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ELECTRON SPIN RESONANCE STUDY OF THE ROLE OF NITROSYL-HEME IN THE ACTIVATION OF GUANYLATE CYCLASE BY NITROSOGUANIDINE AND RELATED AGONISTS

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SUMMARY

The responsiveness of soluble rat hepatic guanylate cyclase to N-methyl-N'-nitro-N-nitrosoguanidine,NO, nitrite and nitroprusside is markedly reduced or abolished with partial purification of enzyme activity, and is subsequently restored by addition of free or protein bound heme, plus a reducing agent. Under conditions required for restoration of enzyme responsiveness, formation of paramagnetic nitrosyl-heme complexes from each enzyme agonist was observed by electron spin resonance spectroscopy. Moreover, preformed nitrosyl-hemoglobin activates purified guanylate cyclase in the absence of both added heme and reducing agents, conditions that do not permit expression of effects of nitrosoguanidine, NO, nitrite or nitroprusside. The capacity of the latter agonists to activate purified enzyme activity correlates with their capacity to generate nitrosyl-heme under different conditions of incubation. These results indicate that formation of nitrosyl-heme may be an obligate step in the activation of guanylate cyclase by nitrosoguanidine, NO, nitrite and nitroprusside.

INTRODUCTION

Several N-nitroso carcinogens, nitric oxide, nitrite and NP[1] markedly increase the activity of guanylate cyclase in the soluble fraction of rat liver (1-4). We have recently observed that partial purification of soluble guanylate cyclase from rat liver results in loss of the responsiveness of the enzyme to these agonists (5,6). Responsiveness is restored by addition of low concentrations (0.25 μM to 50 μM) of free or protein bound heme plus an appropriate concentration of a reducing agent (1 mM dithiothreitol, cysteine,

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[1] Abbreviations used are NP, nitroprusside; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; DTBN, di-t-butylnitroxide; ESR, electron spin resonance.

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ascorbate or glutathione), but not by other free or chelated forms of iron (5,6). A number of observations (7-10) suggest that the paramagnetic nitrosyl-heme complex may be generated from N-nitroso compounds, NO, nitrite or NP under the conditions (heme + reducing agent) required to restore the responsiveness of purified guanylate cyclase to these agonists. In the present study, formation of nitrosyl-heme from agonists of guanylate cyclase was examined by ESR[1] spectroscopy under different incubation conditions and correlated with enzyme activation under the same conditions. The capacity of preformed nitrosyl-hemoglobin to stimulate partially purified enzyme activity was also assessed. The results are consistent with the proposal that nitrosyl-heme is an active intermediate involved in the expression of the actions of N-nitroso compounds, NO, nitrite and NP on guanylate cyclase.

METHODS

Guanylate cyclase activity was partially purified from the 100,000 x g soluble fraction of rat liver by initial precipitation with 40% $(NH_4)_2SO_4$ and DE-52 cellulose chromatography as previously described (11). Fractions from DE-52 cellulose containing peak enzyme activity were combined and concentrated with an Amnicon ultrafiltration apparatus (XM-50 membrane). The concentrated enzyme sample was then applied to a 1.5 x 50 cm agarose (Bio Gel A-0.5) column which had previously been equilibrated with 10 mM Tris, 1 mM dithiothreitol, 1 mM EDTA, 0.1 M NaCl, pH 7.4. Guanylate cyclase activity was then eluted with the same buffer. Using this sequence, a 138-fold purification with 24% recovery of basal enzyme activity from the hepatic supernatant fraction was observed. All buffers used during the purification procedure contained 1 mM dithiothreitol and were equilibrated with N_2 (99.999%, Liquid Carbonic, Pittsburgh, Pa). Fractions containing peak enzyme activity were stored at -70°C under N_2. Residual dithiothreitol was removed by Sephadex G-25 chromatography (10 mM Tris, pH 7.4 at 0°C) before use.

Guanylate cyclase activity was determined from the conversion of [α-^{32}P] GTP to cGMP (2). Standard enzyme assay mixtures contained at final concentration 1 mM GTP and 4 mM $MnCl_2$ (12). Where indicated, NO (99.0%, Liquid Carbonic, Pittsburgh, Pa), purified prior to use by passage through solid KOH to remove higher oxides of N_2 (13), was delivered to some tubes at a rate of approximately 1 ml/min for 2 sec. This was followed by flushing with N_2. cGMP formation was linear with added protein (0.3 - 3 µg) under all conditions shown and with time for 10 min, except when a low concentration of pre-formed NO-hemoglobin (0.1 to 10 µM) was employed as agonist. Under the latter circumstances, reactions were conducted for 2 min.

Hemoglobin (bovine type I, Sigma Chemical Co., St. Louis, Mo.) was equilibrated with N_2 and treated with $Na_2S_2O_4$ (1 mg/ml). The resulting mixture was then chromatographed on Sephadex G-25 before use. Deoxyhemoglobin was prepared from hemoglobin in a Thunberg tube by evacuation and equilibration

with N_2. Conversion of hemoglobin to deoxyhemoglobin was verified spectrophotometrically (14). NO-hemoglobin was prepared according to the procedure of Kon (8). The concentration of NO-hemoglobin was determined spectrophotometrically (E^{418}_{HbNO} = 130 mM^{-1} cm^{-1}) (15).

ESR spectra of incubation mixtures were examined at -180°C using a Varian E-4 ESR spectrometer system equipped with an E-257 variable temperature accessory. Samples were frozen in liquid N_2 in quartz tubes with a 3 mm inner diameter. Scan range, 1000 gauss; modulation amplitude, 5 gauss; modulation frequency, 100 KHz; microwave frequency, 9.08 GHz; microwave power, 10 or 100 mW.

The concentration of NO-hemoglobin formed when deoxyhemoglobin was mixed with agonists of guanylate cyclase was calculated by comparison of the height of the ESR signal obtained with that of standard NO-hemoglobin solutions. Under the conditions described, ESR signal height was linearly related to NO-hemoglobin concentration over the range 0.02-1 mM NO-hemoglobin. When examined separately, deoxyhemoglobin, MNNG[1] and $NaNO_2$ failed to exhibit an ESR spectrum in the presence or absence of cysteine. However, the ESR spectrum of pentacyanonitrosoferrate anion radical $[Fe(CN)_5NO]^{-3}$ (16) was observed when NP $[Fe(CN)_5NO]^{-2}$ was examined in the presence, but not in the absence, of cysteine (Figure 1). Accordingly, in incubations of NP with deoxyhemoglobin containing cysteine, the amount of NO-hemoglobin formed was determined following subtraction of the spectra obtained in the absence of deoxyhemoglobin.

Protein was determined by the Lowry method (17). The significance of differences was assessed by Student's "t" test for unpaired values. The sources of all chemicals have been previously described (2).

RESULTS AND DISCUSSION

Figure 1 shows the ESR spectra of incubation mixtures that were concomitantly examined for their capacity to activate guanylate cyclase. The ESR spectra of pre-formed NO-hemoglobin (Figure 1-A) and DTBN[1] (1-F) were identical to those previously reported (8,18) and are shown for comparison. The characteristic ESR spectra of NO-hemoglobin was also identified in incubation mixtures containing deoxyhemoglobin and cysteine, plus MNNG (1-B), NP (1-D) or nitrite (1-E). In incubation mixtures containing NP and cysteine without added deoxyhemoglobin (1-C), the ESR spectra of $[Fe(CN)_5NO]^{-3}$ was identified (16).

In the absence of added deoxyhemoglobin, MNNG, NO, nitrite and NP all fail to activate partially purified hepatic guanylate cyclase (5,6). This is in contrast to the striking effects of these agents on guanylate cyclase activity in hepatic supernatants (1-4). As shown in Table I, pre-formed NO-hemoglobin (0.1 to 500 μM) stimulated purified guanylate cyclase activity. Optimal expression of the action of pre-formed NO-hemoglobin on guanylate cyclase

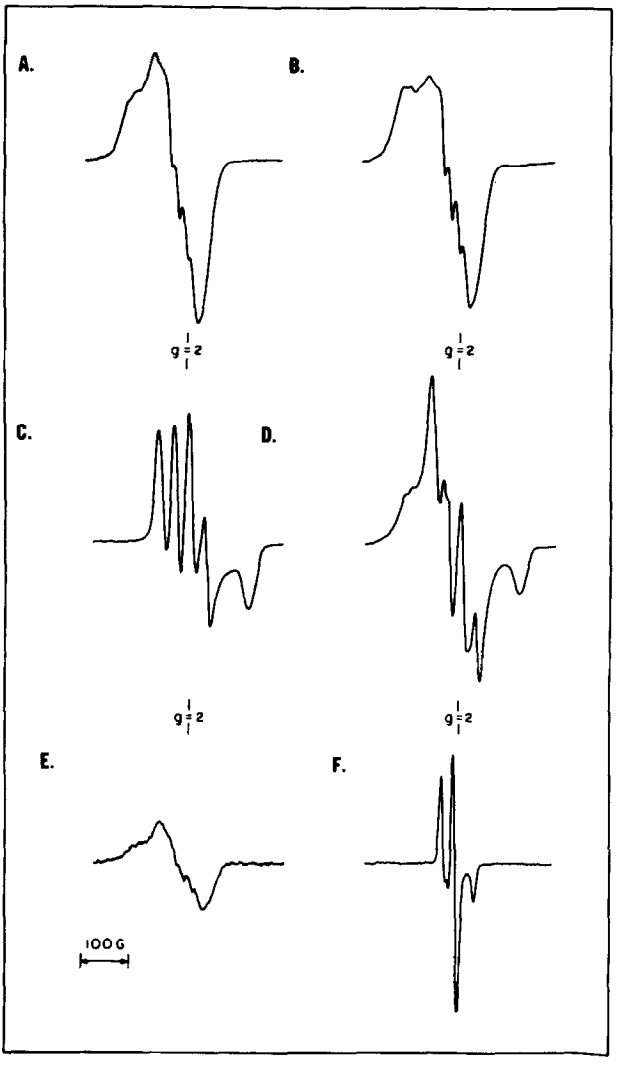
TABLE I

CORRELATION OF THE CAPACITY OF TEST AGENTS TO FORM NO-HEMOGLOBIN WITH THEIR ABILITY TO ACTIVATE PARTIALLY PURIFIED HEPATIC SOLUBLE GUANYLATE CYCLASE

Additions to Partially Purified Enzyme Preparation	Calculated NO-Hemoglobin (μM)	Guanylate Cyclase Activity nmoles/min/mg protein
None	-	5.2 ± 0.6
0.1 μM NO-hemoglobin	-	11 ± 1.2*
5 μM NO-hemoglobin	-	38 ± 5*
+ 45 μM deoxyhemoglobin	-	8.7 ± 1*
10 μM NO-hemoglobin	-	103 ± 10*
50 μM NO-hemoglobin	-	97 ± 11*
500 μM NO-hemoglobin	-	105 ± 12*
5 μM deoxyhemoglobin	-	5.3 ± 0.5
50 μM deoxyhemoglobin	-	3.8 ± 0.4*
+ 1 mM NP	5 ± 1	8.3 ± 1†
+ 1 mM MNNG	6 ± 1	9.2 ± 1†
+ 0.2 mM $K_3Fe(CN)_6$ + 1 mM MNNG	0	3.6 ± 0.4
50 μM deoxyhemoglobin + 1 mM cysteine	-	3.8 ± 0.4*
+ 1 mM MNNG	51 ± 10	95 ± 10†
+ 1 mM NP	49 ± 12	104 ± 11†
+ 1 mM $NaNO_2$	5 ± 1	7.9 ± 1†
+ 1 mM NaN_3	0	3.9 ± 0.4
1 mM DTBN	-	5.3 ± 0.5
1 mM $[Fe(CN)_5NO]^{-3}$	-	5.6 ± 0.5

Test solutions, whose ESR spectra were examined concomitantly, were added in a volume of 20 μl to 180 μl of enzyme. The concentrations of each reactant shown in the table refer to those in the enzyme preincubation mixture. After a 1 min preincubation, a 25 μl aliquot of this mixture was added to 50 μl of a standard enzyme assay mixture. Where indicated, the concentration of NO-hemoglobin generated from the reactions between deoxyhemoglobin and MNNG, NP or nitrite and then added to the enzyme were calculated from the ESR spectra of the concentrated test solutions. Preparation of the latter are described in Figure 1. Results shown are means ± SE of duplicate (guanylate cyclase activity) or single (NO-hemoglobin) determinations from three separate experiments. * $p < 0.01$ compared to values obtained in the absence of an addition to the purified enzyme. † $p < 0.01$ compared to value obtained with 50 μM deoxyhemoglobin in the presence or absence of 1 mM cysteine.

did not require addition of cysteine or deoxyhemoglobin to the enzyme preparation. Maximal effects of NO-hemoglobin (20-fold stimulation) were noted at 10 μM with half maximal increases obtained with 5 μM NO-hemoglobin. Oxyhemoglobin and CO-hemoglobin (5-500 μM) had no effect or inhibited guanylate cyclase activity when tested under conditions identical to those shown for NO-hemoglobin (not shown). The conditions used for the preparation of NO-hemoglobin resulted in quantitative conversion of hemoglobin to the NO form. Thus, no unreacted hemoglobin was present in incubations of preformed NO-hemoglobin with the enzyme. However, consistent with previous reports of inhibitory effects of hemeprotein on guanylate cyclase activation (19-21), the addition of 45 μM deoxyhemoglobin suppressed enzyme stimulation by 5 μM NO-hemoglobin (Table 1). Accordingly, under conditions which result in partial conversion of deoxyhemoglobin to NO-hemoglobin and formation of small quantities of the latter complex, the inhibitory effects of unreacted hemoglobin carried over into the enzyme incubation mixture must be considered. Preincubation of NP or MNNG with deoxyhemoglobin under anaerobic conditions, but without a reducing agent, resulted in only a 10% conversion of deoxyhemoglobin to NO-hemoglobin, as measured by ESR spectroscopy (Table I). The concentration of unreacted hemoglobin carried over into the enzyme reaction mixture was calculated to be approximately 45 μM under these conditions. The stimulation of partially purified guanylate cyclase activity observed when the agonist-deoxyhemoglobin solution was added to the enzyme preparation was comparable to that observed when the same concentration of preformed NO-hemoglobin (5 μM) was added to the enzyme in the presence of 45 μM deoxyhemoglobin (Table I). Addition of $K_3Fe(CN)_6$ to the deoxyhemoglobin-agonist preparation blocked formation of NO-hemoglobin and completely inhibited stimulation of guanylate cyclase activity by the MNNG-deoxyhemoglobin mixture. $K_3Fe(CN)_6$ is known to oxidize heme iron and consequently to suppress formation of NO-hemoglobin (22). The present observations suggest that this action of $K_3Fe(CN)_6$ may explain its ability to block guanylate



Legend to Figure 1: ESR spectra of guanylate cyclase test agents examined at -180°C. A, 0.5 mM NO-hemoglobin pre-formed by the reaction of NO with deoxyhemoglobin (instrument gain = 3.2 x 10^3). B, 0.5 mM deoxyhemoglobin, 10 mM cysteine, 10 mM MNNG (gain = 2 x 10^3). C, 10 mM cysteine, 10 mM nitroprusside (gain = 1.25 x 10^3). D, 0.5 mM deoxyhemoglobin, 10 mM cysteine, 10 mM nitroprusside (gain = 1.25 x 10^3). E, 0.5 mM deoxyhemoglobin, 10 mM cysteine, 10 mM $NaNO_2$ (gain = 8 x 10^3). F, 10 mM DTBN in ethanol. All mixtures were prepared anaerobically. ESR spectra of mixtures B, C, D and E were examined after a 2 min incubation at 37°C. Microwave power was 10 mW.

cyclase responses to a number of recently identified agonists (3,4).

Our previous results have indicated that the effect of free heme or heme-

proteins to restore guanylate cyclase responses to MNNG and related agonists is enhanced in the presence of a reducing agent (5,6). As shown in Table I, preincubation of deoxyhemoglobin with MNNG or nitroprusside in the presence of cysteine leads to complete conversion of deoxyhemoglobin to NO-hemoglobin. Addition of an aliquot of the deoxyhemoglobin-agonist-cysteine solution to the purified enzyme stimulated enzyme activity 20-fold. By contrast, cysteine alone did not alter basal activity or the responses of purified guanylate cyclase to MNNG, NO, nitrite or NP (not shown). As demonstrated in Table I, the concentrations of NO-hemoglobin generated in mixtures of cysteine, deoxyhemoglobin and either MNNG or NP are more than sufficient to account for the stimulatory effects of these solutions on the partially purified enzyme. Solutions of cysteine, deoxyhemoglobin and $NaNO_2$ were much less effective in stimulating guanylate cyclase activity than were those containing MNNG or NP. NO-hemoglobin formation was likewise less with $NaNO_2$ than with either MNNG or NP. The solution of cysteine, deoxyhemoglobin and $NaNO_2$ contained 5 μM NO-hemoglobin and 45 μM unconverted deoxyhemoglobin. Guanylate cyclase stimulation by this solution was identical to that noted with addition of 5 μM preformed NO-hemoglobin plus 45 μM deoxyhemoglobin. As is also shown in Table I, preincubation of NaN_3 with deoxyhemoglobin plus cysteine did not result in either NO-hemoglobin formation or guanylate cyclase activation. Moreover, DTBN and $[Fe(CN)_5NO]^{-3}$, radicals which have ESR spectra in the same region as NO-hemoglobin (Figure 1-C,F),both failed to stimulate partially purified guanylate cyclase activity, implying specificity for the actions of the paramagnetic nitrosyl-heme complex on enzyme activity.

Earlier results from our laboratory have demonstrated that guanylate cyclase activation by MNNG is mediated by a labile decomposition product of this agonist (2). It has been suggested that NO may be the reactive moiety which is generated from N-nitroso compounds and stimulates the enzyme (3,4),but specific identificatio of the reactive intermediate of MNNG action is lacking. We correlated the

decomposition of MNNG with its capacity to form NO-hemoglobin and activate partially purified guanylate cyclase. Nitroso group loss from MNNG was examined at 400 mμ (2) at zero, 15 and 90 min after mixing 2 mM MNNG, 20 mM cysteine in 10 mM Tris, pH 7.6 at 37°C under N_2. At the 90 min time point, the cysteine plus MNNG solution was flushed with N_2 for 5 min before sampling. At each time point, an aliquot of the incubation mixture was mixed with an equal volume of deoxyhemoglobin, to give final concentrations of 0.5 mM deoxyhemoglobin, 10 mM cysteine and 1 mM MNNG. The NO-hemoglobin content of the latter solution was determined by ESR spectroscopy. A second 20 μl aliquot was then added to 180 μl of a partially purified guanylate cyclase preparation to assess the capacity for enzyme stimulation. After 15 min of incubation MNNG is completely decomposed but the solution retains full capacity to generate NO-hemoglobin and to activate partially purified guanylate cyclase. By 90 min, both actions are lost. It should be emphasized that decomposed MNNG has no effect on the activity of partially purified guanylate cyclase, when deoxyhemoglobin is excluded. Thus, these results not only provide evidence for the role of NO formation in guanylate cyclase stimulation by MNNG, but they also indicate that the proximate active moiety responsible for enzyme activation is the paramagnetic nitrosyl-heme complex rather than NO *per se*.

Under enzyme assay condition comparable to those employed here (1 mM GTP, 4 mM $MnCl_2$) previous reports have described a 2 to 4-fold stimulation of partially purified hepatic soluble guanylate cyclase by NO and NP without addition of exogenous heme or hemeprotein (3,4,21). These observations may reflect a heme independent interaction between the agonists and the enzyme. However, they might also be explained by the presence of small amounts of residual heme in the enzyme preparation studied. With regards to the latter possibility, it is notable that the addition of only 20 nM exogenous heme to an unresponsive preparation of the purified enzyme results in a 2 to 4

fold activation by MNNG, NO and NP (6). While some earlier studies have emphasized only the inhibitory effects of hemoglobin and other hemeproteins on guanylate cyclase activation (19-21), examination of fatty acid peroxide stimulation of guanylate cyclase by Hidaka and Asano (23) and our own observations (5,6) indicate that the effects of hemoglobin on enzyme activation are biphasic.

The present study thus provides the first physico-chemical identification of a specific paramagnetic complex capable of activating guanylate cyclase. It is consistent with our original proposal, based on studies with N-nitroso and several related carcinogenic and non-carcinogenic nitro compounds (1,2, 24,26), that the activation of guanylate cyclase is mediated by a free radical event related to the capacity of each of these agonists to form paramagnetic NO-iron complexes in tissue (7-10,27). The relationships between nitrosyl-heme formation, the activation of guanylate cyclase and the expression of the oncogenic actions of MNNG are at present completely unknown. It is of interest that another carcinogen, N-hydroxy-2-acetylaminofluorene, is oxidatively activated by hematin-peroxide systems to the more carcinogenic species 2-nitrosofluorene, with the intermediate formation of the nitroxyl free radical (30,31). The present data raise the possibility that conditions leading to the metabolic conversion of this and other procarcinogens to nitroso derivatives (27,30) could result in formation of nitrosyl-heme or related paramagnetic heme complexes capable of guanylate cyclase activation.

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