METHEMOGLOBIN BLOCKADE OF CORONARY ARTERIAL SOLUBLE GUANYLATE CYCLASE ACTIVATION BY NITROSO COMPOUNDS AND ITS REVERSAL WITH DITHIOTHREITOL

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1. Introduction

Nitric oxide (NO), compounds that contain the nitroso moiety and those which form and/or release NO have been found to activate guanylate cyclase (GC) and elevate tissue concentrations of guanosine cyclic 3′,5′-monophosphate (cyclic GMP) [1–5]. These observations were important because they revealed for the first time that chemical agents capable of elevating tissue levels of cyclic GMP could also activate GC in cell-free systems. Appreciable activation of GC by acetylcholine and other muscarinic agents could never be demonstrated although tissue levels of cyclic GMP could be increased at least several fold. One mechanism by which NO activates GC may be through formation of an intermediate paramagnetic nitrosyl–ferrous heme complex, which presumably binds to and activates the enzyme [6,7]. Compounds such as nitroprusside and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) activate GC by a mechanism which appears to be similar to that of NO, presumably by releasing NO in enzyme reaction mixtures and in intact cells [8,9].

Several inconsistent and inadequately explained observations have been reported with regard to the activation of GC by nitroso compounds, inhibition of such activation with heme proteins and the effects of dithiothreitol (DTT). First, hemoglobin was reported to inhibit activation of GC by NO and related compounds whereas methemoglobin was reported to be ineffective, and myoglobin effects were variable [10,11]. Second, DTT was found to be required for, or to enhance, nitroprusside activation of GC [3], and yet DTT inhibited MNNG activation of this enzyme system [1].

The data here illustrate that:

(1) Methemoglobin is indeed a potent inhibitor of NO activation of GC;
(2) DTT is not required for nitroprusside activation of GC from tissue preparations relatively free of contaminating heme proteins but is required for crude preparations containing appreciable amounts of heme proteins;
(3) DTT reverses methemoglobin blockade of GC activation by nitroso compounds but not by NO;
(4) DTT hastens the inactivation of MNNG.

2. Materials and methods

GC activity was determined in soluble fractions by measuring the formation of cyclic GMP from GTP (0.1 or 0.3 mM) in the presence of 3 mM Mg^{2+} by a modification (double column chromatographic isolation of cyclic GMP using Dowex 50-H^+ followed with neutral alumina) of the methods in [12]. Segments of left descending and circumflex arteries and their branches were dissected from bovine hearts, cleaned of loosely adhering fat and connective tissue, minced finely and homogenized (20% w/v in 50 mM Tris–HCl (pH 7.4) at 4°C) by a two-step procedure involving: (1) cellular disruption with the aid of a Brinkmann Polytron (no. 3 setting for three 10 s intervals with 30 s between); (2) grinding the resulting homogenate

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with a Potter-Elvejhem, Teflon–glass tissue grinder. Homogenates were centrifuged at 110 000 × *g* for 60 min at 4°C and supernatants were removed and stored at −70°C. Soluble GC fractions from rat liver were prepared exactly as in [12].

NO gas (99.99% pure) was obtained from Matheson Gas and dilutions were prepared just prior to use in O_2-free purified N_2, using gas-tight syringes and glass containers. NO was delivered to GC reaction mixtures by direct injection into mixtures in open test tubes at exactly 1 min after initiation of enzyme reactions. An approximate estimation of the upper limit of the NO concentration in reaction mixtures was made, taking into consideration incomplete atmospheric mixing of NO in solution, an NO partition coefficient of 0.05, a fairly even distribution of NO in solution because of slow injections of a very fine stream of bubbles, and the equation $PV = nRT$. Calculations indicated that 0.1 μl NO delivered to 1.0 ml aqueous medium at 37°C yielded a maximal concentration of 0.2 μM. Hemoglobin, methemoglobin, nitroprusside and DTT were obtained from Sigma, and MNNG was from Aldrich.

3. Results and discussion

NO activated bovine coronary arterial soluble GC 70-fold over basal activity in the presence of Mg^{2+} (table 1) and the activation was concentration-dependent (not illustrated; 0.01–1 μl NO activated GC 10–72-fold). NO reacts with and binds to ferrous heme [13] and one mechanism by which NO activates GC may be by forming a paramagnetic nitrosyl–ferrous heme complex, which interacts with the enzyme [6,7]. Although NO forms nitrosyl–ferric heme with methemoglobin [13], this complex is diamagnetic and this may account for its failure to activate GC (unpublished observations). Binding of NO to methemoglobin may also explain the inhibitory effect of this heme protein on NO activation of GC (table 1). Such an explanation may apply also to the hemoglobin tested in these experiments and in those reported by other investigators because commercially available crystalline hemoglobin is actually ~75% methemoglobin, as stated on the product label. Methemoglobin (and hemoglobin) inhibited also the activation of GC by nitroprusside and MNNG (table 1). The mechanism by which methemoglobin inhibits this activation may be the same as that suggested above for NO because both nitroprusside and MNNG activate GC probably by releasing NO [6,7].

DTT enhanced nitroprusside (32% increase) and MNNG (105% increase), but not NO, activation of bovine coronary arterial soluble GC (table 1). Further, although nitroprusside activated coronary arterial GC in the absence of DTT, nitroprusside failed to activate rat liver-soluble GC unless DTT was present in the

Table 1
Effects of dithiothreitol (DTT) and heme proteins on activation of coronary arterial soluble guanylate cyclase by nitroprusside (NP), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and nitric oxide (NO)

Additions	pmol cyclic GMP/min/mg protein[a]		
	0.1 mM NP	0.1 mM MNNG	0.1 μl NO
None	318 ± 14	408 ± 19	592 ± 38
2 mM DTT	419 ± 21	836 ± 31	471 ± 32
2 μM Methemoglobin	9 ± 1	9 ± 1	58 ± 5
2 μM Hemoglobin	12 ± 2	14 ± 2	120 ± 9
2 mM DTT + 2 μM methemoglobin	386 ± 17	778 ± 36	37 ± 4
2 mM DTT + 2 μM hemoglobin	398 ± 19	802 ± 24	85 ± 7

[a] Methemoglobin, hemoglobin, DTT, NP and MNNG were added to reaction mixtures immediately prior to initiation of reactions with tissue; NO was added 1 min later (0.1 mM GTP, 3 mM Mg^{2+} and 150–174 μg tissue protein in 1.0 ml reaction vol. were employed). Basal GC activity was 8.4 ± 0.5 pmol cyclic GMP/min/mg protein. Data represent the mean ± SE, n=3–4

Table 2
Effects of preincubation and dithiothreitol (DTT) on activation of rat liver soluble guanylate cyclase by nitroprusside (NP) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)

Additions	pmol cyclic GMP/min/mg protein[a]		
	No pre-incubation[b]	Preincubation at 0°C for 10 min	
		−Tissue[c]	+Tissue[d]
None	3 ± 0.1	2 ± 0.1	3 ± 0.2
0.1 mM NP	10 ± 0.4	12 ± 0.3	12 ± 0.5
0.1 mM NP + 2 mM DTT	626 ± 23	683 ± 21	654 ± 18
0.1 mM MNNG	880 ± 29	1168 ± 38	1097 ± 31
0.1 mM MNNG + 2 mM DTT	1446 ± 37	8 ± 0.3	1253 ± 44
1.0 μl NO	824 ± 17	4 ± 0.2	616 ± 25
1.0 μl NO + 2 mM DTT	857 ± 26	4 ± 0.1	812 ± 33

[a] Experiments were conducted with 3 mM Mg^{2+}, 0.3 mM GTP and 600–700 μg protein in 1.0 ml reaction vol. Data represent the mean ± SE, n=4
[b] Agents were added together with tissue at start of incubation at 37°C
[c] Agents were preincubated at 0°C for 10 min with substrate (GTP) but without tissue; tissue was added and samples were incubated at 37°C for 10 min
[d] Agents were preincubated at 0°C for 10 min with tissue but without substrate (GTP); GTP was added and samples were incubated at 37°C for 10 min

reaction mixture (table 2). In contrast, MNNG activation of rat liver soluble GC was observed in the absence of DTT and was enhanced by DTT (table 2; no preincubation). These seemingly discrepant observations from the crude soluble fractions of the two different tissues studied may derive from the fact that the crude soluble fraction from coronary artery contained no measurable amounts of methemoglobin or hemoglobin whereas that from rat liver was contaminated with these heme proteins (determined spectrophotometrically). That is, the presence of heme protein in the crude liver soluble fraction may have accounted for the failure of nitroprusside to activate GC in the absence of DTT. DTT not only restored the activation of hepatic GC by nitroprusside (table 2) but also abolished the inhibitory effect of methemoglobin and hemoglobin on nitroprusside activation of coronary arterial GC (table 1). Thus,

Table 3
Inhibition of nitroprusside (NP) activation of coronary arterial soluble guanylate cyclase by rat liver soluble fraction and its reversal with dithiothreitol (DTT)

Additions	pmol cyclic GMP/min[a]		
	Coronary artery[b]	Liver[c]	Coronary artery + liver[d]
None	2 ± 0.1	3 ± 0.2	3 ± 0.2
0.1 mM NP	76 ± 6	2 ± 0.1	12 ± 0.8
0.1 mM NP + 2 mM DTT	93 ± 7	483 ± 24	587 ± 33

[a] Experiments were conducted with 3 mM Mg^{2+}, 0.1 mM GTP and the amounts of protein indicated below, in 1.0 ml reaction vol. Data represent the mean ± SE, n=9
[b] Protein concentration in reaction mixture was 177–194 μg
[c] Protein concentration in reaction mixture was 643–677 μg
[d] Total protein concentration in reaction mixtures was 820–871 μg

DTT restored the activation of hepatic GC by nitroprusside presumably by abolishing the inhibitory effect of contaminating heme protein. Further, the addition of hepatic soluble fraction to coronary arterial soluble fraction abolished nitroprusside activation of GC in the latter, an effect that was reversed with DTT (table 3). Again, the inhibitory effect of the rat liver fraction was probably due to the presence of contaminating heme protein, and this effect was abolished with DTT. In this regard, it may be of interest that the sensitivity of the liver GC fraction to NO activation was 20-fold less than that of the coronary arterial fraction. For example, 0.1 μl NO failed to activate and 1 μl NO activated liver GC to the same extent that 0.05 μl NO activated coronary arterial GC.

Three observations may help to explain the mechanism by which DTT reversed the heme protein blockade of GC activation by nitroprusside and MNNG:

(1) DTT enhanced MNNG and was required for nitroprusside activation of rat liver GC (table 2);
(2) Preincubation of MNNG with DTT for 10 min at 0°C in the absence of tissue fraction abolished the capacity of MNNG to activate rat liver GC (table 2);
(3) DTT abolished heme protein blockage of nitroprusside and MNNG, but not NO, activation of coronary arterial GC (table 1).

These observations suggest that DTT may react with the nitroso compounds to promote release of NO, which may then overcome the inhibitory effect of the heme proteins. Moreover, MNNG may be more sensitive or more prone than nitroprusside to the capacity of DTT to release NO, as is suggested by the observations that MNNG, but not nitroprusside, activates rat liver GC in the absence of DTT (table 2). Preincubation of MNNG and DTT in the absence of tissue fraction resulted in the complete inactivation of MNNG within 10 min at 0°C (table 2). However, in the presence of tissue, preincubation of MNNG and DTT at 0°C did not destroy the reactivity of MNNG and complete capacity to activate GC at 37°C was maintained. If DTT causes NO release from MNNG, then the released NO must react with a soluble tissue component to form a relatively stable substance which is capable of activating GC during incubation at 37°C. Indeed, preincubation of NO in the absence of tissue resulted in inactivation of NO, whereas the inclusion of tissue in the preincubation restored the capacity of NO to activate GC (table 2). Evidence for the formation of a nitrosyl–ferrous heme complex by MNNG in crude hepatic soluble fractions at 4°C has been obtained [1]. Moreover, evidence for the release of NO from nitroso compounds by sulfhydryl reducing agents exists [6,7]. DTT-induced NO release from nitroso compounds may also explain the findings (table 1) that DTT enhanced nitroprusside and MNNG, but not NO, activation of coronary arterial GC.

Preincubation of nitroprusside and DTT and 0°C in the absence of tissue failed to inactivate nitroprusside (table 2). This is in contrast to the findings with MNNG and the reasons for this difference are presently unknown. Possible explanations include a much slower rate of release of NO from nitroprusside, and a direct nonenzymatic transfer of the nitroso moiety to ferrous heme in the presence of suitable reducing agents. Direct determination of the lability of the nitroso moiety on nitroprusside and MNNG in reaction mixtures must be made in order to answer this question. In any event, such an interpretation may be used to explain:

(1) The failure of nitroprusside to activate rat liver GC in the absence of DTT (table 2);
(2) The lesser degree of enhancement by DTT of nitroprusside than of MNNG activation of coronary arterial GC (table 1);
(3) The failure of DTT to reverse the heme protein blockade of GC activation by NO (table 1).

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