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GUANYLATE CYCLASE ACTIVATION BY NITROPRUSSIDE AND NITROSOGUANIDINE IS RELATED TO FORMATION OF S-NITROSOTHIOL INTERMEDIATES

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SUMMARY

The S-nitroso derivatives of cysteine, penicillamine, 2-mercaptoethylamine, glutathione, dithiothreitol, β-D-thioglucose and other thiols markedly activated unpurified and partially purified hepatic soluble guanylate cyclase. Heme was not required for enzyme activation since partially purified enzyme preparations were devoid of detectable heme. Guanylate cyclase activation by S-nitrosothiols was inhibited by hemoproteins and electrophilic agents. The requirement of thiols for activation of partially purified guanylate cyclase, and the marked enhancement by thiols of activation of unpurified guanylate cyclase, by nitroprusside and nitrosoguanidine suggest that the latter compounds reacted with thiols to form S-nitrosothiols which were potent activators of guanylate cyclase.

INTRODUCTION

Studies in this laboratory showed that DTT enhances activation of soluble guanylate cyclase (EC 4.6.1.2.) from coronary artery and liver by nitroprusside and nitrosoguanidine, but only slightly enhances or does not affect activation by NO (1,2). Further, 1 mM glyceryl trinitrate and 3 mM $NaNO_2$ failed to activate soluble coronary arterial guanylate cyclase in the absence of an added thiol (3). Enhancement of guanylate cyclase activation by nitrosoguanidine is likely due to NO because thiols react with aqueous neutral solutions of nitrosoguanidine to liberate NO gas (2). However, thiol enhancement of enzyme activation by nitroprusside cannot be explained similarly because thiols cannot be demonstrated to enhance liberation of NO gas from nitroprusside. In fact, thiols appear to trap the NO gas that is liberated spontaneously from aqueous neutral solutions of nitroprusside (2). The latter observation suggests that NO reacts with thiols to form a third compound. This interpretation is suppor-

Abbreviations: NO, nitric oxide; nitrosoguanidine, N-methyl-N'-nitro-N-nitrosoguanidine; DTT, dithiothreitol; cyclic GMP, guanosine 3',5'-monophosphate.

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ted by reports that thiols react with NO or HONO to form S-nitrosothiols (4-6). Thus, formation of S-nitrosothiols is likely when thiol solutions are exposed to nitroprusside, nitrosoguanidine or NO. The objective of this study was to determine whether S-nitrosothiols activate soluble guanylate cyclase and, if so, whether various chemical agents which are known to inhibit guanylate cyclase activation by nitroso compounds and NO likewise inhibit enzyme activation by S-nitrosothiols.

MATERIALS AND METHODS

Hepatic soluble fraction (unpurified soluble guanylate cyclase) was prepared as described previously (7) and stored at -85°C until used. The preparation of partially purified hepatic soluble guanylate cyclase will be described in detail elsewhere. Briefly, hepatic soluble fraction was prepared as described (7) except that livers were perfused via the portal vein and the liver mince was washed with 10-15 volumes of cold buffer prior to homogenization in order to remove most of the red blood cells. Guanylate cyclase in hepatic soluble fraction, prepared in 10 mM Tris HCl, pH 7.4, 0.25 M sucrose, 2 mM DTT, 0.5 mM EDTA (DTT-Buffer), was precipitated with 40% ammonium sulfate. The precipitate was resuspended and dialyzed in DTT-Buffer and chromatographed on a 1.5 x 25 cm DE52 cellulose column with a linear NaCl gradient (0-0.3 M) in DTT-Buffer. Guanylate cyclase-rich fractions (eluting in 0.18-0.19 M NaCl) were pooled, concentrated and chromatographed on a 1.5 x 25 cm agarose (Bio-Gel A-0.5 m) column with DTT-Buffer containing 0.1 M NaCl. Guanylate cyclase-rich fractions were stored at -85°C until used. Guanylate cyclase activity was determined as described previously (8). Briefly, reaction mixtures containing 50 mM Tris HCl, pH 7.4, 0.3 mM GTP (1 mM GTP for partially purified enzyme), 3 mM Mg^{2+}, 0.3 mM 1-methyl-3-isobutylxanthine and enzyme preparation, were incubated for 10 min at 37°C. Specific basal activities of homogenate and partially purified guanylate cyclase were 2.5 and 776 pmoles cyclic GMP/min/mg protein, respectively, thus indicating a 310-fold enzyme purification.

In view of the report (9) claiming that heme iron is a requirement for guanylate cyclase activation by NO and nitroso compounds, procedures were employed to ensure the absence of detectable heme from partially purified guanylate cyclase. Such procedures included liver perfusion *in situ*, washing the liver mince, and using a 0-0.3 M NaCl gradient in the eluant for the DE52 cellulose column. Employing visible absorption spectroscopy as described by Rossi-Fanelli *et al.* (10), fractions eluted from DE52 cellulose and agarose columns were assayed (540 nm) for heme after addition of dithionite.

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 (99.9%, Matheson Gas) in an O_2-free N_2 atmosphere (4-6). Unreacted NO was completely removed from solution by alternating cycles of vacuum evacuation and N_2 flushing. The thiols studied were L-cysteine, N-acetyl-L-cysteine, glutathione, DL-penicillamine, 2-mercaptoethylamine, 3-mercaptopropionic acid, 2-mercaptoethanol, thioglycolic acid, DL-dithiothreitol, β-D-thioglucose, and the sulfhydryl-rich protein aldolase (obtained from Sigma). Except for the penicillamine solution, which turned from clear to a green color, thiol solutions turned yellow-orange after reaction with NO. S-Nitrosothiols were identified by visible absorption and infrared spectroscopy. The latter method confirmed the presence of an S-nitroso moiety (sharp peak at 1440-1490 cm^{-1}). Absorption

maxima of 10-50 mM thiol solutions after reaction with NO were 540 nm, except those of β-D-thioglucose and penicillamine which were 560 nm and 590 nm, respectively. Values for the S-nitroso derivatives of cysteine, N-acetyl cysteine and penicillamine are in agreement with previously reported values (4,6). Formation of S-nitroso derivatives of the other thiols has not been reported previously. Although it is certain that S-nitrosothiols were formed (because of characteristic absorption spectra of reaction solutions and of crystals isolated from these solutions), the precise amounts formed are unknown (4-6) and consequently, concentrations of S-nitrosothiols tested are expressed as the initial thiol concentration prior to reaction with NO. The instability ($t_{1/2}$ <1 hr) of the dry crystals of S-nitrosothiols precluded their use. Instead, the more stable ($t_{1/2}$ 1-10 days) aqueous reaction solutions were employed immediately after preparation.

RESULTS

Preliminary experiments using unpurified hepatic soluble guanylate cyclase were conducted with S-nitrosocysteine and S-nitrosopenicillamine. Cyclic GMP formation was linear with incubation time and tissue protein concentration in the presence of either Mg^{2+} or Mn^{2+}, under assay conditions (data not illustrated). The product of the enzymatic reaction was verified as cyclic GMP by comparison with authentic compound (Fig. 1). A variety of thiols reacted with NO to form S-nitrosothiols that activated guanylate cyclase (Table 1). The S-nitroso derivatives of 2-mercaptoethylamine, penicillamine and β-D-thioglucose

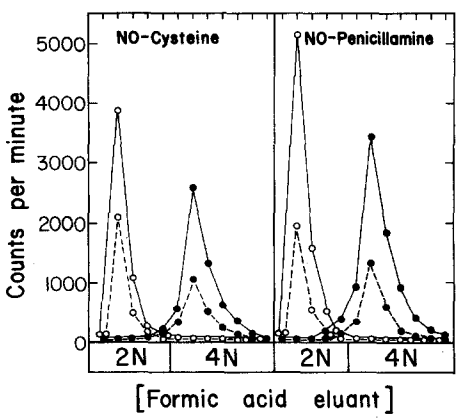

FIGURE 1. Identification of cyclic GMP as product of the hepatic guanylate cyclase reaction by Dowex-1 formate column chromatography and hydrolysis of product to GMP by phosphodiesterase. Reactions using hepatic soluble fraction were conducted as described in the text. Eluates from the double-column chromatographic procedure used to isolate cyclic GMP were further incubated with (○) or without (●) beef heart phosphodiesterase and applied to columns of Dowex-1 formate (8). Solid and dashed lines represent [^{32}P]-product and [^{3}H]cyclic GMP, respectively. GMP and cyclic GMP elute with 2 N and 4 N formic acid, respectively (8). NO-cysteine (10 μM) and NO-penicillamine (10 μM) signify S-nitrosocysteine and S-nitrosopenicillamine, respectively.

TABLE 1. Activation of unpurified hepatic soluble guanylate cyclase by S-nitrosothiols.

S-Nitroso derivative	Guanylate Cyclase Activity (nmoles cyclic GMP/min/mg protein)		
	Concentration of S-nitrosothiol		
	0.1 μM	1 μM	10 μM
Cysteine	0.024 ± 0.002	0.90 ± 0.19	1.85 ± 0.07
N-Acetyl cysteine	—	—	1.37 ± 0.08
2-Mercaptoethylamine	0.85 ± 0.11	1.51 ± 0.08	1.85 ± 0.12
3-Mercaptopropionic acid	—	—	1.35 ± 0.11
Glutathione	—	1.13 ± 0.07	1.78 ± 0.05
Penicillamine	0.73 ± 0.12	1.46 ± 0.04	1.90 ± 0.12
Dithiothreitol	0.16 ± 0.07	1.12 ± 0.05	1.78 ± 0.07
2-Mercaptoethanol	0.003 ± 0.001	0.022 ± 0.002	0.93 ± 0.07
Thioglycolic acid	0.25 ± 0.09	—	1.43 ± 0.08
β-D-Thioglucose	1.22 ± 0.08	1.50 ± 0.08	1.46 ± 0.07

Reaction mixtures, containing 0.3 mM GTP, 3 mM Mg^{2+} and 406 μg protein, were incubated for 10 min at 37°C as described in the text. Basal activity was 0.003 nmoles cyclic GMP/min/mg protein. Data represent the mean ± S.E. of 4-6 determinations from 2-3 separate experiments.

activated guanylate cyclase (0.50-1.1 nmoles cyclic GMP/min/mg protein) at concentrations of 10 nM. NO failed to form active compounds with disulfides (cystine, GSSG), S-methylcysteine, cysteic acid, 5-thioglucose, serine, alanine, insulin or sucrose, none of which possess sulfhydryl moieties (data not shown). However, 10 nM aldolase, a sulfhydryl-rich protein (11), after reaction with NO, activated guanylate cyclase (1.2 nmoles cyclic GMP/min/mg protein). Therefore, free sulfhydryl groups are required to react with NO at neutral pH to form S-nitrosothiols. Five mM concentrations of each of the thiols used to form the corresponding S-nitroso derivatives indicated in Table 1 also potentiated (20-50 fold) nitroprusside and enhanced (1.4-3.6 fold) nitrosoguanidine activation of unpurified hepatic soluble guanylate cyclase (data not shown).

Table 2 compares activation of unpurified and partially purified hepatic guanylate cyclase by nitroprusside, nitrosoguanidine and S-nitrosocysteine in the presence or absence of DTT. Partially purified guanylate cyclase had 310 times the specific basal activity (Mg^{2+}) of the starting homogenate and was devoid of detectable heme (less than 10 nM) as determined by spectral analysis after addition of dithionite. Nitroprusside produced little or no activation

TABLE 2. Effects of dithiothreitol on activation of unpurified and partially purified guanylate cyclase by nitroso compounds.

Activator	Guanylate Cyclase Activity (nmoles cyclic GMP/min/mg protein			
	Unpurified enzyme		Partially purified enzyme	
	-DTT	+2 mM DTT	-DTT	+2 mM DTT
None (basal activity)	0.003	0.003	0.737	0.773
Nitroprusside 100 μM	0.027	1.144	0.726	6.778
Nitrosoguanidine 100 μM	0.520	1.687	0.861	15.93
S-Nitrosocysteine 10 μM	1.465	1.508	21.53	35.98

Reaction mixtures, containing 1 mM GTP, 3 mM Mg^{2+} and 10 μg protein for partially purified enzyme, and 0.3 mM GTP, 3 mM Mg^{2+} and 406 μg protein for unpurified enzyme, were incubated for 10 min at 37°C as described in the text. DTT and nitrosoguanidine signify dithiothreitol and N-methyl-N'-nitro-N-nitrosoguanidine, respectively. Data represent the means of 8 determinations from 4 separate experiments. Standard errors (not shown) were less than 5% of the corresponding means.

of unpurified or partially purified guanylate cyclase unless DTT was also added. On the other hand, nitrosoguanidine activated unpurified guanylate cyclase in the absence of DTT, and addition of DTT enhanced activation about 3-fold. In contrast to the unpurified enzyme, partially purified guanylate cyclase was not appreciably activated by nitrosoguanidine unless DTT was also added. Other thiols could substitute for DTT (data not shown). S-Nitrosocysteine, unlike nitroprusside or nitrosoguanidine, markedly activated both enzyme preparations in the absence of added DTT, although addition of the latter enhanced activation of partially purified guanylate cyclase about 1.3-1.7 fold. The S-nitroso derivatives of 10 μM DTT (Table 3), N-acetyl cysteine, 2-mercaptoethylamine, glutathione, penicillamine, β-D-thioglucose and aldolase activated partially purified guanylate cyclase (12-31 nmoles cyclic GMP/min/mg protein). Thus, unlike NO or other nitroso compounds which have been reported to activate guanylate cyclase only in the presence of heme (9), enzyme activation of S-nitrosothiols does not possess this requirement.

Hemoproteins, thiol alkylating agents and electrophilic agents such as certain oxidants, biological stains and disulfides all inhibit guanylate

TABLE 3. Effects of methemoglobin, electrophilic agents and ethacrynic acid on activation of partially purified hepatic guanylate cyclase by S-nitrosothiols.

Inhibitor	Guanylate Cyclase Activity (nmoles cyclic GMP/min/mg protein)		
	S-Nitroso derivative (10 μM)		0.1 mM Nitrosoguanidine + 2 mM dithiothreitol
	Cysteine	Dithiothreitol	
None	25.1 ± 0.7	32.2 ± 1.8	16.4 ± 0.9
Methemoglobin 25 μM	13.4 ± 0.5	5.1 ± 0.3	4.2 ± 0.2
Ferricyanide 25 μM	9.8 ± 0.6	15.5 ± 0.7	4.9 ± 0.5
Methylene blue 100 μM	3.9 ± 0.2	4.6 ± 0.3	2.1 ± 0.1
Phenazine methosulfate 100 μM	9.9 ± 0.4	14.3 ± 0.5	1.3 ± 0.2
Cystine 2 mM	9.7 ± 0.8	14.8 ± 1.2	3.6 ± 0.3
Ethacrynic acid 2 mM	0.04 ± 0.01	0.06 ± 0.01	0.25 ± 0.03

Reaction mixtures, containing 1 mM GTP, 3 mM Mg^{2+} and 10 μg protein, were incubated for 10 min at 37°C as described in the text. Basal activity was 0.77 nmoles cyclic GMP/min/mg protein and was unaffected by the compounds listed under "Inhibitor", except for ethacrynic acid, which abolished basal activity. Compounds were added to reaction mixtures immediately prior to initiation of reactions with enzyme. Data represent the mean ± S.E. of 4-6 determinations from 2-4 separate experiments.

cyclase activation by NO, nitroso compounds and related agents (1-3,8,9,12, 13). Similarly, methemoglobin, ferricyanide, methylene blue, phenazine methosulfate, cystine and ethacrynic acid inhibited activation of partially purified guanylate cyclase by the S-nitroso derivatives of cysteine and DTT (Table 3). For purposes of comparison with the S-nitrosothiols, the effects of inhibitors on guanylate cyclase activation by nitrosoguanidine plus DTT were determined (Table 3). Again, each of the agents tested inhibited activation of guanylate cyclase by the combination of nitrosoguanidine plus DTT, the latter serving as the required thiol for enzyme activation by nitrosoguanidine.

DISCUSSION

The data in this report indicate that S-nitrosothiols markedly activate hepatic soluble guanylate cyclase. Most of the S-nitrosothiols studied (Table 1) are at least 100 times more potent than either nitroprusside or nitroso-

guanidine (1,2,8), in the absence of added thiols, in activating unpurified hepatic guanylate cyclase. The observations that various thiols markedly enhance activation of unpurified guanylate cyclase by nitroprusside and nitrosoguanidine together with the findings that the latter compounds, but not S-nitrosocysteine, require addition of DTT to activate partially purified guanylate cyclase support the view that S-nitrosothiols act as intermediates in guanylate cyclase activation by certain nitroso compounds. In this regard, it is noteworthy that certain nitrosamides have been reported to react with thiols to form S-nitrosothiols (5,14). Hemoproteins, electrophilic agents or oxidants, and thiol alkylating agents inhibit enzyme activation by S-nitrosothiols (Table 3) as well as by NO and nitroso compounds (1,2,8,9,12,13). Similarly, these inhibitors attenuate the activation of partially purified guanylate cyclase by the combination of nitrosoguanidine and DTT, which may activate the enzyme via formation of the S-nitroso derivative of DTT. Thus, although the precise mechanism is unknown, guanylate cyclase activation by S-nitrosothiols is likely similar to that by NO.

Nitrosoguanidine and, to a limited extent, nitroprusside activate unpurified guanylate cyclase in the absence of added thiols but both require thiol addition to activate the partially purified enzyme. One interpretation of these findings is that whereas thiols are present in the crude soluble fraction, thiols may be absent from the partially purified fraction. Since S-nitrosocysteine does not require added thiols to activate partially purified guanylate cyclase, nitroprusside and nitrosoguanidine may react with thiols to form S-nitrosothiols which may be the intermediate species responsible for guanylate cyclase activation. The striking observation that the magnitude of guanylate cyclase activation by nitroprusside plus DTT, nitrosoguanidine plus DTT, or S-nitrosocysteine, when compared with basal enzymatic activity, is consistently 10-50 fold greater with unpurified than with partially purified enzyme suggests that one or more components (other than thiols or heme) are removed from the crude soluble fraction during enzyme purification. Preliminary data indicate

that one or more heat stable (90°C for 15 min) components, present in the crude soluble fraction, markedly enhance activation of partially purified guanylate cyclase by NO and various nitroso compounds.

The present findings indicate that thiols could serve as a requirement for guanylate cyclase activation by inorganic and organic nitroso compounds. These observations together with those that thiols enable inorganic nitrite and organic nitrates to activate guanylate cyclase (3) suggest that endogenous thiols may be essential for the full expression of cyclic GMP accumulation in tissues as well as any cyclic GMP-dependent pharmacological effect of a wide variety of oxides of nitrogen ranging from NO to organic nitrates.

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