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Induction of Ornithine Decarboxylase and Tumor Promotion by *N*-Methyl-*N*'-Nitro-*N*-Nitrosoguanidine, Sodium Chloride, and Dimethyl Itaconate

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Abstract—The possible tumor-promoting activities of sodium chloride (NaCl) and dimethyl itaconate (DMI), one of the quinone reductase inducers, were examined on stomach of male Wistar rats treated with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG). Administrations of NaCl and DMI after the initiation by MNNG resulted in various sized masses in the rat forestomach. Histopathologic studies showed that the combination of NaCl and DMI made an enhancing effect on the MNNG-induced carcinogenesis, resulting in papilloma in 5 weeks and squamous cell carcinoma in 20 weeks in submucosal area of forestomach. We also used an *in vivo* short-term method for evaluating possible tumor-promoting activity with ornithine decarboxylase (ODC) as a marker. The markable inductions of the ODC activities by MNNG, NaCl, and DMI were found in the pyloric mucosa of rat stomach in time-dependent manners. A single administration of MNNG induced ODC activity up to 288 pmol CO_2/hr/mg protein at 24 hr after the administration. NaCl caused induction of ODC with a maximum of 179 pmol CO_2/hr/mg protein at 8 hr after the administration. ODC was induced up to 539 pmol CO_2/hr/mg protein at 16 hr after the administration of DMI. Additional treatment of NaCl and NaCl plus DMI caused 2 fold and 7 fold increases, respectively, in the ODC activity of the MNNG-alone group at 24 hr after the administration. These results suggest that NaCl and DMI have promoting activities in the rat gastric carcinogenesis initiated by MNNG.

Keywords □ *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, sodium chloride, dimethyl itaconate, gastric carcinogenesis, tumor promotion, quinone reductase inducer, ornithine decarboxylase.

MNNG is one of the chemical carcinogens which act as alkylating agents. It has been shown that MNNG initiates the gastric carcinogenesis in rats. Dietary habit has been suggested to be a major environmental factor in gastric carcinogenesis (Habs and Schmahl, 1980). Some studies have been made on the effects of NaCl on experimental gastric carcinogenesis in rats. Takahashi *et al.* (1983) showed that 10% NaCl in the diet given concomitantly with MNNG increased both the incidence and the size of gastric tumors. Shirai *et al.* (1982) reported that administration of 1 m*l* of saturated NaCl solution before a single dose of MNNG increased the incidence of tumors in the glandular stomach. These results indicated that NaCl acted as a cocarcinogen in the initiation stage of gastric carcinogenesis by MNNG in rats. It has been also shown that administration of 10% NaCl in diet in the promotion stage enhanced the gastric carcinogenesis initiated by MNNG (Takahashi *et al.*, 1984). Ohgaki *et al.* (1984) also showed that the incidence of adenomatous hyperplasia in the glandular stomach was higher in rats given NaCl after MNNG treatment than in those given MNNG alone. These results suggested that NaCl also played a role in the promotion stage of gastric carcinogenesis by MNNG.

DMI is one of the anticarcinogenic enzyme inducers which elevates a phase II xenobiotic metabolizing enzyme, quinone reductase (Talalay *et al.*, 1988). We have shown previously, however, that additional treatment with DMI after MNNG plus 10% NaCl treatment markedly enhanced the incidences of papilloma and squamous cell carcinoma in rat forestomach, suggesting that DMI made a promoting effect on the forestomach

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carcinogenesis by NaCl in rats pretreated with MNNG (Kim *et al.*, 1990 and 1991). In this study, we examined the modifying effects of DMI and different concentrations of NaCl in the MNNG-initiated carcinogenesis.

Polyamines are cationic molecules produced in all living organisms. Synthesis of polyamines is associated with cell proliferation (Pegg, 1988). The first enzyme of the polyamine-biosynthetic pathway, ODC, is a highly regulated enzyme. ODC activity increases in response to various physiological stimuli (Persson *et al.*, 1984). Induction of ODC is one of the early events altered by most of the tumor promoters, including the well-reported skin tumor promoter 12-O-tetradecarnoylphobol-13-acetate(TPA) (Gupta and Mehrotra, 1987). Furihata *et al.* (1984 and 1987) reported that MNNG and NaCl induced the ODC activity in the pyloric mucosa of rats. They found that the ODC activity was not changed in the fundic mucosa and the forestomach epidermis. In the present study, we used a short-term *in vivo* method for evaluating the tumor-promoting activity of possible stomach tumor promoters, MNNG, NaCl, and DMI, with ODC activity as a marker.

Materials and Methods

Animals and Chemicals

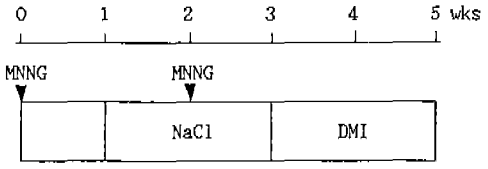
Male, 6-week-old Wistar rats, which were supplied from National Institute of Safety Research, Korea, were used. The rats were housed in polycarbonate cages with hard wood chips in an air-conditioned room (23± 2℃, 55± 10% R.H.) with a 12h light/12h dark cycle. Diets (Jeil sugar Co., Seoul, Korea) and drinking water were available *ad libitum*. MNNG was obtained from Fluka Chemie (Switzerland), and dissolved in 10 % dimethyl sulfoxide (DMSO) solution for intragastric intubation. NaCl, DMI, *E. coli* ornithine decarboxylase, NADPH, FAD, dicoumarol and 2,6-dichloroindophenol were purchased from Sigma Chem. Co. (St. Louis, MO, USA). L-[1-^{14}C] ornithine (50~60 mCi/mmol) was from Amersham (Buckinghamshire, England). Centerwell incubation flasks were from Kontes (Vineland, NJ, USA). Solvable and Atomlight were from NEN (Boston, MA, USA).

Pathological Observations

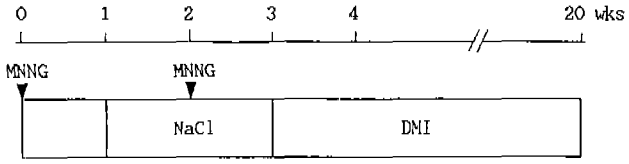
Text-Fig. 1 shows the experimental design and treatment of each animal group for pathological study. The rats were given MNNG solution (200 mg/kg b.w.) by gastric intubation twice at week 0 and 2 of the experiment. Starting at week 1, the animals were given 10% or 5% NaCl in diet for 2 weeks. After the treatment of NaCl, 0.2% DMI in drinking water was administered

Text-Fig. 1. Experimental design and treatment for pathological observations.

A. 5-week scheme



B. 20-week scheme



C. Animal treatment

Group	No. of animals	[a]Treatment
1	8	MNNG − 10% NaCl − 0.2% DMI
2	8	MNNG − 5% NaCl − 0.2% DMI
3	8	MNNG − − − 0.2% DMI
4	8	− − 10% NaCl − 0.2% DMI
5	8	− − 5% NaCl − 0.2% DMI
6	8	− − − 0.2% DMI
7	8	MNNG − 10% NaCl − −
8	8	MNNG − 5% NaCl − −
9	8	MNNG − − − −

[a]The detailed strategy for each treatment is described in Materials and Methods.

until the end of the experiments, for 2 weeks (in a 5-week experiment) or for 17 weeks (in a 20-week experiment). All rats were sacrificed at the end of each experiment. Immediately upon death, excised forestomachs were cut longitudinally into 4 strips and were fixed in 10% neutral buffered formalin solution for hematoxylin and eosin (H & E) staining.

Determination of Quinone Reductase Activity

Cytosol fractions of forestomach and liver of group 1 rats and forestomach of group 7 rats were prepared at the end of 5-week experiment as described in Benson *et al.* (1980). The dicoumarol-sensitive NADH: quinone reductase activity of the rat tissue cytosols was measured with 2,6-dichloroindophenol according to the method of Benson *et al.* (1980).

Determination of Ornithine Decarboxylase Activity

The rats were given a reduced amount of diet overnight. The following day they were treated with the chemicals written below by gastric intubation: MNNG in DMSO (200 mg/kg b.w.); NaCl in water (1.5 g/kg b.w.); DMI in corn oil (1.1 g/kg b.w.); 10% DMSO or corn oil as controls. DMI was diluted with 4 volumes of

corn oil to reduce the irritating effect of DMI on stomach. The rats were sacrificed by cervical dislocation under ether anesthesia after 0, 4, 8, 16, 24 and 48 hours. ODC activity was determined with L-[1-^{14}C] ornithine as substrate by a slight modification of the method of Furihata *et al.* (1985). The stomach was removed, opened along the greater curvature and washed with saline. The pyloric mucosa, fundic mucosa and forestomach epidermis were scraped off with a razor blade and homogenized in 50 mM sodium phosphate buffer (pH 7.2) containing 0.1 mM pyridoxal phosphate and 0.1 mM EDTA. A Potter Elvehjem-type homogenizer with a Teflon pestle was used for the pyloric and fundic mucosa and a sonic dismembrator (Fisher, model 300) for forestomach epidermis. The homogenates were centrifuged at 30,000 g for 20 min at 4℃, and the supernatants were used as enzyme extracts. The enzyme reaction was performed in a 10-ml center-well incubation flask with a side arm and a disposable center well. Portions (200 μl) of the enzyme extract were incubated for 1 hour after addition of 200 μl of L-[1-^{14}C] ornithine (0.25 μCi diluted with 0.2 μmol of cold L-ornithine), 200 μl of buffer solution containing 160 mM sodium phosphate (pH 7.2), 0.7 mM pyridoxal phosphate and 1.6 mM dithiothreitol to the bottom of the flask. The enzyme reaction was terminated by addition of 300 μl of 2M citric acid to the reaction mixture *via* the side arm by syringe, followed by an additional incubation for 30 min. The released $^{14}CO_2$ was trapped in a piece of Whatman GF/C glass fiber filter containing 50 μl of Solvable (NEN) which was placed in the center well. The filter was removed and its radioactivity was determined in 10 ml of a cocktail solution, Atomlight (NEN), with a Packard 2000-CA liquid scintillation counter. The protein content of the enzyme extract was determined by the method of Bradford (1976).

Results

Pathological Observations

As shown in Fig. 1, treatment of NaCl and DMI after initiation by MNNG resulted in various sized masses in the forestomach of the rats both in the 5-week and the 20-week experiments. The lesions were found neither in the glandular nor in the pylorus area of the stomach. Rats treated with MNNG, NaCl, and DMI (groups 1 and 2) showed stronger gross lesions than those treated with either MNNG plus DMI (group 3) or MNNG plus NaCl (groups 7 and 8) or MNNG only (group 9). These results suggest the synergistic effects

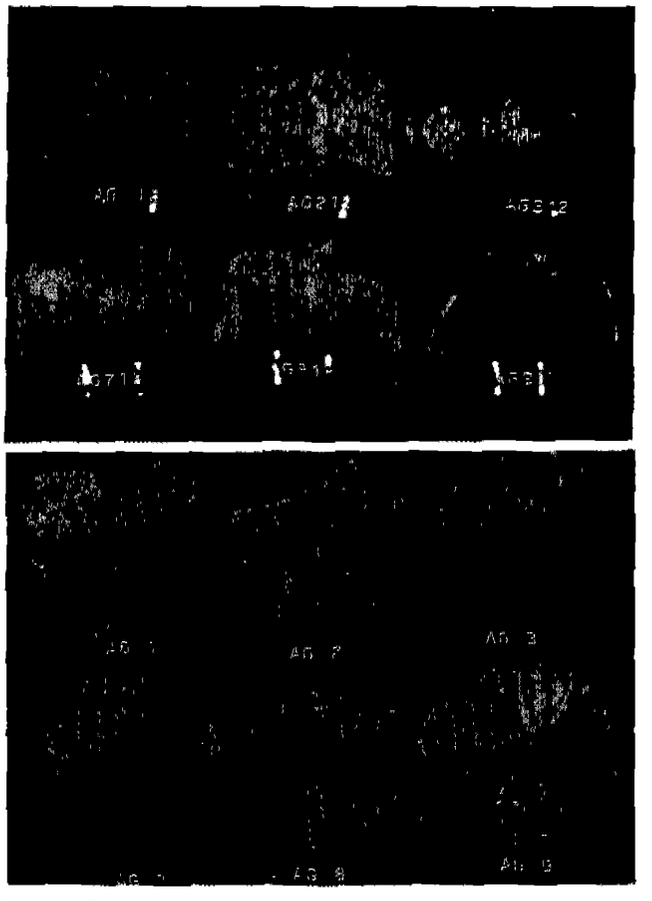


Fig. 1. Gross findings observed in the rat stomach. (A) 5-week experiment. (B) 20-week experiment. Each number represents the following experimental group as described in Materials and Methods; AG1 and AG112: group 1; AG2 and AG112: group 2; AG3 and AG312: group 3; AG7 and AG713: group 7; AG8 and AG812: group 8; AG9 and AG912: group 9.

of NaCl and DMI on MNNG-induced carcinogenesis. Rats in the groups 4, 5, and 6, which were not treated with MNNG, did not show any gross lesion in the stomach (data not shown). Microscopically, the lesions in the forestomach could be classified into hyperplasia (Fig. 2B) and papilloma (Fig. 2C) in the 5-week experiment, and squamous cell carcinoma (Fig. 2D) in the 20-week experiment.

Effect of DMI on the Quinone Reductase Activity

The dicoumarol-sensitive NADH: quinone reductase activity of rat tissue cytosols, measured with 2,6-dichloroindophenol, is shown in Table I. Administration of 0.2% DMI in drinking water for 2 weeks resulted in significant increase of cytosol quinone reductase specific activity in the forestomach of the rats pretreated with MNNG and NaCl. The specific activity of quinone reductase in the forestomach of the rats treated with DMI was increased twice as the control value.

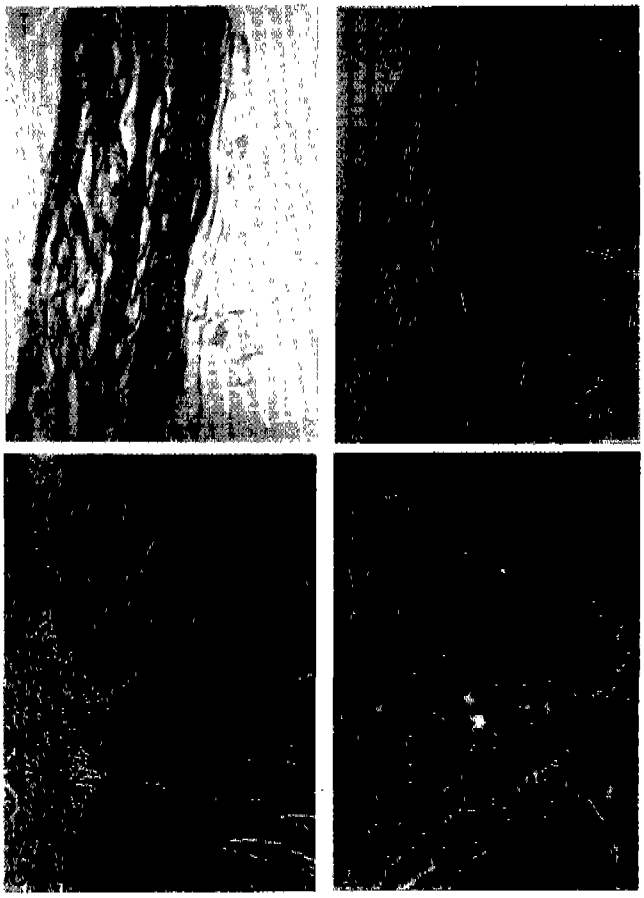


Fig. 2. Microscopic observations. (A) Forestomach epithelium in a normal rat. H & E stain, ×200. (B) Hyperplasia. Hyperkeratosis and basal cell hyperplasia observed in the forestomach of a rat in group 3, 5 weeks. H & E stain, ×40. (C) Papilloma. Down growth of epithelial cells found in the forestomach of a rat in group 2, 5 weeks. H & E stain, ×100 (D) Squamous cell carcinoma. Keratin pearl formation in submucosal area of the forestomach of a rat in group 1, 20 weeks. H & E stain, ×100.

This result indicates that DMI acted as an inducer of the quinone reductase in the present experimental protocol for a gastric carcinogenesis model. The activity of quinone reductase in the forestomach was twelve times higher than that in the liver of the same-treated rats.

Induction of ODC Activity

Fig. 3 shows the induction of ODC activity in the pyloric mucosa of rat stomach in a time-dependent manner. The ODC activity was not changed in the forestomach epidermis and the fundic mucosa (data not shown). The ODC activity of the control rats in each experiment is represented as 0 time points. A single administration of MNNG induced ODC activity up to 288 pmol CO_2/hr/mg protein at 24 hr after the administration. NaCl caused induction of ODC with a maximum of 179 pmol CO_2/hr/mg protein at 8 hr after the administration. ODC was induced up to 539 pmol CO_2

Table I. NADH: quinone reductase specific activities (means± S.E.) in tissue cytosols from two to three rats

Group	Tissue	Treatment	Quinone reductase specific activity, nmol/min/mg protein
[a]1F	Forestomach	MNNG 10% NaCl 0.2% DMI	666± 42
[b]7F	Forestomach	MNNG 10% NaCl	321± 58*
[c]1L	Liver	MNNG 10% NaCl 0.2% DMI	55± 5**

* Significantly different from Group 1F at $p<0.05$.
** Significantly different from Group 1F at $p<0.01$.
[a] Forestomach of group 1 rats as in Text-Fig. 1.
[b] Forestomach of group 7 rats as in Text-Fig. 1.
[c] Liver of group 1 rats as in Text-Fig. 1.

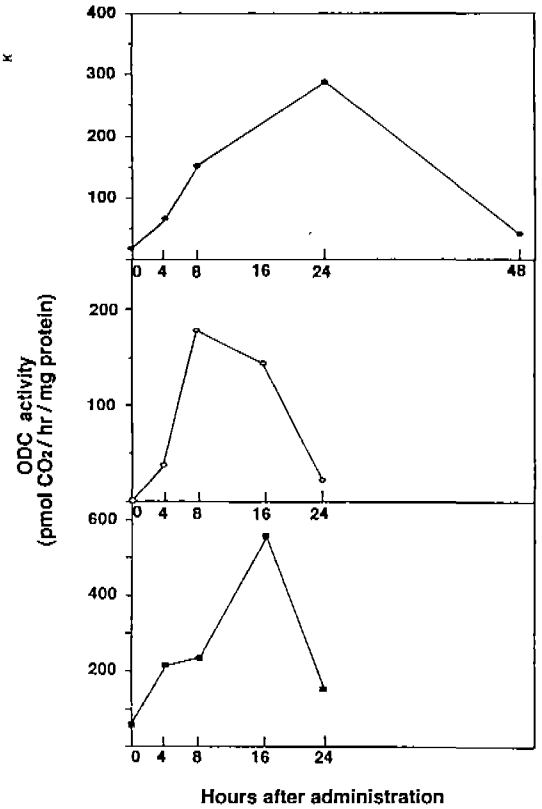


Fig. 3. Induction of ODC activity in the pyloric mucosa of rat stomach after administrations of MNNG (200 mg/kg b.w., closed circles), NaCl (1.5 g/kg b.w., open circles), and DMI (1.1 g/kg b.w., closed squares) by gastric intubation. Results are means for duplicate assays on pooled materials from four rats.

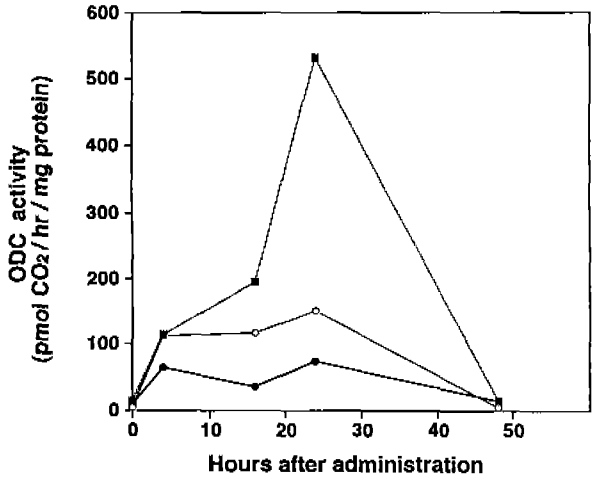


Fig. 4. Induction of ODC activity in the pyloric mucosa of rat stomach. Closed circles: single administration of MNNG; open circles: combined administration of MNNG and NaCl; closed squares: combined administration of MNNG, NaCl and DMI. Doses of the chemicals were as in Fig. 3. Results are means for duplicate assays on pooled materials from four rats.

/hr/mg protein at 16 hr after the administration of DMI. The ODC activities returned to the orignal levels after 24~48 hr. The time-dependent induction of ODC activity was seen neither in the glandular stomach nor in the forestomach region of rats. Additional treatment of NaCl and NaCl plus DMI caused 2 fold and 7 fold increases, respectively, in the ODC activity of the MNNG-alone group at 24 hr after the administration (Fig. 4). These results suggest that NaCl and DMI have tumor-promoting activities in the gastric carcinogenesis initiated by MNNG.

Discussion

The results of the pathological study and the biochemical assay in the present experiment clearly demonstrated that additional treatment with NaCl and 0.2% DMI exerted synergistic effects on the gastric carcinogenesis initiated by MNNG in rats. In the pathological observations, combination of NaCl and DMI caused severe gross and histologic lesions in the forestomach of rats treated with MNNG. This result correlates well with the findings of Kim *et al.* (1991). Concentrations of NaCl did not cause a significant difference in the present experiment, suggesting that a concentration of 5% might have been high enough for NaCl to act as a tumor promotor. MNNG and NaCl induced ODC activities in the pylorus mucosa of rat stomach as reported previously by Furihata *et al.* (1984 and 1987). Possible mechanisms for the enhancing effects of NaCl on gastric carcinogenesis are considered to be disturbance of the mucous barrier, resulting in increased penetration of carcinogens to the garstric mucosa, and/or increased cell proliferation due to repeated injury by NaCl (Ohgaki *et al.*, 1984).

Induction of ODC activity by DMI was also found, indicating the possible tumor-promoting activity of DMI. The finding that the additional treatment of NaCl and DMI caused a marked increase in the ODC activity of pyloric mucosa of rats treated with MNNG demonstrates the possible enhancing effect of NaCl and DMI on the MNNG-induced carcinogenesis. Inductions of ODC activity, demonstrated in Fig. 3 and 4, were not statistically analyzed since the ODC activity varied significantly upon the stomach content of rats when the chemicals were administered as previously noted by Furihata *et al.* (1985). Although the rats were given similar amounts of diet, the amounts of remaining dietary constituents varied in each experiment.

Quinone reductase, an anticarcinogenic enzyme, was activated in the forestomach of rats upon the treatment of DMI. It demonstrates that DMI acted as a quinone reductase inducer in the rats pretreated with MNNG and NaCl. The activity of quinone reductase was much higher in forestomach than in liver of the same-treated rats. It correlates well with the findings of Benson *et al.* (1980) that higher activity was observed in tissues of the gastrointestinal tract than in other tissues. They suggested that these high activities might be an evolutionary adaptation in response to the exposure of these tissues to high levels of toxic, mutagenic, and carcinogenic substances.

On the contrary to the expected anticarcinogenic effect of DMI, DMI exerted a strong promoting effect on the gastric carcinogenesis initiated by MNNG. A possible mechanism of inhibition of MNNG-induced mutagenesis by quinone reductase inducers would be that quinone reductase scavenges reactive electrophilic MNNG degradation products, thereby preventing their action on critical cellular targets. For the inhibition, however, the concurrent treatment of MNNG and the inhibitors was demonstrated to be necessary (Chan *et al.*, 1986). The fact that the time at which DMI was administered was not concurrent with the MNNG treatment in the present study might be the reason for the unexpected result. It seems plausible that DMI is caustic and even though it was diluted with corn oil, it may cause damage to the mucosal membrane of the stomach, which leads to the increased cell proliferation.

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