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ACTIVATION OF GUANYLATE CYCLASE DURING THE OXIDATION OF ARYLAMINE CARCINOGENS

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SUMMARY

When added alone, the arylamine procarcinogens N-acetyl-aminofluorene, 4-acetyl-aminobiphenyl or their N-hydroxy derivatives failed to alter partially purified soluble guanylate cyclase from rat liver or particulate guanylate cyclase activity from colonic mucosa. However, addition of linoleic acid hydroperoxide to the enzyme preparation in the presence N-OH-acetyl-aminofluorene or N-OH-acetyl-aminobiphenyl significantly increased guanylate cyclase activity. With linoleic acid hydroperoxide plus N-OH-acetyl-aminofluorene, both the activation of hepatic guanylate cyclase and the formation of the carcinogen oxidation product 2-nitrosofluorene required hematin but not molecular O_2. Both processes were inhibited by ascorbic acid. These data strongly imply that guanylate cyclase activation was dependent upon hematin catalyzed oxidation of N-OH-acetyl-aminofluorene by the lipid peroxide. The results provide the first evidence that guanylate cyclase activation can occur during the conversion of a procarcinogen to a more reactive chemical species, and thereby emphasize the importance of examining carcinogen interaction with the GC system under conditions which permit such chemical conversion.

INTRODUCTION

Mammalian guanylate cyclases are stimulated by a number of chemical carcinogens (1-10). Current evidence indicates that enzyme activation by both carcinogenic and non-carcinogenic compounds is mediated through free radical or redox reactions (1-5,11-23). N-nitroso carcinogens such as MNNG[1], which undergo rapid spontaneous decomposition to reactive intermediates (5,21), are the most potent activators of guanylate cyclase in broken cell preparations (1-5). Specifically, in the case of MNNG and related agonists of guanylate cyclase, the

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[1] Abbreviations used are MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; AAF, N-acetyl-aminofluorene; AABP, 4-acetylaminobiphenyl (AABP); LAHP, linoleic acid hydroperoxide; NOF, 2-nitrosofluorene.

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generation of nitric oxide and the paramagnetic nitrosyl heme complex have been implicated in enzyme activation (20,21,23). These reactions occur in cell free systems (21,23). By contrast, several chemical carcinogens that are known to require enzymatic conversion to a reactive chemical form have failed to stimulate guanylate cyclase in subcellular preparations (6,9,10). In the present study, the response of guanylate cyclase to two arylamine procarcinogens was examined under in vitro conditions that result in oxidation of these agents to more reactive chemical forms. The study demonstrates that guanylate cyclase is activated during the oxidation of the N-hydroxy derivatives of AAF[1] and AABP[1], and thus provides the first evidence of stimulation of this enzyme system by reactive derivatives of procarcinogens.

MATERIALS AND METHODS

Guanylate cyclase was partially purified (approximately 250-fold) from the rat (200 g male Sprague-Dawley, Zivic Miller, Pittsburgh, PA) hepatic 100,000 x g soluble fraction by $(NH_4)_2SO_4$ precipitation, DEAE cellulose and agarose column chromatography as previously described in detail (20). The 100,000 xg particulate fraction of colonic mucosa was prepared from whole homogenates of full thickness scrapings of rat mucosa (100 mg/ml of 10 mM Tris, pH 7.4) as previously described (3). The particulate fraction was washed twice and resuspended in five volumes of Tris buffer for assay. The soluble form of the enzyme predominates in liver (70 to 80% of total hepatic activity), whereas the particulate form accounts for 90 to 95% of total activity in colonic mucosa (3). These sources of enzyme were also chosen for study because of the established carcinogenic activity of AAF in liver (24,25) and of aminobiphenyl derivatives in colon (26).

Guanylate cyclase activity was determined from the conversion of [α-^{32}P]-GTP to cGMP. The composition of standard assay mixtures (4 mM $MnCl_2$, 1 mM [α-^{32}P]GTP) and the modified procedure for isolation of cGMP from the final assay mixture has been previously described in detail (27,28). Reaction rates were linear with time for at least 10 min and with protein (0.3-3 µg for the soluble enzyme and 25-250 µg for the colon particulate fraction) under all conditions studied.

LAHP[1] was prepared by incubation of 1 ml of a 500 µM solution of linoleic acid in 10 mM Tris, pH 7.4 at 0°C with 150 µg/ml of soy bean lipoxidase as previously described (29). The mixture was diluted 10 x in 10 mM Tris, pH 7.4 and the concentration of LAHP formed determined at 233 mµ (E = 25.25 mM^{-1} cm^{-1}) (29). Approximately 40% of the linoleic acid was converted by LAHP under these conditions. The concentration of NOF [1]formed from reaction of LAHP with N-OH-AAF in the presence of hematin was determined spectrophotometrically by absorption at 362 mµ (E = 2.4 mM^{-1} cm^{-1} (30). Protein was determined by the method of Lowry (31).

Results shown are means ± SE of duplicate (guanylate cyclase) or single (LAHP and NOF) determinations pooled from three separate experiments. Significance of differences between mean values was determined by Student's t test.

For purposes of statistical analysis the average of duplicates from each experiment was entered as a single number (n = 4, comparing data from 2 conditions by t test for unpaired data).

N-OH-AABP was a gift from Dr. James Miller, McArdle Laboratory, Madison, Wisconsin. N-OH-AAF, AAF and AABP were obtained from Dr. James Keith, Carcinogen Clearing House, IITRI, Chicago, Illinois. Linoleic acid (grade III) and soy bean lipoxidase (type I) were obtained from Sigma Chemical Co., St. Louis, MO. The sources of all other chemicals have previously been described (5). AAF, AABP and their N-hydroxy derivatives were added to the enzyme preparations as acetone solutions. The final concentration of acetone employed (1%) did not alter guanylate cyclase activities.

RESULTS AND DISCUSSION

Under conditions which support the action of MNNG on the enzymes, AAF, AABP or their N-hydroxy derivatives failed to activate 100,000 x g soluble guanylate cyclase partially purified from rat liver or 100,000 x g particulate activity prepared from colonic mucosal homogenates (Table I). Results with only a single concentration (50 μM) of AAF, AABP or their N-hydroxy derivatives are shown in Table I. However, these agents were without effect on guanylate cyclase when tested over a concentration range of 1 μM to 250 μM. By contrast, addition of N-OH-AAF or N-OH-AABP in combination with the LAHP generating system, which yielded a concentration of 50 μM pre-formed lipid peroxide in the cyclase mixture, stimulated partially purified hepatic soluble guanylate cyclase 3-fold. Combined addition of AAF or AABP plus the preformed lipid peroxide was without effect on guanylate cyclase (Table I). Guanylate cyclase stimulation was not further enhanced by exposure of the enzyme to the concentration of N-OH-AAF up to 250 μM plus pre-formed LAHP up to 100 μM. Moreover, direct incubation of soluble hepatic guanylate cyclase (± 5 μM hematin) with the lipid peroxid generating system, which resulted in exposure of the enzyme to 200 μM or more LAHP, or incubation of this enzyme with 500 μM linoleic acid or 150 μg/ml of lipoxidase did not alter activity. By contrast, the LAHP generating system containing 50 μM pre-formed lipid peroxide increased the activity of particulate guanylate cyclase of colonic mucosa. However, as shown in Table I, the activation of the colonic particulate enzyme system observed in the presence of the LAHP generating system was significantly potentiated by the presence of N-OH-AAF or N-OH-AABP, whereas enzyme activity was not further increased by

TABLE 1

EFFECTS OF ACETYLAMINOFLUORENE (AAF), ACETYLAMINOBIPHENYL (AABP) AND THEIR N-OH DERIVATIVES ON GUANYLATE CYCLASE ACTIVITIES IN THE PRESENCE OF LINOLEIC ACID HYDROPEROXIDE (LAHP)

	Initial Additions					
	None	MNNG	AAF	N-OH-AAF	AABP	N-OH-AABP
Final Additions	100,000 xg Soluble Enzyme (Liver)					
	nmoles cGMP/min/mg protein					
Buffer	5.6 ±0.7	83* ±10	5.4 ±0.5	5.5 ±0.6	5.3 ±0.5	5.4 ±0.7
LAHP	5.2 ±0.6	75* ±9	5.5 ±0.5	16.2*† ±2.1	5.6 ±0.7	11.3*† ±1.4
	100,000 xg Particulate Enzyme (Colonic Mucosa)					
	pmoles cGMP/min/mg protein					
Buffer	256 ±34	832* ±99	273 ±44	236 ±38	247 ±42	245 ±24
LAHP	382† ±46	914* ±102	369† ±48	657*† ±73	361† ±31	692*† ±18

The partially purified 100,000 xg soluble enzyme from liver (50 µg/ml) or the 100,000 xg particulate fraction of colonic mucosa (2 mg/ml) was preincubated where indicated for 1 min at 0°C with 50 µM AAF, N-OH-AAF, AABP, N-OH-AABP or 0.1 mM MNNG. All enzyme preparations contained 5 µM hematin. The LAHP generating system was then added to the cyclase preparation to yield a concentration of 50 µM preformed LAHP in the enzyme preincubation mixture. Controls received an equal volume of Tris buffer. The guanylate cyclase assay was begun 30 sec later by addition of 25 µl of the enzyme mixture to 50 µl of standard guanylate cyclase reaction mixture. Reactions were conducted at 37°C for 7 min.

* p at least < 0.01 compared to corresponding value in the absence of an initial addition of a carcinogen.

† p at least < 0.01 compared to corresponding value in the absence of LAHP.

TABLE II

EFFECTS OF O_2 EXCLUSION AND ASCORBATE ON ACTIVATION OF HEPATIC SOLUBLE GUANYLATE CYCLASE BY N-OH-AAF: CORRELATION WITH LAHP AND 2-NITROSOFLUORENE FORMATION

	Room Air			N_2			10 mM Ascorbate	
	Guanylate Cyclase	LAHP μM	NOF μM	Guanylate Cyclase	LAHP μM	NOF μM	Guanylate Cyclase	NOF μM
Additions to Partially Purified Soluble Enzyme								
None	5.1 ±0.6	nd	nd	5.3 ±0.7	nd	nd	5.5 ±0.5	nd
N-OH-AAF	5.4 ±0.7	nd	nd	5.6 ±0.6	nd	nd	5.7 ±0.8	nd
+ linoleic acid and lipoxidase	15.8* ±2.1	46 ±5	10.7 ±0.9	5.7† ±0.9	nd	nd	5.6† ±0.7	nd
+ LAHP	17.3* ±1.9	48 ±4	11.4 ±1.2	18.6*$ ±2.4	52 ±7	10.9 ±1.4	5.9† ±0.6	nd

The partially purified soluble enzyme containing 5 μM hematin was equilibrated in room air or with 99.9% N_2 at 0°C. Sodium ascorbate was added to some enzyme preparations at a concentration of 10 mM. Where indicated, 50 μM N-OH-AAF was added alone or followed in 30 sec by sequential additions of linoleic acid (120 μM) and lipoxidase (40 μg/ml). Other cyclase preparations received the LAHP generating system containing approximately 50 μM of preformed LAHP. The enzyme preincubations were conducted at 0°C. Guanylate cyclase assays were begun 30 sec later by addition of 25 μl of the enzyme preincubation mixture to 50 μl of standard cyclase assay mixture which had been equilibrated with either room air or 99.9%, as indicated. Reactions were conducted at 37°C for 7 min. Guanylate cyclase activity is expressed as nmoles cGMP/min/mg protein. The concentration of NOF formed was determined under incubation conditions identical to those used for the preincubation of the enzyme except that the guanylate cyclase preparation was omitted. The concentration of LAHP was determined from the reaction between linoleic acid and lipoxidase alone in the presence and absence of O_2.

exposure to 200 μM LAHP, 500 μM linoleic acid or 150 μg/ml of lipoxidase in the absence of an N-OH-arylamine. Thus, the results with both the partially purified hepatic soluble and colonic particulate enzyme systems imply that guanylate cyclase activation is mediated by products of the reaction between the N-OH-arylamine carcinogens and the lipid peroxide generating system.

In the presence of hematin, LAHP has been shown to convert N-OH-AAF via a nitroxyl free radical intermediate to NOF and N-acetoxy-N-acetyl-2 aminofluorene (32). Conversion of linoleic acid to LAHP by lipoxidase requires molecular O_2, whereas oxidation of N-OH-AAF by pre-formed lipid peroxide is an O_2-independent reaction inhibited by reducing agents (32,33). As shown in Table II, when linoleic acid, lipoxidase, hematin and N-OH-AAF were first combined in the presence of hepatic soluble guanylate cyclase, the activation of the cyclase required molecular O_2. This was correlated with O_2-dependent formation of both LAHP and NOF (Table II). The concentrations of linoleic acid (120 μM) and lipoxidase (40 μg/ml) to which the cyclase preparation was exposed under these conditions of incubation approximated those resulting from transfer of the LAHP generating system containing pre-formed lipid peroxide. However, when LAHP was first generated from linoleic acid in air and the pre-formed peroxide then incubated with N-OH-AAF and guanylate cyclase the activation of guanylate cyclase and the formation of NOF both occurred under anaerobic conditions. Sodium ascorbate (10 mM), but not NaCl, inhibited the action of pre-formed LAHP to convert N-OH-AAF to NOF, and also prevented guanylate cyclase activation (Table II). Similar inhibition was observed with 10 mM dithiothreitol. These results provide support for the suggestion that guanylate cyclase stimulation is linked to the oxidation of N-OH-AAF by the lipid peroxide.

Legend to Table II, continued.

* p at least < 0.01 compared to corresponding value in the absence of additions.

† p at least < 0.01 compared to corresponding value observed in room air and without ascorbate

$ p at least < 0.01 comparing value obtained with addition of preformed LAHP to that with sequential addition of linoleic acid and lipoxidase to the cyclase preparation.

n.d., not detectable.

As shown in Table III, hematin was required for activation of guanylate cyclase mediated by the N-OH-AAF - LAHP system and by MNNG, but was not required for stimulation of enzyme activity observed when Mn^{2+} is present in excess of substrate (1 mM MnGTP). Hematin and hemoglobin are known to catalyze the oxidation of N-OH-AAF by lipid peroxide (32). This action may explain the requirement for hematin in the expression of the action of the N-OH-AAF - LAHP system on guanylate cyclase. Consistent with this possibility, NOF formation was not detectable in the absence of exogenous hematin or hemoglobin (not shown). Of interest, earlier studies of the heme-dependent action of MNNG on guanylate cyclase have indicated that formation of the paramagnetic nitrosyl-heme is an obligate step in enzyme stimulation mediated by N-nitroso compounds, nitric oxide and related nitrogenous activators (20,21,23). Since this pathway is not excluded by the current data, it is possible that the presence of heme subserves more than one essential function in the process by which the N-OH-AAF-lipid peroxide system activates guanylate cyclase. The requirement for heme in the expression of the actions of both the N-nitroso and arylamine carcinogens on guanylate cyclase clearly differs from the heme-independent stimulation of the enzyme by excess Mn^{2+}, and accordingly suggests the existence of more than one mechanism of cyclase activation.

The specific chemical moiety generated from hematin-catalyzed LAHP oxidation of N-hydroxy arylamine carcinogens (NOF, nitroxyl free radical, etc) which mediates guanylate cyclase activation is not identified in this study. Nevertheless, the present observations provide the first evidence that guanylate cyclase may be stimulated during the metabolic conversion of a procarcinogen to more reactive chemical forms. The extent to which the guanylate cyclase system may be a target for activation by reactive derivatives of other classes of procarcinogens, and the role, if any, of stimulation of this enzyme system in the expression of the oncogenic action of arylamines remain to be defined. Several observations suggest that the ultimate carcinogenic metabolite of N-OH-AAF is the sulfate ester (34). However, AAF is also carcinogenic in tissues which are not capable

TABLE III

EFFECTS OF HEMATIN ON THE STIMULATION OF HEPATIC SOLUBLE GUANYLATE CYCLASE BY Mn^{2+}, N-OH-AAF + LAHP OR MNNG

	$MnCl_2$ in Assay	
	1 mM	4 mM
Additions to Partially Purified Enzyme	nmoles/min/mg protein	
None	1.2 ± 0.2	5.3 ± 0.6$^{\$}$
50 μM N-OH-AAF + 50 μM LAHP	1.4 ± 0.2	5.6 ± 0.5$^{\$}$
0.1 mM MNNG	2.3 ± 0.4	6.9 ± 0.6$^{\$}$
5 μM Hematin	1.3 ± 0.2	5.4 ± 0.5$^{\$}$
+ N-OH-AAF + LAHP	6.8 ± 0.8*†	16.7 ± 1.4$^{*\dagger\$}$
+ MNNG	78 ± 9*†	109 ± 9$^{*\dagger\$}$

The partially purified soluble enzyme was preincubated for 1 min at 0°C with N-OH-AAF or MNNG at the final concentration shown in the enzyme preparation. Where indicated, hematin was present in the incubate at a concentration of 5 μM. LAHP or buffer alone was then added to the enzyme mixture and the guanylate cyclase assay begun 30 sec later by addition of 25 μl of the enzyme to 50 μl of guanylate cyclase assay mixture which contained $MnCl_2$ at a final concentration of 1 or 4 mM (1 mM GTP).

* p at least < 0.01 compared to value obtained in the absence of addition.

† p at least < 0.01 compared to value obtained with hematin alone or to corresponding value without hematin.

$^{\$}$ p at least < 0.01 compared to corresponding value at 1 mM $MnCl_2$.

of enzymatic conversion of AAF to the sulfate ester form (35). Therefore, the reactive moieties generated from N-OH-AAF oxidation by lipid peroxides and implicated here as agonists of guanylate cyclase have been proposed as possible mediators of the oncogenic actions of arylamines (36-38). There is evidence that at least two of these reactive derivatives, NOF and nitrosyl free radicals, are formed during the microsomal metabolism of AAF (39-41).

In summary, the current results strongly emphasize the importance of examining the potential interaction of procarcinogens with guanylate cyclase systems under conditions which lead to the conversion of these agents to more chemically reactive forms. They also suggest that stimulation of guanylate cyclase by chemical carcinogens may be a more common event than previously recognized.

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