Effects of Thiols, Sugars, and Proteins on Nitric Oxide Activation of Guanylate Cyclase*

(Received for publication, March 30, 1979, and in revised form, August 7, 1979)

J. Mark Braughler,‡ Chandra K. Mittal, and Ferid Murad
From the Division of Clinical Pharmacology, Departments of Pharmacology and Medicine, University of Virginia, Charlottesville, Virginia 22908

Purification of soluble guanylate cyclase from rat liver resulted in an apparent loss of enzyme activation by nitric oxide that could be restored by dithiothreitol, methemoglobin, bovine serum albumin, or sucrose. Although hemoglobin also permitted some activation with nitric oxide, the effect of other agents to restore enzyme activation was prevented with hemoglobin. As a result of enzyme purification, there is an alteration of the dose-response relationship for nitric oxide activation. After partial enzyme purification, relatively high concentrations of nitric oxide that were stimulatory in crude enzyme preparations had no effect on enzyme activity. However, partially purified or homogeneous enzyme was activated by lower concentrations of nitric oxide. The bell-shaped dose-response curve for nitric oxide was shifted to the left with guanylate cyclase purification. The addition of dithiothreitol, methemoglobin, bovine serum albumin, or sucrose to enzyme markedly broadens the dose-response curve for nitric oxide. Thus, the apparent loss of responsiveness to nitric oxide with purification is a function of increased sensitivity of guanylate cyclase to nitric oxide. Increased sensitivity to nitric oxide with enzyme purification probably results from the removal of heme, proteins, and small molecules that can serve as scavengers or sinks for nitric oxide and prevent excessive oxidation of the enzyme.

It has become increasingly apparent that free radicals and/or processes involving oxidation and reduction are involved in the modulation of guanylate cyclase (GTP pyrophosphatase-lyase, EC 4.6.1.2.) (1–4). A common pathway for activation of guanylate cyclase by compounds such as azide, nitrite, nitroprusside, nitroglycerin, and N-methyl-N′-nitro-N-nitroso-guanidine may be the formation of nitric oxide (3, 5). Hydroxyl radical (2), unsaturated fatty acids (6), prostaglandin endoperoxides, and fatty acid hydroperoxides (7) are also thought to activate the enzyme.

The precise mechanism for guanylate cyclase activation by this diverse group of agents is not known. Nor is it known whether these materials activate the enzyme through similar or different processes. The recent purification of soluble guanylate cyclase from rat liver (8, 9) and lung (10) should provide an opportunity for studying the mechanism of enzyme activation by free radicals and other agents. Soluble rat liver guanylate cyclase purified about 9,000-fold to apparent homogeneity is activated by nitric oxide, nitroprusside, hydroxyl radical, unsaturated fatty acids, and stable prostaglandin endoperoxide analogues (8). Such observations suggest there are no other protein requirements for activation and that free radicals and unsaturated fatty acids may interact directly with the enzyme. The observation that highly purified guanylate cyclase is activated by nitric oxide and nitroprusside in contrast to an earlier report (11) demonstrating that partial enzyme purification resulted in the apparent loss of responsiveness of guanylate cyclase to nitric oxide, nitroprusside, MNNG, and related compounds. In the latter report (11), the responsiveness to nitric oxide could be restored by the addition of heme proteins. Another report has indicated that low concentrations (0.25 to 5 mM) of hemoglobin inhibit or reverse azide, nitric oxide, and nitroprusside activation of guanylate cyclase, whereas higher concentrations of methemoglobin or ferrieycetochrom e c do not (12).

In this communication, we report that the apparent loss of responsiveness of guanylate cyclase to nitric oxide and related materials is due to changes in the dose-response curve which can be reversed by dithiothreitol, bovine serum albumin, methemoglobin, or sucrose. Thus, the apparent loss of responsiveness of guanylate cyclase to higher concentrations of nitric oxide during purification is probably a function of the sensitivity of the enzyme to nitric oxide, the biphasic nature of the dose-response curve, and the removal of scavengers or sinks for nitric oxide during purification.

**MATERIALS AND METHODS**

Guanylate cyclase was partially purified through chromatography on Sepharose 6B or purified to homogeneity from the soluble fraction of rat liver homogenates as described (8). In brief, guanylate cyclase in 100,000 × g supernatant fractions was purified with isoelectric precipitation, ammonium sulfate precipitation, DEAE-Sephadex chromatography, gel filtration with Sepharose 6B, chromatography on hexane/agarose, and preparative polyacrylamide gel electrophoresis (8). These procedures generally increased the specific activity from soluble fractions of homogenates 60- to 100-fold through Sepharose 6B and 800- to 3000-fold through electrophoresis (8). Dithiothreitol added during purification was removed from the Sepharose 6B enzyme by passing the preparation through a column of Sephadex G-25, equilibrated, and run with 20 mM Tris-HCl, pH 7.6. The enzyme after chromatography on Sephadex G-25 was used immediately. Homogeneous guanylate cyclase requires the presence of sucrose and dithiothreitol for stability (8). Experiments described in this report with purified enzyme were conducted following dilution of the purified enzyme to final concentrations of 0.1 mM dithiothreitol and 7 mM sucrose in the guanylate cyclase assay.

* These studies were supported with research grants from the National Institutes of Health (AM15316, HL18260, and AM22125), The Council for Tobacco Research—U.S.A., Inc., and the Virginia Heart Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported with individual National Research Service Award GM06653. Present address, Department of Pharmacology, North-eastern Ohio University, Rootstown, Ohio 44272.

‡ The abbreviation used is: MNNG, N-methyl-N′-nitro-N-nitroso-guanidine.
Nitric Oxide Activation of Guanylate Cyclase

Guanylate cyclase activity was determined as described (2, 5, 8, 12). Incubations (100 µl for 10 min at 37°C) contained 50 mM Tris-HCl buffer (pH 7.6), 10 mM theophylline, 4 mM MnCl₂, 1 mM GTP, and 0 ng to 15 µg of enzyme protein. Theophylline was omitted from assays with homogenous guanylate cyclase.

With partially purified or highly purified guanylate cyclase, basal activity with Mg²⁺ as cofactor was about 10 to 15% of the activity using Mn²⁺. However, with activation, the activities were comparable with either cofactor (8).

Nitric oxide gas was vented into some incubations prior to the guanylate cyclase assay as described (5). Cyclic GMP formed was determined by radioautography (15) with some modification (14). All values are the means of duplicate incubations from representative experiments. Protein was measured by the method of Lowry et al. (15) using bovine serum albumin as standard. All reagents were obtained as described previously (6, 8, 12).

RESULTS

Partial purification of soluble liver guanylate cyclase resulted in the apparent loss of responsiveness to nitric oxide when 165 µl of gas was vented into incubations (Table I). Dithiothreitol, methemoglobin, and albumin, but not sucrose, increased basal guanylate cyclase activity. The stimulatory effects of thiols (16) and albumin (10, 17) on basal guanylate cyclase activity have been reported previously. All of the agents tested restored the responsiveness of partially purified guanylate cyclase to nitric oxide (Table I). The magnitude of nitric oxide stimulation (1.7- to 4-fold) was a function of the material added and its concentration. Dithiothreitol, methemoglobin, albumin, and sucrose also restored enzyme responsiveness to nitric oxide (3, 5).

Hemoglobin at 2.5 nM slightly decreased basal guanylate cyclase activity as reported previously (12) and permitted nitric oxide to activate the enzyme (Table II). The stimulatory effects of thiols (16) and albumin (10, 17) on basal guanylate cyclase activity have been reported previously. All of the agents tested restored the responsiveness of partially purified guanylate cyclase to nitric oxide (Table I). The magnitude of nitric oxide stimulation (1.7- to 4-fold) was a function of the material added and its concentration. Dithiothreitol, methemoglobin, albumin, and sucrose also restored enzyme responsiveness to nitric oxide (3, 5).

Partial purification of soluble liver guanylate cyclase resulted in the apparent loss of responsiveness to nitric oxide when 165 µl of gas was vented into incubations (Table I). Dithiothreitol, methemoglobin, and albumin, but not sucrose, increased basal guanylate cyclase activity. The stimulatory effects of thiols (16) and albumin (10, 17) on basal guanylate cyclase activity have been reported previously. All of the agents tested restored the responsiveness of partially purified guanylate cyclase to nitric oxide (Table I). The magnitude of nitric oxide stimulation (1.7- to 4-fold) was a function of the material added and its concentration. Dithiothreitol, methemoglobin, albumin, and sucrose also restored enzyme responsiveness to nitric oxide (3, 5).

Hemoglobin at 2.5 nM slightly decreased basal guanylate cyclase activity as reported previously (12) and permitted nitric oxide to activate the enzyme (Table II). The stimulatory effects of thiols (16) and albumin (10, 17) on basal guanylate cyclase activity have been reported previously. All of the agents tested restored the responsiveness of partially purified guanylate cyclase to nitric oxide (Table I). The magnitude of nitric oxide stimulation (1.7- to 4-fold) was a function of the material added and its concentration. Dithiothreitol, methemoglobin, albumin, and sucrose also restored enzyme responsiveness to nitric oxide (3, 5).

TABLE I

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>Ratio</th>
<th>+NO</th>
<th>−NO</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 nM</td>
<td>2.9</td>
<td>5.9</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>0.5 µM</td>
<td>2.9</td>
<td>5.9</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 µM</td>
<td>2.8</td>
<td>6.9</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µM</td>
<td>2.9</td>
<td>9.3</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 µM</td>
<td>2.6</td>
<td>9.6</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Methemoglobin</td>
<td>0.5 µM</td>
<td>2.0</td>
<td>4.5</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 µM</td>
<td>2.0</td>
<td>4.5</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µM</td>
<td>2.0</td>
<td>4.5</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 µM</td>
<td>2.0</td>
<td>4.5</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>50 µg/ml</td>
<td>2.1</td>
<td>5.6</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µg/ml</td>
<td>2.1</td>
<td>5.6</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 µg/ml</td>
<td>2.1</td>
<td>5.6</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>400 µg/ml</td>
<td>3.1</td>
<td>7.6</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>800 µg/ml</td>
<td>3.2</td>
<td>7.8</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>10 mM</td>
<td>1.7</td>
<td>2.9</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 mM</td>
<td>1.8</td>
<td>3.4</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>250 mM</td>
<td>1.9</td>
<td>3.4</td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>

Nitric Oxide to activate the enzyme (Table III). The stimulatory effects of thiols (16) and albumin (10, 17) on basal guanylate cyclase activity have been reported previously. All of the agents tested restored the responsiveness of partially purified guanylate cyclase to nitric oxide (Table I). The magnitude of nitric oxide stimulation (1.7- to 4-fold) was a function of the material added and its concentration. Dithiothreitol, methemoglobin, albumin, and sucrose also restored enzyme responsiveness to nitric oxide (3, 5).

TABLE II

The effects of dithiothreitol, methemoglobin, bovine serum albumin, and sucrose on the responsiveness of guanylate cyclase to nitric oxide

Incubations contained 14 µg of enzyme preparation purified through Sepharose 6B and the concentrations of dithiothreitol, methemoglobin, bovine serum albumin, or sucrose indicated. Some incubations were exposed to 165 µl of nitric oxide gas prior to assay (5).

TABLE III

Effects of hemoglobin on the ability of dithiothreitol, methemoglobin, bovine serum albumin, and sucrose to restore nitric oxide responsiveness to partially purified guanylate cyclase

Guanylate cyclase purified through Sepharose 6B was assayed in the presence or absence of hemoglobin and the concentrations of the agents indicated. Some incubations were exposed to 167 µl of NO prior to assay. In Experiment 1, 10 µg of the guanylate cyclase preparation was used per incubation and hemoglobin when present was 2.5 nM. In Experiment 2, 7 µg of enzyme and 10 nM hemoglobin were used.
The apparent loss of responsiveness to nitric oxide is due to a change in the dose-response curve for NO. The partially purified enzyme was, in fact, activated by low concentrations of nitric oxide (Fig. 2). However, higher concentrations of nitric oxide were without effect. In some experiments, the higher concentrations of nitric oxide were inhibitory (not shown). Crude supernatant preparations of guanylate cyclase from liver or lung display a much broader sensitivity to NO than the partially purified enzyme (5). With partially purified enzyme, dithiothreitol, bovine serum albumin, sucrose, and methemoglobin broaden the dose-response curve for nitric oxide (Fig. 2). However, for this preparation, the maximal degree of activation by nitric oxide (about 3.5-fold) was the same regardless of whether or not other agents were added. An exception was observed with sucrose where the degree of activation was less at all concentrations of nitric oxide examined.

Somewhat similar results were obtained with homogeneous guanylate cyclase (Fig. 3). As described previously, 165 µl of nitric oxide activated purified guanylate cyclase with Mg²⁺ as cation cofactor, but not when Mn²⁺ was the cofactor (8). Guanylate cyclase with Mn²⁺ as cofactor was activated, however, by small amounts (4 to 8 µl) of nitric oxide. Sucrose, dithiothreitol, albumin, and methemoglobin all broadened the dose-response curve for nitric oxide with Mn²⁺ as cofactor. When Mg²⁺ was cofactor, these agents increased the ceiling of activation by nitric oxide, but did not alter the shape of the dose-response curve. The reason for the difference between Mn²⁺ and Mg²⁺ activities of purified enzyme is unclear. Due to stability requirements, the control enzyme in Fig. 3 contained 0.1 mM dithiothreitol and 7 mM sucrose. These concentrations were sufficient to facilitate nitric oxide activation of partially purified enzyme (Table I). Perhaps the sensitivity to nitric oxide activation for Mn²⁺ and Mg²⁺ activities differs. Unlike the partially purified enzyme, the degree of activation
Nitric Oxide Activation of Guanylate Cyclase

of purified enzyme by nitric oxide varied, depending upon the agent tested. The reasons for these differences with purified and partially purified enzyme are also unclear. The differences may be related to the presence of some dithiothreitol and sucrose under all conditions since, as summarized in Fig. 1, combinations of agents can alter the degree of activation by nitric oxide. These questions cannot be resolved until the stability of purified enzyme can be maintained in the absence of agents that effect nitric oxide activation. It is clear, however, that the homogeneous soluble guanylate cyclase from liver can be activated by nitric oxide and that this activation can be modified by dithiothreitol, methemoglobin, albumin, and sucrose. These compounds also increased the basal activity of purified enzyme with either Mn$^{2+}$ or Mg$^{2+}$ as cofactor (data not shown).

**DISCUSSION**

In this communication, we report that materials such as thiols, heme proteins, non-heme proteins, and sugars can restore the apparent loss in nitric oxide responsiveness to guanylate cyclase. Restoration of the nitric oxide response by these agents can also be partially blocked by low concentrations (2.5 nM) and totally prevented by higher concentrations (10 nM) of hemoglobin. These results suggest that a nitroheme complex is not a requirement for activation of guanylate cyclase since non-heme materials can substitute for the heme effect. This conclusion is in contrast to that of others (11, 18). However, in these other studies, the effects of non-heme-containing materials were not examined for their ability to restore nitric oxide or nitroso activation of partially purified enzyme (11).

With purification, guanylate cyclase becomes more sensitive to nitric oxide such that concentrations that were stimulatory in crude supernatant fractions are without effect or inhibitory in more purified preparations (Ref. 6 and Fig. 2).

Soluble guanylate cyclase from rat liver purified about 9,000-fold to apparent homogeneity is still responsive to nitric oxide (Fig. 3 and Ref. 8). The responsiveness of purified guanylate cyclase to nitric oxide is observed in the presence of sucrose and dithiothreitol which are required for stabilization of the purified enzyme (8). The presence of a heme moiety associated with guanylate cyclase is unlikely since spectrophotometric studies of purified enzyme have indicated a peak absorbance at 280 nm without shoulders or absorbance in the range of 500 to 650 nm characteristic of heme containing materials. 2

We wish to propose that the concentration of free radicals such as nitric oxide required to activate guanylate cyclase is quite low (less than 1 to 10 μM). Using crude preparations, it has been suggested that oxidation of key sulfhydryl groups on the protein may lead to activation (7). Other studies have indicated that while unsaturated fatty acids, nitric oxide, or compounds which can form nitric oxide can stimulate guanylate cyclase, higher concentrations of these materials are inhibitory (8, 19, 20). It is likely, therefore, that partial oxidation of the enzyme increases activity, whereas excessive oxidation 1

1 The absorption of pure enzyme was maximal at a wavelength of 280 nm, and the absorption for a 0.1 mg/ml solution was 0.29. A detailed study of the physicochemical properties of purified guanylate cyclase is currently underway in this laboratory.

2 Assuming complete atmospheric mixing, a partition coefficient of 0.05, and even distribution in guanylate cyclase assays, the addition of 4 μl of NO to incubations would result in a calculated concentration of NO in solution of about 2.8 μM (5). In all probability, the concentration of NO is considerably less since the brief exposure (1 s) to gas probably does not result in equilibrium. By way of comparison, the concentration of purified guanylate cyclase in Fig. 3 is about 0.53 μM.

Of the protein leads to a loss of activity. Compounds such as hemoglobin, sucrose, dithiothreitol, and bovine serum albumin may protect the enzyme from excessive oxidation by binding nitric oxide or becoming oxidized themselves and, thus, serve as scavengers or sinks for NO. Such interactions would lower the concentration of nitric oxide available for guanylate cyclase activation and prevent excessive oxidation of the protein. Craven and DeRubertis reported that the nitric oxide-hemoglobin complex will activate soluble guanylate cyclase from rat liver and proposed that a nitrosyl-heme complex was required for activation (11). However, we have found that a number of derivatives containing nitric oxide besides heme-containing materials can be prepared by exposing sucrose, glucose, dithiorthreitol, and related compounds to nitric oxide. Following lyophilization, such nitric oxide derivatives can be rehydrated and will markedly activate many purified and partially purified guanylate cyclase preparations in a dose-dependent manner. The mechanism for activation by such nitric oxide derivatives is not clear at this time. It is likely to involve the direct effect of released nitric oxide and does not require the presence of a heme moiety. Thiols may also function to maintain the integrity of sulfhydryl groups on the protein not related to activation. The apparent loss of responsiveness of guanylate cyclase to nitric oxide during purification is probably due to a shift in the dose-response curve for NO resulting from the loss or proteins, heme, and other materials that might serve as nitric oxide scavengers or sinks in cruder systems.

The precise mechanism of guanylate cyclase activation by nitric oxide is not known. Observations with highly purified guanylate cyclase would seem to indicate that a direct interaction with the enzyme is possible. The potential for other more complex modes of interaction cannot be discounted at present. It is clear, however, that although heme, proteins, thiols, and sugars can modulate activation they may not be true requirements for nitric oxide activation of guanylate cyclase. Further studies in this regard with homogeneous enzyme have been hampered by the lability of purified preparations.

**Acknowledgments**—We wish to thank Julia Ruffin, Robert Pintner, Allen Schae, and Larry Carbine for their excellent technical assistance.

**REFERENCES**

Effects of thiols, sugars, and proteins on nitric oxide activation of guanylate cyclase.
J M Braughler, C K Mittal and F Murad


Access the most updated version of this article at http://www.jbc.org/content/254/24/12450

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/24/12450.full.html#ref-list-1