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Enhancing effects of a high fat diet on 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline-induced lung tumorigenesis in female A/J mice

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Abstract. Both heterocyclic amines and a high fat diet are associated with an increased risk of cancer in many organs. Female A/J mice were fed a diet supplemented with 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and a high fat diet to test for the development of lung tumors. In experiment 1, the mice were divided into 6 groups. Groups 1, 2, 3 and 4 were fed a diet supplemented with MeIQx at a concentration of 600 ppm for 0-12 weeks. A high fat diet containing 20% corn oil was given to Groups 1 and 5 for 0-32 weeks, Group 2 for 12-32 weeks and Group 3 for 0-12 weeks. Group 6 was fed a basal diet without supplements. MeIQx-treated groups (Groups 1, 2, 3 and 4) showed a significant increase in macroscopic and microscopic lung nodules compared with the control (Group 6). Areas of adenomas were increased dependent on the duration of exposure to the high fat diet. In experiment 2, Group 1 mice were fed MeIQx and a high fat diet, Group 2 a MeIQx alone diet, Group 3 a high fat alone diet, and Group 4 a basal diet without supplements. CYP1A2 mRNA in the liver was significantly decreased by a high fat diet (Group 3). The MeIQx alone group (Group 2) showed a tendency towards increased CYP1A2 expression, which was partially reduced in the MeIQx + high fat-treated group (Group 1). In the lungs, CYP1A2 mRNA expression was at an extremely low level, with no intergroup differences. In conclusion, MeIQx exerts tumorigenic potential in the lungs, and a high fat diet increases the size of induced lesions. The expression level of CYP1A2 in relation to MeIQx and a high fat diet may be associated with lung carcinogenesis.

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Introduction

Lung cancer is one of the most common causes of mortality and morbidity in the world (1,2). Though the main risk factor for the disease is tobacco smoking, non-smokers are also at risk. Nevertheless, risk factors other than tobacco use have yet to be fully elucidated. It has been suggested that diet exerts an effect, possibly interacting with smoking and genetic susceptibility (3).

Previous studies have demonstrated that heterocyclic amines generated in cooked meat and fish are highly mutagenic and carcinogenic in rodents (4-6). Case control studies in humans have further provided evidence that high-temperature-cooked meat is associated with the risk of colon (7,8), breast (9,10), gastric (11) and lung (12,13) cancer. MeIQx, one of the major amines contained in cooked meat, is activated by cytochrome P450 (CYP)1A2 after being ingested, and is reported to induce liver and lung tumors as well as lymphomas and leukemias in CDF_1 mice (14). Although the incidence of MeIQx-induced liver tumors was high, the values for lung and hematopoietic system tumors were much lower, and only females were affected among the lung cases. Therefore, whether the lung is indeed a target organ for MeIQx is controversial.

Fat consumption is associated with the risk of cancer in several organs (15-19), including the lungs (13,16,20-22). One epidemiological study linked a high fat diet to an increased risk of human lung adenocarcinomas, but specific fats were found to lack significant influence after adjustment for total fat intake (13). Experimental studies in rats have shown that fats containing ω-6 fatty acids (for example, corn oil) enhance, while ω-3 fatty acids (for example, fish oil and mustard oil) reduce, the development of chemically-induced colon tumors (23,24). Previously, we reported that a high fat diet (20% corn oil) enhanced 4-nitroquinoline 1-oxide (4NQO)-induced pulmonary tumorigenesis in male ICR mice (25). Therefore, in the present study, female A/J mice, which are highly susceptible to lung carcinogens, were fed a diet supplemented with MeIQx

and a high concentration of corn oil, to test for the development of lung tumors. The influence of cytochrome P450 (CYP)1A2 induction was also examined in a separate experiment.

Materials and methods

Chemicals and animals. MeIQx was purchased from the Nard Institute (Osaka, Japan). Female A/J mice (5 weeks of age) were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan) and maintained at the Kagawa University Animal Facility according to the institutional animal care guidelines. The animals were housed for feeding in polycarbonate cages with white wood chips, and were given free access to a powdered basal diet (CE-2; Clea Japan Inc., Tokyo, Japan) and tap water under controlled conditions of humidity (60±10%), lighting (12-h light/dark cycle) and temperature (24±2˚C). Experimentation was commenced when the mice were 7 weeks of age.

Experiment 1

Protocol. The experimental protocol is shown in Fig. 1. Starting at 7 weeks of age, mice in Groups 1, 2, 3 and 4 were fed a diet supplemented with MeIQx at a concentration of 600 ppm for 0-12 weeks, while Groups 5 and 6 received a diet lacking the carcinogen. A high fat diet containing 20% corn oil was fed to Groups 1 and 5 for 0-32 weeks, Group 2 for 12-32 weeks, and Group 3 for 0-12 weeks. Group 6 was fed the basal diet CE-2 without supplements. The mice were maintained on their respective diets under controlled conditions of humidity (60±0%), lighting (12-h light/dark cycle) and temperature (24±2˚C) until termination of the experiment at week 32. All surviving mice were then sacrificed under ether anesthesia. At autopsy, the lungs, livers and kidneys were excised and weighed. Lungs were infused through the bronchi with 10% neutral buffered formalin and carefully inspected grossly to determine the numbers of macroscopic lung nodules using a stereomicroscope.

Histopathological analysis. The excised tissues were routinely processed for embedding in paraffin, serially sectioned (3.5 μm) and stained with hematoxylin and eosin for histopathological examination. Lung nodules, alveolar hyperplasias and adenomas were diagnosed according to established criteria (26), and their numbers were counted under a microscope.

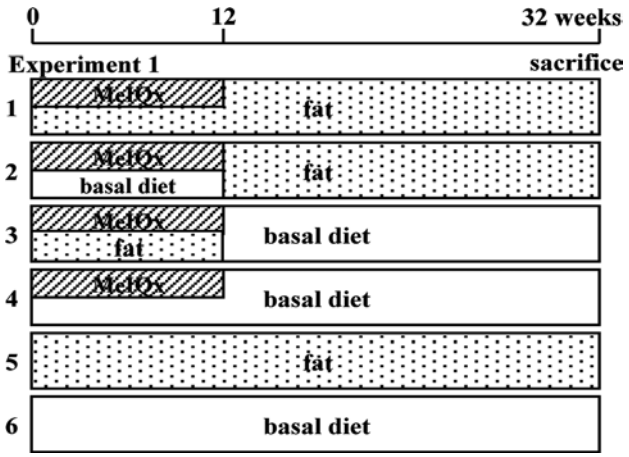

Figure 1. Protocol for experiment 1. Animals were 7-week-old female A/J mice. MeIQx (600 ppm) or fat (corn oil 20%) was added to the pelleted diet. CE-2 was used as the basal diet.

Areas of lung proliferative lesions were measured with the assistance of an image analyzer (IPAP, image processor for analytical pathology; Sumika Technoservice Co., Hyogo, Japan). The total area (mm^2) of lung proliferative lesions per mouse was used for statistical analysis.

Experiment 2

Protocol. The second experiment was conducted to determine the effects of MeIQx and a high fat diet on CYP1A2 mRNA expression. MeIQx and a high fat diet were administered by the same routes as those used in experiment 1. Mice in Groups 1 and 2 were fed a diet supplemented with MeIQx for 2 weeks, while Groups 1 and 3 received a high fat diet for 2 weeks. Group 4 was fed the basal diet CE-2 without supplements. The surviving mice were sacrificed at the end of week 2, and their lungs, livers and kidneys were excised for RNA isolation and the quantitative analysis of CYP1A2. Total RNA was isolated from 30 mg of whole lung and liver tissues using RNAlater RNA Stabilization Reagent and an RNeasy Mini Kit (both from Qiagen Corp., Hilden, Germany). The concentration of RNA was measured at an absorbance of 260 nm. First-strand cDNA was synthesized from 400 ng of total RNA using TaqMan Reverse Transcription Reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

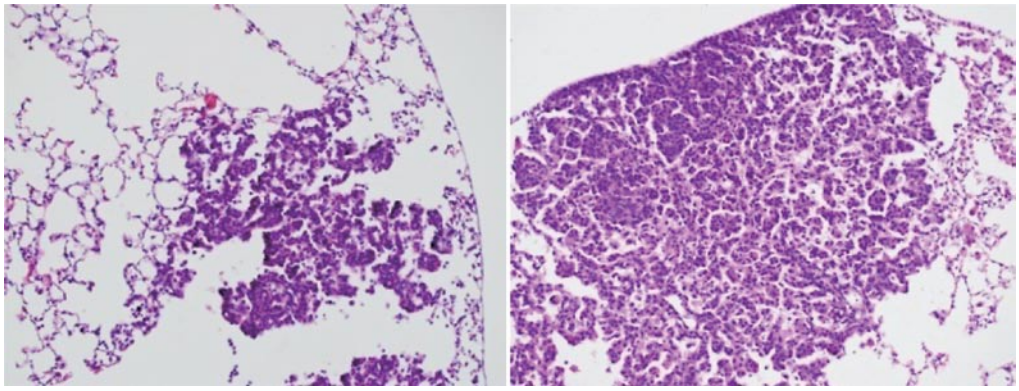

Figure 2. Representative images of lung proliferative lesions. Left, hyperplasia; right, adenoma.

Quantitative real-time RT-PCR. Optimal primers and probes were purchased from the Assays-on-Demand System of ABI (Applied Biosystems). TaqMan Rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Control Reagent (Applied Biosystems) was used for PCR to measure GAPDH mRNA levels as an internal control.

Quantitative real-time RT-PCR was performed with the ABI PRISM 7000 Sequence Detection System using specific primers and a TaqMan probe for CYP1A2. PCR was carried out in 50-μl reaction mixtures containing 25 μl of 2X TaqMan Universal PCR Master Mix, 50 ng of cDNA, 100 nM of each primer and 200 nM of TaqMan probe. Cycling conditions were as follows: 2 min at 50˚C, 10 min at 95˚C, and then 40 cycles of 15 sec at 95˚C followed by 1 min at 60˚C. TaqMan PCR products were detected as an increase in fluorescence from cycle to cycle. The amplification plots of the PCR reaction were used to determine the threshold cycle (Ct). The Ct value represented the PCR cycle at which an increase in reporter fluorescence (ΔRn) above the line of the optimal value was first detected. The initial copy number of the target mRNA was calculated from plots of the Ct against the input target quantity.

Since the precise amount and quality of total RNA are difficult to assess, we quantified transcripts of the GAPDH gene as an internal control according to a quantitative RT-PCR assay. Normalization of the data was achieved by quantitating the cycle number at an arbitrary fluorescence intensity in the linear exponential phase using the ratio of the cycle number of each enzyme relative to that of GAPDH.

Statistical analysis. Data for final body and organ weights were analyzed by the Student's t-test. The incidence of lung proliferative lesions was analyzed by Fisher's exact probability test, and data for multiplicity and area by the Student's t-test. CYP1A2 mRNA levels were analyzed by the Mann-Whitney U test. Areas of lung lesions were also analyzed by the Spearman's rank correlation coefficient.

Results

Final body and organ weights are shown in Table I. Final body weight was significantly increased in the high fat alone group (Group 5) compared to the control (Group 6). MeIQx + high fat-treated groups (Groups 1 and 2) also showed an increase in final body weight compared to the MeIQx alone group (Group 4).

The relative organ weight of the lungs, liver and kidneys in the high fat alone group (Group 5) and the MeIQx + high fat groups (Group 1, 0-32 weeks and Group 2, 12-32 weeks) was significantly decreased. These changes in relative organ weight were mainly due to an increase in body weight in Groups 1, 2 and 5. The MeIQx alone group (Group 4) showed no significant change in organ weight. Absolute organ weight showed no treatment-related change.

Results for the lung lesions are shown in Table II. Whitish nodules were macroscopically detected in all groups, including the basal diet controls. The MeIQx-treated groups (Groups 1, 2, 3 and 4) exhibited a significant increase in macroscopic lung nodules compared to the control (Group 6), while only a few lung nodules were observed in the high fat alone group (Group 5). Microscopically, the lung nodules were hyperplasias

Table I. Final body and organ weights of mice in experiment 1 .

Group	No.[a]	Body weight (g)	Lung weight		Liver weight		Kidney weight	
			Absolute (g)	Relative (%)	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
1 MeIQx + fat (0-32 weeks)	20	33.00±4.79^{bc}	0.19±0.03	0.59±0.11^{bc}	0.96±0.11	2.94±0.40^{bc}	0.29±0.02	0.90±0.10^{bc}
2 MeIQx + fat (12-32 weeks)	20	31.74±3.53^{bc}	0.20±0.03	0.63±0.12^{c}	0.98±0.08	3.11±0.47^{bc}	0.30±0.02	0.95±0.13^{bc}
3 MeIQx + fat (0-12 weeks)	20	27.16±2.35	0.20±0.02	0.74±0.09	0.96±0.10	3.53±0.34	0.29±0.02	1.07±0.11^{b}
4 MeIQx	20	26.46±3.19	0.20±0.02	0.78±0.11	0.94±0.09	3.56±0.33	0.29±0.02	1.12±0.12
5 Fat (0-32 weeks)	14	36.10±4.60^{bc}	0.20±0.02	0.56±0.12^{bc}	0.99±0.11	2.78±0.57^{bc}	0.20±0.03	0.82±0.10^{bc}
6 Control	10	25.62±2.24	0.18±0.02	0.71±0.06	0.95±0.11	3.71±0.15	0.31±0.03	1.20±0.01

Data are presented as the mean ± SD values. [a]No. of animals. [b]P<0.05 compared with Group 6. [c]P<0.05 compared with Group 4.

Table II. Macroscopic and microscopic results for lung lesions of mice in experiment 1.

Group	No.[a]	Macroscopic incidence of nodules (%)	Macroscopic lung nodules/mouse	Microscopic lung nodules/mouse			Area of hyperplasia (mm^2)	Area of adenoma (mm^2)	Area of hyperplasia + adenoma (mm^2)
				Hyperplasia	Adenoma	Total (hyperplasia + adenoma)			
1 MeIQx + fat (0-32 weeks)	20	15/20 (75.0)[b]	1.30±0.98[b]	0.15±0.37	0.70±0.80[b]	0.85±0.81[b]	0.02±0.05	0.35±0.45[bc]	0.37±0.45[b]
2 MeIQx + fat (12-32 weeks)	20	14/20 (73.7)[b]	1.79±1.44[b]	0.37±0.50	0.95±0.91[b]	1.32±1.00[b]	0.08±0.16	0.34±0.37[bc]	0.42±0.39[b]
3 MeIQx + fat (0-12 weeks)	20	17/20 (85.0)[b]	1.80±1.24[b]	0.55±0.61	0.85±0.99[b]	1.40±1.10[b]	0.11±0.20	0.30±0.34[bc]	0.41±0.35[b]
4 MeIQx	20	15/20 (78.9)[b]	1.32±1.11[b]	0.37±0.68	0.79±0.54[b]	1.16±0.90[b]	0.09±0.21	0.24±0.22[bc]	0.34±0.31[b]
5 Fat (0-32 weeks)	14	3/14 (21.4)	0.21±0.43	0.14±0.36	0.07±0.27	0.21±0.58	0.01±0.04	0.02±0.09	0.04±0.13
6 Control	10	2/10 (25.0)	0.38±0.74	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Data are presented as the mean ± SD values. [a]No. of animals. [b]P<0.05 compared with Group 6. [c]P<0.02, R=0.36, Spearman's rank correlation coefficient.

Table III. Relative quantification of CYP1A2 mRNA in the liver and lungs of A/J mice in experiment 2.

Group	No.[a]	CYP1A2 mRNA in the liver	CYP1A2 mRNA in the lung (x10^{-3})
1 MeIQx + fat	5	1.13±0.23	4.21±6.54
2 MeIQx	5	1.98±0.89	3.06±2.41
3 Fat	5	0.55±0.14[b]	3.38±4.03
4 Control	5	1.08±0.30	5.92±2.70

Data are presented as the mean ± SD values. [a]No. of animals. [b]P<0.05 compared to Groups 1, 2 and 4.

and adenomas (Fig. 2). Values for microscopic hyperplasias and adenomas in the MeIQx-treated groups (Groups 1, 2, 3 and 4) were significantly higher than the respective data for the group without MeIQx treatment (Group 6). Additionally, areas of adenomas and hyperplasias + adenomas were significantly elevated in the MeIQx-treated groups (Groups 1, 2, 3 and 4), with the degree of increase dependent on the duration of the high fat diet. Microscopically, the liver and kidney showed no treatment-related changes.

Data regarding the relative quantification of CYP1A2 mRNA in the liver and lungs of A/J mice are summarized in Table III. Expression levels of CYP1A2 mRNA in the liver were significantly decreased by a high fat diet (Group 3). The MeIQx alone group (Group 2) showed a tendency toward an increase in CYP1A2 mRNA compared to the controls (Group 4). By contrast, the MeIQx + high fat-treated group (Group 1) showed a tendency toward a decrease compared to Group 2, though without statistical significance. In the lungs, CYP1A2 mRNA expression levels were extremely low, with no intergroup differences.

Discussion

In agreement with a previous study (14), this study confirms that MeIQx induces mouse lung tumors, and corroborates the epidemiological finding that MeIQx is associated with the risk of lung cancer. Furthermore, a high concentration of corn oil in the diet significantly increased the areas of MeIQx-induced lung adenomas, without any influence on the incidence and multiplicity of lung tumors. Notably, the enhancing effect of the high fat diet was duration-dependent. In this study, MeIQx-induced lung lesions exhibited almost the same histopathological features as lesions induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (33,34), though with relatively low numbers.

It is well known that MeIQx is converted to genotoxic metabolites by liver CYP1A2 (27,28), resulting in DNA adduct formation. Previous research revealed expression levels of CYP1A2 mRNA in the liver to be increased approximately 1.5- to 2-fold by treatment with MeIQx (29); in the present study, values were elevated 1.83-fold. A high fat diet caused a significant decrease in CYP1A2 mRNA, suggesting that it has the potential to reduce the metabolic activation of MeIQx, consequently inhibiting MeIQx-induced lung tumorigenesis

during the initiation phase. On the other hand, the observed enhancing effect on areas of MeIQx-induced lung adenomas indicates the promotion of lung tumor development. In this study, CYP1A2 mRNA levels in the lungs were very low compared to findings in humans, where CYP1A2 is mainly detected in the liver (30). The results indicate that CYP1A2 in the liver may play an important role in MeIQx-induced lung tumorigenesis.

In a previous study, CDF_1 mice exhibited a high incidence of liver tumor development when fed a diet containing MeIQx at 600 ppm for 84 weeks (14), while in this study no liver tumors were observed in any of the groups. This is in line with the known resistance of the A/J mouse strain to hepatocarcinogenesis (31). By contrast, while male and female CDF_1 mice exhibited a lung tumor incidence of 43% (14), the figure for A/J female mice in this study was 78.9%, confirming that the A/J strain is highly susceptible to lung tumorigenesis (32).

Epidemiological studies suggest a relationship between the risk of cancer and increased consumption of ω-6 fatty acids found in corn oil (15-19). It has been reported that a decreased ω-6/ω-3 fatty acid ratio reduces the invasive potential of human lung cancer cells by downregulating cell adhesion/invasion-related molecules such as MMP-1, integrin-α2 and nm23-H4 (35). The enhancing effect of a corn oil diet on MeIQx-induced lung tumors in the present study might therefore be due to the high levels of ω-6 included.

In conclusion, MeIQx shows tumorigenic potential in female A/J mouse lungs, and a high fat diet increases the areas of MeIQx-induced lung adenomas, implying a promotion potential. Our results also suggest a role for CYP1A2 in relation to MeIQx and the effects of a high fat diet on lung carcinogenesis.

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