Biochemical Pharmacology, Vol. 27, pp. 1671–1675.

0006-2952/78/0615-1671$02.00/0

INVOLVEMENTS OF CYCLIC NUCLEOTIDE SYSTEMS IN ENLARGED MICE LUNGS PRODUCED BY BUTYLATED HYDROXYTOLUENE*

J. F. KUO, NANCY L. BRACKETT, JOSEPH W. STUBBS, MAMORU SHOJI and DAVID M. HELFMAN

Departments of Pharmacology and Medicine (Hematology and Oncology), Emory University School of Medicine, Atlanta, GA 30322, U.S.A.

(*Received* 13 *April* 1977; *accepted* 4 *November* 1977)

Abstract—A single intraperitoneal injection of butylated hydroxytoluene (BHT), at a dose of 400 mg/kg of body weight, to mice caused enlargement of their lungs, which was found to be associated with some modifications in certain parameters of the cyclic nucleotide systems. These include: (a) increased levels of cyclic GMP, with cyclic AMP levels remaining unaffected, (b) increased levels of phosphodiesterases for cyclic GMP and cyclic AMP, with the lowest ratio of these activities seen at the beginning of the tissue mass increase, (c) increased levels of both cyclic GMP-dependent and cyclic AMP-dependent protein kinases, and (d) increased levels of stimulatory modulator of the cyclic GMP-dependent enzyme, with unaltered levels of inhibitory modulator of the cyclic AMP-dependent enzyme. Since increases in levels of cyclic GMP, the cyclic GMP target enzyme and its stimulatory modulator, as well as of the activity of DNA polymerase, occurred prior to or coincided with the pulmonary hypertrophy and hyperplasia, it is suggested that the cyclic GMP-mediated processes may be particularly crucial for cell proliferation after toxic injury of the lung by BHT.

It has been shown that intraperitoneal injection of the antioxidant, butylated hydroxytoluene (BHT), to mice produces lung damage which is followed by extensive hyperplasia, hypertrophy and general disorganization of cellular components [1, 2]. The initial toxic injury of the lung by BHT and by a variety of pulmonary toxicants involves damage and death of type I alveolar cells; the subsequent tissue repair is characterized by proliferation of type II alveolar cells [2–5]. Determination of biochemical parameters indicative of the presence of toxic lung damage has been unsuccessful. One reason may be that, in the lung, cell death is often quickly followed by cell proliferation. Thus, increases in the content of DNA and in the activities of thymidine kinase, uridine kinase and DNA polymerase in the lung have been demonstrated shortly after BHT injection [2].

An increased cyclic GMP level has been shown to be associated with tumors [6, 7] and rapidly growing tissues [8], whereas an increased cyclic AMP level has been implicated in decreased proliferation and increased differentiation of cells [9]. The present investigation was undertaken to examine whether any changes in certain cyclic nucleotide-related parameters may occur in the lung in response to toxic injury by BHT. We found that modifications in cyclic nucleotide systems favoring expression of the cyclic GMP effect have indeed occurred in lung undergoing hyperplasia and hypertrophy.

* This work was supported by Grants HL-15696, CA-16255 and T32-GM-07594 from USPHS.

MATERIALS AND METHODS

Materials. Male C57B1/6Sp inbred mice, weighing about 25 g, were purchased from Charles River, Wilmington, MA; [γ-^{32}P]ATP, cyclic [8,5′-^{3}H]GMP (39.7 Ci/m-mole), cyclic [G-^{3}H]AMP (27.9 Ci/m-mole) and [methyl-^{3}H]TMP were from New England Nuclear, Boston MA; arginine-rich histone (HA) was from Worthington; cyclic AMP, cyclic GMP and butylated hydroxytoluene (BHT) were from Sigma, St. Louis, MO.

Methods. The mice were given a single intraperitoneal injection of BHT dissolved in 0.5 ml corn oil in a dose of 400 mg/kg of body weight. The control animals received only 0.5 ml corn oil. The mice were killed at periodic intervals, as indicated in the tables. The lungs were immediately dissected and were homogenized in 10 vol. of ice-cold 50 mM Tris–Cl buffer, pH 7.5, containing 3 mM mercaptoethanol. The homogenates were centrifuged for 20 min at 30,000 *g*. Appropriate aliquots of extracts were used for assaying enzymes so that the enzyme activities were linear with reaction time and amount of enzyme protein employed.

DNA polymerase was assayed by the method of Guzzo and Glazer [10]. The assay method for phosphodiesterases was a modification [11] of that described by Thompson and Appleman [12], using 1 μM concentration of either radioactive cyclic AMP or cyclic GMP as substrate. One unit of the phosphodiesterase is defined as that amount of activity that hydrolyzes 1 pmole cyclic nucleotide/min. The standard assay system [13, 14] for protein kinases contained, in a final volume of 0.2 ml,

Table 1. Effects of BHT treatment on certain parameters in mice lungs*

Day of BHT injection	Lung weight (mg)	Lung protein (mg)	Lung DNA (mg)	DNA polymerase (units)†	Cyclic AMP (pmoles/mg protein)	Cyclic GMP (pmoles/mg protein)
Control‡	182 ± 12	22 ± 3	1.1 ± 0.1	12.4 ± 0.6	23.3 ± 0.9	5.3 ± 0.5
1	185 ± 32	24 ± 2	1.2 ± 0.1	13.2 ± 0.8	ND§	ND
2	196 ± 10	26 ± 3	1.3 ± 0.1	19.1 ± 1.2‖	19.9 ± 2.2	4.6 ± 0.2
3	208 ± 12	30 ± 4	1.4 ± 0.1	25.0 ± 1.6‖	26.7 ± 3.6	6.9 ± 0.2‖
4	261 ± 10‖	31 ± 2‖	1.8 ± 0.1‖	42.1 ± 0.9‖	22.2 ± 3.7	7.3 ± 0.4‖
5	275 ± 19‖	32 ± 3‖	2.5 ± 0.1‖	46.5 ± 1.8‖	22.5 ± 3.1	5.5 ± 0.6
6	360 ± 35‖	31 ± 2‖	2.5 ± 0.1‖	57.6 ± 1.9‖	24.9 ± 1.3	5.5 ± 0.3
7	352 ± 21‖	32 ± 3‖	2.6 ± 0.1‖	ND	27.7 ± 2.7	4.2 ± 0.8

* Assay methods for the parameters measured were as described in the text. The data presented are mean ± S.E. of the values obtained from three to eight mice/group.

† Defined as pmoles [^{3}H]TMP incorporated/min.

‡ Combined from three mice each from groups receiving corn oil for 0, 2, 4 and 6 days (oil injection had no effect on all parameters measured).

§ Not determined.

‖ Significantly different from the control groups ($P < 0.05$ to < 0.005).

potassium phosphate buffer, pH 7.0, 10 μmoles; theophylline, 0.5 μmole; arginine-rich histone, 40 μg; $MgCl_2$, 2 μmoles; [γ-^{32}P]ATP, 1 nmole, containing about 1.1×10^6 cpm; either cyclic GMP or cyclic AMP, 80 pmoles; appropriate amounts (5–30 μg) of enzyme proteins; and stimulatory modulator [14, 15] of cyclic GMP-dependent protein kinase, 20–40 μg. Since stimulatory modulator has no effect on cyclic AMP-dependent protein kinase, the present system is suitable for assaying both classes of protein kinases in tissue extracts or crude enzyme preparations in the same incubation solution [16, 17]. The reaction was carried out for 10 min at 30°C. One unit of the enzyme is that amount of activity that transfers 1 pmole ^{32}P from [γ-^{32}P]ATP to recovered histone.

Crude protein kinase modulator was prepared from lung extracts by heating and precipitating from 5% trichloroacetic acid [14, 18]. The activity of stimulatory modulator in the crude modulator preparation thus obtained was assayed based upon its ability to specifically stimulate purified cyclic GMP-dependent protein kinase [14, 15], whereas the activity of inhibitory modulator was assayed based upon its ability to specifically inhibit cyclic AMP-dependent protein kinase [14, 15], under the assay conditions used for protein kinases. Histone was used as the substrate for either case. One unit of stimulatory modulator is defined as that amount of activity that stimulates 20 units of the guinea pig fetal lung cyclic GMP-dependent enzyme 20 per cent in the presence of 0.5 μM cyclic GMP. One unit of inhibitory modulator is defined as that amount of activity that inhibits 20 units of the bovine heart cyclic AMP-dependent enzyme 20 per cent in the presence of 0.5 μM cyclic AMP.

Cyclic AMP and cyclic GMP were assayed by the protein kinase catalytic methods reported elsewhere [19]. DNA was determined by the method of Dische [20], and protein was determined by the method of Lowry *et al.* [21].

RESULTS

Significant increases in the weight as well as in the protein and DNA contents were noted for the lungs of mice on day 4 after a single dose (400 mg/kg) of an intraperitoneal injection of BHT (Table 1). An increase in the pulmonary DNA polymerase activity and the cyclic GMP content, however, was noted as early as days 2 and 3, respectively (Table 1). These results confirm earlier findings reported by others [2] that BHT causes hyperplasia and hypertrophy in mice lungs. It appeared that the cyclic AMP content was not significantly affected by the BHT treatment.

Phosphodiesterases for cyclic GMP and cyclic AMP hydrolysis in the lung were higher in the mice on day 5 (Table 2). The ratio of cyclic GMP to cyclic AMP phosphodiesterase activity, however, was lowest 4 days after treatment, when the enlargement of the lung became evident, and the value returned to the control level on day 6. Kinetic analysis revealed that the pulmonary phosphodiesterases from the BHT-treated mice had higher V_{max} values for both cyclic GMP and cyclic AMP without altering the K_m values for both cyclic nucleotides (Table 3), indicating that BHT caused increases in the amounts of the enzymes rather than affecting the species of the enzymes.

The protein kinase activity, assayed either in the absence or presence of cyclic GMP or cyclic AMP, was elevated as early as 2 days after the BHT injection, reaching a peak level around day 6 (Table 4). The ratio of protein kinase activity stimulated by cyclic GMP to that stimulated by cyclic AMP, however, remained rather constant throughout the entire experimental period. A pronounced increase in stimulatory modulator (of cyclic GMP-dependent protein kinase) was noted in the BHT-treated mice (Table 5). Inhibitory modulator (of cyclic AMP-dependent protein kinase), in contrast, remained practically unaffected by the agent, resulting in an

Table 2. Effects of BHT treatment on phosphodiesterases for cyclic GMP and cyclic AMP in mice lungs*

Day of BHT injection	Phosphodiesterase activity (units/mg protein in extracts)		Activity ratio
	Cyclic GMP	Cyclic AMP	
Control†	197 ± 24	134 ± 16	1.48 ± 0.10
1	211 ± 20	169 ± 49	1.28 ± 0.25
2	197 ± 22	178 ± 21	1.10 ± 0.11
3	209 ± 16	167 ± 10	1.29 ± 0.09
4	170 ± 16	167 ± 10	1.04 ± 0.05‡
5	289 ± 16‡	257 ± 8‡§	1.12 ± 0.03‡
6	382 ± 15‡§	228 ± 22‡§	1.72 ± 0.22§
7	299 ± 17‡§	208 ± 12‡	1.50 ± 0.24§

* The concentration of cyclic GMP or cyclic AMP used as substrate for phosphodiesterase was 1 μM. The amounts of extracts used for enzyme assay ranged from 20 to 25 μg. The data presented are the means (± S.E.) of the values obtained from three mice/group.

† Combined from three mice each from groups receiving corn oil for 0, 2, 4 and 6 days (oil injection had no effect on the enzyme activities).

‡ Significantly different from the control groups ($P < 0.05$ to < 0.0005).

§ Significantly different from the 4-day group ($P < 0.05$).

approximately 4-fold increase in the ratio of the two types of modulator activities. In order to verify the unilateral increase in stimulatory modulator activity in the enlarged lung (Table 5), the two modulator activities in the crude preparations were separated on Sephadex G-100 columns, and their activities quantitated, according to the method described earlier [14, 15]. The results shown in Fig. 1 confirm the observations shown above.

DISCUSSION

The most striking observation made in the present studies is the specific increase in stimulatory modulator in the lung after BHT injection (Table 5 and Fig. 1), while inhibitory modulator remains unaltered in the enlarged lung. Thus, this change, coupled with a specific increase in cyclic GMP (Table 1), would allow a unilateral augmentation of the cyclic GMP-dependent protein kinase activity, which is also increased in the enlarged lung (Table 4). It seems, therefore, that pulmonary hyperplasia and hypertrophy induced by BHT may be characterized as a process of enhanced cyclic GMP expression. This contention is in line with earlier observations made by others that greatly elevated cellular cyclic GMP contents, or increased ratios of cyclic GMP to cyclic AMP, are related to cell proliferation, exemplified by rapidly growing hepatomas [6], psoriatic lesions [24] and rapidly growing neonatal kidneys [8]. We recently observed that the activity of cyclic GMP-phosphodiesterase is greatly reduced, whereas that of cyclic AMP-phosphodiesterase is elevated, in the fast-growing Morris hepatoma 3924A [25]. Our earlier findings [22] that the level of cyclic GMP-dependent protein kinase,

Table 3. Comparison of kinetic parameters of phosphodiesterases for cyclic GMP and cyclic AMP in lung extracts of mice with or without BHT treatment*

Mice	Phosphodiesterases for hydrolysis of: Cyclic GMP		Cyclic AMP	
	K_m (μM)	V_{max} (units)	K_m (μM)	V_{max} (units)
Control†	1.2	2.0	0.9	2.4
BHT-treated	1.2	4.6	0.9	6.7

* Lung extracts pooled from the controls (oil injection) and the BHT-injected (5 and 6 days after treatment) mice were assayed for phosphodiesterase activities. The same amount of extracts (25 μg protein) from the two groups of mice was used as the enzyme source. The concentrations of either cyclic GMP or cyclic AMP used as substrates for kinetic analysis were 0.8, 1.0, 1.5, 3.0 and 5.0 μM.

† Same control mice from Table 2.

Table 4. Effects of BHT treatment on protein kinases stimulated by cyclic GMP and cyclic AMP in the lung extracts of mice*

Day of BHT injection	Protein kinase activity (units/mg protein in extracts)			Activity ratio†
	Basal	+ Cyclic GMP	+ Cyclic AMP	
Control‡	117 ± 8	210 ± 15	276 ± 26	0.60 ± 0.03
1	101 ± 13	178 ± 33	234 ± 36	0.56 ± 0.06
2	172 ± 7§	293 ± 10§	372 ± 22§	0.61 ± 0.03
3	189 ± 4§	286 ± 12§	353 ± 17§	0.60 ± 0.01
4	184 ± 15§	288 ± 30§	360 ± 41	0.60 ± 0.01
5	294 ± 7§	413 ± 4§	492 ± 20§	0.62 ± 0.08
6	286 ± 19§	452 ± 13§	531 ± 12§	0.68 ± 0.03
7	296 ± 22§	425 ± 30§	491 ± 32§	0.69 ± 0.07

* Aliquots (0.02 ml, containing 20–25 μg protein) of extracts were assayed for protein kinase activity in the presence and absence of 0.4 μM cyclic GMP or cyclic AMP, and in the presence of 25 μg of stimulatory modulator, according to the method described in the text. The data presented are the means (± S.E.) of the values obtained from three mice/group.

† Defined as: cyclic GMP-stimulated activity/cyclic AMP-stimulated activity [22, 23].

‡ Combined from three mice each from groups receiving corn oil for 0, 2, 4 and 6 days (oil injection had no effect on the enzyme activities).

§ Significantly different from the control groups ($P < 0.05$ to < 0.005).

Table 5. Effects of BHT treatment on stimulatory and inhibitory modulators of protein kinases in mice lungs*

Day of BHT injection	Protein kinase modulator activity (units/g fresh lung) Stimulatory (S)	Inhibitory (I)	S/I ratio
Control†	50 ± 5	220 ± 9	0.23 ± 0.01
1	56 ± 13	225 ± 5	0.25 ± 0.03
2	62 ± 10	241 ± 8	0.26 ± 0.03
3	79 ± 10‡	238 ± 14	0.33 ± 0.03‡
4	221 ± 18‡	247 ± 16	0.89 ± 0.08‡
5	204 ± 15‡	244 ± 12	0.84 ± 0.07‡
6	205 ± 18‡	247 ± 19	0.83 ± 0.08‡

* Assay methods for the modulator activities were as described in the text. The data presented are the means (± S.E.) of the values obtained from three to five mice/group.

† Combined from three mice each from groups receiving corn oil for 0, 2, 4 and 6 days (oil injection had no effect on the modulator activities).

‡ Significantly different from the control groups ($P < 0.05$ to < 0.005).

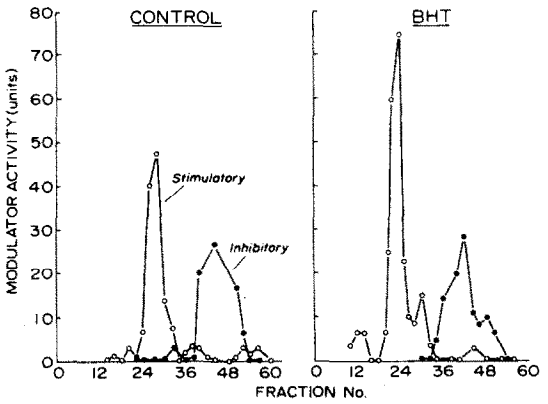

Fig. 1. Sephadex G-100 gel filtration of crude protein kinase modulator prepared from the lung of mice injected with or without BHT. Aliquots (1 ml, equivalent to 1 g of fresh lung) of the crude modulator from the control and the BHT-injected (6 days) groups of mice were applied to Sephadex G-100 columns (2.2 × 30 cm), which had been previously equilibrated with 5 mM potassium phosphate buffer, pH 7.0. Separation of the stimulatory and inhibitory modulator components was achieved by eluting the columns with the same buffer, as described elsewhere [14, 15]. (The fraction size was 1.5 ml.) Aliquots (0.1 ml) of the fractions were assayed for the modulator activities by the procedures described in the text.

relative to that of the cyclic AMP-dependent enzyme in the guinea pig lung and heart, is highest in the fetus, lowest in the adult, and has an intermediate value in the neonate, lend further support to a prominent role of the cyclic GMP system in cell division and tissue growth. Our present findings indicate that increases in cyclic GMP protein kinase and stimulatory modulator activities, as in the case of DNA polymerase activity, preceded or coincided with increases in weight, protein and DNA of the lung of mice. It is conceivable, therefore, that protein kinase activity, especially that of the cyclic GMP-dependent enzyme, may be crucial in pulmonary cell proliferation after toxic damage of the tissue by BHT. This contention, however, has to be considered as a working hypothesis, since the effect of stimulatory modulator in augmenting the phosphotransferase activity of cyclic GMP-dependent protein kinase to date has been observed only when histones (by far the most effective phosphate acceptors) were used as substrates [26], and histones have not yet been identified as endogenous substrates for this class of protein kinase.

The proliferation-related alterations in the cyclic nucleotide systems mentioned above may be compared with those of cardiac hypertrophy (devoid of significant cardiac hyperplasia) produced under different conditions. In the case of cardiac hypertrophy secondary to hypertension in the spontaneously hypertensive rat, one adaptive modification involved depression of the level of cyclic GMP-dependent protein kinase, associated with an unchanged level of cyclic AMP-dependent protein kinase [23]. In the case of cardiac hypertrophy in the rat induced by repeated administration of isoproterenol, the biochemical modifications involved increased levels of cyclic AMP-phosphodiesterase and stimulatory modulator of cyclic GMP-dependent protein kinase, accompanied by an unaltered level of both cyclic AMP-dependent and cyclic GMP-dependent protein kinases [27].

Acknowledgement—The authors thank Dr. Robert I. Glazer for performing DNA polymerase assay.

REFERENCES

1. A. A. Mario and J. T. Mitchell, *Proc. Soc. exp. Biol. Med.* **140**, 122 (1972).
2. H. P. Witschi and M. G. Cote, *Fedn Proc.* **35**, 89 (1976).
3. I. Y. R. Adamson and D. H. Bowden, *Lab. Invest.* **30**, 35 (1974).
4. R. L. Hackett and E. W. Sunderman, Jr., *Archs Envir. Hlth* **16**, 349 (1968).
5. S. L. Kauffman, *Am. J. Path.* **68**, 317 (1972).
6. E. W. Thomas, F. Murad, W. B. Looney and H. P. Morris, *Biochim. biophys. Acta* **297**, 564 (1973).
7. F. R. DeRubertis, R. Cahoth and J. B. Field, *J. clin. Invest.* **57**, 641 (1976).
8. D. Schlondorff and H. Weber, *Proc. natn. Acad. Sci. U.S.A.* **73**, 524 (1976).
9. W. L. Ryan and M. L. Heidrick, *Science, N.Y.* **162**, 1484 (1968).
10. G. Guzzo and R. I. Glazer, *Cancer Res.* **36**, 1041 (1976).
11. C. W. Davis and J. F. Kuo, *Biochim. biophys. Acta* **444**, 554 (1976).
12. W. J. Thompson and M. M. Appleman, *Biochemistry* **10**, 311 (1971).
13. J. F. Kuo, W. N. Kuo, M. Shoji, C. W. Davis, V. L. Seery and T. E. Donnely, Jr., *J. biol. Chem.* **251**, 1759 (1976).
14. W. N. Kuo and J. F. Kuo, *J. biol. Chem.* **251**, 4283 (1976).
15. W. N. Kuo, M. Shoji and J. F. Kuo, *Biochim. biophys. Acta* **437**, 142 (1976).
16. M. Shoji, J. G. Patrick, C. W. Davis and J. F. Kuo, *Biochem. J.* **161**, 213 (1977).
17. M. Shoji, J. G. Patrick, J. Tse and J. F. Kuo, *J. biol. Chem.* **252**, 4347 (1977).

18. D. A. Walsh, C. D. Ashby, C. Gonzalez, D. Calkins, E. H. Fischer and E. G. Krebs, *J. biol. Chem.* **246,** 1977 (1971).
19. J. F. Kuo and P. Greengard, *Adv. Cyclic Nucleotide Res.* **2,** 41 (1972).
20. Z. Dische, in *The Nucleic Acid* (Eds E. Charguff and J. N. Davidson), Vol. 1, pp. 285–305. Academic Press, NY (1955).
21. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 262 (1951).
22. J. F. Kuo, *Proc. natn. Acad. Sci. U.S.A.* **72,** 2256 (1975).
23. J. F. Kuo, C. W. Davis and J. Tse, *Nature, Lond.* **261,** 335 (1976).
24. J. Voorhees, M. Stawiski, E. Duell, M. Haddox and N. D. Goldberg, *Life Sci.* **13,** 639 (1973).
25. M. Shoji, H. P. Morris, C. W. Davis, N. L. Brackett and J. F. Kuo, *Biochim. biophys. Acta* **500,** 419 (1977).
26. J. F. Kuo, M. Shoji and W. N. Kuo, *Adv. Cyclic Nucleotide Res.* **9,** 199 (1978).
27. J. Tse, N. L. Brackett and J. F. Kuo, *Biochim. biophys. Acta* (in press).