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SOLUBLE GUANYLATE CYCLASE ACTIVATION BY NITRIC OXIDE AND ITS REVERSAL

INVOLVEMENT OF SULFHYDRYL GROUP OXIDATION AND REDUCTION*

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Abstract—Pre-incubation of either crude or purified nitric oxide-stimulated soluble lung guanylate cyclase resulted in a temperature-dependent decay of enzyme activity. The decay of nitric oxide-stimulated activity during pre-incubation was associated with a reduced responsiveness of the enzyme to reactivation by a second exposure to nitric oxide. This loss of enzyme responsiveness to reactivation by nitric oxide was greater with purified guanylate cyclase than with the crude enzyme and was highly dependent upon the nitric oxide dose. The addition of dithiothreitol or other thiols to nitric oxide-stimulated enzyme markedly accelerated the decay of activity in a dose-dependent manner. In addition, thiols prevented the loss of responsiveness of guanylate cyclase to reactivation by nitric oxide. Nitric oxide-stimulated enzyme activity was, therefore, reversed by the addition of thiol reducing agents. The addition of the thiol oxidizing agents, diamide or oxidized glutathione, to nitric oxide-stimulated guanylate cyclase caused a rapid and irreversible loss of activity. The effects of diamide or oxidized glutathione on the crude enzyme were prevented by excess dithiothreitol. Dithiothreitol did not prevent the destruction of purified nitric oxide-stimulated guanylate cyclase activity by diamide or oxidized glutathione, however. The results suggest that nitric oxide activation and its reversal are linked to the reversible oxidation and reduction, respectively, of sulfhydryl groups on guanylate cyclase which are involved in enzyme activation. The results further suggest the existence of a second class of sulfhydryl groups involved in the maintenance of enzyme activity.

The regulation of guanylate cyclase [GTP pyrophosphate lyase (cyclizing) EC 4.6.1.2] by oxidative mechanisms has been the subject of intense investigation over the past several years. It is now clear that soluble guanylate cyclase activity can be dramatically altered by the apparent oxidation of some enzyme component(s). A variety of nitro-containing compounds, such as sodium nitroprusside or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, activate guanylate cyclase by an oxidative mechanism that probably involves nitric oxide [1–3]. A number of laboratories have now purified the soluble guanylate cyclase to apparent homogeneity from several tissues [4–8]. Although the purified enzyme from liver or lung is highly labile in the absence of thiols, it can be directly activated by nitric oxide [2, 5, 7] indicating that there are no special requirements for such activation. In addition to nitric oxide, oxidative free radicals such as the hydroxyl radical [9] and superoxide anion [10], as well as unsaturated fatty acid hydroperoxides [11, 12], prostaglandin endoperoxides [12], dehydroascorbate [13] and manganese ion [14], have been shown to stimulate crude or purified preparations of guanylate cyclase. Although the precise mechanism for activation is unclear, each of these activators shares the potential for oxidative modification of the cyclase.

Despite a large amount of evidence that guanylate cyclase activity can be altered by redox events, the importance of this type of regulation in cell function is not yet clear. It is known that compounds which can precipitate redox events or generate the intracellular formation of oxidative free radicals can elevate the guanosine 3′,5′-monophosphate (cyclic GMP) content of tissues [10, 13, 15]. In addition, recent studies have shown that hormonal stimulation of cyclic GMP formation in cells may be associated with the Ca^{2+}-dependent release of fatty acids from the membrane and their subsequent metabolism to fatty acid oxygenation products [16–18]. Observations such as these strongly suggest that oxidative activation of guanylate cyclase does, in fact, play an important role in the *in vivo* regulation of the activity of the enzyme.

Although the activation of guanylate cyclase by oxidative mechanisms has been studied in some detail, the reversal of the activation process has not been examined to any extent. If redox regulation of guanylate cyclase is important to cellular function, this implies that both a "turn on" and "turn off" mechanism must exist. Such an activation/inactivation cycle would be necessary if guanylate cyclase activity were coupled to the dynamic regulation of cyclic GMP levels in cells. Evidence suggests that activation of guanylate cyclase by nitric oxide or other free radicals involves the oxidation of key sulfhydryl groups on the enzyme [11, 13]. The present study describes the reversal of nitric oxide acti-

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vation and the apparent involvement of sulfhydryl groups in the activation/inactivation process.

METHODS

The preparation of crude soluble guanylate cyclase from rat lung was slightly modified from the procedure described previously for liver [5]. Male Sprague–Dawley rats (150–200 g) were anesthetized with sodium pentobarbital (30 mg/kg, i.p.), and the thoracic and abdominal cavities were opened. The lungs were then perfused *in situ* through the right ventricle with approximately 50 ml of cold 0.9% NaCl. The pure white lungs were carefully removed, rinsed in saline to remove any superficial blood, and homogenized with a Potter–Elvehjem homogenizer in 9 vol. of 20 mM Tris–HCl, pH 8.0, containing 250 mM sucrose, 1 mM dithiothreitol and 1 mM EDTA. The homogenate was centrifuged at 100,000 *g* for 60 min to obtain the soluble fraction which was frozen at −70° until use. Some soluble lung guanylate cyclase was further purified from the soluble fraction to apparent homogeneity as described [5] with slight modification [7].

Immediately prior to each experiment, enzyme preparations were passed through a column of Sephadex G-25 (1.5 × 6 cm) equilibrated with 20 mM Tris–HCl, pH 7.6, to remove thiols and other stabilizing agents added to the enzyme for storage. Mg^{2+}-supported guanylate cyclase activity in crude enzyme preparations was determined in a 10-min incubation at 37° as described [5] in a reaction (100 μl) containing 50 mM Tris–HCl, pH 7.6, 1 mM GTP, 4 mM $MgCl_2$, 15 mM creatine phosphate, 20 μg creatine phosphokinase (115 units/mg), 10 mM theophylline and 0.001 to 0.05 mg enzyme protein. The assay of purified enzyme was at 37° in a 100-μl reaction comprised of 50 mM Tris–HCl, pH 7.6, containing 1 mM GTP, 4 mM $MgCl_2$, 0.02% bovine serum albumin, and 0.02 to 0.05 μg enzyme protein. Reactions were started by the addition of enzyme to the otherwise complete reaction mixture. Cyclic GMP formed was determined by radioimmunoassay as described [2, 5, 14]. Protein was measured by the method of Lowry *et al.* [19]. All values represent the means of duplicate or triplicate incubations from representative experiments.

The reversal of nitric oxide activation was examined by following the decay of nitric oxide-stimulated enzyme activity during a pre-incubation. Guanylate cyclase was activated with nitric oxide essentially as described [1, 2]. Following chromatography on Sephadex G-25, 100-μl aliquots of enzyme in 10 × 75 mm borosciliate glass test tubes were flushed with N_2, exposed to nitric oxide gas for 5–10 sec (flow rate = 0.5 to 2 ml/min, 2 cm above the enzyme surface), and again flushed extensively with N_2. Exposure conditions (time and flow rate) were varied for crude or purified enzyme preparations to result in consistent and maximal activation of all preparations. Following activation, various additions were made as indicated to the active enzyme which was then pre-incubated in a water bath prior to assay. Crude and purified enzyme activities with Mg^{2+} as the cation cofactor were maximally increased 220- and 27-fold, respectively, by nitric oxide. Average Mg^{2+} supported basal and maximum nitric oxide-stimulated activities of crude guanylate cyclase preparations before pre-incubation were 29 ± 5 and 6240 ± 120 (mean ± S.E.) pmoles cyclic GMP per mg protein per min, respectively, and 38 ± 4 and 1020 ± 280 nmoles cyclic GMP per mg protein per min, respectively, for purified preparations. In some experiments, pre-incubated enzyme was re-exposed to nitric oxide immediately prior to assay.

Rats were obtained from the Zivic Miller Co., Pittsburgh, PA. Diamide and reduced and oxidized glutathione were from the Sigma Chemical Co., St. Louis, MO. Nitric oxide gas was from the Union Carbide Corp., East Chicago, IN. All other materials were obtained as described [2, 5, 14].

RESULTS

In early studies on the activation of guanylate cyclase by nitric oxide, Arnold *et al.* [1] reported that activation of the crude soluble rat liver enzyme by nitric oxide was slowly reversible ($T_{\frac{1}{2}}$ 3–4 hr) at 4°. Pre-incubation of crude soluble lung guanylate cyclase activated with nitric oxide resulted in a temperature-dependent decay of enzyme activity that was markedly accelerated by the addition of dithiothreitol to the active enzyme (Fig. 1). In the absence of dithiothreitol, nitric oxide-stimulated activity remained constant during a 60-min pre-incubation at 4°. Pre-incubation at 4° in the presence

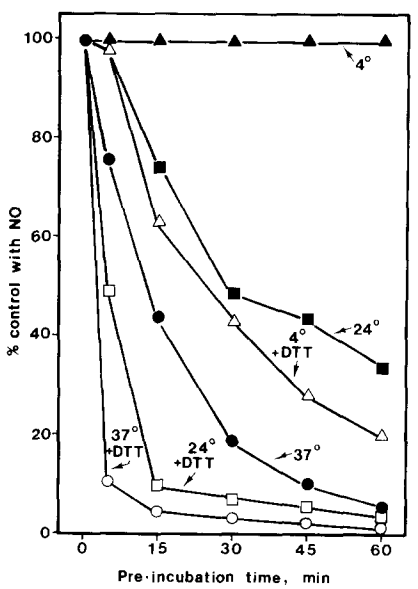

Fig. 1. Effects of temperature and dithiothreitol on the decay of nitric oxide-stimulated activity with the crude enzyme. Crude soluble lung guanylate cyclase after Sephadex G-25 chromatography was exposed to 200 μl of nitric oxide and preincubated as described in Methods at 37° (circles), 24° (squares) or 4° (triangles) in the absence (closed symbols) or after the addition (open symbols) of 10 mM dithiothreitol (DTT). At the times indicated, aliquots were removed from the pre-incubation and assayed for guanylate cyclase activity. The results are expressed as the percent of control activity with nitric oxide before pre-incubation. Control activities with or without dithiothreitol were 6240 ± 120 (mean ± S.E.) pmoles cyclic GMP per mg protein per min.

of dithiothreitol, however, caused a precipitous fall in activity. At 24° and 37°, nitric oxide-stimulated activity in the absence of dithiothreitol decayed with half-times of 12.5 and 28 min respectively. Dithiothreitol markedly accelerated the loss of nitric oxide-stimulated activity and converted a roughly first-order decay process to one which was biphasic in nature. Dithiothreitol had no effect on basal or nitric oxide-stimulated activities of crude enzyme that was not pre-incubated. It did, however, prevent the spontaneous activation of basal activity during pre-incubation at 37° (approximately 2-fold in 60 min) in agreement with studies by White *et al.* [20]. It is unlikely that the decay of nitric oxide-stimulated activity was due to the loss of dissolved nitric oxide gas from the incubation. Mixing of a non-pre-incubated enzyme preparation exposed to nitric oxide with enzyme not previously exposed to nitric oxide caused minimal activation of the unexposed preparation (data not shown). This suggested that the amount of nitric oxide initially remaining in solution following exposure was not sufficient to support significant enzyme activation.

In the absence of dithiothreitol, the decay of nitric oxide-stimulated activity was associated with an irreversible loss of enzyme activity. Figure 2 shows

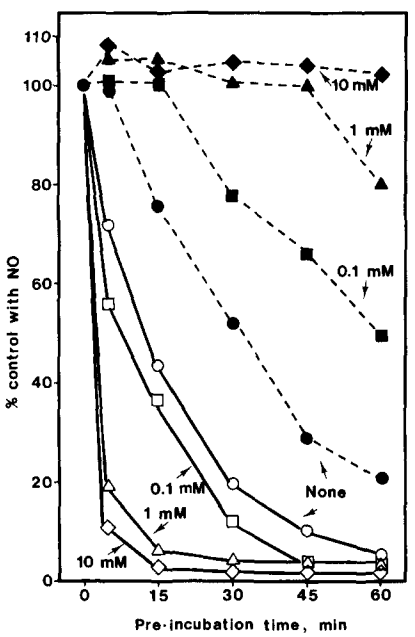

Fig. 2. Effects of dithiothreitol on the reversal of nitric oxide activation with the crude enzyme. Crude soluble lung guanylate cyclase after Sephadex G-25 chromatography was exposed to 200 μl of nitric oxide and preincubated as described in Methods at 37° in the absence (○—○) or after the addition of 0.1 mM (□—□), 1 mM (△—△) or 10 mM (◇—◇) dithiothreitol. At the times indicated, aliquots were removed from the pre-incubation and assayed for guanylate cyclase activity. In some cases, enzyme was re-exposed to nitric oxide immediately before assay (closed symbols - - -). The results are expressed as the percent of control activity with nitric oxide before pre-incubation. Control activities were similar to those reported in the legend to Fig. 1.

the decay of nitric oxide-stimulated activity and the subsequent reactivation of enzyme by re-exposure to nitric oxide. At 37° in the absence of dithiothreitol, there was a progressive decline in the ability to reactivate guanylate cyclase with a second exposure to nitric oxide. The loss of responsiveness to nitric oxide was prevented by the addition of dithiothreitol in a dose-dependent manner. This effect of dithiothreitol was not due to an enhancement of the absolute level of activation since dithiothreitol does not potentiate nitric oxide activation of crude soluble enzyme [21, 22]. The loss of responsiveness of activated enzyme to a second exposure to nitric oxide was less at 24° (Table 1) as was the loss of nitric oxide-stimulated activity. Enzyme exposed to nitric oxide and kept at 4° in the absence of dithiothreitol was not inhibited by a second exposure to nitric oxide (Table 1). This indicated that re-exposure to nitric oxide at the dose used did not cause inhibition of enzyme activity that could be mistakenly interpreted as unresponsiveness. Therefore, in the absence of dithiothreitol, true reversal of nitric oxide activation was accompanied with the loss of some enzyme activity. The addition of dithiothreitol potentiated the reversal of nitric oxide activation and decreased the lability of the active enzyme.

The ability of dithiothreitol to accelerate the reversal of nitric oxide activation, and to prevent the further loss of enzyme responsiveness to nitric oxide, was independent of the time of its addition following nitric oxide activation (Fig. 3). The addition of dithiothreitol to a decaying nitric oxide-stimulated preparation caused an immediate acceleration of the reversal process. Although the addition of dithiothreitol prevented further loss of enzyme responsiveness to re-activation, it did not restore the level of activation to pre-incubation values. These observations support the contention that in the absence of dithiothreitol there was an irreversible loss of some active enzyme.

Qualitatively similar results were obtained using soluble lung guanylate cyclase purified to homogeneity. Purified guanylate cyclase activity following exposure to 20 μl of nitric oxide did not decay during a 60-min pre-incubation at 4° (Fig. 4). Pre-incubation at 37°, however, caused the activity to decline with

Table 1. Effects of temperature on the decay of crude nitric oxide-stimulated guanylate cyclase activity*

Pre-incubation (temp)	% Control with NO	
	60 min	Re-exposed after 60 min
37°	5	21
24°	33	66
4°	100	102

* Crude soluble lung guanylate cyclase after Sephadex G-25 chromatography was exposed to nitric oxide and pre-incubated at the temperature indicated as described in Methods. After 60 min, an aliquot was transferred to the guanylate cyclase assay either before or after re-exposure to nitric oxide. Results are expressed as the percent of control activity with nitric oxide activity before pre-incubation. Control activities prior to pre-incubation were 6240 ± 120 (mean ± S.E.) pmoles cyclic GMP per mg protein per min.

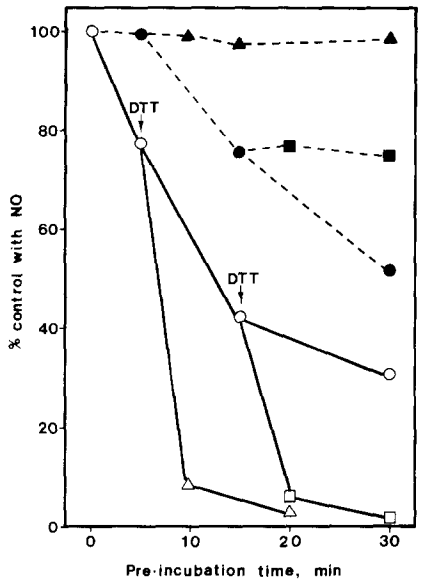

Fig. 3. Effects of dithiothreitol on the time course of the reversal of nitric oxide activation with the crude enzyme. Crude soluble guanylate cyclase after Sephadex G-25 chromatography was exposed to 200 μl nitric oxide and pre-incubated as described in Methods at 37° in the absence of dithiothreitol (○—○). At 5 (△—△) or 15 (□—□) min, 10 mM dithiothreitol (DTT) was added to the pre-incubation. At the times indicated, aliquots were removed from the pre-incubation and assayed for guanylate cyclase activity. In some cases, enzyme was re-exposed to nitric oxide immediately before assay (closed symbols – – –). The results are expressed as the percent of control activity with nitric oxide before pre-incubation. Control activities were similar to those reported in the legend to Fig. 1.

time in a manner that was accelerated by the addition of dithiothreitol. If purified nitric oxide-stimulated enzyme was pre-incubated in the absence of dithiothreitol, it could not be reactivated by a second exposure to nitric oxide, indicating that an irreversible loss of enzyme activity had occurred. In the presence of 10 mM dithiothreitol, however, partial reactivation could be achieved with a second nitric oxide exposure. Previous studies had shown that dithiothreitol did not enhance the absolute activation of Mg^{2+}-supported purified guanylate cyclase by low concentrations of nitric oxide [2], such as those used in the present study. The partial reactivation of nitric oxide-stimulated enzyme in the presence of 10 mM dithiothreitol was not due to enhanced activation by the thiol, but rather represented true reactivation.

Despite partial protection by dithiothreitol, the purified nitric oxide-stimulated enzyme was highly labile. Even 10 mM dithiothreitol would not totally prevent the permanent loss of some nitric oxide-stimulated enzyme activity during pre-incubation. In addition, with the purified enzyme, the loss of activity was highly dependent upon the dose of nitric oxide. Although no decay of nitric oxide-stimulated activity occurred at 4° in the absence of dithiothreitol following 20 μl of nitric oxide (Fig. 4), exposure of the purified enzyme to larger amounts of nitric oxide resulted in a time-dependent irreversible loss of activity (Fig. 5). With the higher doses of nitric oxide (100 and 200 μl), pre-incubation of the activated enzyme at 37° resulted in a more rapid (within 5 min) and complete inactivation of guanylate cyclase which could not be prevented by dithiothreitol (data not shown). Thus, although purified guanylate cyclase behaved much like the crude soluble enzyme in that activation by nitric oxide could be reversed by dithiothreitol, the extreme lability of the purified enzyme compromised its ability to be reactivated by nitric oxide even in the presence of a thiol reducing agent. Because of this problem, the majority of experiments were conducted using the crude soluble enzyme so that complete reversal of activation could be examined without complications associated with enzyme lability.

The addition of other thiols or hemoglobin to a nitric oxide-stimulated preparation was also found to accelerate the reversal of nitric oxide activation (Table 2). Dithiothreitol, however, was the most effective in preventing the irreversible loss of a significant amount of enzyme activity during pre-incubation. Although both reduced or oxidized glutathione accelerated the decay of nitric oxide-stimulated enzyme, only reduced glutathione prevented the irreversible loss of activity. Oxidized glutathione, in fact, potentiated the irreversible loss of nitric oxide-stimulated enzyme activity. These findings suggested that the oxidation of certain enzyme sulfhydryl groups not involved in the activation process resulted in inhibition of enzyme activity rather

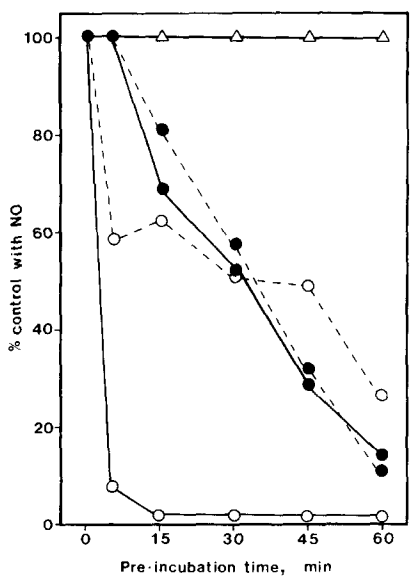

Fig. 4. Effects of dithiothreitol on the time course of the reversal of nitric oxide activation with the purified enzyme. Purified soluble guanylate cyclase after Sephadex G-25 chromatography was exposed to 20 μl of nitric oxide and pre-incubated as described in Methods at 4° (△—△) or 37° in the absence (●—●) or after the addition (○—○) of 10 mM dithiothreitol. At the times indicated, aliquots were removed and assayed for guanylate cyclase activity. In some cases, enzyme was re-exposed to 20 μl of nitric oxide immediately before assay (broken lines). The results are expressed as the percent of control activity with nitric oxide before pre-incubation which was 1020 ± 280 nmoles cyclic GMP per mg protein per min.

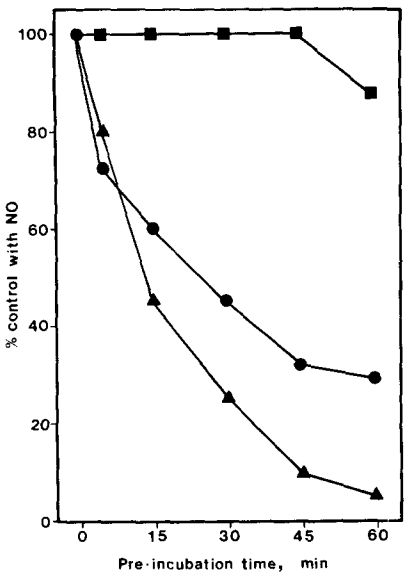

Fig. 5. Effects of nitric oxide dose on the decay of nitric oxide-stimulated activity with the purified enzyme. Purified guanylate cyclase after Sephadex G-25 chromatography was pre-incubated at 4° following exposure to 50 μl nitric oxide (■—■), 100 μl nitric oxide (●—●) or 200 μl nitric oxide (▲—▲). At the times indicated, aliquots were removed from the pre-incubation and assayed for guanylate cyclase activity. The results are expressed as the percent of control activity with nitric oxide before pre-incubation. Due to the biphasic nature of the dose–response curve for nitric oxide [2, 7], control activities with nitric oxide were 910, 575 and 312 nmoles cyclic GMP per mg protein per min with 50, 100 and 200 μl nitric oxide respectively.

than reversal of activation. Because the addition of hemoglobin to a preparation of active enzyme could alter the decay of nitric oxide activity, it was important in studies with the crude enzyme to use a preparation from lungs which had been perfused with saline prior to homogenization to remove blood components. In addition, although hemoglobin or dithiothreitol could accelerate the decay of nitric oxide-stimulated activity if added after enzyme activation by nitric oxide, if they were present in the enzyme preparation prior to its initial exposure to nitric oxide, the reversal of the active enzyme was actually slowed (data not shown). This was probably due, in part, to the formation of nitrosyl heme or *S*-nitrosothiols [21–25] which could serve to maintain enzyme in an activated state.

The effects of diamide or oxidized glutathione on the reversal of nitric oxide activation with crude enzyme are shown in Table 3. Although the addition of diamide or oxidized glutathione to activated enzyme accelerated and enhanced the irreversible decay of nitric oxide-stimulated activity, they slowed, to some degree, the reversal of nitric oxide activity in the presence of 1 or 10 mM dithiothreitol. In the presence of 1 mM dithiothreitol, diamide or oxidized glutathione still caused an irreversible loss of enzyme activity. The irreversible loss of crude active enzyme caused by diamide or oxidized glutathione was, however, prevented by 10 mM dithiothreitol. On the other hand, pre-incubation of purified nitric oxide-stimulated guanylate cyclase in the presence of 1 mM oxidized glutathione or diamide resulted in the immediate and complete inactivation of the enzyme which could not be prevented by 1 or 10 mM dithiothreitol (data not shown). Diamide or oxidized glutathione also completely inhibited basal activity of crude or purified guanylate cyclase during pre-incubation in a manner that was prevented by excess dithiothreitol in agreement with similar reports from other laboratories [26, 27]. The reason why excess dithiothreitol could prevent the complete inactivation by diamide or oxidized glutathione or purified basal activity and not purified nitric oxide-stimulated activity probably relates to the striking increase in lability of the active enzyme.

Table 2. Effects of different thiol reagents and hemoglobin on the reversal of nitric oxide activation with crude guanylate cyclase*

Addition (mM)	% Control with NO		
	5 min	30 min	Re-exposed after 30 min
None	77	18	52
Dithiothreitol (0.1)	72	10	78
Dithiothreitol (1)	15	5	101
Dithiothreitol (10)	11	3	100
2-Mercaptoethanol (1)	74	4	51
2-Mercaptoethanol (10)	64	3	82
Reduced glutathione (1)	14	2	66
Reduced glutathione (10)	5	2	87
Oxidized glutathione (1)	12	0	0
Oxidized glutathione (10)	5	0	0
Hemoglobin (0.1)	6	1	0

* Crude soluble lung guanylate cyclase after Sephadex G-25 chromatography was exposed to nitric oxide and pre-incubated at 37° with the additions indicated as described in Methods. After 5 or 30 min of pre-incubation, an aliquot was removed and assayed for guanylate cyclase activity. Some enzyme was re-exposed to nitric oxide after 30 min of pre-incubation before transfer to the enzyme assay. Results are expressed as the percent of control activity with nitric oxide activity before pre-incubation. Control activities with or without additions were similar to those reported in the description of Table 1.

Table 3. Effects of diamide and oxidized glutathione on crude nitric oxide-stimulated guanylate cyclase*

Addition	% Control with NO		
	5 min	30 min	Re-exposed after 30 min
None	78	17	51
Diamide	10	0	0
Diamide plus dithiothreitol	49	9	9
Diamide plus dithiothreitol (10 mM)	34	9	100
Oxidized glutathione	12	0	0
Oxidized glutathione plus dithiothreitol	23	0	0
Oxidized glutathione plus dithiothreitol (10 mM)	24	7	100

* Crude soluble lung guanylate cyclase after Sephadex G-25 chromatography was exposed to nitric oxide and pre-incubated at 37° with the additions indicated as described in Methods. After 5 or 30 min of pre-incubation, an aliquot was removed and assayed for guanylate cyclase activity. Some enzyme was re-exposed to nitric oxide after 30 min of pre-incubation before transfer to the enzyme assay. Concentrations of additions were 1 mM unless indicated otherwise. Results are expressed as the percent of control activity with nitric oxide before pre-incubation. Control activities with or without additions were similar to those reported in the description of Table 1.

DISCUSSION

Studies by a number of investigators have suggested that oxidation of key sulfhydryl groups on guanylate cyclase is responsible for enzyme activation. Activation of splenic cell soluble guanylate cyclase by dehydroascorbate or prostaglandin endoperoxides, for example, was shown to be inhibited or reversed by thiol reagents such as dithiothreitol [12, 13]. In other studies, Hidaka and Asano [11], using guanylate cyclase from human platelets, demonstrated that a number of sulfhydryl reagents would inhibit enzyme activation by fatty acid hydroperoxides. This inhibition was accomplished either by covalently complexing enzyme sulfhydryl groups with *N*-ethylmaleimide or maintaining them in the reduced form with dithiothreitol or 2-mercaptoethanol. DeRubertis and Craven [24] have shown that activation of soluble hepatic guanylate cyclase by *N*-methyl-*N'*-nitro-*N*-nitroguanidine can be inhibited or reversed by thiol reducing agents such as dithiothreitol.

That sulfhydryl groups are important for the maintenance of guanylate cyclase activity and its regulation has been suggested by the recent studies of Brandwein *et al.* [26] using the highly purified soluble enzyme from rat lung. These investigators have shown that several disulfides, such as cystamine and cystine, can form mixed disulfides with the purified cyclase. Mixed disulfide formation resulted in the inactivation of guanylate cyclase and was reversed by dithiothreitol. Accompanying the disulfide-induced inactivation was a reduced responsiveness of the enzyme to nitric oxide activation. These investigators suggested that the reduced responsiveness of guanylate cyclase to nitric oxide following the formation of mixed disulfides was due to either: (1) the complexing of one or more enzyme sulfhydryl groups required for activation, or (2) the complete inactivation of a population of enzyme molecules. Interestingly, the formation of mixed disulfides between proteins and thiols has been described in tumors [28]. This laboratory has reported recently that guanylate cyclase prepared from diethylstilbestrol-induced renal tumors cannot be activated by nitric oxide or other oxidative compounds [29]. Whether or not these two observations are related remains to be determined.

The results of the present study are consistent with the hypothesis that oxidation and reduction of enzyme sulfhydryl groups play a role in the activation and inactivation of guanylate cyclase by nitric oxide. The inactivation of nitric oxide-stimulated guanylate cyclase was temperature dependent and was accelerated by the addition of thiols or hemoglobin. The observations with hemoglobin are in agreement with earlier studies demonstrating the reversal of NaN_3 activation by heme proteins [30, 31]. Agents such as dithiothreitol, however, not only reverse nitric oxide activation but also prevent the irreversible loss of active enzyme during pre-incubation.

The addition of diamide or oxidized glutathione following nitric oxide activation resulted in the irreversible inhibition of the activated enzyme. The inhibitory effects of the thiol oxidants on the crude enzyme were prevented by excess dithiothreitol. The purified enzyme, particularly following activation, however, was apparently considerably more sensitive to oxidation than was the enzyme in crude preparations. This, in part, may be due to the presence of other proteins and cell components in crude preparations which may act as radical sinks or antioxidants [2]. The fact that dithiothreitol can potentiate the reversal of nitric oxide activation, as well as prevent enzyme inactivation by excessive oxidation, suggests that the oxidation of certain enzyme sulfhydryl groups can enhance, while oxidation of other sulfhydryl groups can inhibit, enzyme activity. This hypothesis is supported by the work of others who have demonstrated an involvement of sulfhydryl

groups in the reversible inactivation of basal guanylate cyclase activity [26, 27] and the activation of the enzyme by dehydroascorbate, fatty acid hydroperoxides and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [11–13, 24]. It is not yet clear what physicochemical changes in guanylate cyclase result in activation, its reversal, or inactivation. Current studies with the purified enzyme and the production of monoclonal antibodies to guanylate cyclase [7], however, should be helpful in this regard.

Based upon the findings of the present study and reports by other investigators, it is possible to propose a hypothetical model for the role of sulfhydryl groups in the regulation of guanylate cyclase activity (Fig. 6). Such a model may serve as the basis for future experiments designed to examine the role of sulfhydryl groups in the dynamic regulation of guanylate cyclase activity. In this hypothetical model there exist two classes of enzyme sulfhydryl groups. One class, $-SH^A$, is involved in the activation of guanylate cyclase by nitric oxide and other oxidative activators as well [11, 12, 24]. Oxidation of $-SH^A$ results in the enhancement of enzyme activity, and subsequent reduction of these groups returns active guanylate cyclase to its basal state. A second class of sulfhydryl groups, $-SH^B$, is perhaps less accessible and is critical for the maintenance of enzyme activity. Oxidation of these ($-SH^B$) by excessive exposure to nitric oxide [2, 7], sulfhydryl reactive agents such as diamide, oxidized glutathione, or *p*-hydroxymercuribenzoate [27], or mixed disulfide formation with cystamine or cystine [26], results in the inactivation of guanylate cyclase. Apparently, either active or basal guanylate cyclase can be affected since diamide or oxidized glutathione will inhibit both basal or nitric oxide-stimulated enzyme. Clearly, from the studies in this report the active enzyme is more labile in this regard. Inactive guanylate cyclase can be returned to basal by the addition of thiols. It has not yet been possible to demonstrate direct conversion of inactive enzyme to active. The purified enzyme is apparently particularly sensitive to the inactivation process. This suggests that some irreversible inactivation may be independent of any sulfhydryl group modification. Alternatively, excessive oxidation of protein sulfhydryl or other functional groups may result in protein denaturation.

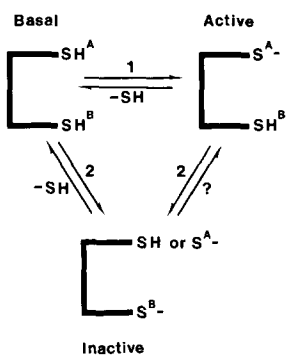

Fig. 6. Proposed model for the involvement of sulfhydryl groups in the regulation of guanylate cyclase activity. Conversion of basal to active enzyme (1) by nitric oxide or other oxidative activators involves the oxidation of sulfhydryl groups designated as $-SH^A$. Reversal of activation is accomplished by reduction of $-S^A-$ with thiol reducing agents such as dithiothreitol. Inactivation of basal or active guanylate cyclase (2) involves the oxidation or complexing of sulfhydryl groups designated as $-SH^B$. Conversion of inactive to basal may occur by the reduction of $-S^B-$ with thiols.

Recent reports have suggested that soluble guanylate cyclase may exist in two forms, one containing a heme moiety and another form which is heme deficient [32, 33]. Although preliminary studies have provided evidence that the heme moiety may participate in enzyme activation by nitric oxide, the exact nature of the role of heme is, at present, unclear since heme is not an absolute requirement for nitric oxide activation [2, 34]. Nitric oxide is known to react with heme-containing compounds and enzymes to form nitrosyl–heme complexes which themselves are potent activators of both crude and purified guanylate cyclase [21, 34]. Such observations suggest that a heme moiety on guanylate cyclase may merely act as an intermediate in the activation process by complexing nitric oxide subsequent to sulfhydryl group oxidation by either the nitrosyl–heme moiety or free nitric oxide itself. This conclusion is supported by the fact that thiol alkylating agents such as ethacrynic acid can inhibit guanylate cyclase activation by both nitric oxide and a variety of nitrosyl–heme complexes as well [34].

From the studies described in this report it is apparent that sulfhydryl group modification is critically involved in the dynamic modulation of soluble guanylate cyclase by nitric oxide *in vitro*. Other recent studies from this laboratory have demonstrated a similar role for sulfhydryl groups in the regulation of particulate guanylate cyclase activity [35]. However, the important question is, does this translate into a mechanism by which a variety of drugs and hormones alter cyclic GMP levels in intact tissues? Studies from this laboratory have clearly demonstrated that sulfhydryl oxidants such as diamide or oxidized glutathione can block the elevation of cyclic GMP in tissues exposed to a variety of agents including nitric oxide, and hormones such as norepinephrine [36]. These data suggest that enzyme sulfhydryl groups may indeed be important in the *in vivo* regulation of guanylate cyclase activity.

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