HYDRAZINE ACTIVATION OF GUANYLATE CYCLASE: POTENTIAL APPLICATION TO TOBACCO CARCINOGENESIS

David L. Vesely and Gerald S. Levey

Division of Endocrinology and Metabolism,
Department of Medicine, University of Miami School of Medicine
Miami, Florida 33152

Received December 3, 1976

SUMMARY

Hydrazine, a chemical carcinogen in tobacco and tobacco smoke, has been shown to induce the production of a variety of tumors in rats. The present report demonstrates that hydrazine stimulates the enzyme guanylate cyclase (EC 4.6.1.2) which catalyzes the production of guanosine 3',5'-monophosphate. At a maximal concentration of 100 mM, hydrazine activated guanylate cyclase 45-fold in liver, 39-fold in colon, 22-fold in stomach, 19-fold in heart, 18-fold in pancreas, 8-fold in lung and kidney, and 4-fold in spleen. Since recent evidence suggests a role for guanosine 3',5'-monophosphate in malignant transformation, these data may help explain the tumor inducing capacity of this tobacco carcinogen.

Guanylate Cyclase (EC 4.6.1.2) catalyzes the conversion of guanosine triphosphate to guanosine 3',5'-monophosphate (cyclic GMP). The enzyme is found in virtually all mammalian cells (1,2) and its product, cyclic GMP, is thought to be involved in cell growth. Cyclic GMP has been reported to increase DNA synthesis (3,4,5), increase protein synthesis (6,7) and increase growth of fibroblasts (4) and thymocytes (8). Moreover, cyclic GMP may be involved in malignant transformation (3) and the nucleotide has been reported to be increased in rat hepatomas (9) and in human adenocarcinomas (10).

Hydrazine, a carcinogen which occurs in nature in tobacco and tobacco smoke (11) has been shown to produce tumors in lung (12) liver (13), cecum (14), stomach (14,15), thyroid (14), adrenal (14), and pancreas (15). The present report demonstrates that hydrazine markedly activates a soluble preparation of guanylate cyclase from many tissues including lung. The stimulation of guanylate cyclase by hydrazine is the most pronounced yet reported for an in vitro preparation of this enzyme.

TABLE 1

EFFECT OF HYDRAZINE ON GUANYLATE CYCLASE IN VARIOUS TISSUES

Tissue	Cyclic GMP (pmoles accumulated/mg protein/10 minute incubation)* Control	Hydrazine, 100 mM	P†
Liver	190 ± 6	8592 ± 17	< .001
Lung	1151 ± 29	8726 ± 29	< .001
Kidney	344 ± 12	2877 ± 51	< .001
Heart	163 ± 6	3139 ± 17	< .001
Stomach	561 ± 6	12634 ± 12	< .001
Colon	435 ± 6	16960 ± 19	< .001
Spleen	858 ± 29	3122 ± 57	< .001
Pancreas	191 ± 6	3539 ± 57	< .001

* Each value represents the mean ± S.E.M. of 6 individual determinations.
† Significance of comparison with control was determined by Student t test.

METHODS AND MATERIALS

Tissues used in these experiments were obtained from male Sprague-Dawley rats, weighing 150-200 grams, which had been maintained ad libitum on Purina laboratory chow. Hydrazine was purchased from Eastman Kodak Chemicals. Alumina oxide, neutral activity I for column chromatography was obtained from E. Merck (Darmstadt, Germany). The alpha [^{32}P] GTP was purchased from New England Nuclear Corp. (Boston, Mass.). Guanylate cyclase activity was measured as previously described (16,17) utilizing a modification of the original method of White and Aurbach (2) and Hardman and Sutherland (1). The various tissues were homogenized in cold 0.03 M Tris HCl, pH 7.6, and centrifuged at 37,000 g in a Sorval refrigerated centrifuge at 4^{o}C for 15 minutes. The supernatant was then assayed at 37^{o} for 10 minutes for guanylate cyclase activity, using a reaction mixture consisting of 20 mM Tris HCl, pH 7.6; 5 mM $MnCl_2$, 2.67 mM cyclic GMP (used to minimize destruction of [^{32}P] GTP; a GTP regenerating system (5 mM creatine phosphate, 11.25U creatine phosphokinase), 100 µg bovine serum albumin, 20 mM caffeine, [alpha-^{32}P] GTP, approximately 5x10^{5} cpm, and the enzyme preparation having 0.2 to 0.6 mg protein. The reaction was terminated by the addition of 10 µl of 0.1 M EDTA, pH 7.6, containing about 30,000 cpm of [^{3}H] cyclic GMP (to estimate recovery in the subsequent steps) and

TABLE 2

DOSE RESPONSE OF HYDRAZINE ON HEPATIC GUANYLATE CYCLASE

Concentration	Cyclic GMP (pmoles accumulated/mg protein/10 minute incubation)* Hydrazine (NH_2NH_2)
0	200 ± 6
100 mM	8592 ± 17†
75 mM	3833 ± 12†
50 mM	1893 ± 12†
20 mM	576 ± 12†
10 mM	183 ± 6^{ns}

* Mean ± S.E.M. of 6 samples.
† $P < .001$ compared to control with Student t test.
ns = not significantly different than control.

boiling for three minutes. After cooling in an ice bath, each reaction mixture was applied to one gram of neutral alumina oxide columns and washed with 0.03 M Tris HCl, pH 7.6. The first ml of elutant from the column was discarded since the majority of the cyclic GMP appeared in the second, third and fourth mls of elutant. The above three mls of elutant from the column were collected directly into scintillation vials containing 15 mls of Bray's solution (18). The elutants were then counted in a Packard Tri-Carb Liquid Scintillation spectrometer. All of the [^{32}P] containing material was identifiable as cyclic GMP as determined by thin layer chromatography on Cellulose (PEI, Brinkman) using 1 M formic acid, 1 M LiCl as solvent and chromar sheets (Mallinchrodt, St. Louis, Mo.) developed with absolute alcohol and concentrated NH_4OH (5:2v/v). In several experiments the product of the incubations was assayed additionally using the cyclic GMP radioimmunoassay method of Steiner et al. (19), in order to provide further confirmation that the material was cyclic GMP. Protein was determined by the method of Lowry et al. (20).

RESULTS AND DISCUSSION

The tobacco carcinogen, hydrazine (H_2N-NH_2), activated guanylate cyclase in all the various tissues tested (Table 1). The resulting stimulation of cyclic GMP accumulation was 45-fold in liver, 39-fold in colon, 22-fold in stomach, 19-fold in heart, 18-fold in pancreas, 8-fold in lung and kidney, and

4-fold in spleen. The increases in cyclic GMP accumulation secondary to guanylate cyclase activation were highly significant in all these tissues. The dose response curve to hydrazine in rat lung is shown in Table 2. Hydrazine produced a maximal stimulation of the enzyme at 100 mM and approached non-stimulated levels at 10 mM.

These results demonstrate that hydrazine activates the enzyme guanylate cyclase which catalyzes the production of cyclic GMP. Hydrazine has been shown to produce tumors in four of the tissues tested here, namely lung (12), liver (13), stomach (14,15), and pancreas (15). Of relevance to the tumor-inducing capacity of these agents is the fact that cyclic GMP is involved in several processes related to cell growth (3-10) and has recently been implicated in cells undergoing malignant transformation (3). Kimura and Murad found elevated cyclic GMP levels in rat hepatomas (9) and DeRubertis et al. (10) reported increased concentrations of cyclic GMP in adenocarcinoma of the human colon. Thus, if cyclic GMP is a significant factor in malignant transformation, the ability of hydrazine to induce tumors may be related to hydrazine's capacity to activate guanylate cyclase which would in turn increase the production of cyclic GMP. It should be pointed out that hydrazine activates guanylate cyclase in some tissues (colon, kidney, spleen, and heart) in which hydrazine-induced tumors have not been reported. This may be related to variations in the metabolism of hydrazine <u>in vivo</u>. Also of note is the fact that lung tissue has an eight-fold activation of guanylate cyclase, a finding which may provide additional biochemical evidence linking tobacco smoking with lung cancer.

Finally, the nitrosoamine group of chemical carcinogens have also been reported to activate guanylate cyclase in rat tissues (17,21). Kimura and Murad have reported that sodium azide, NaN_3 a structure which possesses nitrogen bonds, is also a potent activator of liver guanylate cyclase (22). Since hydrazine, $H_2N\text{-}NH_2$, activates guanylate cyclase as well as or better than the nitrosoamines and sodium azide, it appears that the N-N bond, or in

some cases possibly the N-O bond, is critical for guanylate cyclase activation and perhaps tumor induction by these agents.

ACKNOWLEDGEMENTS

This study was supported by NIH grant HL 13715-06 and by a grant from the Juvenile Diabetes Research Foundation. Dr. Levey is an Investigator of the Howard Hughes Medical Institute.

REFERENCES

1. Hardman, J. G., and Sutherland, E. W. (1969) J. Biol. Chem, 244, 6363-6370.
2. White, A. A., and Aurbach, G. D. (1969) Biochim. Biophys. Acta, 191, 686-697.
3. Kram, R., and Tomkins, G. M. (1973) Proc. Natl. Acad. Sci. U.S.A., 70, 1659-1663.
4. Rudland, P. S., Gospodarowicz, D., and Seifert, W. (1974) Nature, 250, 741-724, 773-774.
5. Seifert, W. E., and Rudland, P. S. (1974) Nature, 248, 138-140.
6. Watson, J., Epstein, R., and Cohn, M. (1973) Nature, 246, 405-409.
7. Lanzani, G. A., Giannattasio, M., Manzocchi, L. A., Bollini, R., Soffientini, A. N., and Macchia, V. (1974) Biochem. Biophys. Res. Comm., 58, 172-177.
8. Whitfield, J. F., MacManus, J. P., Franks, D. J., Gillan, D. J., and Youdale, T. (1971) Proc. Soc. Exp. Biol. Med., 137, 453-458.
9. Kimura, H., and Murad, F. (1975) Proc. Natl. Acad. Sci. U.S.A., 72, 1965-1969.
10. DeRubertis, F. R., Chayoth, R., and Field J. B. (1976) J. Clin. Invest., 57, 641-649.
11. Liu, Y. Y., Schmeltz, I., and Hoffman, D. (1974) Anal. Chem., 46, 885-889.
12. Biancifiori, C., and Ribacchi, R. (1962) Nature, 194, 488-489.
13. Severi, L., and Biancifiori, C. (1968) J. Nat. Cancer Inst., 41, 331-340.
14. Toth, B. (1972) Cancer Res., 32, 804-807.
15. Toth, B. (1972) Int. J. Cancer, 9, 109-118.
16. Vesely, D. L., Chown, J., and Levey, G. S. (1976) J. Mol. and Cellular Cardiology, in press.
17. Vesely, D. L., Rovere, L. E., and Levey, G. S. (1977) Cancer Res, in press.
18. Bray, G. A. (1960) Anal. Biochem., 1, 279-285.
19. Steiner, A. L., Parker, C. W., and Kipnis, D. M. (1972) J. Biol. Chem., 247, 1106-1113.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem., 193, 265-275.
21. DeRubertis, F. R., and Craven, P. A. (1976) Science, 193, 897-899.
22. Kimura, H., Mittal, C. K., and Murad, F. (1975) J. Biol. Chem., 251, 8016-8022.