Possible Tumor-initiating and -promoting Activity of *p*-Methylcatechol and Methylhydroquinone in the Pyloric Mucosa of Rat Stomach

Chie Furihata, Shinobu Oguchi and Taijiro Matsushima

Department of Molecular Oncology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108

The possible tumor-promoting and tumor-initiating activities of *p*-methylcatechol and methylhydroquinone in the pyloric mucosa of male F344 rats were studied. Ornithine decarboxylase (ODC) and replicative DNA synthesis (RDS) were used as markers of tumor promotion and DNA single strand scission and unscheduled DNA synthesis (UDS) as markers of tumor initiation. The compounds were administered by gastric intubation and results were compared with those after administration of catechol. *p*-Methylcatechol at doses of 60 to 180 mg/kg body weight dose-dependently induced up to 20-fold increase in ODC activity and 9-fold increase in RDS with maxima 24 h after its administration, but it did not induce significant DNA single strand scission or UDS. Methylhydroquinone at a dose of 200 mg/kg body weight induced up to 6-fold increase in ODC activity 24 h, and 5-fold increase in RDS 16 h after its administration, but the induction was not dose-dependent. At a dose of 200 mg/kg body weight it induced DNA single strand scission, but not UDS. These results and previous findings show that the possible tumor-promoting activities of catechol are several times higher than those of *p*-methylcatechol and 10 times higher than those of methylhydroquinone.

Key words: *p*-Methylcatechol — Methylhydroquinone — Replicative DNA synthesis — Ornithine decarboxylase — DNA damage

Phenolic antioxidants are important constituents of plants, foodstuffs, cosmetics, cigarette smoke, hair dyes and photographic developer, and are used industrially.¹⁻⁶⁾

Recently, Hirose *et al.* found that catechol and its analogue *p*-methylcatechol (Fig. 1) induced adenocarcinoma in the pyloric mucosa of rats and strongly enhanced two-step stomach carcinogenesis initiated by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), whereas another analogue at a similar dose, methylhydroquinone (Fig. 1), did not.⁷⁻¹⁰⁾

Previously we developed *in vivo/in vitro* short-term methods for evaluating possible tumor-initiating activity, using unscheduled DNA synthesis (UDS)¹¹⁾ and DNA single strand scission determined by an alkaline elution method¹²⁾ as markers, and also for evaluating possible tumor-promoting activity with ornithine decarboxylase (ODC), which is thought to be a key enzyme in cell proliferation, and replicative DNA synthesis (RDS) as markers.^{13,14)} Using these methods we showed that catechol induced ODC and RDS, but not UDS or DNA single strand scission in the pyloric mucosa of F344 rat stomach after its administration by gastric intubation.¹⁵⁾ These results suggested that catechol may have tumor-promoting activity, but not tumor-initiating activity.

In the present study, we examined the possible tumor-initiating and -promoting activities of *p*-methylcatechol and methylhydroquinone, analogues of catechol, in the pyloric mucosa of rat stomach after their administration to rats by gastric intubation, and compared the results with those obtained with catechol.

MATERIALS AND METHODS

Animals and treatment Male Fischer rats (F344/DuCrj; Charles River Japan, Kanagawa) of 7 weeks old, were given limited amount of diet overnight (4 g/rat of commercial pellet diet, Nihon Clea, Tokyo) to reduce their stomach contents. The following day they were given 1 ml of an aqueous solution of *p*-methylcatechol (Tokyo Kasei Kogyo Co.) or methylhydroquinone (99% pure, Aldrich Chemical Co., Milwaukee, WI) by gastric intubation. Under the present conditions, 180 mg/kg body weight of *p*-methylcatechol and 300 mg/kg body weight of methylhydroquinone were the highest tolerated doses. Control animals were given distilled water only.

ODC ODC activity in crude enzyme extracts of the pyloric mucosa of the stomach was determined with L-[1-¹⁴C]ornithine (2 GBq/nmol, New England Nuclear, Boston, MA) as a substrate, as described previously.^{13,14)} The protein content of the extracts was determined by micro-assay as described¹⁶⁾ with bovine serum albumin as a standard. Results are shown as means of duplicate assays on pooled materials from four rats. The time-dependent induction of ODC activity was examined at a dose of 0.6 to 0.8 times the highest tolerated dose.

RDS and UDS RDS and UDS were determined in small pieces of pyloric mucosa of the stomach in *in vitro* organ culture in the presence of tritiated thymidine ([³H]dThd, 3 TBq/nmol, New England Nuclear) without and with 10 mM hydroxyurea, an inhibitor of RDS, as described previously.^{11,14)} The DNA fraction was extracted from

Catechol p-Methylcatechol Methylhydroquinone

Fig. 1. Chemical structures of catechol, *p*-methylcatechol and methylhydroquinone.

the tissue, an aliquot was dissolved in ACS II (Amersham Corp., Arlington Heights, IL), and the incorporation of [^3H]dThd into DNA was determined in a Packard Tricarb 1500 liquid scintillation counter. The DNA content of the DNA fraction was determined with 3,5-diaminobenzoic acid (Tokyo Kasei Kogyo Co., Tokyo) as described¹⁷⁾ with calf thymus DNA (Worthington Biochemicals Co., Freehold, NJ) as a standard. Values for five individual rats were determined in each experiment. The time-dependent increase in RDS was examined at a dose of 0.6 to 0.8 times the highest tolerated dose.

DNA single strand scission DNA single strand scission was determined by the alkaline elution method described previously.¹²⁾ Samples of 5 to 10 mg of pyloric mucosa of individual rats were lysed on a filter (Fluorinert, 1.0 μm pore size, 25 mm, Japan Millipore Co., Tokyo) with 3.0 ml of lysis solution, pH 9.7, in the presence of proteinase K (1 mg/ml, Sigma Chemical Co.) for 1 h in the dark, and then DNA was eluted in the dark at 20 to 25°C with 21 ml of solution of pH 12.1 at a flow rate of 0.05 ml/min. Fractions of 3 ml were collected and the DNA contents of the fractions and on the filters were determined with 3,5-diaminobenzoic acid¹⁷⁾ after hydrolysis in 5% trichloroacetic acid at 80°C for 30 min. The elution rate constant (/ml) of DNA was calculated from a plot (log scale) from the start to the 5th fraction. The results were analyzed by the use of Welch's or Student's *t* test.

RESULTS

p-Methylcatechol

ODC Figure 2 shows the time-dependent induction of ODC activity in the pyloric mucosa of rat stomach after administration of *p*-methylcatechol at a dose of 150 mg/kg body weight. Increase in ODC activity induced by *p*-methylcatechol showed a broad peak between 4 and 24 h with a maximum at 24 h and the activity returned to the control level within 48 h. A 20-fold increase in ODC activity was observed 24 h after *p*-methylcatechol

Fig. 2. Time-dependent induction of ornithine decarboxylase activity in the pyloric mucosa of rat stomach after administration of catechol (▲), *p*-methylcatechol (●) or methylhydroquinone (■) at doses of 75, 150 and 200 mg/kg body weight, respectively. Results are means for duplicate assays on pooled materials from four rats.

Fig. 3. Dose-dependent induction of ornithine decarboxylase activity in the pyloric mucosa of rat stomach by catechol (▲), 8 h after its administration, and by *p*-methylcatechol (●) and methylhydroquinone (■) 24 h after their administrations.

administration. Figure 3 shows the dose-dependent induction of ODC activity in the pyloric mucosa 24 h after administration of *p*-methylcatechol at doses of 50 to 180 mg/kg body weight. A dose of 180 mg/kg body weight induced a 20-fold increase in ODC activity. The dose of *p*-methylcatechol necessary to induce ODC activity was 4-fold that of catechol. The level of ODC activity in the

pyloric mucosa of control rat stomach was close to the lowest level detectable by the present assay method (4 ± 4 pmol CO₂/30 min/mg protein).

RDS Figure 4 shows the time-dependent increase in RDS (DNA synthesis measured in the absence of hydroxyurea) in the pyloric mucosa of rat stomach after administration of *p*-methylcatechol at a dose of 150 mg/kg body weight. In control stomach, RDS is always observed in cells of the proliferative zone of the pyloric mucosa, where the cells are renewed. DNA synthesis at time 0 in Fig. 4 represents the control level of DNA synthesis in the pyloric mucosa (807 ± 404 dpm [³H]-dThd/ μ g DNA). RDS increased to a maximum of 9 times ($P < 0.01$) the control value after 24 h. Figure 5 shows the dose-dependent stimulation of RDS in the pyloric mucosa 24 h after administration of *p*-methylcatechol at doses of 30 to 180 mg/kg body weight. A dose of 180 mg/kg body weight induced a 7-fold increase ($P < 0.05$) in RDS. The dose of *p*-methylcatechol necessary to stimulate RDS was 2-fold that of catechol, as catechol induced an 8-fold increase in RDS at a dose of 75 mg/kg body weight.¹⁵⁾

DNA single strand scission Figure 6 shows that the elution rate constant, an index of DNA single strand scission, in the pyloric mucosa 2 h after administration of *p*-methylcatechol at doses of 90 to 180 mg/kg body weight was not significantly different from the control value (0 dose), although the values for two of seven rats

at a dose of 180 mg/kg body weight were increased. These results show that *p*-methylcatechol at doses of 90 to 180 mg/kg body weight did not induce significant DNA single strand scission in the pyloric mucosa 2 h after treatment.

Fig. 5. Dose-dependent stimulation of replicative DNA synthesis in the pyloric mucosa 24 h after administration of *p*-methylcatechol. Values at 60 ($P < 0.05$), 100 ($P < 0.02$) and 180 ($P < 0.05$) mg/kg body weight were significantly different from those at dose 0 by Student's *t* test.

Fig. 4. Time-dependent stimulation of replicative DNA synthesis in the pyloric mucosa of rat stomach after administration of *p*-methylcatechol at a dose of 150 mg/kg body weight. Results are for five individual rats at each time, and horizontal lines are means of the five values. Values at 16 ($P < 0.02$) and 24 ($P < 0.01$) h were significantly different from those at time 0 by Student's *t* test.

Fig. 6. Values for the elution rate constant, an index of DNA single strand scission, in the pyloric mucosa 2 h after administration of *p*-methylcatechol at various doses.

UDS Figure 7 shows that *p*-methylcatechol at doses of 60 to 180 mg/kg body weight did not affect DNA synthesis in the pyloric mucosa determined in the presence and absence of hydroxyurea 2 h after its administration compared with the control level (0 dose). Thus, UDS was not induced in the pyloric mucosa by *p*-methylcatechol at doses of 60 to 180 mg/kg body weight 2 h after its administration.

Methylhydroquinone

ODC Administration of methylhydroquinone at a dose of 200 mg/kg body weight induced a 6-fold increase in ODC activity in the pyloric mucosa 24 h after its administration (Fig. 2). Significant induction of ODC activity was observed only at a dose of 200 mg/kg body weight (Fig. 3). The potency of methylhydroquinone in induction of ODC activity was less than one-tenth of that of catechol.

RDS Figure 8 shows the time-dependent increase in RDS in the pyloric mucosa of rat stomach after administration of methylhydroquinone at a dose of 200 mg/kg body weight. RDS was stimulated 5-fold ($P < 0.02$) 16 h

after its administration and then decreased gradually. Figure 9 shows values for RDS 16 h after administration of methylhydroquinone at doses of 90, 200 and 300 mg/kg body weight. A slight, but not significant, increase of RDS was observed at a dose of 300 mg/kg body weight.

DNA single strand scission Figure 10 shows that methylhydroquinone induced dose-dependent increase in the elution rate constant in the pyloric mucosa at doses of 90 to 200 mg/kg body weight 2 h after its administration,

Fig. 7. DNA synthesis determined in the presence and absence of hydroxyurea (HU) in the pyloric mucosa 2 h after administration of *p*-methylcatechol.

Fig. 8. Time-dependent stimulation of replicative DNA synthesis in the pyloric mucosa of rat stomach after administration of methylhydroquinone at a dose of 200 mg/kg body weight. Values at 16 h were significantly different from those at time 0 by Student's *t* test ($P < 0.02$).

Fig. 9. Replicative DNA synthesis in the pyloric mucosa of rat stomach 16 h after administration of methylhydroquinone.

Fig. 10. Increase in elution rate constant, an index of DNA single strand scission, in the pyloric mucosa 2 h after administration of methylhydroquinone. Values at 200 mg/kg body weight were significantly different from those at dose 0 by Student's *t* test ($P < 0.05$).

a dose of 200 mg/kg body weight inducing a 7-fold increase ($P < 0.05$). A dose of 300 mg/kg body weight induced increase in the elution rate constant, but the mean value was less than that at a dose of 200 mg/kg body weight and the increase was not statistically significant. A dose of 300 mg/kg body weight may be slightly toxic.

UDS Figure 11 shows DNA synthesis determined in the presence and absence of hydroxyurea in the pyloric mucosa 2 h after administration of methylhydroquinone at doses of 100 to 250 mg/kg body weight. Slight increases in DNA synthesis in the presence and absence of hydroxyurea were observed at doses of 100, 200 and 250 mg/kg body weight. Consequently, the slight increases in the presence of hydroxyurea can be explained as reflections of increased DNA synthesis in the absence of hydroxyurea, not as due to UDS, because hydroxyurea inhibits about 95% of RDS, but about 5% is insensitive to hydroxyurea. Thus increase in DNA synthesis in the absence of hydroxyurea (RDS) causes an increase in DNA synthesis in the presence of hydroxyurea.

DISCUSSION

The present results showed that *p*-methylcatechol is likely to have tumor-promoting activities (effects on ODC and RDS), but is unlikely to have significant tumor-initiating activities (effects on UDS and DNA

Fig. 11. DNA synthesis determined in the presence and absence of hydroxyurea (HU) in the pyloric mucosa 2 h after administration of methylhydroquinone.

single strand scission) in the pyloric mucosa of rat stomach. Although DNA single strand scission was observed at a dose of 180 mg/kg body weight in two of seven rats, this change was not statistically significant.

In contrast, methylhydroquinone showed very weak tumor-promoting activities and some tumor-initiating activities in the pyloric mucosa of rat stomach. Weak induction of ODC activity was observed in both time-dependent and dose-dependent studies. Significant stimulation of RDS was observed in time-dependent, but not dose-dependent studies. Significant induction of DNA single strand scission was observed, but induction of UDS was not observed.

p-Methylcatechol at doses of 150 to 180 mg/kg body weight induced similar effects to catechol in rats at doses of 40 to 75 mg/kg body weight¹⁵; namely, (a) ODC activity was 97.4 pmol CO₂/30 min/mg protein with 150 mg *p*-methylcatechol/kg body weight, and 71.2 pmol CO₂/30 min/mg protein with 40 mg catechol/kg body weight, and (b) RDS was increased 9-fold by 150 mg *p*-methylcatechol/kg body weight, and 8-fold by 75 mg

catechol/kg body weight. These results suggest that the possible tumor-promoting activity of catechol is several times greater than that of *p*-methylcatechol. Catechol did not show any tumor-initiating activity.¹⁵⁾ Results on induction of DNA single strand scissions by *p*-methylcatechol at a dose of 180 mg/kg body weight (the highest tolerated dose) were ambiguous and not statistically significant.

Methylhydroquinone at a dose of 200 mg/kg body weight induced similar possible tumor-promoting activities to catechol in rats at a dose of 20 mg/kg body weight; namely, (a) ODC activity induced by 200 mg methylhydroquinone/kg body weight was 41.1 pmol CO₂/30 min/mg protein, whereas that induced by 40 mg catechol/kg body weight was 71.2 pmol CO₂/30 min/mg protein; (b) with 200 mg methylhydroquinone/kg body weight RDS increased to 1.7–5 times the control value and with 20 mg catechol/kg body weight it increased 4-fold. These results suggest that the possible tumor-promoting activity of catechol is 10 times that of methylhydroquinone. Although catechol did not show any tumor-initiating activity at doses of up to 90 mg/kg body weight (the highest tolerated dose), methylhydroquinone showed significant induction of DNA single strand scission ($P < 0.05$) at a dose of 200 mg/kg body weight. However, methylhydroquinone did not induce UDS at doses of up to 250 mg/kg body weight. Inductions of DNA single strand scission and UDS in the stomach pyloric mucosa by MNNG, a typical glandular stomach carcinogen, were detected at doses as low as 1 mg/kg body weight and 50 mg/kg body weight, respectively.^{11, 12)} The UDS method is generally less sensitive than the alkaline elution method, which might explain why methylhydroquinone induced no detectable UDS.

The time-dependencies of induction of ODC by *p*-methylcatechol and methylhydroquinone were similar but different from that by catechol. The time-dependen-

cies of induction of ornithine decarboxylase in the pyloric mucosa differ with different chemicals: the times of maximal induction of ODC are reported to be 4 h with taurocholate,¹⁸⁾ 6–9 h with NaCl,¹³⁾ 16 h with glyoxal¹⁹⁾ and 24 h with MNNG.¹³⁾ However, there are no corresponding differences in the time of maximal stimulation of RDS, which is uniformly between 16 and 24 h.

Hirose *et al.* reported that catechol at a dose of 1.5% in powdered diet for the initial 4 weeks and 0.8% for the following 47 weeks, and *p*-methylcatechol at a dose of 1.5% in powdered diet for 51 weeks caused similar strong enhancements of two-step stomach carcinogenesis initiated by MNNG.^{8, 9)} Hirose *et al.* also reported that methylhydroquinone at a dose of 1.5% in powdered diet for 51 weeks after initiation with MNNG induced an adenocarcinoma in the pyloric region in one of 20 rats, although this induction was not statistically significant. No adenocarcinoma was found in the control group given basal diet after initiation with MNNG. The potency of catechol in induction of ODC and stimulation of RDS is several times that of *p*-methylcatechol and 10 times higher than that of methylhydroquinone. Thus, the present results correlated well with those on the long-term tumor-promoting activities of these three chemicals. These good correlations between long-term experiments and our short-term assays suggest that our *in vivo/in vitro* short-term method is useful for evaluating possible tumor-promoting and tumor-initiating activities of compounds and for studies on action mechanisms.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan and a grant from the Japanese Society for Promotion of Cancer Research.

(Received September 14, 1992/Accepted December 22, 1992)

REFERENCES

- 1) IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, Vol. 38. "Tobacco Smoking," pp. 83–126 (1986). IARC, Lyon.
- 2) IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man, Vol. 15. "Some Fumigants, the Herbicides 2,4-D and 2,4,5-T, Chlorinated Dibenzodioxines and Miscellaneous Industrial Chemicals," pp. 155–175 (1977). IARC, Lyon.
- 3) Raff, R. and Ettling, B. V. "Hydroquinone, Resorcinol and Pyrocatechol. Encyclopedia of Chemical Technology," Ed. 2, Vol. 11, pp. 462–492 (1966). John Wiley and Sons, Inc., New York.
- 4) Rahn, W. and Köning, W. A. GC/MS investigations of the constituents in a diethyl ether extract of an acidified roast coffee infusion. *J. High Resolut. Chromatogr.*, **1**, 69–71 (1979).
- 5) Lepri, L., Desideri, P. G. and Coas, V. Separation and identification of colouring agents in the oxidation-type hair dyes by ion-exchange thin-layer chromatography. *Ann. Chim.*, **66**, 451–460 (1976).
- 6) Smyth, R. B. and McKeown, G. G. The analysis of arylamines and phenols in oxidation-type hair dyes by paper chromatography. *J. Chromatogr.*, **16**, 454–459 (1964).
- 7) Hirose, M., Kurata, Y., Tsuda, H., Fukushima, S. and Ito, N. Catechol strongly enhances rat stomach carcinogenesis: a possible new environmental stomach carcinogen.

- Jpn. J. Cancer Res.*, **78**, 1144–1149 (1987).
- 8) Hirose, M., Fukushima, S., Kurata, Y., Tsuda, H., Tatematsu, M. and Ito, N. Modification of N-methyl-N'-nitro-N-nitrosoguanidine-induced forestomach and glandular stomach carcinogenesis by phenolic antioxidants in rats. *Cancer Res.*, **48**, 5310–5315 (1988).
- 9) Hirose, M., Yamaguchi, S., Fukushima, S., Hasegawa, R., Takahashi, S. and Ito, N. Promotion by dihydroxybenzene derivatives of N-methyl-N'-nitro-N-nitrosoguanidine-induced F344 rat forestomach and glandular stomach carcinogenesis. *Cancer Res.*, **49**, 5143–5147 (1989).
- 10) Hirose, M., Fukushima, S., Shirai, T., Hasegawa, R., Kato, T., Tanaka, H., Asakawa, E. and Ito, N. Stomach carcinogenicity of caffeic acid, sesamol and catechol in rats and mice. *Jpn. J. Cancer Res.*, **81**, 207–212 (1990).
- 11) Furihata, C., Yamawaki, Y., Jin, S.-S., Moriya, H., Kodama, K., Matsushima, T., Ishikawa, T., Takayama, S. and Nakadate, M. Unscheduled DNA synthesis in rat stomach mucosa induced by glandular stomach carcinogens. *J. Natl. Cancer Inst.*, **72**, 1327–1334 (1984).
- 12) Furihata, C., Hatta, A., Sato, Y. and Matsushima, T. Alkaline elution of DNA from stomach pyloric mucosa of rats treated with glyoxal. *Mutat. Res.*, **213**, 227–231 (1989).
- 13) Furihata, C., Sato, Y., Hosaka, M., Matsushima, T., Furukawa, F. and Takahashi, M. NaCl induced ornithine decarboxylase and DNA synthesis in rat stomach mucosa. *Biochem. Biophys. Res. Commun.*, **121**, 1027–1032 (1984).
- 14) Furihata, C., Sato, Y., Matsushima, T. and Tatematsu, M. Induction of ornithine decarboxylase and DNA synthesis in rat stomach mucosa by methylglyoxal. *Carcinogenesis*, **6**, 91–94 (1985).
- 15) Furihata, C., Hatta, A. and Matsushima, T. Inductions of ornithine decarboxylase and replicative DNA synthesis but not DNA single strand scission or unscheduled DNA synthesis in the pyloric mucosa of rat stomach by catechol. *Jpn. J. Cancer Res.*, **80**, 1052–1057 (1989).
- 16) Heil, A. and Zilling, W. Reconstitution of bacterial DNA-dependent RNA-polymerase from isolated subunits as a tool for the elucidation of the role of the subunits in transcription. *FEBS Lett.*, **11**, 165–168 (1970).
- 17) Kissane, J. M. and Robins, E. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem.*, **233**, 184–188 (1958).
- 18) Furihata, C., Takezawa, R., Matsushima, T. and Tatematsu, M. Potential tumor-promoting activity of bile acids in rat glandular stomach. *Jpn. J. Cancer Res.*, **78**, 32–39 (1987).
- 19) Furihata, C., Yoshida, S. and Matsushima, T. Potential initiating and promoting activities of diacetyl and glyoxal in rat stomach mucosa. *Jpn. J. Cancer Res.*, **76**, 809–814 (1985).