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Inhibitory Effects of Antioxidants on N-Bis(2-hydroxypropyl)nitrosamine-induced Lung Carcinogenesis in Rats

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Potential second-stage modifying effects of 8 antioxidants on lung tumorigenesis initiated by N-bis(2-hydroxypropyl)nitrosamine (DHPN) were examined in male F344 rats. After an initial 2-week treatment with DHPN (0.1% in drinking water), rats were administered one of the antioxidants supplemented in the diet for 30 weeks. Although the incidences of lung adenomas were not affected, those of carcinomas were lowered by 2% butylated hydroxyanisole (BHA, 2 rats/20 rats), 1% butylated hydroxytoluene (BHT, 1/20), 0.8% ethoxyquin (EQ, 3/20) and 1% α-tocopherol (α-TP, 2/20) treatments as compared to the control level (9/20), while 5% sodium L-ascorbate (SA), 0.8% catechol (CC), 0.8% resorcinol (RN), and 0.8% hydroquinone (HQ) did not exert any significant effect on incidence. Quantitative analysis of adenomas and carcinomas (numbers and areas of lesions per unit area of lung section) revealed obvious inhibitory effects of SA, CC, and RN as well as BHA, BHT, EQ, and α-TP. Among the antioxidants, BHT exerted the strongest inhibitory activity. In contrast, DHPN-induced thyroid tumorigenesis was significantly enhanced by BHT (14/20) and EQ (20/20) treatments (control=5/20). Thus the antioxidants showed opposite effects on lung and thyroid carcinogenesis in the rat.

Key words: Antioxidant — Lung tumorigenesis —Inhibition — Rat

Antioxidants have been widely used as food additives in various processed foods to prevent autoxidation of fatty acids. Neither mutagenic nor clastogenic,[1] they have been reported to inhibit chemical carcinogenesis in many organs.[2,3] These properties have been demonstrated not only for synthetic antioxidants but also for naturally occurring or physiological antioxidants,[4,5] and the potential use of antioxidants in the prevention of human cancer has been extensively discussed.[2,3,6-8] However, the inhibitory effects are target-organ dependent and determined by dosing schedules, and there are many reports that antioxidants may also enhance tumor yield in animals given chemical carcinogens.[6,7]

Furthermore, carcinogenic potency of BHA[4] for the forestomach of rats[9] and hamsters[10] has been demonstrated, resulting in extensive studies of the carcinogenic or tumor-modifying effects of various antioxidants mainly using rats. In a series of studies, it has been revealed that many antioxidants possess tumor-modifying potential and that their effects on carcinogenesis, either inhibition or enhancement, depend on and vary with the target organ. The extensive results on antioxidants have recently been summarized in a number of review articles,[7,11-13]

The present paper describes modifying effects of 8 antioxidants on lung and thyroid carcinogenesis induced by a wide-spectrum carcinogen, DHPN in male rats.[14,15] Antioxidants chosen for the investigation were BHA, BHT, EQ, SA, α-TP, CC, RN, and HQ since the tumor-modifying effects of these compounds have been extensively studied for other organs. BHA, BHT and EQ are synthetic antioxidants and the others are naturally occurring.

MATERIALS AND METHODS

Animals and chemicals A total of 260 male F344/DuCrj rats were obtained from Charles River Japan, Inc., Atsugi, and maintained on basal diet (CRF-1, Charles River Japan, Inc.) *ad libitum*. The rats were housed in plastic cages in an air-conditioned room at 22±2°C and 55±5% humidity. DHPN was obtained from Iwai Chemical Co., Tokyo. SA, CC, RN, and HQ were from Wako Pure Chemical Industries, Ltd., Osaka, BHA from Nikki Universal Co., Tokyo, BHT from Takeda

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[4] Abbreviations used are: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DHPN, N-bis(2-hydroxypropyl)nitrosamine; EQ, ethoxyquin; SA, sodium L-ascorbate; α-TP, α-tocopherol; CC, catechol; RN, resorcinol; HQ, hydroquinone.

Pharmaceutical Co., Osaka, EQ from Kokin Chemical Co., Osaka, α-TP from Eisai Co. Ltd., Tokyo, and vitamin K_3 from Sigma Chemical Co., St. Louis, MO.

Treatment of animals The rats were divided into 3 groups. Group 1 was given 0.1% DHPN in the drinking water for 2 weeks and then administered one of the antioxidants supplemented into powder diet for the following 30 weeks. Group 2 animals received DHPN alone and group 3 rats were given one of the antioxidants for 30 weeks without prior DHPN administration as controls.

The doses were chosen based on the results of our previous studies as follows: 5% for SA, 2% for BHA, 1% for BHT and α-TP, and 0.8% for EQ, CC, RN, and HQ. The animals given BHT were also treated with vitamin K_3 at a dose of 7 ppm in the drinking water to prevent loss of animals due to BHT toxicity. The rats had free access to their diet and water. Food and water intakes and body weights were periodically measured. At the end of week 32 of the experiment, all rats were killed under light ether anesthesia after overnight fasting. No rats died before the termination of the experiment.

Immediately after death, the lungs were removed with other thoracic tissues and weighed together. The lungs were inflated by injection of 10% phosphate-buffered formalin solution (about 5 ml) through the trachea, which was then pinched by a Pean's forceps to keep the lungs inflated. After 10 min of fixation, the heart, trachea, esophagus and connective tissues were separated from the lungs and weighed to obtain lung weight. The lung lobules were sliced at 2 mm thickness, giving 3 slices from the left lung and 6 from the right.

Livers and kidneys were removed and weighed. Urinary bladder and stomach were inflated with fixative and then removed. Thyroids were also sampled. All removed organs were placed in fixative, and then processed for routine staining with hematoxylin and eosin for histopathological examination.

Quantitative analysis Numbers of lung lesions were microscopically counted. Areas of lung lesions and total areas of lung sections examined were measured using a color video image processor (VIP-21 C, Olympus-Ikegami Tsushin Co., Tokyo). Statistical analysis was carried out by using Student's t test and Cochran's t test in combination with the F test for variability, and the Fisher exact test.

RESULTS

Body and organ weights Food intake was almost the same in all groups. Final body weights and absolute lung and liver weights are summarized in Table I. The body weights of rats were significantly lowered by BHA, BHT, EQ, CC, RN and HQ treatments in DHPN-initiated groups when compared with non-initiated groups. BHA, RN and HQ selectively lowered the final body weights in DHPN-initiated groups. In each chemical-treated group, lung weights were generally higher in DHPN-treated groups as compared to non-initiated animals except in the case of BHA. Lung weights were lowest in BHT-treated animals. The increase in lung weight was correlated with macroscopic findings: localized dark or whitish nodular lesions, that were microscopically hyperplastic and/or neoplastic, were grossly observed in these lungs. Liver weights were significantly increased by BHT, EQ, CC and HQ and were not affected by BHA, SA, α-TP and RN in the DHPN-initiated groups.

Histological findings in the lungs Histologically, proliferative, hyperplastic or neoplastic lesions in the lung were classified into hyperplasia, adenoma and carcinoma categories. Hyperplastic lesions are characterized by interalveolar septa of slightly to moderately increased thickness and cellularity, with the epithelial cells being columnar or cuboidal, larger than normal cells but organized as single cell lining layers with apparent alveolar space formation. This type of lesion has also been designated as bronchiolo-alveolar adenomatosis. Adenomas and carcinomas are lesions composed of papillar and interlacing cords of cuboidal cells or solidly growing cells. Cell atypism is slight to moderate in adenomas (Fig. 1), whereas carcinomas are composed of cells with atypical nuclei and increased mitoses, usually observed as solid lesions. Squamous cell differentiation is frequently evident in carcinomas (Fig. 2).

Incidences of lung lesions are summarized in Table II. No lung tumors were induced in rats that were not administered DHPN (group 3). DHPN alone induced lung adenomas in all rats (20/20) and carcinomas in 45% of the animals (9/20). Incidence of adenomas was not affected by the chemical treatment. However, the incidences of carcinomas were significantly decreased by administration of BHA (10%, 2/20), BHT (5%, 1/20), EQ (15%, 3/20), and α-TP (10%, 2/20). This inhibitory effect of the antioxidants was more evident when the neoplastic lesions were quantitatively analyzed as numbers and areas of lesion per unit area of the lung section (Fig. 3). The numbers of adenomas were lowered from the control value (0.63 adenomas/cm^2) in groups given BHA, BHT, EQ, CC, and RN, and the total areas were smaller than the control value (1.36 mm^2/cm^2) in all groups except for HQ. With carcinomas, the numbers were significantly lowered by BHT treatment and there seemed to be a tendency for them to be lowered by other antioxidants examined except for RN. The total area of carcinomas was significantly reduced by BHA, BHT, and EQ, and was also decreased, but not significantly, by the others. Among the antioxidants examined, BHT was

Table I. Body and Organ Weights of Rats Treated with Various Antioxidants

Group	Treatment	No. of rats	Body (g)	Lungs (g)	Liver (g)
1.	DHPN/BHA	20	$303\pm32^{a,b,e)}$	1.64 ± 0.25	$8.02\pm1.06^{f)}$
	DHPN/BHT	20	$308\pm12^{b)}$	$1.49\pm0.13^{b,e)}$	$13.10\pm0.88^{b)}$
	DHPN/EQ	20	$321\pm24^{b)}$	$1.80\pm0.39^{g)}$	$11.33\pm1.37^{b,g)}$
	DHPN/SA	20	361 ± 24	$1.84\pm0.22^{e)}$	7.57 ± 0.72
	DHPN/α-TP	20	380 ± 25	$1.90\pm0.15^{d,e)}$	8.17 ± 0.49
	DHPN/CC	20	$329\pm19^{b)}$	1.72 ± 0.16	$8.48\pm0.69^{c)}$
	DHPN/RN	20	$349\pm25^{d,g)}$	$1.82\pm0.14^{e)}$	7.82 ± 0.86
	DHPN/HQ	20	$355\pm26^{f)}$	$1.85\pm0.25^{f)}$	$8.57\pm1.10^{d,f)}$
2.	DHPN/None	20	370 ± 26	1.78 ± 0.20	7.77 ± 0.82
3.	BHA	10	340 ± 15	1.63 ± 0.19	9.22 ± 0.55
	BHT	10	305 ± 12	1.24 ± 0.09	12.90 ± 0.64
	EQ	10	321 ± 35	1.54 ± 0.11	12.34 ± 0.95
	SA	10	376 ± 19	1.53 ± 0.15	7.85 ± 0.42
	α-TP	10	376 ± 28	1.53 ± 0.14	8.18 ± 0.80
	CC	10	340 ± 20	1.61 ± 0.22	9.04 ± 0.87
	RN	10	375 ± 32	1.52 ± 0.18	8.23 ± 0.90
	HQ	10	386 ± 20	1.61 ± 0.15	9.62 ± 0.58

a) Data are mean $\pm$ SD values.
b) Significantly different from group 2 at $P<0.001$.
c) Significantly different from group 2 at $P<0.01$.
d) Significantly different from group 2 at $P<0.05$.
e) Significantly different from non-initiated group at $P<0.001$.
f) Significantly different from non-initiated group at $P<0.01$.
g) Significantly different from non-initiated group at $P<0.05$.

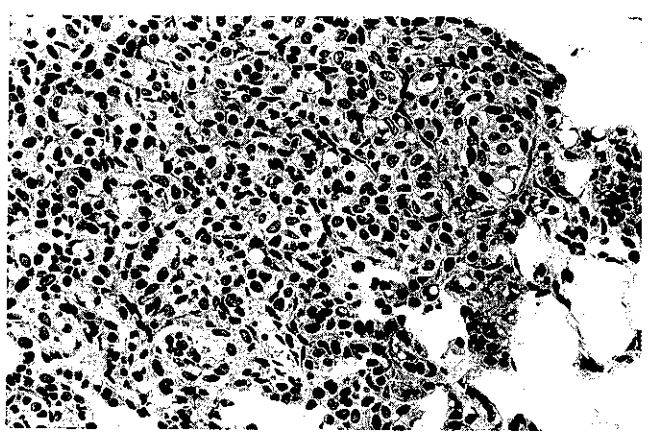

Fig. 1. Lung adenoma induced by DHPN alone. H-E. $\times$250.

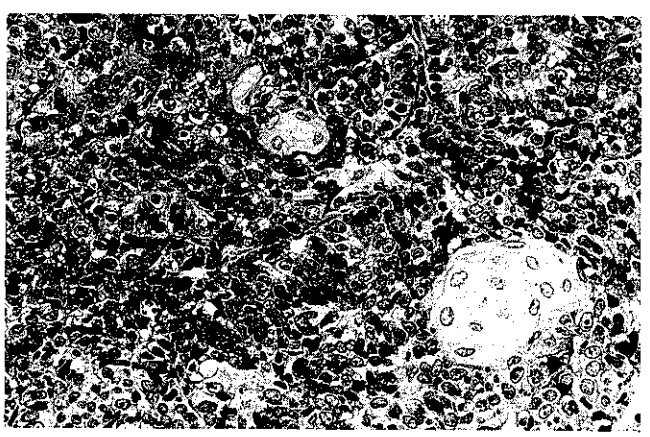

Fig. 2. Lung carcinoma induced by DHPN alone. Nuclear atypism, many mitoses and squamous differentiation are observed. H-E. $\times$250.

most effective for inhibition of lung neoplasia development. Histopathologically, however, BHT-specific or toxic lesions were not observed in the lung.

Other organs Histopathologically evident neoplastic changes are summarized in Table III. The thyroid is one of the target organs of DHPN. Thyroid tumors were classified into adenoma and carcinoma based on cellular and architectural atypism. Five of 20 rats bore thyroid tumors (25%) in group 2, 1 with an adenoma (5%) and 4 with follicular carcinomas (20%). The thyroid ade-

noma induction was significantly increased by subsequent treatment with BHT to 60% (12/20), and with EQ to 95% (19/20). SA and RN also increased the incidence, but not significantly. EQ non-significantly increased the carcinoma induction in this organ from 20% to 50%. None of the antioxidants induced any thyroid lesions by themselves.

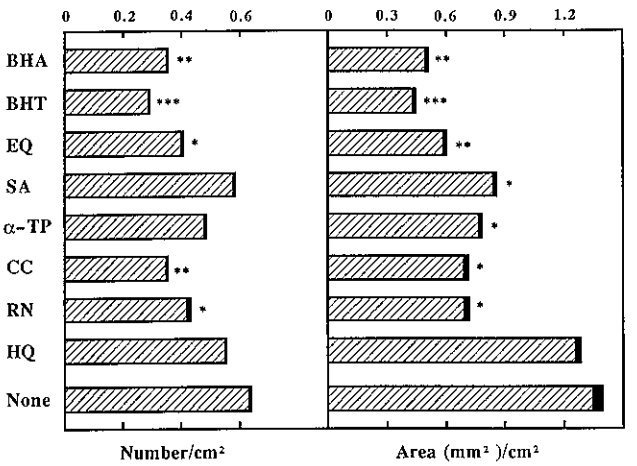

Fig. 3. Numbers and areas of lung neoplastic lesions per unit tissue section in groups pretreated with DHPN. Inhibitory effects are evident for all antioxidants, except for HQ, in terms of either number or area of lesions. No lung tumors were induced by antioxidants alone (group 3). ▨: adenoma, ■: carcinoma. *** $P<0.001$, ** $P<0.01$, * $P<0.05$.

In the urinary bladder, transitional cell papillomas were induced by BHA or BHT treatment, but the increases in lesion development were not significant. EQ also exerted slight tumor-promoting potential. A variety of kidney tumors were induced in DHPN-treated animals, including renal cell adenomas, nephroblastomas, and transitional cell papillomas and carcinomas in the pelvis. As can be seen in Table III, induction of these renal tumors seemed to be enhanced by several of the antioxidants, but the differences were not statistically significant.

Table II. Incidence of Lung Neoplasias in Rats Treated with DHPN and Various Antioxidants[a, b]

Group/ Subgroup		No. of rats	Adenoma	Carcinoma	Combined tumors
1.	BHA	20	16	2 (10)[d]	16 (80)
	BHT	20	17	1 (5)[c]	17 (85)
	EQ	20	19	3 (15)[d]	19 (95)
	SA	20	20	4 (20)	20 (100)
	α-TP	20	20	2 (10)[d]	20 (100)
	CC	20	19	4 (20)	20 (100)
	RN	20	18	8 (40)	18 (90)
	HQ	20	18	5 (25)	18 (90)
2.	None	20	20	9 (45)	20 (100)

a) No neoplastic lesions were observed in group 3.
b) Numbers in parentheses are percentage values.
c) Significantly different from group 2 at $P<0.01$.
d) Significantly different from group 2 at $P<0.05$.

Table III. Histological Findings in Thyroids, Urinary Bladder and Kidneys of Rats Treated with DHPN and Various Antioxidants[a]

Group/ Subgroup		No. of rats	Thyroid gland			Urinary bladder		Kidney			
			Adenoma	Carcinoma	Combined tumors	PN[b] hyperplasia	Papilloma	Adenoma	TCC[c] or papilloma	Nephro-blastoma	Combined tumors
1.	BHA	20	1	0	1	1	2	0	2	4	6
	BHT	20	12[d]	3	14[e]	1	2	0	1	1	2
	EQ	20	19[d]	10	20[d]	3	0	1	2	5	6
	SA	20	6	2	8	1	0	2	2	3	6
	α-TP	20	2	2	4	1	0	1	3	0	4
	CC	20	1	1	2	0	0	0	1	1	2
	RN	20	6	5	11	0	0	2	2	5	7
	HQ	20	4	5	9	0	1	0	1	3	4
2.	None	20	1	4	5	1	0	1	1	1	2

a) No neoplastic lesions were observed in group 3.
b) Papillary or nodular.
c) Transitional cell carcinoma.
d) Significantly different from group 2 at $P<0.001$.
e) Significantly different from group 2 at $P<0.01$.

In the other organs, BHA itself induced epithelial hyperplasia of the forestomach in all rats, independently of prior DHPN treatment. DHPN plus BHA or HQ induced squamous cell carcinomas of the forestomach in one rat each. CC itself induced submucosal and adenomatous hyperplasia in the pyloric region of the glandular stomach.

DISCUSSION

In the present experiment, male F344 rats were initially treated with DHPN, a propylnitrosamine which possesses strong carcinogenic activity in a variety of organs,[15] including the lung and thyroid.[14] As with a previous report,[14] the incidence of lung carcinomas in the group treated with DHPN alone was 45% in the present experiment, the assay model therefore being suitable for examination of both enhancing and inhibitory effects of test chemicals which were given after carcinogen administration. Using this system, the inhibitory influence of antioxidants, including BHT, on rat lung carcinogenesis was clearly demonstrated.

We have earlier extensively examined the effects of various antioxidants in rodent bioassay models for carcinogenicity, tumor-promoting activity and inhibitory potential. In our studies, antioxidants are usually given in the second stage after carcinogen treatment. BHA is carcinogenic for the forestomach of rats and hamsters[9, 10] and promotes tumorigenesis in the forestomach[16–18] and urinary bladder,[17, 18] while inhibiting liver[19, 20] and mammary[21] tumor development in the rat. BHT has been reported to enhance carcinogenesis in several organs[6, 7] including the lung of the mouse[22, 23] and the urinary bladder and thyroid in the rat.[17, 18] EQ enhances colon,[24] urinary bladder[11] and kidney[19] carcinogenesis, and inhibits liver carcino-genesis.[19, 20] It causes severe damage in the kidney and it may itself exert carcinogenic effects in this organ.[25] SA enhances bladder carcinogenesis[17, 26] like other sodium salt compounds, as well as forestomach lesion development.[17] α-TP has been used for several studies on the prevention of tumor formation and its deficiency has been reported to cause an enhancement of mammary tumorigenesis.[27] It slightly inhibited bladder carcinogenesis in an experiment carried out in our laboratory.[28] CC, which is contained in vegetables, wood tar and cigarette smoke, has recently been suggested to be carcinogenic in glandular stomach of rats,[29] also positively influencing forestomach and glandular stomach carcinogenesis induced by other agents in rats.[30] Other phenolic compounds such as RN and HQ, isomers of CC, have not been shown to exert tumor-enhancing activities in the forestomach of rats.[31] Recently, CC, RN and HQ were also found to enhance tongue and esophagus carcinogenesis in rats pretreated with methyl-*n*-amylnitrosamine.[32]

Thus, antioxidants exert myriad complicated effects in chemical carcinogenesis. It could be concluded that inhibitory effects of antioxidants on tumorigenesis have generally been found when the antioxidants were administered simultaneously with carcinogens.

In the present study, antioxidants were administered during the second stage of carcinogenesis and many, including BHT, exerted inhibitory effects on lung tumor development. The effects seem to be related to lowered body weight gain. However, the most retarded body weight gain was observed with BHA treatment and the strongest inhibitory effect was seen with BHT treatment. Furthermore, SA and α-TP did not affect body weight gain, but showed inhibitory potential in this organ. The findings indicate that the observed inhibition might not be related solely to the reduced body weight gain.

In view of the fact that BHT was earlier reported to strongly promote mouse lung carcinogenesis,[23, 24] the finding that it was the most effective among the antioxidants which exerted inhibitory activity is unexpected. BHT is toxic to mouse lung and induces cell proliferation in this organ within 2–4 days after intraperitoneal injection.[23] Tumor-enhancing effects of BHT in mice have been observed for both pretreatment (6 h before carcinogen injection) and chronic treatment after a single urethane injection. Later work by Witschi revealed that the observed enhancement of lung tumor production by BHT post-treatment might not be due to the production of diffuse alveolar cell hyperplasia.[33] The effect apparently depends on the mouse strain, and whether the animals are sensitive or resistant to urethane carcinogenicity.[34] In the present experiment, BHT showed general toxicity as reflected by the reduced body weight gain, although histological examination revealed no antioxidant-specific lung damage. Whether the reduction of BHT toxicity by vitamin K supplementation may be one reason for the differential results for mice and rats remains unclear. Nevertheless, it is still possible that vitamin K itself inhibited lung tumorigenesis, although no evidence for this has been reported to our knowledge.

Inhibition of lung tumorigenesis has been reported for antioxidants such as BHA, EQ and even BHT in mice[6] and for vitamin A in rats[35] when administered simultaneously with carcinogen and for ethinyl estradiol in rats given subsequent to carcinogen exposure.[36] In the inhibition case, trapping of carcinogenic radical species by antioxidants suggests itself as an attractive hypothesis because by definition these compounds react with free radicals.[2, 6] Inhibitory activity has been very often demonstrated in a number of organs when antioxidants are co-administered with carcinogens, but the situation

is complicated by their potential effects on carcinogen metabolism due to induction of drug-metabolizing enzymes.[6]

In recent work, diethylmaleate inhibited BHA promotion of forestomach carcinogenesis when simultaneously administered to rats after N-methyl-N′-nitro-N-nitrosoguanidine initiation.[16] Diethylmaleate is a glutathione-depleting agent, and we observed that 0.2% diethylmaleate in the diet inhibited BHA-induced forestomach hyperplasia dose-dependently.[37] However, it paradoxically increased the reduced glutathione level in the forestomach epithelium, although it decreased that in the liver of rats (unpublished data). These data suggest that glutathione may in some way be involved in the modification by BHA, and probably other antioxidants, of experimental tumorigenesis, including that of the lung.

In the present experiment, tumor-inhibitory effects were observed with several antioxidants of different structures, without any possibility of interaction at the initiation level. It is possible that the anti-free radical properties play a major role in this phenomenon.

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