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INVOLVEMENT OF SULFHYDRYL GROUPS IN THE OXIDATIVE MODULATION OF PARTICULATE LUNG GUANYLATE CYCLASE BY NITRIC OXIDE AND *N*-METHYL-*N'*-NITRO-*N*-NITROSOGUANIDINE*

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Abstract—Particulate guanylate cyclase from rat lung was activated by nitric oxide or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in a dose-dependent manner that was enhanced by dithiothreitol. Nitric oxide-stimulated guanylate cyclase activity decayed during a 60-min preincubation at 37°, but did not decay at 24° or 4°. Dithiothreitol enhanced the decay of nitric oxide-stimulated enzyme at all temperatures by potentiating the reversal of nitric oxide activation. Following the reversal of nitric oxide activation at 24° by dithiothreitol, the particulate enzyme could be reactivated by a second exposure to nitric oxide. Preincubation of basal particulate guanylate cyclase activity at 37° resulted in the loss of enzyme responsiveness to activation by nitric oxide or MNNG that was potentiated by diamide or oxidized glutathione. The inhibitory effects of the thiol oxidants on enzyme responsiveness to activation by MNNG were prevented by dithiothreitol. The results suggest that activation of particulate guanylate cyclase by nitric oxide or MNNG involves the oxidation of key enzyme sulfhydryl groups.

Guanylate cyclase [GTP pyrophosphate lyase (cyclizing), EC 4.6.1.2], the enzyme responsible for the synthesis of cyclic GMP in cells, exists in both soluble and particulate fractions of cell homogenates. Immunological studies with antibodies produced to the purified enzymes [1–3], as well as other physical and kinetic data [4, 5], suggest that the two forms represent different enzyme species.

It has become apparent that guanylate cyclase activity may be regulated by redox events [4]. Sodium azide, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and other *N*-nitroso compounds activate guanylate cyclase and increase cyclic GMP levels in intact tissues by a mechanism that probably involves nitric oxide [4, 6]. A number of other free radicals and oxidative compounds have also been shown to stimulate guanylate cyclase and increase cyclic GMP levels in cells [4, 5, 7–10]. Most of the information on the regulation of guanylate cyclase by redox has been obtained from studies on the modulation of soluble enzyme activity. Little is known about the regulation of the particulate enzymic form. The present report describes the activation of particulate rat lung guanylate cyclase by nitric oxide and MNNG and suggests the involvement of sulfhydryl groups in the activation process.

MATERIALS AND METHODS

Male Sprague–Dawley rats (150–200 g) were anesthetized with sodium pentobarbital (30 mg/kg, i.p.), and the abdominal and thoracic cavities were opened. The inferior vena cava was cut and the lungs were perfused through the right ventricle with 50 ml of cold 0.9% NaCl. The pure white lungs were 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 (homogenizing buffer). The homogenate was centrifuged at 100,000 *g* for 60 min. The resulting particulate fraction was resuspended in a volume of homogenizing buffer equal to the original homogenate and was recentrifuged at 100,000 *g* for 60 min. The final 100,000 *g* pellet was again resuspended in homogenizing buffer as before, centrifuged at 1500 *g* for 15 min to remove large, poorly suspended particulate matter, and frozen at −70° until use.

Immediately prior to each experiment, the particulate enzyme preparation was passed through a column of Sephadex G-25 (1.5 × 6 cm) equilibrated with 20 mM Tris–HCl (pH 7.6) to remove dithiothreitol, sucrose, and EDTA added during homogenization. Guanylate cyclase was assayed in a 10-min incubation at 37° as described [6, 11] in a 100 μl reaction 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 0.02 mg 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 [6, 11, 12]. Protein was measured as described by Lowry *et al.* [13]. All values are the means of duplicate or triplicate incubations from representative experiments.

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The activation of particulate guanylate cyclase by nitric oxide was essentially as described for the soluble enzyme [6]. The reversal of nitric oxide activation was examined by following the decay of active enzyme during a preincubation. Following G-25 chromatography, 100 μl aliquots of enzyme were flushed with N_2, exposed to nitric oxide gas for 10 sec (flow rate = 600 μl/min, 2 cm above the enzyme surface), and again flushed extensively with N_2. These conditions resulted in consistent and maximum activation of all preparations. Following activation, various additions were made, as indicated, to the active enzyme which was then preincubated in a water bath under the conditions described prior to assay. Some enzyme was re-exposed to nitric oxide as just described following the preincubation period.

Rats were from the Zivic Miller Co., Pittsburgh, PA. Nitric oxide gas was from the Union Carbide Corp., East Chicago, IN. MNNG, diamide, and oxidized and reduced glutathione were from the Sigma Chemical Co., St. Louis, MO. All other materials were obtained as described previously [6, 11, 12].

RESULTS

Nitric oxide (Fig. 1) or MNNG (Fig. 2) increased particulate lung guanylate cyclase activity in a dose-dependent manner that was enhanced by dithiothreitol. Although low concentrations of nitric oxide or MNNG produced near maximum activation, excessive amounts decreased the particulate enzyme activity. Similar findings have been reported for the activation of the purified soluble enzyme from rat liver or lung by nitric oxide [1, 6, 11]. The results with MNNG are qualitatively similar to those reported by Lad and White [14] for the particulate lung enzyme assayed with Mn^{2+} as the cation cofactor. These investigators demonstrated slight activation (1- to 2-fold) of Mn^{2+}-supported particulate guanylate cyclase that was potentiated by 2-mercaptoethanol and/or filipin [14]. They did not, however, examine the dose–response relationship for MNNG with Mg^{2+} as cofactor. Although both the soluble and particulate enzymes can utilize either Mn^{2+} or Mg^{2+} as the cation cofactor, oxidative activation is most dramatic with Mg^{2+}-supported activity [1, 6, 11, 15]. The physiological importance of Mn^{2+}-supported activity is of some question due to the almost non-existent levels of free Mn^{2+} in cells and the recent observation that free Mn^{2+} may oxidatively stimulate or inhibit soluble guanylate cyclase [12]. For these reasons only the activation of Mg^{2+}-supported particulate guanylate cyclase activity by nitric oxide and MNNG was examined in this study.

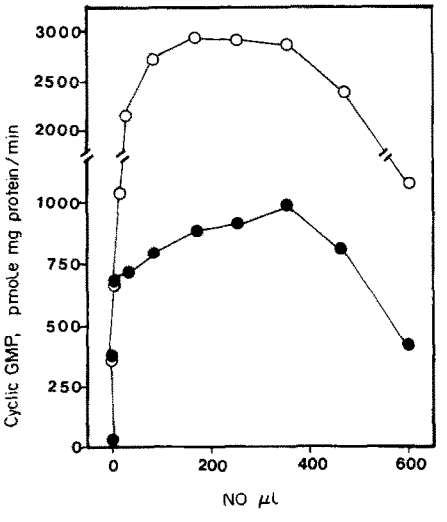

Fig. 1. Dose–response relationship for the activation of particulate guanylate cyclase by nitric oxide. Aliquots (100 μl) of particulate lung guanylate cyclase, prepared as described in Materials and Methods, were exposed to the amounts of nitric oxide indicated either in the absence (●——●) or presence (○——○) of 1 mM dithiothreitol. Following exposure, a 10 μl aliquot was transferred to the guanylate cyclase assay and the activity was determined. The final concentration of dithiothreitol in the enzyme assay was 0.1 mM. Basal activities in the absence or presence of dithiothreitol were 7.8 pmoles cGMP·(mg protein)$^{-1}$·min^{-1}.

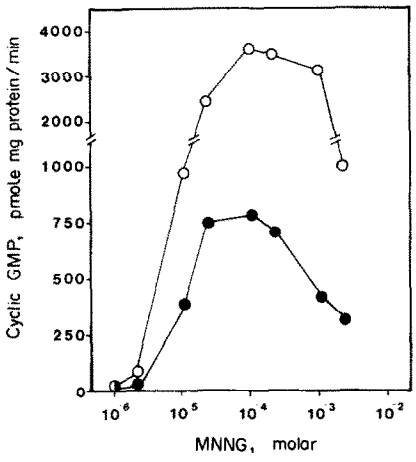

Fig. 2. Dose–response relationship for the activation of particulate guanylate cyclase by MNNG. Particulate guanylate cyclase, prepared as described in Materials and Methods, was assayed in the absence (●——●) or presence (○——○) of 1 mM dithiotreitol and the concentrations of MNNG indicated. Basal activities were similar to those reported in Fig. 1.

Preincubation of particulate guanylate cyclase at 37°, following activation with nitric oxide, decreased the nitric oxide-stimulated activity (Fig. 3). No decay of nitric oxide-stimulated activity, however, was seen at 24° or 4° during a 60-min preincubation. The addition of 10 mM dithiothreitol to nitric oxide-stimulated enzyme significantly enhanced the decay of activity during preincubation. This effect was most apparent at the lower preincubation temperatures. Basal activity did not decay during a 60-min preincubation at 37° (data not shown).

The decay of nitric oxide-stimulated activity at 37° was due, in part, to the irreversible loss of some enzyme activity. Figure 4 shows the decay during preincubation and the subsequent reactivation of nitric oxide-stimulated particulate guanylate cyclase.

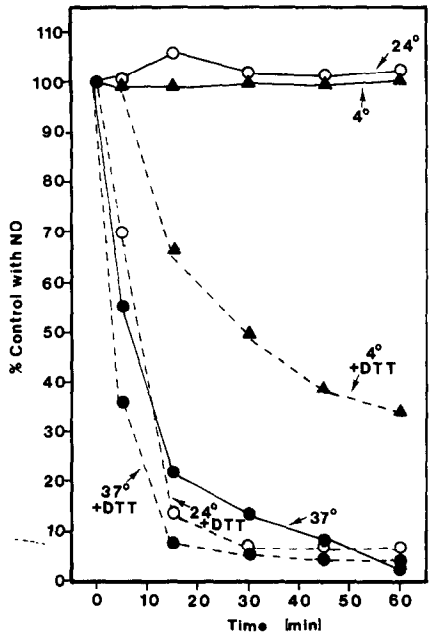

Fig. 3. Effect of temperature and dithiothreitol on the decay of nitric oxide-stimulated particulate guanylate cyclase. Particulate guanylate cyclase following chromatography on Sephadex G-25 was exposed to 100 μl of nitric oxide as described in Materials and Methods. Following exposure to nitric oxide, 10 mM dithiothreitol was added to some tubes (broken lines) and the nitric oxide-stimulated enzyme was preincubated at 37° (●——●), 24° (○——○) or 4° (▲——▲). At the times indicated following preincubation, a 10 μl aliquot was removed and transferred to the guanylate cyclase assay. The dithiothreitol was diluted 10-fold in the final enzyme assay. Results are expressed as the percentages of the control activity with nitric oxide. The activity of nitric oxide-stimulated enzyme before preincubation was 961 pmoles cyclic GMP·(mg protein)$^{-1}$·min^{-1} and was not altered by the addition of dithiothreitol following nitric oxide exposure.

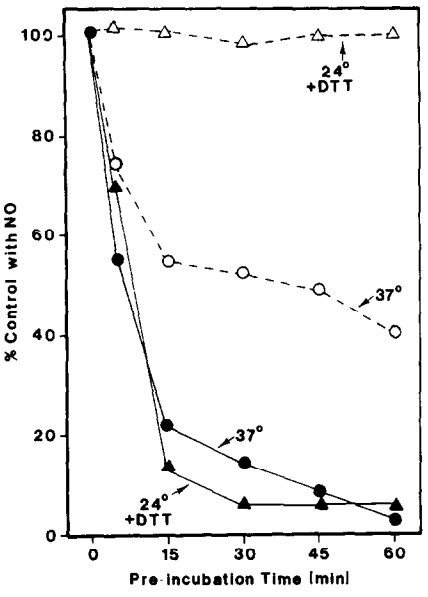

Fig. 4. Reversal of nitric oxide activation during preincubation of nitric oxide-stimulated enzyme. Particulate guanylate cyclase following chromatography on Sephadex G-25 was exposed to 100 μl of nitric oxide as described in Materials and Methods. Following exposure to nitric oxide, the enzyme was preincubated at 37° in the absence of dithiothreitol (●——●) or at 24° in the presence of 10 mM dithiothreitol (▲——▲). At the times indicated, a 10 μl aliquot was transferred to the guanylate cyclase assay either before (●——●; ▲——▲) or after (○- - -○; △ - - △) re-exposure to nitric oxide. Results are expressed as the percentages of control activity with nitric oxide before preincubation. The non-preincubated nitric oxide-stimulated activity before or after re-exposure to nitric oxide was 961 pmoles cyclic GMP·(mg protein)$^{-1}$·min^{-1} in the absence of dithiothreitol. The non-preincubated activity after re-exposure to nitric oxide in the presence of 10 mM dithiothreitol was 3101 pmoles cyclic GMP·(mg protein^{-1}·min^{-1}.

During preincubation at 37° there was a progressive decline in the responsiveness of nitric oxide-stimulated enzyme to activation by a second exposure of nitric oxide. The loss of responsiveness to nitric oxide reactivation was not an artifact resulting from the inhibition of enzyme activity by excess nitric oxide, since re-exposure of non-preincubated nitric oxide-stimulated enzyme did not result in enzyme inhibition. It could also be seen from the dose–response curve for nitric oxide (Fig. 1) that a nitric oxide dose of 200 μl (i.e. 100 μl nitric oxide for initial exposure followed by a second 100 μl exposure) was on the plateau of the dose–response relationship. Although dithiothreitol enhanced the absolute level of activation (Fig. 1), it did not prevent the loss of responsiveness during preincubation at 37° to reactivation by nitric oxide (data not shown). At 24° in the presence of dithiothreitol, however, the decay of nitric oxide-stimulated guanylate cyclase activity was completely reversible upon re-exposure to nitric oxide (Fig. 4). Since no decay of active enzyme occurred at 24° in the absence of dithiothreitol (Fig. 3), these findings suggested that, at this temperature, dithiothreitol caused the reversal of nitric oxide activation. Other thiols would also reverse nitric oxide activation (data not shown); however, dithiothreitol was the most effective. The observations are consistent with the hypothesis that the reversible oxidation and reduction of sulfhydryl groups on particulate guanylate cyclase are involved in its activation by nitric oxide.

Preincubation of basal particulate lung guanylate cyclase activity at 37° prior to the guanylate cyclase assay reduced enzyme responsiveness to MNNG activation (Table 1). Responsiveness of basal enzyme activity to activation by nitric oxide was also reduced during preincubation (data not shown). Dithiothreitol, 2-mercaptoethanol and reduced gluthathione prevented the loss of responsiveness of particulate guanylate cyclase to MNNG during the preincubation. The various thiol antioxidants varied in their ability to enhance the activation of the particulate enzyme by MNNG. They were, however, similarly effective in preventing the decay of enzyme responsiveness to MNNG during preincubation. These results were in contrast to those with nitric oxide-

Table 1. Effects of various thiol reagents on the loss of enzyme responsiveness to activation by MNNG*

Preincubation addition (mM)	Cyclic GMP [pmoles·(mg protein)$^{-1}$·min^{-1}] Control	60 min
None	678	291
Dithiothreitol (1)	2925	3087
Dithiothreitol (10)	3665	3497
2-Mercaptoethanol (1)	736	697
2-Mercaptoethanol (10)	1174	1099
Reduced glutathione (1)	1153	1150
Reduced glutathione (10)	1914	1916
Diamide (1)	693	34
Diamide (1) plus dithiothreitol (1)	1804	45
Diamide (1) plus dithiothreitol (10)	3107	3095
Oxidized glutathione (1)	893	43
Oxidized glutathione (1) plus dithiothreitol (10)	3551	3509

* Particulate guanylate cyclase, prepared as described in Materials and Methods, was preincubated at 37° in the absence or presence of the additions indicated. Either before (control) or after 60 min of preincubation, a 10 μl aliquot was transferred to the guanylate cyclase assay containing 0.1 mM MNNG and the activity was determined. The additions indicated were diluted 10-fold in the final guanylate cyclase assay.

stimulated enzyme which exhibited an irreversible loss of responsiveness to re-activation during preincubation at 37° even in the presence of dithiothreitol. Observations such as these suggested that the active form of particulate guanylate cyclase was more labile than was the enzyme under basal conditions. Increased lability is also a characteristic of the active soluble enzyme [4, 16].

Although the loss of enzyme responsiveness to activation by MNNG was prevented by thiol antioxidants, it was potentiated by the thiol oxidants, diamide or oxidized glutathione (Table 1). The presence of thiol oxidants did not affect the activation of particulate guanylate cyclase by MNNG in the absence of preincubation; however, they markedly accelerated the loss of enzyme responsiveness when preincubated with the enzyme. The thiol oxidants also reduced the responsiveness of enzyme to activation by nitric oxide (data not shown). The inhibitory effects of diamide or oxidized glutathione on MNNG activation were prevented by dithiothreitol. Although equal-molar concentrations of dithiothreitol relative to sulfhydryl oxidant would not prevent the loss of responsiveness to MNNG, concentrations of dithiothreitol in excess of the sulfhydryl oxidant were effective.

Basal particulate guanylate cyclase activity underwent spontaneous activation during preincubation that was prevented by dithiothreitol (Table 2). These findings were similar to the spontaneous activation of soluble guanylate cyclase reported by White *et al.* [17]. Although preincubation of particulate guanylate cyclase with diamide inhibited the activation of the enzyme by MNNG (Table 1), it enhanced the spontaneous activation of basal enzyme activity (Table 2). Both the inhibition of MNNG activation and the increased spontaneous activation of basal activity by diamide were blocked by dithiothreitol.

Table 2. Effects of diamide and dithiothreitol on the spontaneous activation of basal enzyme activity during preincubation*

Preincubation addition (mM)	Cyclic GMP [pmoles·(mg protein)$^{-1}$·min^{-1}] Control	60 min
None	7.5	22.6
Dithiothreitol (10)	7.6	7.8
Diamide (1)	7.5	46.3
Diamide (1) plus dithiothreitol (10)	7.4	7.4

* Particulate guanylate cyclase, prepared as described in Materials and Methods, was preincubated at 37° in the absence or presence of the additions indicated. Either before (control) or after 60 min of preincubation a 10 μl aliquot was transferred to the guanylate cyclase assay and the activity was determined. The additions indicated were diluted 10-fold in the final guanylate cyclase assay.

DISCUSSION

The majority of mammalian tissues contain both soluble and particulate forms of guanylate cyclase. Although the soluble enzyme is the predominant form in the liver, lung, and kidney, certain tissues such as the small intestinal mucosa contain the particulate form almost exclusively [18]. A number of studies have demonstrated that the ratio of soluble to particulate guanylate cyclase within a tissue can be altered, particularly during periods of rapid cell growth. For example, in a variety of transplantable or carcinogen-induced tumors as well as during liver regeneration, the ratio of soluble to particulate guanylate cyclase is decreased [19–22]. This, coupled with

the observation that many of these systems have elevated levels of cyclic GMP [19–22], has suggested that the particulate enzyme may be more important than the soluble in terms of the regulation of the levels of cyclic GMP in cells [4]. The relative contribution of soluble and particulate guanylate cyclases to the cellular pool of cyclic GMP, however, remains uncertain.

Much of the particulate guanylate cyclase activity in tissue homogenates is latent and is only expressed following solubilization with detergents [18]. Treatment of particulate fractions with detergents is also thought to cause some enzyme activation as well [4, 5, 18]. Detergent-treated particulate or soluble guanylate cyclase loses its ability to be activated by agents such as nitric oxide or MNNG [4, 23, 24]. Nevertheless, in the absence of detergents, particulate guanylate cyclase can be activated by various *N*-nitroso compounds. The degree of activation, however, is generally less than that observed with the soluble enzyme [23].

Particulate guanylate cyclase which had been activated by nitric oxide decayed during preincubation at 37° but not at 24° or 4°. Dithiothreitol accelerated the decay of nitric oxide-stimulated activity at all temperatures and potentiated the reversal of nitric oxide activation. Particulate guanylate cyclase which had been activated with nitric oxide was apparently quite labile, since at 37° there was an irreversible loss of a considerable amount of activated enzyme that was not prevented by dithiothreitol. At lower temperatures, however, dithiothreitol promoted the reversal of nitric oxide activation and prevented the irreversible loss of activated enzyme.

These findings suggest that the particulate enzyme contains sulfhydryl groups which are involved in the activation of the enzyme by nitric oxide. Oxidation of these groups results in activation which can be reversed by subsequent reduction with dithiothreitol. In this sense the particulate enzyme is perhaps similar to the soluble. Studies with the soluble guanylate cyclase from spleen and platelets have suggested that the oxidation of key sulfhydryl groups on the soluble enzyme results in activation [9, 10, 25]. Recent studies in this laboratory have also indicated that the oxidation and reduction of one class of enzyme sulfhydryl groups is responsible for the reversible activation of the soluble lung enzyme by nitric oxide (J. M. Braughler, manuscript in preparation).

That dithiothreitol and other thiols enhance activation of particulate lung guanylate cyclase by nitric oxide or MNNG is in agreement with the studies of Lad and White [14]. These investigators also showed that treatment of the lung particulate fraction with the neutral polyene antibiotic filipin enhanced the activation of particulate guanylate cyclase by sodium nitroprusside or other *N*-nitroso compounds. The fact that thiols enhance the activation of the particulate enzyme by nitric oxide, however, is in contrast to studies with soluble guanylate cyclase. Ignarro *et al.* [26] have shown that dithiothreitol will potentiate the activation of the crude soluble enzyme from bovine coronary artery by a number of *N*-nitroso compounds but not by nitric oxide. If the soluble enzyme is partially purified, however, dithiothreitol or other thiols can potentiate its activation by nitric oxide [6, 27]. The reason for these apparent differences between the soluble and particulate enzymes is not clear at this time. Purification of the soluble mammalian enzyme to homogeneity from several sources has been accomplished by a number of laboratories [1, 2, 11, 28, 29]; however, the only particulate enzyme to be purified thus far is that from sea urchin sperm [30]. Recently, however, Waldman *et al.* [31] have obtained a highly purified (500-fold) preparation of rat lung particulate guanylate cyclase. The highly purified particulate enzyme is activated by nitric oxide and other activators. Continued studies with purified particulate enzyme should be helpful in defining the apparent differences between soluble and particulate guanylate cyclase.

Preincubation of particulate guanylate cyclase in the absence or presence of diamide or oxidized glutathione inhibits the activation of particulate guanylate cyclase by MNNG. The loss of responsiveness of preincubated enzyme is enhanced significantly by the thiol oxidants. The spontaneous activation of basal activity during preincubation, on the other hand, is enhanced by diamide. Dithiothreitol blocks the spontaneous activation of basal activity, the loss of enzyme responsiveness to MNNG, and their potentiation by diamide. These results suggest that the oxidation of some sulfhydryl groups on particulate guanylate cyclase may block enzyme activation by MNNG. At the same time, sulfhydryl group oxidation may lead to some spontaneous enzyme activation. The reason why diamide can potentiate spontaneous enzyme activation and at the same time prevent activation by MNNG is not clear. It is likely, however, that multiple classes of sulfhydryl groups are important for the regulation of particulate guanylate cyclase activity. Studies with the soluble guanylate cyclase from rat liver or lung have indicated that preincubation with diamide, oxidized glutathione, or disulfides leads to an inhibition of basal activity that is prevented by dithiothreitol [32, 33]. These findings are in contrast to those reported here for the particulate enzyme. On the other hand, Brandwein *et al.* [33] have shown that the responsiveness of purified rat lung soluble guanylate cyclase to activation by nitric oxide is reduced by the formation of mixed protein disulfides. Studies by others have also suggested that sulfhydryl group oxidation can block activation of soluble guanylate cyclase by unsaturated fatty acid peroxides [10, 25]. These findings are in agreement with those reported here that sulfhydryl group oxidants can block activation of the particulate enzyme by MNNG.

The role of the oxidative modulation of guanylate cyclase activity in cell function is not yet clear. It is apparent, however, that both the soluble and particulate forms of the enzyme can be regulated by redox events. This regulation apparently involves the oxidation and reduction of key enzyme sulfhydryl groups that may result in enzyme activation or inhibition. The relative importance of the two forms of guanylate cyclase in the dynamic regulation of cyclic GMP levels in cells cannot be judged at this time. Continued study of the two forms and their regulation should provide important information in this regard.

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