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Harman and Norharman Suppressed but NaNO₂ Enhanced the Development of Preneoplastic Liver Cell Foci in 2-Amino-3,8-Dimethylimidazo[4,5-*f*]Quinoxaline (MeIQx)-Treated Rats

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Abstract: The modifying potentials of two heterocyclic amines, harman and norharman, as well as sodium nitrite (NaNO₂), on 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx)-induced rat liver carcinogenesis were investigated using a medium-term liver bioassay system. Harman (500 ppm in diet), norharman (500 ppm in diet) or NaNO₂ (0.1% in drinking water) were given with or without MeIQx (300 ppm in diet) for 6 weeks after initiation with a single dose of dimethylnitrosamine (DEN). The appearance of MeIQx-induced glutathione S-transferase placental form positive foci in the liver was significantly decreased in the harman and norharman cases ($p < 0.001$), but it was significantly increased by NaNO₂ ($p < 0.001$). These chemicals, however, did not modify DEN-liver carcinogenesis in the absence of MeIQx. Neither harman nor norharman affected mRNA expression of CYP1A1 and 1A2 in the liver, with or without MeIQx administration, whereas NaNO₂ significantly enhanced CYP1A1 mRNA levels together with MeIQx. (J Toxicol Pathol 2005; 18: 99–104)

Key words: rat, liver, MeIQx, harman, norharman, NaNO₂

Introduction

Human beings are exposed to multiple agents present in the environment. There are many natural carcinogenic and co-carcinogenic chemicals which originate in our food¹, one example being 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), a heterocyclic amine generated with broiling of fishes and meats¹. It induces carcinomas in the liver as well as Zymbal glands of rats^{2,3} and in the stomach and liver of mice². Harman and norharman, β -carboline derivatives which are found in some vegetables and in pyrolysis products of tobacco and meat^{4,5}, lack mutagenicity themselves, but have shown co-mutagenic potential in several *in vitro* assays^{6–10}. Also, norharman caused DNA strand breaks and mutations in Chinese hamster V79 cells^{8,10}. Levitt *et al.* found both harman and norharman

inhibit the mutagenicity and binding to DNA of benzo[*a*]pyrene metabolites¹¹. Furthermore, Zajdela *et al.* reported that norharman inhibited the induction of skin tumors induced by dibenzofluoranthene 3,4-dihydrodiol and benzo[*a,e*]fluoranthene *in vivo*⁹. No carcinogenicity of norharman was evident in an *in vivo* study¹², despite its mutagenicity to *Salmonella typhimurium* TA98 with S9 mix when non-mutagenic aromatic amines like aniline or *o*- or *m*-toluidine were added¹³. Norharman and aniline together have been found to form a mutagenic rat hepatocarcinogen, 9-(4'-aminophenyl)-9H-pyrido[3,4-*b*]indole (aminophenylnorharman)^{14,15}.

Sodium nitrite (NaNO₂) is secreted from the salivary glands but mainly comes from vegetables¹⁶ and also from food additives used as colors and preservatives in meats and fishes. Although NaNO₂ is mutagenic in the *S. Typhimurium* strain TA100, no carcinogenicity was evident either in rats and mice in long-term studies¹⁷.

In the present study, we investigated the modifying effects of harman, norharman and sodium nitrite on MeIQx hepatocarcinogenesis to explore interactions among natural food chemicals. For this purpose, we utilized a medium-term rat liver bioassay system established in our laboratory^{18–20}. This features a relatively short-period and is

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very sensitive for detection of the modification potential of chemicals, using quantitative assessment of glutathione S-transferase placental form positive liver foci as end-point marker lesions.

Materials and Methods

Animals and chemicals

Five-week-old male F344 rats were obtained from Charles River Japan Inc. (Atsugi, Japan), and kept in the Experimental Animals Science Center, Nagoya City University Graduate School of Medical Sciences. They were randomly distributed three animals per cage on hardwood chip bedding in an environment-controlled room, maintained at $23 \pm 2^\circ\text{C}$ and $50 \pm 5\%$ humidity with a 12-hr light and dark cycle. The use of animals in this study followed the Guidelines for the Care and Use of Laboratory Animals of Nagoya City University Graduate School of Medical Sciences and the study was approved by the Institutional Animal Care and Use Committee.

MeIQx (>99.9% purity) was purchased from the Nard Institute (Osaka, Japan), harman (1-methyl-9H-pyrido[3,4-*b*]indole) and norharman (9H-pyrido[3,4-*b*]indole) from Sigma Chemical Co. (St. Louis, MO), and NaNO_2 from Tokyo Kasei Kogyo (Tokyo, Japan).

Experimental protocol

The experimental protocol for the medium-term rat liver bioassay employed is shown in Fig. 1. At the age of 6 weeks, animals were given a single intraperitoneal injection of diethylnitrosamine (DEN, >99% purity, Tokyo Kasei Kogyo Co., Ltd, Tokyo) dissolved in saline at a dose of 200 mg/kg b.w. to initiate hepatocarcinogenesis, and divided into 8 groups. Starting 2 weeks later, the rats of Groups 1, 2 and 5, 6 were administered diet containing harman or norharman at 500 ppm, with or without MeIQx at 300 ppm, for 6 weeks, respectively. Rats in Groups 3 and 7 were administered 0.1% NaNO_2 through drinking water with or without diet containing MeIQx at 300 ppm. Group 4 was given a diet containing only MeIQx at 300 ppm and Group 8 received DEN alone without further test chemical administration as a control. The doses of each chemical selected were based on previous studies^{21–23}. Three weeks after the beginning of the experiment, two-thirds partial hepatectomy was performed on all animals. A few animals were lost due to surgical failure (Table 1). Body weights and food and water consumption were recorded once a week. All survivors were sacrificed under ether anesthesia for examination at week 8. At the autopsy, the livers and kidneys were immediately excised and weighed, and organ to body weight ratios were calculated. The livers were cut into sections 2–3 mm thick with a razor blade and fixed in ice-cold acetone for immunohistochemical examination of glutathione S-transferase (GST-P) expression and routine hematoxylin and eosin (H&E.) staining. Parts of the remaining livers were immediately frozen in liquid nitrogen and stored at -80°C until use. The kidneys were fixed in 10% phosphate-

Fig. 1. Experimental protocol. Animals, 6-week-old F344 male rats; ↓, diethylnitrosamine (DEN) 200 mg/kg b.w., i.p.; ▽, two-thirds partial hepatectomy; ▨, MeIQx 300 ppm in diet; ■, Harman or Norharman 500 ppm in diet, or NaNO_2 0.1% in drinking water.

buffered formalin solution for routine staining with H&E.

Immunohistochemistry

Liver sections were treated with rabbit anti-rat GST-P antibody (MBL, Nagoya, Japan) and then sequentially with secondary antibody and the avidin-biotin complex reaction (Vectastain ABC elite kit, Vector Laboratories Inc., CA, USA). The sites of peroxidase binding were demonstrated with diaminobenzidine and then sections were counterstained with hematoxylin for microscopic examination. Areas of GST-P positive foci larger than 0.2 mm in diameter in liver were quantitatively measured with an Image Processor for Analytical Pathology (IPAP, Sumika Technos Co., Osaka, Japan).

Semi-quantitative reverse transcription-polymerase chain reactions

Total RNAs were isolated using ISOGEN (Wako, Tokyo, Japan), according to the manufacturer's instructions. One microgram of RNA was converted to cDNA with avian myoblastosis virus reverse transcriptase (Takara, Otsu, Japan) in 20 μl of reaction mixture. Aliquots of 2 μl of cDNA samples were subjected to quantitative PCR in 20 μl reactions using FastStart DNA Master SYBR Green I and a Light Cycler (Roche Diagnostics, Mannheim, Germany). Primers for rat cytochrome P450s (CYP1A1, 1A2, 2B1/2, 2C11, 2E1, 3A1, 3A2, 4A1) and cyclophilin were used from a Rat cytochrome P450 Competitive RT-PCR Set (Takara, Otsu, Japan). Initial denaturation at 95°C for 10 min was followed by 40 cycles with denaturation at 95°C for 15 s, annealing at 56°C for 5 s and extension at 72°C for 30 s. The fluorescence intensity of the double-strand specific SYBR Green I, reflecting the amount of formed PCR product, was monitored at the end of each extension step. Data are expressed relative to cyclophilin expression.

Statistical analysis

The significance of differences between the means of the treatment and control groups with respect to body and organ weight, food and water intake, and quantitative data for liver GST-P positive foci were analyzed using Student's

Table 1. Body and Relative Organ Weights, Average Food and Water Intake

Treatment	No. of Rats		Final Body Weight (g)	Relative Organ Weights		Average Food Intake (g/rat/day)	Average Water Intake (ml/rat/day)
	Initial	Effective		Liver (%)	Kidney (%)		
MeIQx + Harman	16	15	254.4 ± 11.9 ^{a,***}	3.62 ± 0.15 ^{***}	3.35 ± 0.18 ^{##}	14.3 ± 2.8	20.4 ± 2.8
MeIQx + Norharman	16	14	254.1 ± 9.5 ^{***}	3.74 ± 0.13 ^{***}	3.34 ± 0.15 ^{###}	12.8 ± 2.6 [*]	18.3 ± 2.9 [*]
MeIQx + NaNO ₂	16	14	238.2 ± 10.6 ^{***,###}	3.66 ± 0.15 ^{***}	3.62 ± 0.19 ^{***,##}	12.6 ± 3.7 [*]	16.2 ± 2.8 ^{***,#}
MeIQx	16	15	256.4 ± 8.7 ^{***}	3.66 ± 0.19 ^{***}	3.48 ± 0.12 [*]	13.3 ± 3.7	18.7 ± 2.8 [*]
Harman	16	12	263.1 ± 13.7	3.33 ± 0.18	3.25 ± 0.44	15.9 ± 4.7	21.2 ± 2.6
Norharman	16	14	270.1 ± 13.0	3.29 ± 0.21	3.21 ± 0.18 ^{***}	14.2 ± 3.6	20.4 ± 2.3
NaNO ₂	16	16	265.6 ± 9.2 [*]	3.10 ± 0.12 ^{***}	3.26 ± 0.16 ^{**}	14.0 ± 3.5	19.5 ± 2.5
Control	15	14	272.0 ± 7.4	3.34 ± 0.08	3.39 ± 0.18	15.7 ± 3.2	21.0 ± 3.2

a: mean ± SD.

*, **, ***: $p < 0.05, 0.01, 0.001$ vs. Control group.#, ##, ###: $p < 0.05, 0.01, 0.001$ vs. MeIQx group.**Table 2.** Quantitative Analysis of GST-P Positive Foci

Treatment	Effective No. of Rats	GST-P positive foci	
		Number (No./cm ²)	Area (mm ² /cm ²)
MeIQx + Harman	15	7.50 ± 1.85 ^a [34.4] ^{#,***}	0.57 ± 0.12 [25.9] ^{***}
MeIQx + Norharman	14	13.47 ± 2.68 [61.7] ^{###,***}	1.13 ± 0.47 [49.3] ^{###,***}
MeIQx + NaNO ₂	14	42.26 ± 9.12 [193.7] ^{###,***}	6.08 ± 2.05 [265.5] ^{###,***}
MeIQx	15	21.82 ± 4.16 [100.0] ^{###}	2.29 ± 0.83 [100.0] ^{###}
Harman	12	4.60 ± 1.69	0.29 ± 0.12
Norharman	14	4.25 ± 2.26	0.28 ± 0.16
NaNO ₂	16	5.46 ± 2.04	0.33 ± 0.16
Control	14	5.76 ± 2.62	0.48 ± 0.40

a: mean ± SD.

[]: data represent percentage of MeIQx group value.

#, ###: $p < 0.05, 0.001$ vs. Control group.***: $p < 0.001$ vs. MeIQx group.

t-test and Welch's *t*-test in combination with the *F*-test.

Results

Significant suppression of body weight gain was observed in all groups of rats receiving MeIQx, compared with the controls (Table 1). There was no suppression in body weight gain in rats given harman or norharman alone, but a significant difference was found in the NaNO₂ group. More suppression in body weight gain was also observed in rats given combined treatment with MeIQx and NaNO₂. This combination regimen also suppressed food and water intake (Table 1). Increase of relative liver weights was evident in rats given MeIQx, but NaNO₂ alone caused decrease.

Quantitative data for rat liver GST-P positive foci, putative preneoplastic lesions, are summarized in Table 2. MeIQx induced statistically significantly increased numbers and areas of GST-P positive foci as compared to the controls. Harman, norharman and NaNO₂ themselves did not modify the development of GST-P positive foci; however, these compounds modified the development of the foci induced by

MeIQx. Harman and norharman suppressed the numbers and areas of the foci to 34 and 26%, and 62 and 49% of the control values (MeIQx alone), respectively. In contrast, NaNO₂ significantly enhanced foci development, the average number and area being 194% and 266%, respectively, of the control values.

Figure 2 shows mRNA expression levels for CYP1A1 and 1A2 in the liver samples taken at the end of the experiment. The data are expressed as multiples of the control level. None of the compounds examined increased CYP1A1 expression levels when used as alone, but surprisingly the combination of MeIQx and NaNO₂ resulted in a 17.2 fold increase. CYP1A2 expression levels were slightly elevated by MeIQx treatment alone, but no enhancement by additional administration of the other compounds was evident. Expression of other CYPs (2B1/2, 2C11, 2E1, 3A1, 3A2 and 4A1) were not altered by treatment with any of the compounds (data not shown).

In the kidneys, moderate chronic degenerative and regenerative cortical tubular lesions were found in several rats exposed to harman, with or without MeIQx (data not shown).

Fig. 2. Expression profiles of CYP1A1 and 1A2 mRNA in the rat liver determined by semi-quantitative RT-PCR analysis.

Discussion

The present data clearly show that MeIQx increased the numbers and areas of GST-P positive foci over the control values in rats given DEN, in line with expectation, demonstrating the reliability of the medium-term rat liver bioassay. However, neither harman nor norharman exerted any effects after DEN alone, confirming the data of a previous work with higher doses of the two chemicals²³. Another previous *in vivo* study showed that neither harman nor norharman, when given after initiation of liver carcinogenesis with *N*-ethyl-*N*-hydroxyethylnitrosoamine, changed liver tumor development while kidney tumorigenesis was enhanced, probably due to their renal toxicity¹⁸. It was also reported that administration of norharman to rats for 2 years did not induce liver tumors in rats¹².

Indeed, a significant finding in the present study was that both harman and norharman in fact suppressed MeIQx-associated liver carcinogenesis as assessed with reference to GST-P positive foci development. Norharman has been reported to act as a co-mutagen^{6,7}. However, the present data indicate that neither harman nor norharman exerted co-carcinogenic potential on MeIQx carcinogenicity *in vivo*, and this is consistent with the finding of anti-mutagenic action of the two compounds against dibenzo[*a,h*]anthracene¹⁰. Previous *in vivo* studies indicated that simultaneous treatment with acetaldehyde and norharman did not affect the tumor response in the hamster larynx²⁴. However, norharman strongly inhibited the induction of skin tumors when given together with dibenzofluoranthene-12,13-dihydrodio⁹. Thus, the modification potential of harman or norharman when given simultaneously with a carcinogen or mutagen depends on the paired chemical.

The data from many *in vitro* and *in vivo* studies indicate that MeIQx is metabolically activated by cytochrome P450s, CYP1A2 and CYP1A1, and then *N*-acetyltransferase 1 and/or *N*-acetyltransferase 2^{25–34}. Although CYP1A2 is mainly involved, other enzymes such as CYP1A1, CYP3A4 and CYP2C9/10 may also contribute^{25,34}. However, there are considerable interspecies differences in the metabolism of MeIQx by P450s^{25–27,30,31}, and no conclusions can be drawn about the metabolic pathways of MeIQx. Even though neither harman nor norharman changed the mRNA levels of the CYPs in this study, they modified MeIQx liver carcinogenesis. This finding suggests that protein levels of enzymatic activities might have been modified, as previously reported by Nii³⁵; norharman was found to inhibit CYP-mediated biotransformation of a heterocyclic amine. Hummerich *et al.* demonstrated harman inhibited hepatic CYP1A1³⁴. On the basis of these findings, inhibition of MeIQx hepatocarcinogenicity by harman and norharman in the present study was probably associated with modification of CYP-related enzymatic activities.

Alteration in body and organ weights and food and water intakes observed in this experiment did not appear to be associated with GST-P positive foci development, so a simple mechanism related to toxicity is unlikely.

In this study, moderate chronic degenerative and regenerative cortical tubular lesions were observed in rats exposed to harman, with or without MeIQx, but not norharman. Hagiwara *et al.*²³ reported that both harman and norharman exert renal toxicity at 1000 ppm, but not at 200 ppm, with or without initiation. Our observation is partly in agreement with previous studies.

Sodium nitrite, in the present investigation, significantly increased the appearance of GST-P positive foci in combination with MeIQx. NaNO₂ itself is not carcinogenic to the liver of rats¹⁷. A preliminary biochemical study of analysis of new chemical formation in the gastric contents indicated the presence of a very low level of an unknown nitric compound (data not shown). NaNO₂ did not increase liver weights, suggesting there was no induction of microsomal enzymes in the liver. Although Galenko *et al.*³⁶ reported that sodium nitrite alone decreased liver metabolic activity when given chronically, simultaneous administration of sodium nitrite with MeIQx here caused clear enhancement. The clear increase in CYP1A1 levels with simultaneous exposure to NaNO₂ and MeIQx may be associated with the enhancement of liver carcinogenesis. In contrast, our previous study demonstrated that development of mammary carcinomas of rats given 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, one of the carcinogenic heterocyclic amines, was significantly suppressed by simultaneous administration of NaNO₂³⁷. Therefore, the mechanisms underlying enhancement or suppression of heterocyclic amine carcinogenesis by NaNO₂ are organ dependent and should be clarified in future. Further investigations should cast light on such complex interactions between two or more chemicals in determining carcinogenic effects.

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