INHIBITING EFFECTS OF DIETHYLMALEATE OR NH_4Cl ON NaHCO_3 , BUT NOT BUTYLATED HYDROXYANISOLE, PROMOTION OF URINARY BLADDER CARCINOGENESIS IN MALE F344 RATS INITIATED WITH N-BUTYL-N-(4-HYDROXYBUTYL)NITROSAMINE

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ABSTRACT—The modifying potential of diethylmaleate (DEM) and NH_4Cl on promotion by butylated hydroxyanisole (BHA) or NaHCO_3 of urinary bladder carcinogenesis in rats initiated with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) was investigated. Six week old animals received 0.05% BBN for 4 weeks and then BHA (2%) + DEM (0.15%), BHA + NH_4Cl (1%), NaHCO_3 (3%) + DEM, NaHCO_3 + NH_4Cl , BHA, DEM, NH_4Cl or no supplement, administered during experimental weeks 5–36. BHA and NaHCO_3 clearly amplify the induction of papillary or nodular (PN) hyperplasias and papillomas in rats initiated with BBN. The promoting activity of BHA was not affected by simultaneous administration of DEM or NH_4Cl . The enhancing effects of NaHCO_3 , in contrast, were clearly diminished by concurrent administration of either of these agents. DEM itself did not influence lesion development whereas NH_4Cl reduced the incidence of papillomas.

In a second experiment, rats exposed to the same protocol were killed at week 8, and assessed for levels of lipid peroxides in the bladder tissue. No remarkable alterations were observed in any group.

Thus, the fact that DEM did exert inhibiting effects on tumor promotion by NaHCO_3 without decreasing the urinary sodium ion concentration or pH and influence on lipidperoxide levels, suggests essential differences in the mechanisms of action of different types of bladder promoters.

KEY WORDS: Modifying effects on promotion, Urinary bladder carcinogenesis, Diethylmaleate, NH_4Cl , NaHCO_3 , Butylated hydroxyanisole, Lipid peroxidation.

INTRODUCTION

Butylated hydroxyanisole (BHA), a phenolic antioxidant, is a well established non-genotoxic forestomach carcinogen (Ito *et al.*, 1983 ; Ito *et al.*, 1989), also proven to act as a promoter of urinary bladder carcinogenesis (Imaida *et al.*, 1984 ; Imaida *et al.*, 1983). BHA rapidly induces cell proliferation in the target organ epithelia (Hirose *et al.*, 1986 ; Shibata *et al.*, 1989). It has been considered that its effects might be mediated by generation of free radicals following lipid peroxidation (Ito and Hirose, 1989 ; Ito *et al.*, 1991). Glutathione (L- γ - glutamyl-L-cysteinyl-glycine ; GSH) is a major endogenous reducing substance which protects against lipid peroxidation (Meister, 1983) and there is ample evidence that diethylmaleate (DEM), a known GSH depleting agent, enhances toxicity and/or promotion activity of toxins (Casini *et al.*, 1985 ; Denke *et al.*, 1985 ; Mgbodile *et al.*, 1975 ; Mizutani *et al.*, 1984 ; Richardson *et al.*, 1975). However, contrary to expectation, DEM earlier prevented the induction of squamous cell hyperplasia (Hirose *et al.*, 1987) and promoting activity (Hirose *et al.*, 1989 ; Shibata *et al.*, 1993) in the forestomach when given simultaneously with BHA.

NaHCO₃, a sodium salt type promoter of urinary bladder carcinogenesis, also induces transitional cell hyperplasia and surface changes of the epithelium, in this case possibly mediated by elevated urinary sodium ion level and pH (Fukushima, 1991 ; Fukushima *et al.*, 1991). Administration of NH₄Cl, an acidifier of urine, was found to inhibit urinary bladder tumor promotion by sodium ascorbate (Fukushima, 1991 ; Fukushima *et al.*, 1991).

The objective of the present studies was to further assess the modifying effects of DEM and NH₄Cl on BHA and NaHCO₃ promotion of urinary bladder carcinogenesis in male F344 rats initiated with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN). Lipid peroxidation concentrations were also measured in urinary bladder tissue after administration of agents either alone or in combination.

MATERIALS AND METHODS

Animals : Male F344/DuCrj rats, 5 weeks of age, were obtained from Charles River Japan, Inc., Kanagawa. They were housed five to a plastic cage with hardwood chips for bedding, and fed MF powdered diet (Oriental Yeast, Co., Ltd., Tokyo) and water *ad libitum*. Animals were maintained at a temperature of $22 \pm 2^\circ\text{C}$ and humidity of $55 \pm 10\%$ with a 12-hr light/dark cycle. After a one-week acclimation period, they were used in this investigation.

Chemicals : BBN was purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo. DEM, NH₄Cl, BHA and NaHCO₃ were obtained from Wako Pure Chemical Industries, Ltd., Osaka. Dose levels of test chemicals selected for this investigation were determined on the basis of results from previous studies for DEM (Hirose *et al.*, 1987 ; Hirose *et al.*, 1989) and other chemicals (Fukushima, 1991 ; Fukushima *et al.*, 1991).

Initiation-promotion study (experiment 1) : A total of 135 rats were divided randomly into 9 groups. Rats in all groups were given drinking water with 0.05% BBN for 4 weeks and then treated from weeks 5 to 36 as follows : group 1, 2% BHA in the diet plus 0.15% DEM in the drinking water ; group 2, 2% BHA+1% NH₄Cl in the drinking water ; group 3, 3% NaHCO₃ in the diet+0.15% DEM ; group 4, 3% NaHCO₃+1% NH₄Cl ; group 5, 2% BHA ; group 6, 3% NaHCO₃ ; group 7, 0.15% DEM ; group 8, 1% NH₄Cl ; group 9, untreated. The animals were observed daily for abnormalities and weighed every week for the first 6 weeks, and then at two week intervals. Food and water consumption were measured over a 2-day period before each time of weighing.

At weeks 12, 24 and 36, fresh urine samples were obtained from at least 5 rats in each group, and urinary pH values measured using a pH meter (Hitachi-Horiba Co., Kyoto). In addition, urine samples collected over a 4 hour period (9 a.m.–1 p.m.) were measured for volume and then analyzed for electrolyte concentrations (Na⁺, K⁺, Ca²⁺, Mg²⁺ and Cl⁻).

At week 36, all animals were deprived of food, but not water, overnight and then killed under ether anesthesia. The livers and kidneys

were removed, weighed, and fixed in 10% phosphate buffered formalin solution. The urinary bladders were fixed by inflation with and immersion in the same fixative, halved, weighed and examined under a dissecting microscope. They were then cut into 8 strips, embedded in paraffin, sectioned, stained with hematoxylin and eosin and examined histopathologically. For quantitative analysis, proliferative transitional cell lesions of the urinary bladder were counted microscopically, the total length of the basement membrane being measured with a color video image processor (SPICCA-II; Nippon Avionics Co., Ltd., Tokyo), and numbers of lesions per 10 cm of basement membrane recorded (Fukushima *et al.*, 1983).

Lipid peroxidation (experiment 2): Ninety male rats were randomly divided into 9 equal groups. They were treated as in experiment 1 until the termination at week 8. Treatment period of test chemical for the analysis of tissue lipid peroxide level was decided from our earlier studies, in which morphological alterations were developed (Hirose *et al.*, 1987; Ito *et al.*, 1991). At autopsy, urinary bladders were resected at once, immersed in ice-chilled phosphate buffer solution and immediately homogenized and centrifuged. Obtained supernatant fractions were

assayed for lipid peroxides (LPO; Kyowa Medex Co., Ltd., Tokyo) by catalytic reaction of hemoglobin (Shibata *et al.*, 1991; Yagi, 1976).

Statistical analysis: The significances of inter-group differences in numerical data obtained for final body weights, organ weights, and quantitative histopathological findings were assessed using the two-sided Student's *t*-test. Insufficient homogeneity of variance was corrected with respect to the degrees of freedom according to Welch.

RESULTS

Antemortem examinations: No clinical abnormalities or mortalities related to the test chemical administration were apparent in any group during the experiment period. Data for body weights, BBN and test chemical intakes, and food and water intakes are summarized in Table 1. Body weight gain was clearly retarded in rats given BHA or DEM (groups 1–3, 5 and 7). During the 4 week initiation period, average BBN intakes in groups 1–9 were 51–53 mg/kg/day, with no significant inter-group differences being apparent. Rats given BHA+NH₄Cl (group 2), NaHCO₃+NH₄Cl (group 4), or NaHCO₃ (group 6) drank more water than the controls (group 9),

Table 1. Data for average final body weights, BBN or test chemical intake and food and water intake.

Group and treatment ^a	No. of rats examined	Final Body Weight (g)	Average BBN intake weeks 0–4 (mg/kg/day)	Average test chemical intake weeks 5–36 (mg/kg/day)	Average daily consumption weeks 5–36 (g/rat/day)	
					Food	Water
1 BBN→BHA+DEM	15	340±15**	53	902+ 82	14.6	17.7
2 BBN→BHA+NH ₄ Cl	15	368±19**	51	925+745	15.9	25.7
3 BBN→NaHCO ₃ +DEM	15	385±23**	52	1243+ 82	16.8	19.3
4 BBN→NaHCO ₃ +NH ₄ Cl	15	433±10*	52	1186+708	15.3	27.3
5 BBN→BHA	15	370±18**	52	942 —	16.2	21.2
6 BBN→NaHCO ₃	15	430±19	52	1211 —	15.6	25.3
7 BBN→ DEM	15	373±13**	52	— 65	13.5	15.0
8 BBN→ NH ₄ Cl	15	408±26	52	— 579	14.1	21.5
9 BBN only	15	421±19	52	— —	14.8	20.7

^a BBN, DEM and NH₄Cl were added to the drinking water, and NaHCO₃ and BHA were incorporated into the diet (see Materials and Methods).

Significantly different from control (group 9) values : *P<0.05 ; **P<0.01.

while water consumption in rats receiving BHA + DEM (group 1), or DEM (group 7) were less than the control value. Food consumption by the treated groups was essentially similar to the control level.

Urinalysis : Urinary pH in rats administered NH_4Cl with or without BHA (groups 2 and 8) was remarkably reduced at all time points. BHA and DEM did not affect urinary pH or sodium ion levels. NaHCO_3 markedly increased both of these parameters, and this was normalized by simultaneous treatment with NH_4Cl . Urinary chloride ion levels were obviously increased in rats receiving NH_4Cl with or without other chemicals (groups 2, 4 and 8). Other parameters examined were considered to be within the normal ranges.

Gross findings and urinary bladder weights : Macroscopically, large nodules were observed on the urinary bladder luminal surfaces in rats given BHA, with or without DEM or NH_4Cl (groups 1, 2 and 5), whereas only minute or small nodules were found in rats receiving DEM (group 7), NH_4Cl (group 8) or no supplement (group 9). The severity of gross lesion development in rats which received BHA + DEM (group 1) was similar to that in rats given BHA (group 5). Large nodules were also evident in animals exposed to NaHCO_3 (groups 3, 4 and 6), but the extent of development of such gross lesions in rats given NaHCO_3 + DEM or NaHCO_3 + NH_4Cl (groups 3 and 4) was much less than in the NaHCO_3 alone

case (group 6).

The urinary bladder-to-body weight ratios in rats given BHA + NH_4Cl or BHA alone (groups 2 and 5) were significantly increased as compared to control (group 9) values (Table 2). Rats receiving NaHCO_3 (group 6) similarly showed higher values. The liver- and kidney-to-body weight ratios in rats given either BHA or DEM (groups 1-3, 5 and 7) were also significantly greater than control values, presumably due to the low body weights in these groups.

Histopathology of the urinary bladder : Quantitative data regarding preneoplastic and neoplastic lesions of the urinary bladder epithelium are summarized in Table 3. The lesions observed were classified as simple hyperplasia, PN hyperplasia, papilloma and carcinoma on the basis of the criteria described previously (Fukushima *et al.*, 1983). Simple hyperplasia was evident in all rats initiated with BBN, and was excluded from the quantitative analysis. Significant increases in the incidence of PN hyperplasia were apparent in rats given BHA + DEM (group 1) when compared to the controls (group 9). Quantitative analysis revealed significantly increased densities of papillary or nodular (PN) hyperplasia in rats which received BHA + DEM or BHA alone (groups 1 and 5), as compared to the controls. The density of PN hyperplasias also tended to be increased in rats given BHA + NH_4Cl . The density of papillomas in rats given BHA + DEM (group 1) was significantly increased. The inci-

Table 2. Relative organ weight data.

Group and treatment	No. of rats examined	Relative organ weights		
		Liver (%)	Kidneys (%)	Urinary bladder (%)
1 BBN→BHA + DEM	15	3.11 ± 0.13**	0.73 ± 0.04**	0.052 ± 0.014
2 BBN→BHA + NH_4Cl	15	3.14 ± 0.26**	0.73 ± 0.06**	0.061 ± 0.020*
3 BBN→ NaHCO_3 + DEM	15	2.34 ± 0.10*	0.67 ± 0.03**	0.057 ± 0.022
4 BBN→ NaHCO_3 + NH_4Cl	15	2.25 ± 0.09	0.60 ± 0.02**	0.057 ± 0.027
5 BBN→BHA	15	3.04 ± 0.16**	0.66 ± 0.04**	0.060 ± 0.022*
6 BBN→ NaHCO_3	15	2.15 ± 0.08	0.58 ± 0.03	0.066 ± 0.023
7 BBN→ DEM	15	2.39 ± 0.11**	0.65 ± 0.02**	0.046 ± 0.009
8 BBN→ NH_4Cl	15	2.27 ± 0.08	0.61 ± 0.03**	0.046 ± 0.017
9 BBN only	15	2.23 ± 0.14	0.57 ± 0.03	0.045 ± 0.012

Significantly different from control (group 9) values : * $P < 0.05$; ** $P < 0.01$.

dence of transitional cell papillomas was significantly reduced in rats fed NH_4Cl (group 8), while in the BHA-treated groups a tendency for increase was observed. The incidence of transitional cell carcinomas was significantly increased in rats fed BHA (group 5). No carcinomas were evident in rats exposed to NH_4Cl or receiving no supplement (groups 8 and 9).

The incidences and densities of PN hyperplasias in rats treated with NaHCO_3 (groups 3, 4 and 6) were clearly increased when compared to controls (group 9). However, the densities of PN hyperplasias in rats given $\text{NaHCO}_3 + \text{DEM}$ or

NH_4Cl (groups 3 and 4) were significantly reduced when compared to the NaHCO_3 alone case (group 6). Incidences and densities of papillomas in groups 3, 4 and 6 tended to be increased, the increase in value for density being statistically significant. Incidences of carcinomas were significantly increased in groups 3, 5 and 6.

Analysis of lipid peroxidation: No increases of lipid peroxidation level in urinary bladder tissue were observed in rats treated with BHA, DEM, NH_4Cl , BHA + NH_4Cl or $\text{NaHCO}_3 + \text{DEM}$ (Table 4). A slight but significant lowering was seen in rats exposed to BHA + DEM (group 1) or

Table 3. Histopathological findings for the urinary bladder at week 36 in male F344 rats initiated by BBN and then treated with test chemicals alone or in combination.

Group and treatment	No. of rats	PN hyperplasia		Papillomas		Carcinomas
		Cases (%)	Density ^a	Cases (%)	Density ^a	Cases (%)
1 BBN→BHA + DEM	15	14 (93)*	$3.7 \pm 2.1^*$	10 (67)	$1.0 \pm 1.1^*$	2 (13)
2 BBN→BHA + NH_4Cl	15	11 (73)	4.1 ± 6.1	8 (53)	0.7 ± 1.1	3 (20)
3 BBN→ $\text{NaHCO}_3 + \text{DEM}$	15	14 (93)*	$2.8 \pm 2.1^{*,+}$	9 (60)	0.9 ± 1.3	5 (33)*
4 BBN→ $\text{NaHCO}_3 + \text{NH}_4\text{Cl}$	15	15 (100)**	$3.1 \pm 1.7^{*,+}$	8 (53)	0.8 ± 1.1	4 (27)
5 BBN→BHA	15	13 (87)	$3.3 \pm 2.1^*$	8 (53)	0.8 ± 1.1	5 (33)*
6 BBN→ NaHCO_3	15	15 (100)**	$5.0 \pm 2.4^*$	9 (60)	$1.0 \pm 1.1^*$	8 (53)**
7 BBN→ DEM	15	12 (80)	1.3 ± 1.1	4 (27)	0.4 ± 0.7	1 (7)
8 BBN→ NH_4Cl	15	6 (40)	0.4 ± 0.5	0 (0)*	—	0 (0)
9 BBN only	15	9 (60)	0.7 ± 0.7	5 (33)	0.3 ± 0.5	0 (0)

^a Density is expressed as the number of lesions per 10 cm of basement membrane. Data are mean $\pm$ S.D. values.

*, ** $P < 0.05$, 0.01 vs group 9.

⁺ $P < 0.05$ vs group 6.

Table 4. Levels of lipid peroxidation in bladder tissue at weeks 8 in experiments 2.

Group and treatment	No. of rats	Lipid peroxidation ^a (nmoles MDA/mg protein)
1 BBN→BHA + DEM	10	$0.25 \pm 0.08^*$
2 BBN→BHA + NH_4Cl	10	0.29 ± 0.19
3 BBN→ $\text{NaHCO}_3 + \text{DEM}$	10	0.36 ± 0.09
4 BBN→ $\text{NaHCO}_3 + \text{NH}_4\text{Cl}$	10	$0.61 \pm 0.07^{*,++}$
5 BBN→BHA	10	0.35 ± 0.17
6 BBN→ NaHCO_3	10	$0.25 \pm 0.07^*$
7 BBN→ DEM	10	0.28 ± 0.10
8 BBN→ NH_4Cl	10	0.47 ± 0.09
9 BBN only	10	0.44 ± 0.11

^a Measured as the concentration of malondialdehyde (MDA).

* $P < 0.05$, vs group 9.

⁺⁺ $P < 0.01$ vs group 6.

NaHCO₃ (group 6) as compared to no supplement (group 9). Animals receiving NaHCO₃ + NH₄Cl demonstrated significant increase over control or NaHCO₃ alone levels.

DISCUSSION

In the present study, we confirmed the promoting activity of BHA for urinary bladder carcinogenesis in rats initiated with BBN. This phenolic antioxidant earlier showed striking enhancement of bladder tumor development in rats initiated with either BBN (Imaida *et al.*, 1983) or the wide-spectrum carcinogen N-methyl-N-nitrosourea (Imaida *et al.*, 1984). No modification of BHA action by concurrent treatment with DEM, a tissue glutathione depleting agent (Meister, 1983), or NH₄Cl, a urine acidifier (Fukushima, 1991; Fukushima *et al.*, 1991) was evident in the present data.

During metabolism of BHA, including redox cycling, active oxygen species could be formed, and these free radicals could bind to lipid and induce lipid peroxidation (Ito and Hirose, 1989). There is ample evidence that lipid peroxidation is well correlated with cytotoxicity and/or promotion of carcinogenesis (Casini *et al.*, 1985; Denke *et al.*, 1985; Mgbodile *et al.*, 1975; Mizutani *et al.*, 1984; Richardson *et al.*, 1975). BHA-induced lipid peroxidation in the bladder tissue is therefore a possible mechanism underlying the observation of increased DNA synthesis (Shibata *et al.*, 1989) and strong bladder tumor promotion (Imaida *et al.*, 1984; Imaida *et al.*, 1983). Depletion of intracellular GSH would in this case be expected to potentiate BHA action, since this major endogenous reducing agent protects against lipid peroxidation. However, it was earlier demonstrated that DEM in fact prevents the induction of squamous cell hyperplasia of the forestomach in rats by BHA (Hirose *et al.*, 1987), and also inhibits its promoting activity for forestomach carcinogenesis in rats initiated with N-methyl-N'-nitro-N-nitrosoguanidine (Hirose *et al.*, 1989; Shibata *et al.*, 1993). These results might be related to the finding that DEM could cause an increase in GSH levels in the rat forestomach (Hirose *et al.*, 1989), in clear contrast to the depletion reported in several organs of small rodents (Casini *et al.*, 1985; Denke *et al.*, 1985; Mgbodile *et al.*, 1975; Mizutani *et al.*, 1984; Richardson *et al.*, 1975).

al., 1985; Mgbodile *et al.*, 1975; Mizutani *et al.*, 1984; Richardson *et al.*, 1975).

Lipid hydroperoxide levels in the urinary bladder tissue were not affected by BHA and DEM treatment in the current study, in agreement with previous results for forestomach epithelial tissue (Ito *et al.*, 1991). Unfortunately, GSH levels could not be measured in the present study, because of the limited amounts of epithelial tissue available from rat bladders.

The tumor promoting activity of BHA was also not affected by simultaneous administration of NH₄Cl used as an urine acidifier. On the other hand, NH₄Cl exerted clear inhibiting effects on NaHCO₃ promotion of urinary bladder tumor development in the present rats initiated with BBN. This might have been mediated via reducing effects on urinary sodium ion content and pH (Fukushima, 1991; Fukushima *et al.*, 1991). DEM, which would not be expected to influence these parameters, also showed inhibition of NaHCO₃ promotion of urinary bladder carcinogenesis. This result indicated that its effects on sodium-salt type bladder tumor promoters might be mediated by a different mechanism. To elucidate this point, further investigations are needed.

In conclusion, the present study revealed that BHA promoting effects in the urinary bladder are apparently not mediated by lipid peroxidation. The precise mechanism(s) underlying promotion of urinary bladder carcinogenesis remain future investigation.

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