POSSIBLE INVOLVEMENT OF *S*-NITROSOTHIOLS IN THE ACTIVATION OF GUANYLATE CYCLASE BY NITROSO COMPOUNDS

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1. Introduction

The inhibitory effects of methemoglobin on activation of coronary arterial soluble guanylate cyclase by nitric oxide (NO), nitroprusside and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were described in [1]. Also dithiothreitol (DTT) enhanced activation of coronary arterial and hepatic soluble guanylate cyclase by nitroprusside and MNNG but not by NO, and reversed the methemoglobin blockade of activation by nitroprusside and MNNG but not by NO. Indeed, DTT was required for hepatic guanylate cyclase activation by nitroprusside. These observations suggested that DTT may react directly with the nitroso compounds to promote release of NO, which may then overcome the inhibition by methemoglobin [1]. An alternative explanation for the effects of DTT on guanylate cyclase activation was a direct non-enzymatic transfer of the NO-moiety from the nitroso compound to guanylate cyclase (or a ferroheme intermediate) in the presence of thiols.

Each of the above hypotheses was tested and the data here illustrate that:

(1) Thiols promote release of NO gas from MNNG, but not from nitroprusside, in aqueous neutral buffer.
(2) Inhibition of guanylate cyclase activation by NO varies directly with the concentration of methemoglobin and indirectly with the amount of NO.
(3) Thiols react with NO to form stable *S*-nitrosothiols which are potent activators of guanylate cyclase.

2. Materials and methods

Guanylate cyclase activity was determined in soluble fractions prepared from bovine coronary artery and rat liver by measuring the formation of cyclic GMP from GTP, using Mg^{2+} as the required divalent cation, according to [2]. GTP was 0.1 mM and 0.3 mM for reaction mixtures containing coronary arterial and hepatic soluble fractions, respectively. Soluble fractions from bovine coronary artery and rat liver were prepared exactly as in [1,3] and stored at −80°C. Nitroprusside, thiols, heme proteins, ferricyanide and methylene blue were purchased from Sigma, and MNNG was from Aldrich.

S-Nitrosothiols were synthesized by reacting O_2-free solutions of each thiol in 50 mM Tris–HCl (pH 7.4) at 4°C for 15 min with purified NO gas (99.9%, Matheson gas) in an O_2-free N_2 atmosphere [4,5]. Unreacted NO was completely removed by alternating cycles of vacuum evacuation and N_2 flushing. After reaction with NO, solutions of cysteine, *N*-acetyl cysteine, GSH, DTT and β-D-thioglucose turned from clear to a yellow–rose color, and the penicillamine solution turned green [4–6]. Absorption maxima of 10–50 mM thiol solutions after reaction with NO were 540–550 nm (DTT, β-D-thioglucose), 550–560 nm (cysteine, *N*-acetyl cysteine, GSH) and 590–600 nm (penicillamine). Values for the *S*-nitroso derivatives of cysteine, GSH, DTT, and penicillamine are in excellent agreement with reported values [4,6]. Formation of *S*-nitroso derivatives of *N*-acetyl cysteine and β-D-thioglucose has not been reported yet.

3. Results and discussion

DTT enhanced the activation of coronary arterial and hepatic guanylate cyclase by nitroprusside and MNNG but not by NO [1]. Similarly, cysteine, GSH,

and penicillamine enhanced guanylate cyclase activation by nitroprusside, MNNG, and $NaNO_2$ but not by NO. For example, employing coronary arterial guanylate cyclase, 0.1 mM nitroprusside or MNNG stimulated the formation of 408–482 pmol cyclic GMP . min^{-1} . mg $protein^{-1}$, whereas 3 mM $NaNO_2$ did not alter basal activity (5–8 pmol cyclic GMP . min^{-1} . mg $protein^{-1}$). In the presence of 5 mM cysteine, GSH or penicillamine, activation (pmol cyclic GMP . min^{-1} . mg $protein^{-1}$) by nitroprusside (799–908), MNNG (807–945), and $NaNO_2$ (531–880) was markedly enhanced.

DTT reversed or abolished the methemoglobin blockade of guanylate cyclase activation by nitroprusside and MNNG but not by NO [1]. Similarly, methemoglobin blockade was reversed with cysteine, GSH or penicillamine (unpublished). An explanation proposed earlier for such observations was that thiols may enhance release of NO from nitroso compounds and the increased amount of NO may then overcome the inhibitory effect of methemoglobin [1]. Consistent with this proposal, fig.1 illustrates that increasing concentrations of methemoglobin, hemoglobin or myoglobin produced proportionally greater inhibition of guanylate cyclase activation by NO, and that greater amounts of NO overcame the inhibitory effect of the heme proteins. One explanation of the enhancement, and the reversal of methemoglobin blockade, of guanylate cyclase activation by thiols is that the latter interact with nitroso compounds or $NaNO_2$ to liberate NO gas. Therefore, experiments were conducted to determine the effects of thiols on NO gas

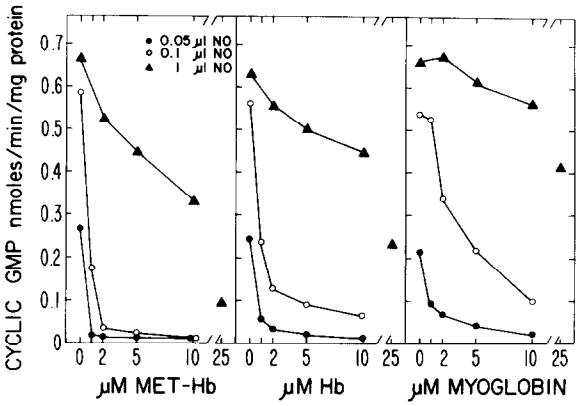

Fig.1. Inhibitory effects of heme proteins on activation of bovine coronary arterial soluble guanylate cyclase by NO. Reaction mixtures (0.1 mM GTP, 3 mM Mg^{2+}, 85–150 µg soluble tissue protein) were incubated for 10 min at 37°C. Heme proteins (MET-Hb, methemoglobin; Hb, hemoglobin) were present in reaction mixtures at initiation of reactions with enzyme fraction whereas NO was delivered at exactly 1 min incubation [1,2]. Basal activity was 6–9 pmol cyclic GMP . min^{-1} . mg $protein^{-1}$. Data represent the mean ± SE, n=6.

release from neutral aqueous solutions of nitroprusside and MNNG. The data in table 1 indicate that whereas cysteine, DTT, and GSH promoted the release of NO gas from MNNG, these thiols either decreased or trapped the NO gas released spontaneously from nitroprusside. $NaNO_2$ did not release NO gas in the absence or presence of the same concentrations of thiols (data not shown). In order to generate detectable amounts of NO gas into the upper

Table 1
Release of NO gas from nitroprusside (NP) and MNNG in the absence and presence of thiols, as detected by guanylate cyclase activation

Nitroso compound	Sample vol. (µl)	pmol cyclic GMP . min^{-1} . mg $protein^{-1}$			
		Alone	20 mM Cysteine	20 mM DTT	20 mM GSH
10 mM NP	10	31 ± 2	7 ± 3	6 ± 1	6 ± 2
	30	226 ± 63	4 ± 1	7 ± 3	161 ± 7
	100	575 ± 27	536 ± 50	462 ± 17	577 ± 36
10 mM MNNG	10	7 ± 2	219 ± 14	9 ± 2	7 ± 3
	30	7 ± 3	431 ± 17	16 ± 4	681 ± 28
	100	18 ± 5	706 ± 8	244 ± 16	790 ± 33

Reactions between nitroso compounds and thiols were conducted in 3 ml of 100 mM Tris–HCl (pH 7.4) for 30 min at 25°C under an atmosphere (2.5 ml) of O_2-free N_2. Gaseous samples were delivered to guanylate cyclase reaction mixtures (0.1 mM GTP, 3 mM Mg^{2+} and 75–90 µg soluble bovine coronary arterial protein) which were incubated for 10 min at 37°C [1]. Basal activity was 4–7 pmol cyclic GMP . min^{-1} . mg $protein^{-1}$. Data represent the mean ± SE, n = 3–6

atmosphere of reaction vessels, concentrations of reactants used in the experiments of table 1 were greater than those used in standard guanylate cyclase reaction mixtures. NO gas release in these experiments was assayed by comparing effects of the gaseous phase of reaction mixtures to those of purified NO on guanylate cyclase activation and coronary arterial relaxation in the absence and presence of methemoglobin and methylene blue, both of which inhibit these effects of NO [2,7]. The above data suggest that thiol enhancement of guanylate cyclase activation by MNNG but not by nitroprusside or $NaNO_2$ may be due, at least in part, to increased liberation of NO gas through direct chemical interaction of thiol and MNNG.

The findings that thiols:

(i) Enhanced guanylate cyclase activation by nitroprusside and $NaNO_2$;
(ii) Did not promote release of NO gas from nitroprusside or $NaNO_2$;
(iii) May have trapped NO gas released from nitroprusside (table 1);

suggested that thiols reacted with nitroso compounds or $NaNO_2$ to form *S*-nitrosothiols, which then activated guanylate cyclase. Indeed, formation of *S*-nitrosocysteine from cysteine plus either $NaNO_2$ [6] or MNNG [5] in aqueous solution has been reported. *S*-Nitrosothiols are easily synthesized by reacting thiols with either $NaNO_2$ at acid pH [6] or NO at neutral pH [4,5]. The latter method was used in this study. The various *S*-nitrosothiols prepared markedly activated coronary arterial and hepatic soluble guanylate cyclase (table 2). Although table 2 presents data obtained with 10–50 μM *S*-nitrosothiols, only 0.1–1 μM *S*-nitroso derivatives of cysteine, penicillamine, and β-D-thioglucose activated guanylate cyclase (224–935 pmol cyclic GMP . min^{-1} . mg $protein^{-1}$). Other thiols which formed active *S*-nitrosothiols were β-mercaptoethylamine, 3-mercaptopropionic acid, thioacetic acid and β-mercaptoethanol. Active compounds were not formed from reactions of NO with cystine, GSSG, oxidized DTT, *S*-methylcysteine, cysteic acid, 5-thioglucose, L-serine or L-alanine, none of which possess free sulfhydryl moieties.

The observations that *S*-nitrosothiols are potent activators of guanylate cyclase and are formed by reaction of thiols with NO [4,5], $NaNO_2$ [6], and MNNG [5] suggest that enhancement of guanylate cyclase activation by nitroprusside, MNNG and $NaNO_2$ in the presence of thiols may be through formation of *S*-nitrosothiol intermediates. Formation of *S*-nitrosothiols may also explain the thiol-induced reversal of methemoglobin blockade of guanylate cyclase activation by nitroprusside and MNNG, because enzyme activation by *S*-nitrosothiols was much more resistant than that by NO to methemoglobin blockade (table 3, fig.1). However, the oxidant ferricyanide inhibited guanylate cyclase activation by *S*-nitrosothiols (table 3) or NO [2] equally well, thus

Table 2
Activation of soluble guanylate cyclase from bovine coronary artery and rat liver by *S*-nitrosothiols

S-Nitroso derivative (μM)	pmol cyclic GMP . min^{-1} . mg $protein^{-1}$	
	Coronary artery	Liver
None (basal activity)	8 ± 0.2	5 ± 0.1
Cysteine (10)	917 ± 26	1215 ± 54
GSH (10)	764 ± 31	524 ± 18
DTT (50)	728 ± 33	1110 ± 46
Penicillamine (10)	993 ± 25	1632 ± 42
N-Acetyl cysteine (10)	588 ± 19	605 ± 24
β-D-Thioglucose (10)	684 ± 21	1633 ± 48

Reaction mixtures for coronary arterial guanylate cyclase contained 0.1 mM GTP, 3 mM Mg^{2+}, 75–95 μg soluble tissue protein and were incubated for 10 min at 37°C. Those for hepatic guanylate cyclase contained 0.3 mM GTP, 3 mM Mg^{2+}, 350–406 μg soluble tissue protein and were incubated for 5 min at 37°C. Reactions were initiated by addition of enzyme fraction followed immediately with *S*-nitrosothiol. Data represent the mean ± SE, n=4–6

Table 3
Effects of methemoglobin and ferricyanide on activation of bovine coronary arterial soluble guanylate cyclase by *S*-nitrosothiols

Additions	pmol cyclic GMP . min^{-1} . mg $protein^{-1}$		
	S-Nitroso derivative		
	Cysteine (10 μM)	Penicillamine (10 μM)	DTT (50 μM)
None	841 ± 29	814 ± 27	536 ± 21
Methemoglobin			
2 μM	747 ± 34	809 ± 22	518 ± 18
10 μM	593 ± 38	729 ± 37	446 ± 16
25 μM	378 ± 30	498 ± 41	412 ± 22
Ferricyanide			
50 μM	14 ± 2	12 ± 1	12 ± 2

Reaction mixtures (0.1 mM GTP, 3 mM Mg^{2+}, 82 μg soluble tissue protein) were incubated for 10 min at 37°C. Methemoglobin and ferricyanide were present in reaction mixtures at initiation of reactions with enzyme fraction whereas *S*-nitrosothiols were added immediately after enzyme. Basal activity was 4–7 pmol cyclic GMP . min^{-1} . mg $protein^{-1}$. Data represent the mean ± SE, n=4–6

suggesting that enzyme activation by these different forms of NO involves a common mechanism, which may be direct or possibly through formation of a nitrosyl–ferroheme complex [8]. In contrast to effects on nitroprusside, MNNG and $NaNO_2$, thiols failed to enhance guanylate cyclase activation by NO and to reverse methemoglobin blockade of NO activation [1]. Although *S*-nitrosothiols are formed during exposure of thiols to large amounts of NO under anaerobic conditions, only negligible amounts of *S*-nitrosothiols are likely formed during the brief exposure of guanylate cyclase reaction mixtures containing added thiols to small amounts (0.1 μl) of NO under aerobic conditions.

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