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Promoting Effect of Sodium Chloride in 2-Stage Urinary Bladder Carcinogenesis in Rats Initiated by N-Butyl-N-(4-hydroxybutyl)-nitrosamine

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Summary. The promoting effect of sodium chloride (NaCl) in 2-stage urinary bladder carcinogenesis in F344 rats initiated by 2 doses of N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) was investigated. The incidences of PN hyperplasia were significantly higher in rats initiated with 0.01 or 0.05% BBN when they were given diet containing 10% NaCl for 32 weeks than when they were given control diet. The incidence of papilloma in rats given 0.05% BBN followed by diet containing 10% or 5% NaCl tended to be higher than that in control rats. The urine of rats given diet containing NaCl was larger in volume and had lower osmolality than that of controls. The total urinary sodium and chloride contents were also increased, whereas those of potassium and phosphorus were decreased. No calculus formation or crystalluria was observed. These data suggest that excess intake of sodium as NaCl has a weak promoting effect in 2-stage urinary bladder carcinogenesis.

Key words: Rat bladder carcinogenesis, Initiation and promotion, N-Butyl-N-(4-hydroxybutyl)nitrosamine, Urinary composition, Sodium chloride.

Introduction

Two-stage carcinogenesis, by initiation and promotion, first shown in mouse skin [1, 2, 23] has also been demonstrated in the urinary bladder [5, 8–14, 17, 20, 22]. There are numerous reports on promoters of bladder carcinogenesis in experimental animals. In the bladder, sodium saccharin has been shown to be a promoter [5, 8, 13], and we recently reported that the sodium salts of L-ascorbate, erythorbate and o-phenylphenate were also promoters in rats, whereas salts from other cations were not [10–12]. These findings suggest that sodium may have a promoting effect in urinary bladder carcinogenesis. In gastric carcinogenesis, previous studies have shown that NaCl promotes N-methyl-N'-nitrosoguanidine (MNNG) induction of tumors of the forestomach [25] and glandular stomach [19, 26] in rats. In the present study, we examined the effect of NaCl as a promoter in two-stage urinary bladder carcinogenesis.

Materials and Methods

Animals

A total of 325 male F344 rats of 6 weeks old (Charles River Japan, Inc., Kanagawa, Japan) was used. The animals were housed 5 to a plastic cage with hardwood chips for bedding and were given free access to water and diet under controlled temperature (22 ± 2 °C), humidity (50 ± 10%) and lighting (12 h – 12 h light-dark cycle).

Chemicals

BBN was obtained from Izumi Chemical Co., Yokohama, Japan. NaCl (for additive use) was obtained from Wako Pure Chemical Industries, Osaka. NaCl was incorporated into Oriental M (Oriental Yeast Co., Tokyo) basal diet at concentrations of 10 and 5%, respectively, and the mixtures were made into pellets.

Experiment 1

Rats were randomly divided into 5 groups of 50 rats each. The rats in groups 1 to 3 were given drinking water containing 0.01% BBN for 4 weeks and then NaCl at dietary concentrations of 10% (group 1), 5% (group 2) and 0% (group 3) for 32 or 64 weeks, respectively. Groups 4 and 5 were given drinking water without BBN for 4 weeks and then 10% (group 4) or 5% (group 5) NaCl in their diet for 32 or 64 weeks, respectively. Half the rats in each group were sacrificed in week 36 and half in week 68 after the beginning of the experiment.

Experiment 2

Rats were randomly divided into 3 groups of 25 rats each. Groups 1 and 2 were given 0.05% BBN in their drinking water for 4 weeks and then diet containing 10 or 5% NaCl, respectively, for 32 weeks. Group 3 acted as a control and was given only 0.05% BBN for 4 weeks. The total observation period was 36 weeks.

Table 1. Average body weights, BBN intake (up to 4 weeks), water consumption (5 to 36 weeks), and NaCl intake (5 to 36 weeks) of rats in Experiments 1 and 2

Group	Treatment		Body wt (g)		BBN intake	Water consumption	NaCl intake
	BBN (%)	NaCl (%)	Initial	Final	(mg/kg/day)	(g/rat/day)	(mg/kg/day)
Experiment 1							
1	0.01	10.0	123	372[a]	11.3	89	5,150
2	0.01	5.0	121	390[b]	11.6	42	2,157
3	0.01	–	120	405	11.2	21	–
4	–	10.0	120	361	–	88	5,154
5	–	5.0	120	395	–	41	2,211
Experiment 2							
1	0.05	10.0	140	368[a]	42.6	69	5,434
2	0.05	5.0	139	382[b]	44.9	34	2,618
3	0.05	–	142	399	44.7	15	–

[a] Significantly different from group 3 at $P < 0.01$
[b] Significantly different from group 3 at $P < 0.05$

For urinary analyses in Experiments 1 and 2, urine samples were obtained from 10 rats in each group in both experiments before their sacrifice. The pH of fresh specimens of urine was determined with pH paper (bromothymol blue and methyl red) (Toyo Roshi Co., Tokyo, Japan). Rats were placed in individual metal metabolic cages without food and water for collection of urine samples over a 4 h period. The weight of urine in the 4 h period was measured. Osmolality was determined by freezing-point depression using an osmometer (Osmette A, Percision System Inc., Mass., USA). Urine-testing strips (Multistix, Miles-Sankyo Co., Ames Division) were used for examination of hematuria. The remainder of urine specimens was centrifuged and the precipitate was examined for epithelial cells, red blood cells, white blood cells, casts and crystals. The urinary bladder was ligated at the neck, inflated by intraluminal injection of 10% phosphate-buffered formalin and excised. After fixation, the urinary bladder was carefully opened and cut longitudinally into 8 strips. The strips were embedded in paraffin, sectioned at 3–5 μm, and stained routinely with hematoxylin and eosin.

For quantitative analyses, urinary bladder lesions were counted by light microscopy, the total length of the basement membrane was measured with a color video image processor (VIP-21CH; Olympus-Ikegami Tsushin Co., Tokyo, Japan), and numbers of lesions per 10 cm of basement membrane were calculated.

Experiment 3

Fifteen male F344 rats which were 7 weeks old at the beginning of the experiment were used for the urine analyses. These rats were divided into groups of 5 rats each and given diets containing 10, 5 and 0% NaCl for 3 weeks. In urine analyses, parameters were measured twice during the 3 weeks period. Fresh urine samples were obtained from rats in each group by forced micturition at 08.00 h. The urinary pH was determined with a pH meter (Hitachi-Horiba, F-8DP, Tokyo) with a microelectrode. In pH-determinations, the portion voided first was excluded, because it might contain bacteria and foreign matter from the external genitalia. Since urinary excretion by rats was shown to be greatest at night [6], values for overnight samples were examined. The rats were placed in individual metal metabolic cages with plastic flasks for 24-h periods (09.00 h–09.00 h) and given free access to water and food. The volume and osmolality of urine samples collected over a 24-h period in each group were measured. Then portions were used for measurements of sodium and potassium by flame photometry (with a Flame-30C photometer; Jasco Medical Industries Inc.), for chloride with a chloride meter (Model CL-12; Jasco Medical Industries, Inc.), calcium (by the cresol-phthalein complexone method), magnesium (by a colorimetric method based on the reaction with Calmagite), and phosphorus (by a modification of the phosphomolybdate method in an Olympus Optical Co., Model ACA-8000 chemical analyser). Analyses of urinary electrolytes were carried out at the Chunichi Clinical Center, Ohgaki, Japan.

Statistical Analysis

The significances of differences between groups in body weights, numbers of bladder lesions per 10 cm of basement membrane and values in urinary analyses were examined by Student's t test. The significances of differences in tumor incidences in different groups were analysed by the one-sided Fisher's exact probability test.

Results

Experiment 1

The general condition of rats given NaCl in their diet was good throughout the study. Apparent effects of NaCl were diarrhoea, and soaking of fur in the perineal region because of polyuria. Data on the body weights, BBN intakes, water consumption, and NaCl intake of rats sacrificed in week 36 are shown in Table 1. The final average body weight of groups 1 and 2 given diet containing NaCl was significantly lower than that of group 3. The final average body weight of groups 4 and 5 given diet containing NaCl without BBN was also lower than that of the control. There was no difference in the average BBN intake of groups given BBN for 4 weeks. Food consumption was similar in the different groups (15 to 20 g/day/rat). Water consumption was in-

Table 2. Histopathological findings in the urinary bladder of rats treated with BBN followed by NaCl

Group	Treatment		Effective No. of rats	Simple hyperplasia	Papillary or nodular hyperplasia		Papilloma		Carcinoma	
	BBN (%)	NaCl (%)		Incidence	Incidence	No./10 cm of BM[a]	Incidence	No./10 cm of BM	Incidence	No./10 cm of BM
Experiment 1										
36 weeks period										
1	0.01	10.0	25	17 (68)[b, c]	15 (60)[d]	0.82 ± 0.88[e]	8 (32)	0.24 ± 0.37	0	0
2	0.01	5.0	25	19 (76)[b]	13 (52)	0.80 ± 1.06	6 (24)	0.24 ± 0.46	0	0
3	0.01	–	25	7 (28)	8 (32)	0.40 ± 0.62	8 (32)	0.29 ± 0.53	0	0
4	–	10.0	25	0	0	0	0	0	0	0
5	–	5.0	25	0	0	0	0	0	0	0
68 weeks period										
1	0.01	10.0	25	25 (100)	22 (88)	1.22 ± 0.95	16 (64)	0.55 ± 0.50	15 (60)	0.54 ± 0.54
2	0.01	5.0	21	20 (95)	18 (86)	1.76 ± 1.30	12 (57)	0.46 ± 0.58	8 (38)	0.31 ± 0.44
3	0.01	–	24	23 (96)	22 (92)	1.95 ± 1.74	15 (63)	1.02 ± 1.24	11 (46)	0.68 ± 0.88
4	–	10.0	25	0	0	0	0	0	0	0
5	–	5.0	25	0	0	0	0	0	0	0
Experiment 2										
1	0.05	10.0	25	25 (100)[b]	24 (96)[b]	1.16 ± 0.70	13 (52)	0.41 ± 0.53	2 (8)	0.04 ± 0.15
2	0.05	5.0	24	23 (96)[b]	19 (79)	3.24 ± 4.14[b]	14 (58)	0.44 ± 0.55	4 (17)	0.09 ± 0.20
3	0.05	–	24	16 (67)	18 (75)	0.73 ± 0.82	7 (29)	0.25 ± 0.43	3 (13)	0.08 ± 0.21

[a] BM, basement membrane
[b] Significantly different from group 3 at $P < 0.01$
[c] Numbers in parentheses are percentages
[d] Significantly different from group 3 at $P < 0.05$
[e] Mean ± S.D.

Table 3. Urinary characteristics of rats fed NaCl in Experiment 3

Group	Treatment	No. of rats	pH	Volume (gram)	Osmolality (m Osm/kg H_2O)	Electrolytes					
						(m Eq/l)				(mg/dl)	
						Na	Cl	K	Ca	P	Mg
1	10% NaCl	5	6.54 ± 0.41[a]	29.7 ± 5.1[b]	1,333 ± 154[b]	438 ± 48[b]	493 ± 58[b]	83.5 ± 13.0[b]	6.4 ± 1.0	100 ± 10[b]	13.2 ± 3.7
2	5% NaCl	5	6.73 ± 0.28	19.3 ± 6.8[b]	1.738 ± 315[b]	498 ± 54[b]	526 ± 58[b]	145.3 ± 52.2[b]	5.5 ± 1.2	120 ± 34[b]	3.7 ± 3.6
3	Basal diet	5	6.84 ± 0.43	4.9 ± 0.8	2,453 ± 150	197 ± 18	248 ± 5	400.5 ± 22.8	8.0 ± 2.7	258 ± 56	22.4 ± 27.9

[a] Mean ± S.D.
[b] Significantly different from group 3 at $P < 0.01$

Fig. 1. The urinary bladders were larger in rats given NaCl than in control rats

creased 4-fold and 2-fold in rats given diets containing 10 and 5% NaCl, respectively, compared with that of the controls from week 5 to the end of experiment. Data on the body weight, BBN intake, water consumption and NaCl intake of rats sacrificed in week 68 showed similar trends to those of the rats sacrificed in week 36.

Urinalyses in weeks 36 and 68 showed significant increases in the urinary volume and a significant decrease of osmolality in groups treated with NaCl. Hematuria was detected grossly or with urine-test strips and by microscopic examination in weeks 36 and 68 in rats in groups 1 to 3 given BBN. The incidence of hematuria was high in rats in groups 1 to 3 in week 68, but there was no apparent difference in the incidences of hematuria in these three groups. No crystalluria or calculus formation was observed.

The histopathological lesions of the urinary bladder observed in each group are summarized in Table 2. Bladder lesions were classified as described previously [10, 16] as simple hyperplasia, papillary or nodular hyperplasia (PN hyperplasia), papilloma and carcinoma.

Histopathology of Urinary Bladder in Week 36. No carcinoma was found in any rats sacrificed in week 36. The incidence of simple hyperplasia and PN hyperplasia was significantly higher in group 1 than in the control group 3. However, there was no difference in the number of PN hyperplasia per 10 cm of basement membrane or the incidence or number of papillomas in groups 1 to 3. The incidence of simple hyperplasia was significantly higher in groups 1 and 2 than in control group 3. Treatment with NaCl alone did not induce any lesions of the urinary bladder.

Histopathology of Urinary Bladder in Week 68. There was no significant difference in the incidence of bladder lesions in experimental and control groups. The bladder was larger in all rats given NaCl (Groups 1, 2, 4, and 5) than in control rats (Fig. 1). Rats given only NaCl diet (groups 4 and 5) had no lesions of the urinary bladder.

Experiment 2

The body weight of rats given 10 or 5% NaCl in their diet was less than that of controls (Table 1). The BBN intake in different groups was almost the same. Water consumption was increased in rats given NaCl in the diet, as in Experiment 1 (Table 1). Results of urinalyses showed similar trends to those in Experiment 1; namely, a significant increase in urine volume and a decrease in osmolality in groups given NaCl. The bladders of rats in groups 1 and 2 were also larger and compared with the bladders of rats given NaCl in Experiment 1.

The lesions of the urinary bladder are shown in Table 2. The incidence of simple hyperplasia was significantly higher in groups 1 and 2 given diet containing NaCl than in control group 3. The incidence of PN hyperplasia was significantly higher in group 1 than in group 3, but that in group 2 was not significantly different from that in group 3, although the number of PN hyperplasia per 10 cm of basement membrane was significantly higher in group 2 than in group 3. The incidence of papilloma was slightly, but not significantly, increased in groups 1 and 2. The incidence or number of carcinoma in groups 1 and 2 did not differ significantly from those in control group 3. Treatment with NaCl alone did not produce any bladder lesions detectable by light microscopy.

Experiment 3

Results of the first urinalyses in Experiment 3 are presented in Table 3. The urinary pH values (measured with a pH meter) were not significantly different between the different groups. The volume of urine was increased 6-fold and 4-fold in rats given diet containing 10% or 5% NaCl, respectively, as compared with that of controls. The osmolalities of the urine of rats given NaCl were significantly lower than that of control animals. Diet containing 10 or 5% NaCl significantly increased the amount of sodium and chloride excreted. In contrast, the amount of potassium and phos-

phorus excreted was decreased dose-dependently by NaCl in the diet. No other differences were observed. Results of the second urinalyses showed similar trends.

Discussion

In the present study, we examined the promoting effect of NaCl in the diet on two-stage urinary bladder carcinogenesis initiated in rats by two doses of BBN. After treatment with either dose of BBN, diet containing 10% NaCl significantly increased the incidence of PN hyperplasia in week 36. After treatment with 0.05% BBN, diet containing 10% NaCl increased the incidence of papilloma slightly, but not significantly, but in rats given diet containing NaCl for 68 weeks, the incidence of urinary bladder lesions was not significantly greater than in controls. Thus, the results showed that NaCl has weak promoting activity in two-stage urinary bladder carcinogenesis.

Studies in this laboratory have demonstrated various bladder promoters [9–12, 17]. We found that sodium saccharin, sodium L-ascorbate, sodium erythorbate and sodium o-phenylphenate were promoters only as their sodium salts [10–12]. The sodium salts of bladder promoters altered the composition of the urine, such as by increasing its pH value, its contents of $MgNH_4PO_4$ crystals and sodium, and decreasing its osmolality [10–12, 24]. Moreover, these sodium salts produced morphological alterations, such as formation of pleomorphic microvilli, short or uniform microvilli and ropy or leafy microridges, on the surface of the urinary bladder of rats, detected by scanning electron microscopy [24]. Rats fed high sodium diet may excrete sodium in the urine, and this urinary sodium may produce alkaline urine, resulting in formation of $MgNH_4PO_4$ crystals. Nevertheless, in the present study no apparent increase of pH value or formation of $MgNH_4PO_4$ crystals was observed in rats fed diet containing NaCl. This may have been because the urine was too dilute due to a high water intake. This high water intake was associated with intake of salts and with diarrhoea. Previous investigations indicated that urinary crystals may be a contributory factor to induction of urinary bladder cancer [6, 7]. However, these crystals are not always associated with promoting activity. For instance, acetazolamide, which is a diuretic agent, had no promoting activity, but caused crystalluria [9, 21].

Previous studies in this laboratory have shown that the membrane potential of epithelium in the early stage of urinary bladder carcinogenesis increases significantly on treatment with BBN or sodium saccharin [15]. Since the apical membrane potential of the cell depends largely on permeability to Na^+, it reflects the activity of Na^+ channels, which play an essential role in Na^+ transport across the urinary bladder epithelium. Therefore, it seems likely that the consistently high excretion of Na^+ by rats in the present study resulted in unbalanced active transport of Na^+ across the urinary bladder epithelium. Thus, urinary sodium might have played an important role in two-step bladder carcinogenesis. A high intracellular sodium concentration is known to be related to rapid proliferation of cells in in vitro [3, 4] and NaCl induces epithelial hyperplasia of renal papilla in rats [18]. A more important factor is that the urinary pH is actually elevated, and these 2 factors cooperate in promoting urinary bladder carcinogenesis. In the present study, the amount of sodium excreted was increased, although the urinary pH was not elevated. Consequently, the development of BBN-induced bladder tumor in rats was not dramatically promoted by an increase in dietary NaCl. Further research is needed on the promoting activity of NaCl in two-stage urinary bladder carcinogenesis.

It is possible that excess intake of sodium as NaCl has a weak promoting effect on urinary bladder carcinogenesis.

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