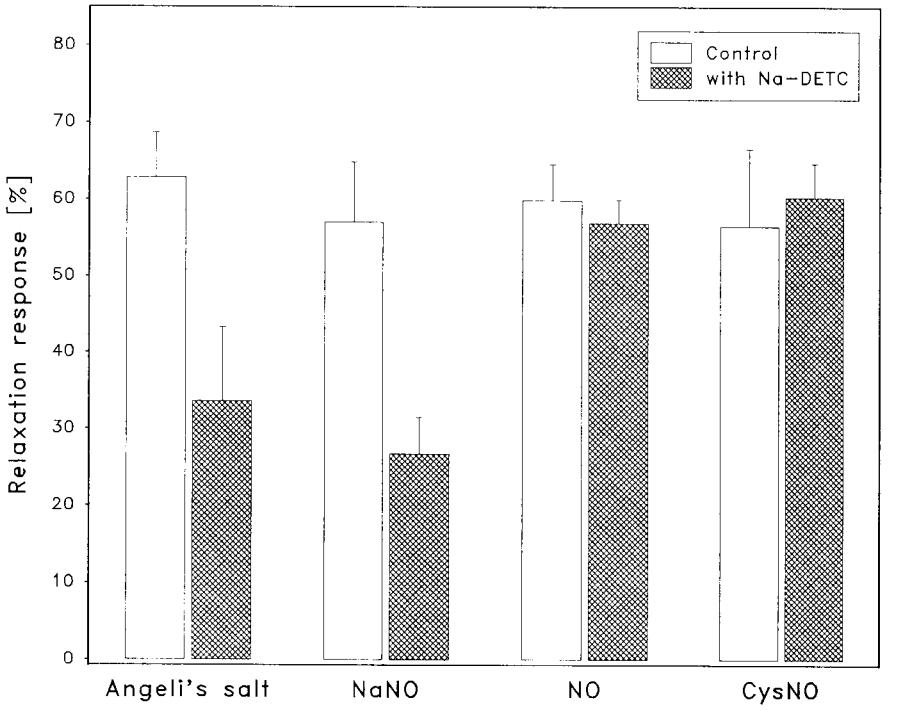

Fig. 3. Inhibitory effect of Na-DETC (0.1 mM) on the relaxation response to Angeli's salt (0.3 μM), NaNO (10 μM), $NO^{\cdot}$ (3 μM) and to CysNO (0.3 μM). Results are means ± S.E.M. of 3 experiments.

[25]. The use of concentrations of Na-DETC >0.1 mM were not considered specific for the differentiation between $NO^{\cdot}$ and NO^{-} as these concentrations also attenuated the vasorelaxation elicited by $NO^{\cdot}$ and CysNO (n=2, not shown), presumably by trapping of $NO^{\cdot}$ by DETC/Fe^{2+} complexes formed in the tissue [26].

Another possible explanation for the inhibitory action of cysteine on Angeli's salt and NaNO may be a direct reaction between L-cysteine and NO^{-}. Indeed, the species formed from the spontaneous decomposition of Angeli's salt can be trapped in part by thiols [27], yielding hydroxylamine (NH_2OH) as a final product:

$$NO^{-} + H^{+} \rightleftharpoons HNO \quad (1)$$

$$HNO + RSH \longrightarrow RSNHOH \quad (4)$$

$$RSNHOH + RSH \longrightarrow RSSR + NH_2OH \quad (5)$$

NH_2OH itself also has vasorelaxant activity (see Tab. 1), but this requires the action of catalase [28], an enzyme which is also inhibited by thiols [25]. Thus, high concentrations of L-cysteine may have inhibited both SOD and catalase, which may explain the decrease of the tissue response to NH_2OH, if it were actually formed. Alternatively, NH_2OH may have trapped the NO^{-} generated [29] with formation of nitrogen (N_2), which has no vasodilator activity:

$$NH_2OH + HNO \longrightarrow N_2 + 2\,H_2O \quad (6)$$

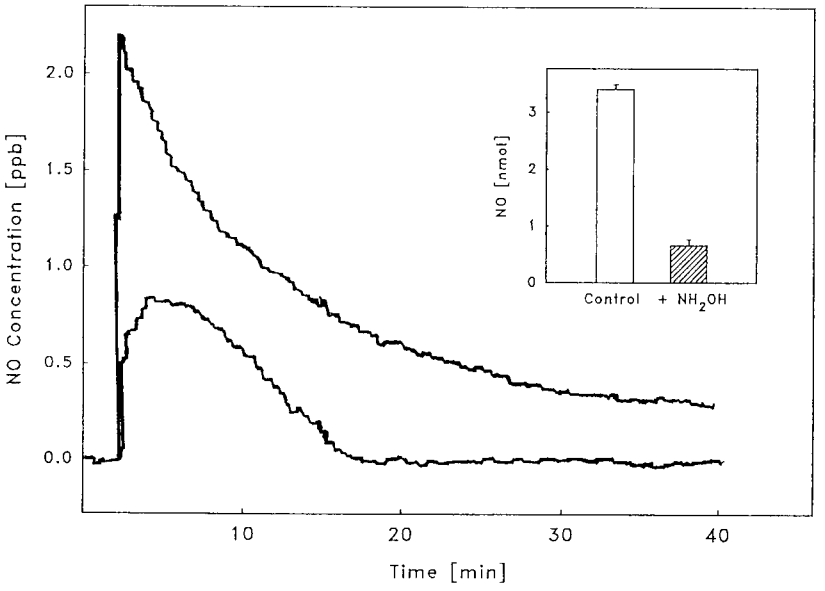

Fig. 4. Effect of NH_2OH (5 mM) on the release of $NO^{\cdot}$ from Angeli's salt (0.5 mM) at 37°C in phosphate buffer as detected by chemiluminescence. The inset shows the amount of $NO^{\cdot}$ detected after incubation of Angeli's salt for 35 min. Means ± S.E.M. of 3 experiments.

Using chemiluminescence we could indeed demonstrate a decrease in the formation of NO· from Angeli's salt in the presence of an excess of NH_2OH (Fig. 4).

Collectively, the results of the present study demonstrate that L-cysteine is a useful tool for the discrimination by bioassay of NO^- from NO·. Furthermore, the present data confirm that NO^--generating compounds such as Angeli's salt can be considered a new class of nitrovasodilators [20], which act via intracellular conversion of generated NO^- to NO·. Whether or not such compounds will have therapeutic potential as NO· donors will not least depend on the extent of interaction with enzymes susceptible to inhibition by NO^-, such as aldehyde dehydrogenase [12], which may limit their usefulness by interference with the metabolism of other drugs, ethanol and certain food constituents.

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REFERENCES

1. Palmer, R.; Ferrige, A.G.; Moncada, S.: Nature (1987), 327, 524-526.
2. Moncada,S.; Palmer, R.; Higgs, E.A.: Pharmacol. Reviews (1991), 43, 109-142.
3. Stamler, J.S.; Singel, D.J.; Loscalzo, J.: Science (1992), 258, 1898-1901.
4. Fukuto, J.M.; Chiang, K.; Hszieh, R.; Wong, P.; Chaudhuri, G.: J. Pharmacol. Exp. Ther. (1992), 263 (2), 546-551.
5. Grätzel, M.; Tanaguchi, S.; Henglein, A.: Ber.Bunsen-Ges. Phys. Chem. (1970), 74, 1003-1010.
6. Bonner, F.T.; Hughes, M.N.: Coments Inorg. Chem. (1988), 7 (4), 215-234.
7. Bazylinski, D.A.; Hollocher, T.C.: J. Am. Chem. Soc. (1985), 107 (26), 79828-7986.
8. Mahapatro, S.N.: J.Cell. Biol. (1988), 107, 6 Pt 3, 619 A.
9. Hughes, M.N.; Wimbledon, P.E.: J. Chem. Soc. Dalton Trans. (1977), 17, 1650-1653.
10. Bonner, F.T.; Ko, Y.: Inorg. Chem. (1992), 31, 2514-2519.
11. Lee, M.J.C.; Nagasawa, H.T.; Elberling, J.A; De Master, E.G.: J. Med.Chem. (1992), 35, 3648-3652.
12. Nagasawa, H.T.; De Master, E.G.; Redfern, B.; Shirota, F.N., Goon, D.J.W.: J. Med.Chem. (1990), 33, 3120-3122.
13. Fukuto, J.M.; Wallace, G.C.; Hszieh, R.; Chaudhuri, G.: Biochem. Pharmacol. (1992), 43 (3), 607-613.
14. Feelisch, M.; te Poel, M.; Zamora, R.; Deussen, A.; Moncada, S.: Nature (1994), 368, 62-65.
15. Smith, P.A.S.; Hein, G.E.: J. Am. Chem. Soc. (1960), 82, 5731-5740.
16. Zintl, E.; Harder, A.: W. Chem. Ber. (1933), 66, 760-761.
17. Feelisch, M.: J. Cardiovasc. Pharmacol. (1991), 17, S25-S33.
18. Knüttel, K.; Feelisch, M.: Endothelium (1993), 1, s32.
19. Rinden, E.; Maricq, M.M.; Grabowski, J.J.: J. Am. Chem. Soc. (1989), 111 (4), 1203-1210.
20. Fukuto, J.M.; Hobbs, A.J.; Ignarro, L.J.: Biochem. Biophys. Res. Comm. (1993), 196 (2), 707-713.
21. Misra, H.P.: J. Biol. Chem. (1974), 269, 2151-2155.
22. Mc Aninly, J.; Williams, D.L.H.; Askew, S.C.; Butler, A.R.; Russell, C.: JCS Chem. Commun. (1993), 23, 1758-1759.