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Dose-dependent Induction of 8-Hydroxyguanine and Preneoplastic Foci in Rat Liver by a Food-derived Carcinogen, 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, at Low Dose Levels

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Male F344 rats were administered 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in the diet at doses of 200, 50, 12.5, 3.2, 0.8, 0.2 and 0.05 ppm for 6 weeks, and partially hepatectomized 1 week after the beginning of MeIQx administration. Quantitative values for glutathione S-transferase placental form (GST-P)-positive foci in the liver were dose-dependently increased by the MeIQx treatment. 8-Hydroxyguanine (8-OHG) levels assessed after 1 week of dietary MeIQx administration were also dose-dependently increased, although the effect was no longer observed at the end of the treatment period. The correlation between numbers of GST-P-positive foci at week 6 and 8-OHG levels at week 1 was linear, values for both parameters being higher than the control levels even in the 0.8 ppm dose group. These findings indicate that, in addition to the previously reported MeIQx-DNA adduct formation, DNA modifications due to oxidative damage may play an important role in MeIQx liver carcinogenesis in rats.

Key words: GST-P-positive foci — 8-Hydroxyguanine — MeIQx — Liver carcinogenesis — Rat

2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) is one of 10 heterocyclic amines that have been demonstrated to be carcinogenic in rodents and/or non-human primates.[1–3] MeIQx, first isolated from fried beef,[4] shows mutagenicity not only in bacteria, but also in cultured mammalian cells[2,5] and is a carcinogen in both the mouse[6] and the rat.[7,8]

Under most cooking conditions MeIQx is the second most prevalent heterocyclic amine, with levels ranging between 100 and 28 pg/g of cooked food.[2,9] MeIQx has been reported to be excreted into the urine of healthy volunteers eating a normal diet, and the daily exposure level to this compound is estimated to be 0.2–2.6 μg/person.[10] Layton *et al.*[11] have concluded that MeIQx is the second most important incremental risk factor for human cancer among 5 heterocyclic amines investigated, based on data on concentrations of heterocyclic amines in cooked foods, a dietary survey of the US population, and the cancer potencies of the individual compounds in animal bioassays.

Regarding the mechanisms of carcinogenesis by heterocyclic amines, DNA adduct formation has been considered to be a major causal factor. All heterocyclic amines so far identified are strongly mutagenic[2,3,9] and dose-dependent increase of DNA adducts in target organs has been demonstrated for MeIQx,[12–15] the relationship being linear for the rat liver.[12,14] However, since similar types and levels of DNA adducts have also usually been observed in non-target organs for MeIQx[12] and other carcinogens,[16,17] it can be concluded that DNA-chemical adduct formation may be a prerequisite, but itself is not sufficient, for their carcinogenic action.

Recently, we have found significant inhibition of MeIQx liver carcinogenesis by simultaneous administration of 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ), a synthetic antioxidant[18,19] to rats,[20] indicating that oxygen radicals may be important for heterocyclic amine-induced tumor development. It is well known that 8-hydroxyguanine (8-OHG) is a major species of oxidative DNA damage which can result in specific types of mutation.[21–24] It has therefore been suggested to participate in the pathogenesis of various disorders such as neoplasia and other aging-associated diseases.[25–28]

The present experiment was designed to examine whether no-effect levels for MeIQx exist in terms of histopathological and biological parameters in the rat liver using our medium-term bioassay system.[29,30] Preneoplastic lesions and levels of 8-OHG were evaluated.

MATERIALS AND METHODS

Chemicals and animals Male F344 rats (Charles River Japan, Inc., Atsugi), 8 weeks old at the commencement, were used. The rats were housed in an air-conditioned

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animal room at 23±2°C and 50±10% humidity and food and water were available *ad libitum*. The animals received MeIQx (Nard Institute, Osaka) in powdered diet (Oriental M, Oriental Yeast Co. Ltd., Tokyo) at doses of 200, 50, 12.5, 3.2, 0.8 or 0 ppm in the first experiment and at 0.2, 0.05 or 0 ppm in the second experiment for 6 weeks, along with 2% corn oil as a moistener. The second experiment was conducted since the lowest dose group in the first experiment exhibited apparent effects in 8-OHG and foci analysis. All rats were subjected to two-thirds partial hepatectomy (PH) at week 1 under light ether anesthesia, and killed at week 6. The livers were removed, weighed, and processed for immunohistochemical and biological analyses as follows. Kidneys were also weighed.

Evaluation of glutathione S-transferase placental form (GST-P)-positive foci Liver tissues obtained at week 6 were fixed in ice-cold acetone, routinely embedded in paraffin and immunohistochemically stained for GST-P as previously reported.[31] Numbers of GST-P-positive hepatic lesions categorized in size as single cells, foci of 2–4 cells, foci of 5–10 cells, foci of 11–20 cells, and foci of more than 20 cells were counted under the light microscope and the total areas of liver sections examined were measured using a video image processor, VIP 21C (Olympus-Ikegami Tsushin Co., Tokyo).

8-OHG analysis Liver samples (about 0.5 g) obtained at PH (week 1) and at week 6 were immediately frozen in liquid nitrogen and kept in a deep freezer at −80°C until analysis.

Ribonuclease (EC 3.1.27.5) A, alkaline phosphatase (EC 3.1.3.1) and 2′-deoxyguanosine (2′-dG) were obtained from Sigma Chemical Co., St. Louis, MO. Nuclease P1 (EC 3.1.30.1) and 8-hydroxydeoxyguanosine (8-OHdG) were purchased from Yamasa Shoyu Co., Ltd., Chiba, and Wako Pure Chemical Industries, Ltd., Kyoto, respectively. A Sepagene kit for DNA extraction was obtained from Sanko Junyaku Co., Ltd., Tokyo. Isopropyl alcohol, ethyl alcohol and methyl alcohol were all of HPLC grade (Wako Pure Chemical Industries, Ltd.). All other reagents were of analytical grade obtained from either Wako Pure Chemical Industries, Ltd. or Nacalai Tesque, Inc., Kyoto. Water used throughout the analysis was double-distilled by Fi-Stream (Barnstead, Division of Sybron, Boston, MA), purified by Nanopure II (Barnstead), ultrafiltered to eliminate pyrogens through a Hollow Fiber Cartridge (Barnstead), deoxygenated by argon-sparging and autoclaved at 120°C for 30 min. Experimental tools were also autoclaved in a similar manner. Light and air contamination were avoided whenever possible.

DNA was extracted from liver samples using the Sepagene kit as previously described[32] and then digested into deoxynucleosides by a method adapted from that of Takagi *et al.*[33] 8-OHG levels in the resultant samples were quantified by high-performance liquid chromatographically using electrochemical detection by the method of Kasai *et al.*[34] with modifications as described elsewhere.[32, 35] The 8-OHG levels were calculated by calibration against curves from HPLC runs of standard samples, containing known amounts of authentic 8-OHdG and dG, and expressed as the number of 8-OHdGs per 10^5 total dGs.

Statistics Statistical analysis of differences between means was carried out using Student's t test or Welch's t test after application of the preliminary F-test for equal variance for each appropriate pair. The relationship between GST-P and 8-OHG values was analyzed using Pearson's correlation coefficient.

RESULTS

Essentially, the control values for weights, foci and 8-OHG in the two experiments were the same, and it was

Table I. Final Body and Liver Weights of Rats Given MeIQx in the Diet

Dose of MeIQx (ppm)	No. of rats	Body weight (g)	Weight relative to body (%)		Average food consumption (g/rat/day)
			Liver	Kidney	
200	6	277.0±16.1[a)]	3.64±0.10	0.65±0.03	12.8
50	4	304.8±5.3	3.62±0.11	0.64±0.02	13.7
12.5	6	299.1±8.4	3.35±0.16	0.66±0.03	12.3
3.2	6	302.5±4.9	3.30±0.12	0.68±0.03	12.8
0.8	4	311.1±13.3	3.34±0.09	0.64±0.03	14.2
0	4	307.8±24.2	3.38±0.28	0.65±0.03	14.1
0.2	6	296.0±10.5	3.35±0.07	0.63±0.02	13.5
0.05	6	301.9±15.5	3.46±0.09	0.65±0.03	14.0
0	6	300.5±12.4	3.37±0.09	0.65±0.02	14.2

a) Significantly different from the 0 ppm group at $P<0.05$.

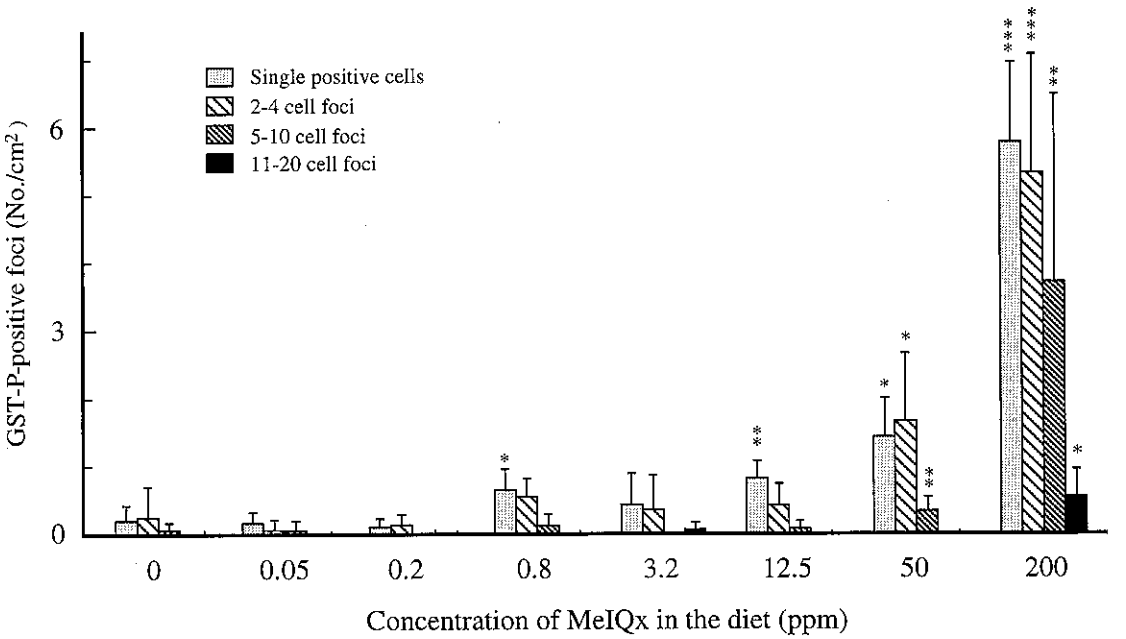

Fig. 1. Numbers of GST-P-positive liver cells or foci per cm^2 of liver section. Data from the two experiments were combined. Although a statistically significant increase was only observed in groups administered MeIQx at doses higher than 50 ppm in the diet, it is clear that the values in the 0.8 ppm group are higher than the control levels and that there is a dose-dependent increase of foci. *, **, ***; Significantly different from the 0 ppm level for each corresponding size of foci at $P<0.05$, $P<0.01$, and $P<0.001$, respectively.

therefore considered that the data from experiments 1 and 2 could be combined.

Data for final body and organ weights and food consumption are summarized in Table I. A few rats died, possibly due to the surgery, during the experimental period. Final body weights were almost the same in different dose groups. Only the highest MeIQx dose group exhibited a significantly lower body weight. The treatment with MeIQx resulted in a dose-dependent increase of liver weight in the higher dose range, but did not affect the kidney weight. Food consumption was almost the same in all groups; the values were between 12.3–14.2 g/rat/day, and average daily MeIQx intakes were 2.6 mg/rat for the highest dose group and 0.7 μg/rat for the lowest dose group.

To analyze the low-dose effects of MeIQx precisely, positive lesions were categorized into single cells, 2–4 cells, 5–10 cells, 11–20 cells, more than 20 cells and expressed as numbers per unit area of liver section. The results for groups from the two experiments are illustrated in Fig. 1. Control values from the two experiments were combined. Foci of more than 20 cells were not observed. Although a statistically significant increase was generally observed in the higher-dose groups, numbers of single cells were significantly greater than the basal diet group values even in the 12.5 and 0.8 ppm cases. Larger foci also appeared mainly in the high-dose groups. In the groups receiving less than 0.8 ppm dosage, data for each size of foci were almost the same as in the basal diet group. When foci were divided into those comprising 4 cells or less and those with 5 cells or more, dose-dependent induction of foci could be clearly demonstrated for smaller foci and significantly increased levels were found for the 0.8, 12.5, 50, and 200 ppm dose groups,

Table II. Levels of 8-OHG in the Livers

Dose of MeIQx (ppm)	At week 3		At week 8	
	No. of rats	8-OHG[a)]	No. of rats	8-OHG[a)]
200	3	10.84±0.25[b)]	6	1.21±0.11
50	3	3.49±0.18[b)]	4	2.50±3.30
12.5	3	2.43±0.21[b)]	6	1.07±0.67
3.2	3	1.01±0.16[c)]	6	0.94±0.37
0.8	3	0.56±0.10[d)]	4	1.01±0.26
0	3	0.24±0.07	4	1.44±0.86
0.2	6	0.31±0.03	6	0.73±0.11
0.05	6	0.25±0.02	6	0.76±0.10
0	6	0.24±0.02	4	0.72±0.08

a) No. of 8-OHG/10^5 dG are expressed as mean±SD.
b, c, d) Significantly different from the 0 ppm group at $P<0.001$, $P<0.01$, $P<0.05$, respectively.

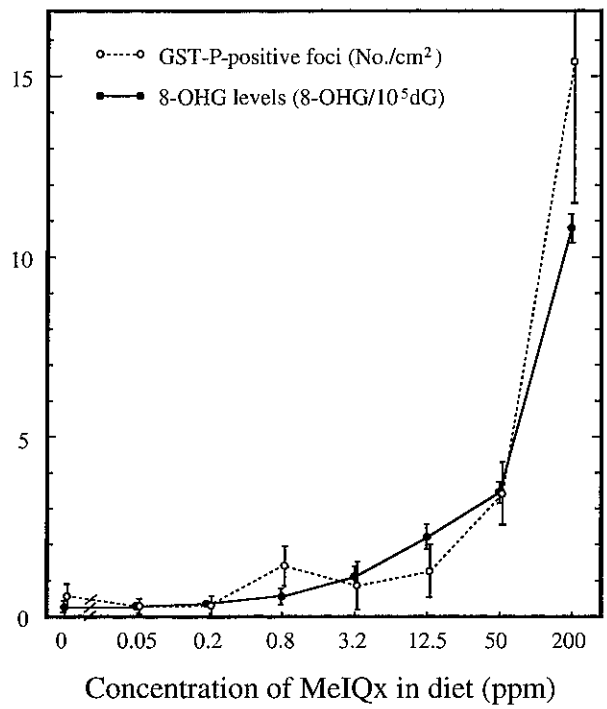

Fig. 2. The relationship between the total number of GST-P-positive single cells and foci per cm^2 at week 6 and the 8-OHG level in the liver at week 1. These values increase with the dose of MeIQx, but not linearly. However, the correlation coefficient between the two parameters is 0.984 ($n=8$), linearity being significant at $P<0.001$. Thus the development of GST-P-positive lesions was well correlated with the induction of 8-OHG.

with large foci increasing in number with higher doses of MeIQx, such as 50 and 200 ppm (data not shown).

Measured levels of 8-OHG in the liver are summarized in Table II. At week 1, the samples for the analysis were obtained from the resected livers at PH. The 8-OHG levels were dose-dependently increased along with the concentration of MeIQx in the diet, and the value for the 0.8 ppm dose group was significantly higher than the background level. However, 8-OHG levels at week 8 did not show any consistent trend of alteration. The value in the 50 ppm group was higher than in the other dose groups, but it was not significantly greater than the background level at week 6.

Although the two parameters, total numbers of GST-P-positive foci at week 6 and 8-OHG levels at week 1, were not linear with regard to the logarithmic concentration of MeIQx as illustrated in Fig. 2, the correlation between the two was linear. The correlation coefficient value was 0.984 ($n=8$), the correlation being statistically significant ($P<0.001$).

DISCUSSION

MeIQx is a heterocyclic amine which has recently been extensively examined from a variety of viewpoints. It is abundant in our diet and human beings are actually exposed to this compound on a daily basis.[2, 3, 9–11, 36, 37] MeIQx is a potent carcinogen in rodents with a clear dose-response relationship.[7, 8] Incidences of liver cell tumors found in male F344 rats were 100% for 400 ppm, 90% for 200 ppm, and 17% for 100 ppm in 56-week and 62-week experiments.[7, 8]

Quantitative evaluation of GST-P-positive foci is considered the most reliable method for rapid assessment of carcinogenic potential in rat liver,[29–31] and a dose-dependent induction of these lesions was demonstrated in the present study for MeIQx. Using 4 or 6 animals per group, the lowest effective dose for induction of GST-P-positive foci was 0.8 ppm when only smaller foci were evaluated. With doses less than 0.8 ppm, GST-P-positive foci values were essentially the same as the control levels. Based on the weight data, however, significant toxic effects of MeIQx were identified only with the highest dose levels. Thus, analysis of foci is more sensitive than examination of body and organ weights to distinguish adverse chemical effects. The concentration of MeIQx that unequivocally induced preneoplastic lesions in the liver after only a 6-week exposure was 8 ppm (10–11 μg/rat/day). This is 960–12000 times higher than the human exposure level (0.2–2.6 μg/person/day)[10] when the respective body weights are taken into consideration (rat=250 g : human =60 kg). However, the no-observed-effect level might be decreased when animals receive longer periods of treatment and further investigations of MeIQx carcinogenicity at much lower concentrations in the diet are now on-going in Japan using more than 2000 rats.

A clear dose-dependent increase of 8-OHG formation was demonstrated for MeIQx administration after 1 week of treatment. Interestingly, the correlation between GST-P levels at the end of the experiment (week 6) and 8-OHG levels examined in the removed liver at PH at week 1 was significantly linear. Previous studies indicated that MeIQx-DNA adduct levels in the rat liver directly parallel the dose of MeIQx in the diet,[12, 14] in contrast to the threshold observed for induction of GST-P-positive foci. Although it is generally believed that the genotoxicity and carcinogenicity of MeIQx are strongly related to carcinogen-DNA adduct formation, and a variety of MeIQx-DNA adduct forms have been proposed as critical for carcinogenesis,[38, 39] the results of the present experiment indicate that DNA damage due to oxidative stress is another important factor for MeIQx liver carcinogenesis in the rat.

The dose-dependent inhibitory effects of HTHQ, a potent synthetic antioxidant,[18, 19, 40] on MeIQx liver carci-

nogenesis[20] provide indirect support for a role for oxygen radicals in the carcinogenic process. Similar inhibition was observed for HTHQ against 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) liver carcinogenesis[20] and against 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) mammary carcinogenesis.[41] We have demonstrated that HTHQ has antimutagenic activity in the *Salmonella* strain TA 98 against MeIQx, Glu-P-1, PhIP and other heterocyclic amines.[40] The observed antimutagenic effects were greater than those of *t*-butylhydroquinone, propyl gallate, butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol.[33] Oxidative liver injury caused by either carbon tetrachloride[42] or a choline-deficient diet[43] has been reported to produce a condition susceptible to hepatocarcinogenesis. While liver injury and consequent regenerative processes must be taken into consideration, it is also possible that damage due to oxidative stress is an enhancing factor for MeIQx liver carcinogenesis, along with DNA adduct formation.

The findings that induction of 8-OHG was no longer observed after 6 weeks of feeding and that background levels were markedly increased at this time point requires explanation. The fact that the rats were subjected to partial hepatectomy at week 1 may have significance in this respect, along with age-dependent increase of oxidative modifications.[26, 44, 45] Since the final 8-OHG levels were lower than those at PH, especially in the higher-dose groups, it is also possible that 8-OHG was more effectively repaired due to induction of repair enzymes.

The mechanism of dose-dependent induction of 8-OHG by MeIQx and other heterocyclic amines in the diet clearly warrants further investigation.

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