

Different threshold levels for 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) initiation of lung and colon carcinogenesis and the effects of an additional initiation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in A/J mice

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Abstract. The existence of possible threshold dose levels of genotoxic carcinogens for carcinogenesis is of particular interest for human risk assessment. Recently, no observed effect levels (NOELs) for various hepatocarcinogens have been reported. However, reports on threshold levels for lung carcinogenesis have hitherto been lacking. In the present study, we first investigated low dose response lung and colon carcinogenesis with a food-derived genotoxic carcinogen, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) (0, 0.01, 0.1, 1, 10 and 100 ppm in the diet) alone for 32 weeks using female A/J mice. The endpoints were histopathologically diagnosed hyperplasias and adenomas in the lung, and aberrant crypt foci (ACF) in the colon. The results showed NOELs of 100 and 1 ppm, respectively. We next investigated the effect of additional pre-treatment with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (2 mg/mouse, single dose, intraperitoneal injection) prior to the low-dose application of MeIQx (0, 0.01, 0.1, 1, 10, 100 and 600 ppm in the diet) for 32 weeks. Lung lesions were significantly increased in the NNK + MeIQx (1 ppm) group, but not in the NNK + MeIQx (≥ 10 ppm) groups. Since the dose-response curve was not of so-called 'hockey stick type', it was not possible to determine a NOEL for lung tumorigenesis. Significant increase in the mRNA expression of CYP2A5, a major metabolic enzyme

for NNK, was also observed in the NNK + MeIQx (1 ppm) group, and a similar pattern was noted for O⁶-methylguanine DNA methyltransferase (MGMT). By contrast, the formation of colon ACF showed a dose-dependent increase. The NOEL for the formation of colon ACF was considered to be 10 ppm MeIQx with NNK. These results suggest that MeIQx may have different threshold dose levels for the induction of lung tumorigenic lesions and ACF formation in the colon. Pre-treatment with NNK, a potent lung carcinogen, concealed the effects of MeIQx in the lung, but exerted minimal influence in the colon. CYP2A5 and MGMT expression may be of importance, particularly in the lung. The present study provides critical suggestions for the human risk assessment of genotoxic carcinogens.

Introduction

Throughout life, humans are exposed to thousands of natural and artificial chemicals. Some of these pose serious threats to human health, raising the need for risk assessment. Recently, the threshold of toxicological concern (TTC) has been proposed, as a principle to establish a generic human exposure threshold value for chemicals below which there is no appreciable risk to human health (1-3). Genotoxic carcinogens have traditionally been considered to have no threshold in exerting their potential for cancer induction. However, there have now been reports indicating the possibility of a threshold level for hepatocarcinogenesis caused by genotoxic agents (4,5). This is clearly of great general importance for risk assessment.

2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) is a well known genotoxic food-derived heterocyclic amine (HCA) carcinogen (6,7). The carcinogenicity of MeIQx in the liver has been established in rats and monkeys (7-10), and in the liver and lungs of CDF1 mice (11). In particular, numerous studies on low-dose-response liver carcinogenesis have been conducted using rat models, and as a consequence the existence of a threshold level has been proposed. No observed effect levels (NOELs) for MeIQx-induced hepatocellular foci and *fas* gene expression have been reported (4,5). In general,

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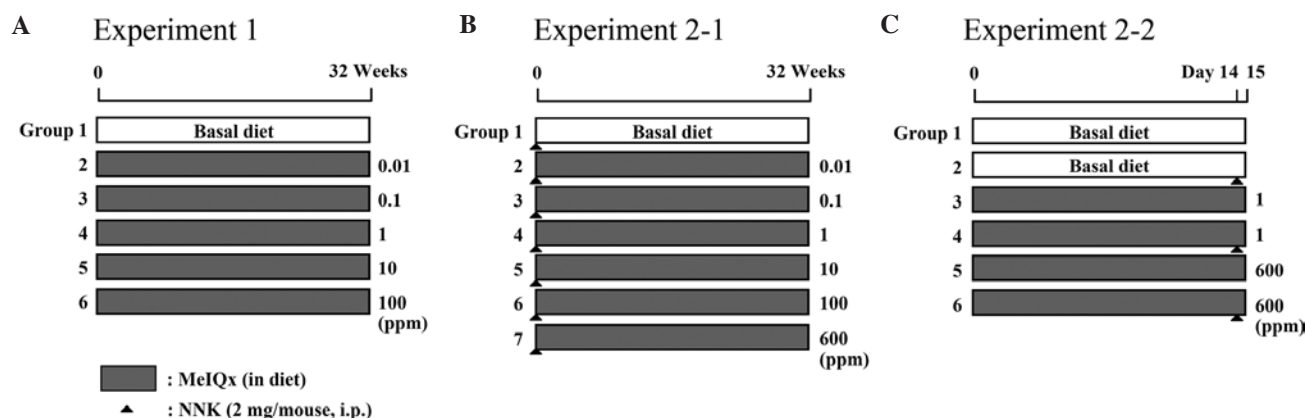


Figure 1. Experimental design for Experiments 1, 2-1 and 2-2. Mice aged ~7 weeks were administered MeIQx alone or MeIQx and NNK. (A) MeIQx alone at concentrations of 0 (basal diet), 0.01, 0.1, 1, 10 and 100 ppm for 32 weeks in the diet. (B) MeIQx at concentrations of 0 (basal diet), 0.01, 0.1, 1, 10, 100 and 600 ppm for 32 weeks subsequent to a single dose of NNK (2 mg/0.1 ml saline/mouse, i.p.) on Day 0. (C) MeIQx at concentrations of 0 (basal diet), 1 and 600 ppm for 2 weeks, after groups 2, 4 and 6 were administered a single dose of NNK (2 mg/0.1 ml saline/mouse, i.p.) on Day 14. Surviving mice were sacrificed on Day 15.

the colon is recognized as a prime target of most HCAs (12,13), but an absence of colon carcinogenicity in mice for MeIQx has been reported (14). On the other hand, proliferative lesions were induced by MeIQx in a dextran sulfate sodium model for tumor promotion, suggesting initiation activity of MeIQx in the colon (14). We also reported lung tumorigenesis for MeIQx in A/J mice (15,16), a strain which is commonly considered to be highly susceptible to the induction of lung lesions (15,17).

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a tobacco-specific N-nitrosamine genotoxic carcinogen with an important role in tobacco-related human lung cancer, given its strong potential to induce lung carcinogenesis in rodents (18). We earlier reported a bioassay model for the chemoprevention of lung cancer induced by a single intraperitoneal injection (i.p.) of NNK in female A/J mice (19,15), in which lung tumors arise in almost all animals after only 13 weeks. This has clear advantages for studies on dose-dependence.

Since, to the best of our knowledge, there have been no reports on threshold levels for lung carcinogenesis, we examined low-dose carcinogenesis induced by MeIQx in A/J mice, as well as the effects of additional initiation with NNK. Organ differences (lung vs. colon) in threshold levels for MeIQx with/without NNK pre-treatment were also examined.

Materials and methods

Chemicals. MeIQx was purchased from Wako Pure Chemical Industries (Osaka, Japan) for Experiment 1, and from the NARD Institute (Osaka, Japan) for Experiment 2. NNK was from Toronto Research Chemicals (Toronto, Canada).

Animals. Female A/J mice (5 weeks of age) were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). The mice were housed in polycarbonate cages with white wood chips for bedding, given free access to drinking water, and fed a basal diet under controlled conditions of humidity (60±10%), lighting (12-h light/dark cycle) and temperature (24±2°C). After 32 weeks, surviving mice were sacrificed

under ether anesthesia. The animals were maintained at the Kagawa University Animal Facility according to the Institutional Rules for Animal Experimentation. The protocols for the experiments were approved by the Animal Care and Use Committee for Kagawa University.

Experimental design

Experiment 1. The first experiment was conducted to evaluate the NOEL for lung carcinogenesis induced by low-dose MeIQx administration (Fig. 1A). Mice aged 7 weeks were fed diets supplemented with MeIQx at concentrations of 0 (control), 0.01, 0.1, 1, 10 and 100 ppm (groups 1, 2, 3, 4, 5 and 6, respectively) for 32 weeks. Surviving animals were sacrificed at the end of week 32. At necropsy, the lungs were excised and weighed, infused with 10% neutral buffered formalin, and carefully inspected grossly. Macroscopically detected lesions and each lung lobe were examined histopathologically. The colons were also excised and infused with 10% neutral buffered formalin, and examined after methylene blue staining under a stereomicroscope.

Experiment 2-1. This experiment was conducted to evaluate the NOEL for lung carcinogenesis with low-dose MeIQx after a single NNK administration (Fig. 1B). Mice aged 7 weeks were fed diets supplemented with MeIQx at concentrations of 0 (control), 0.01, 0.1, 1, 10, 100 and 600 ppm (groups 1, 2, 3, 4, 5, 6 and 7, respectively) for 32 weeks. Each group was administered a single dose of NNK (2 mg/0.1 ml saline/mouse, i.p.) at the commencement of MeIQx administration (Day 0). Surviving animals were sacrificed at the end of week 32. At necropsy, the lungs were excised and weighed, infused with 10% neutral buffered formalin, and carefully inspected grossly. Macroscopically detected lesions and each lung lobe were examined histopathologically. The colons were also excised and infused with 10% neutral buffered formalin, and examined after methylene blue staining under a stereomicroscope. Portions of liver were sampled and frozen in liquid nitrogen for subsequent RNA isolation and quantitative analysis of CYP1A2 and CYP2A5.

Experiment 2-2. The second part of the experiment was conducted to evaluate the alteration of enzymes, including

Table I. Incidence and multiplicity of lung proliferative lesions (Experiment 1).

Group	Treatment	Macroscopic lesions		Histopathological lesions	
		Incidence (%) ^a	Incidence (%) ^a	Hyperplasia	Adenoma
				Incidence (%) ^a	Incidence (%) ^a
1	Basal diet	4/24 (16.7)	4/24 (16.7)	4/24 (16.7)	0/24 (0.0)
2	MeIQx (0.01 ppm)	7/20 (35.0)	2/20 (10.0)	2/20 (10.0)	3/20 (15.0)
3	MeIQx (0.1 ppm)	4/20 (20.0)	3/20 (15.0)	3/20 (15.0)	1/20 (5.0)
4	MeIQx (1 ppm)	3/20 (15.0)	1/20 (5.0)	1/20 (5.0)	2/20 (10.0)
5	MeIQx (10 ppm)	6/20 (30.0)	3/20 (15.0)	3/20 (15.0)	2/20 (10.0)
6	MeIQx (100 ppm)	7/20 (35.0)	2/20 (10.0)	2/20 (10.0)	1/20 (5.0)
7	Basal diet	6/19 (31.6)	0/19 (0.0)	0/19 (0.0)	6/19 (31.6)
8	MeIQx (600 ppm)	17/20 (85.0) ^c	7/20 (35.0) ^c	7/20 (35.0) ^c	14/20 (70.0) ^b

^aNumber of mice observed with each lesion (%) ^bP<0.05 and ^cP<0.01, significantly different compared to group 7. Groups 7 and 8, based on citation data (16).

CYP1A2, CYP2A5 and MGMT, in the lungs and/or liver following NNK and MeIQx administration (Fig. 1C). Mice aged 7 weeks were fed a basal diet alone or supplemented with MeIQx at concentrations of 1 ppm (groups 3 and 4) and 600 ppm (groups 5 and 6) for 14 days. Groups 2, 4 and 6 were administered a single dose of NNK (2 mg/0.1 ml saline/mouse, i.p.) on Day 14.

Surviving mice were sacrificed under ether anesthesia on Day 15 (5 animals/group). At necropsy, the lungs and liver were excised, weighed, and then sampled and frozen in liquid nitrogen for subsequent RNA isolation and quantitative analysis of CYP and MGMT mRNA expression.

Histopathological analysis. All five lobes of the formalin-fixed lungs were embedded in paraffin, and the slides were stained with H&E for the histopathological assessment of proliferative lesions (hyperplasia and adenoma) based on previously established criteria (20,21).

RNA isolation. Total RNA was extracted from 30-mg samples of frozen whole liver tissue using an RNeasy mini kit plus (Qiagen Corp., Hilden, Germany) according to the manufacturer's instructions. The concentration of RNA was measured with absorbance at 260 nm, and first-strand cDNA was synthesized from 200 ng of total RNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Quantitative real-time RT-PCR. Optimal primers and probes were selected using Software Primer Express Ver. 1.7 (Applied Biosystems). As an endogenous control for the PCR of r18S mRNA, r18S Control Reagent (Applied Biosystems) was applied. The sequences of primers and TaqMan probes for mouse CYP2A5 mRNA were as follows: forward, 5'CCAACGTTATGGTCCTGTATTCAC3'; reverse, 5'TCCACCAGAGCTTCCTTGACT3'. The sequences of primers and probes for CYP1A2, MGMT and r18S mRNA

were unknown, as they were purchased from the Assays-on-Demand System of Applied Biosystems. The following assays were used: Mm00487224_m1 for CYP1A2 mRNA, Mm00485014_m1 for MGMT mRNA, and 4310893E for r18S mRNA.

Quantitative real-time RT-PCR was performed with the ABI PRISM 7000 Sequence Detection System using specific primers and TaqMan probes for mouse CYP1A2, CYP2A5 and MGMT. PCR was carried out in 50- μ l reaction mixtures containing 25 μ l 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. PCR amplification of r18S mRNA was similarly carried out. TaqMan PCR products were detected as an increase in fluorescence from cycle to cycle, and 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 (DRn) above the line of the optimal value was first detected. The initial copy number of the target mRNA was calculated by plotting the Ct against the input target quantity. r18S gene transcripts were also quantified as an endogenous control according to quantitative RT-PCR. Normalization of the data was achieved by quantitating the cycle number at an arbitrary fluorescence intensity in the linear exponential phase, and by calculating the ratio of the cycle number of each enzyme relative to that of r18S.

Statistical analysis. The body and organ weights, the incidence and multiplicity of lung and colon lesions (including macroscopic and histopathological lesions), and the relative ratios of CYP1A2, CYP2A5 and MGMT mRNA levels were analyzed by the parametric or non-parametric Dunnett's multiple comparison test. The incidences of lung proliferative lesions and colon ACF were analyzed by Fisher's exact probability test.

Table II. Incidence and multiplicity of aberrant crypt foci in the colon (Experiment 1).

Group	Treatment	Aberrant crypt foci	
		Incidence (%) ^a	No./mouse ^b
1	Basal diet	2/24 (8.3)	0.08±0.28
2	MeIQx (0.01 ppm)	2/20 (10.0)	0.10±0.31
3	MeIQx (0.1 ppm)	4/20 (20.0)	0.20±0.41
4	MeIQx (1 ppm)	5/20 (25.0)	0.30±0.57
5	MeIQx (10 ppm)	9/20 (45.0) ^c	0.80±1.40 ^c
6	MeIQx (100 ppm)	15/20 (75.0) ^c	1.55±1.43 ^d

^aNumber of mice observed with each lesion (%). ^bMean ± SD. ^cP<0.05, ^dP<0.01 and ^eP<0.001, significantly different compared to group 1.

Table III. Multiplicity of lung proliferative lesions (Experiment 2-1).

Group	Treatment	Macroscopic lesions	Histopathological lesions		
		No./mouse ^a	Hyperplasia No./mouse ^a	Adenoma No./mouse ^a	Adenoma + Hyperplasia No./mouse ^a
1	NNK + Basal diet	6.75±3.34	3.54±2.00	1.79±1.64	5.33±2.68
2	NNK + MeIQx (0.01 ppm)	6.92±4.42	2.23±1.42	2.23±1.69	4.46±2.40
3	NNK + MeIQx (0.1 ppm)	9.67±2.87	3.08±2.07	3.00±2.00	6.08±2.87
4	NNK + MeIQx (1 ppm)	10.08±2.64 ^b	4.42±2.07	3.33±1.07 ^b	7.75±2.63 ^b
5	NNK + MeIQx (10 ppm)	8.25±2.64	4.17±2.20	1.00±0.74	5.17±2.59
6	NNK + MeIQx (100 ppm)	7.62±4.01	4.69±1.84	0.77±0.93	5.46±2.40
7	NNK + MeIQx (600 ppm)	5.77±2.71	2.92±1.44	0.92±0.86	3.85±1.57

^aMean ± SD. ^bP<0.05, significantly different compared to group 1. NNK dose, 2 mg/mouse.

Results

Experiment 1. During the 32-week experimental period, the body weights of the mice in the treated group were similar to those of the basal diet group, although elevation was sporadically observed. No significant differences were observed between the liver and lung weights.

Data regarding the incidence and multiplicity of lung macroscopic lesions are summarized in Table I. Histopathologically, the lung lesions were categorized as hyperplasia or adenoma based on previously established criteria (20,21). Lung lesions containing areas of both hyperplasia and adenoma were categorized as adenoma. No significant differences were observed between the incidence and multiplicity of lung macroscopic lesions with up to 100 ppm of MeIQx exposure. However, lung lesions were increased at a dose of 600 ppm in our previous study using the same protocol (16). Since the incidences of hyperplasia and adenoma separately were not altered, histopathological examination for multiplicity was not performed. No significant findings were obtained for the liver.

Data on the incidence and multiplicity of colon lesions are summarized in Table II. Values for ACF were significantly increased in the groups treated with 10 and 100 ppm MeIQx (P<0.05 and P<0.001, respectively).

Experiment 2-1. During the 32-week experimental period, the body weights of the mice in the treated group were similar to those of the basal diet group. No significant differences were observed between the liver and lung weights.

Data regarding the multiplicity of lung macroscopic and microscopic lesions are summarized in Table III and Fig. 2. As in Experiment 1, the lung lesions were histopathologically categorized as hyperplasia or adenoma based on previously established criteria (20,21). A significant increase in the incidence and multiplicity of the lung lesions was noted in the NNK + MeIQx (1 ppm) group (P<0.05) compared to the NNK + Basal diet group. By contrast, no increase was evident when NNK was combined with MeIQx (≥10 ppm).

Data regarding the incidence and multiplicity of colon lesions are summarized in Table IV. Values for ACF were significantly higher in the NNK + MeIQx (600 ppm) group than in the NNK + Basal diet group (P<0.001). The absolute values of incidence and multiplicity of colon ACF in the NNK + MeIQx (10 ppm) and NNK + MeIQx (100 ppm) groups were similar to the results of Experiment 1 (MeIQx alone). Thus, the initiation effect of MeIQx in Experiments 1 and 2-1 appeared to be similar, with no increase in colon ACF with NNK treatment.

Table IV. Incidence and multiplicity of aberrant crypt foci in the colon (Experiment 2-1).

Group	Treatment	Aberrant crypt foci	
		Incidence (%) ^a	No./mouse ^b
1	NNK + Basal diet	5/22 (22.7)	0.32±0.65
2	NNK + MeIQx (0.01 ppm)	2/10 (20.0)	0.20±0.42
3	NNK + MeIQx (0.1 ppm)	2/12 (16.7)	0.25±0.62
4	NNK + MeIQx (1 ppm)	3/11 (27.3)	0.27±0.47
5	NNK + MeIQx (10 ppm)	3/11 (27.3)	0.55±1.04
6	NNK + MeIQx (100 ppm)	7/10 (70.0) ^c	1.60±1.51
7	NNK + MeIQx (600 ppm)	10/10 (100) ^d	73.80±36.6 ^d

^aNumber of mice observed with each lesion (%). ^bMean ± SD. ^cP<0.05 and ^dP<0.001, significantly different compared to group 1. NNK dose, 2 mg/mouse.

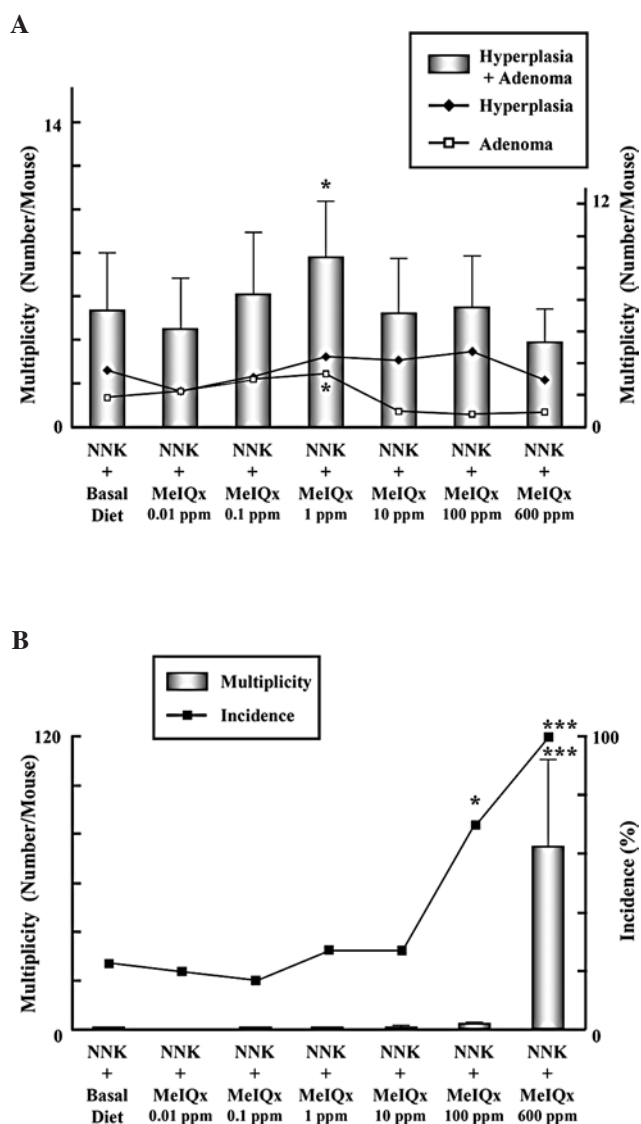


Figure 2. Incidence and multiplicity of (A) histopathological lung lesions, and (B) colon ACF in Experiment 2-1. (A) Histopathological lung lesions were indicative of a significant increase (*P<0.05) in lung tumorigenesis in group 3 [MeIQx (1 ppm) with NNK]. (B) ACF in the colon were significantly increased (*P<0.05, ***P<0.001) in group 6 and 7 in a dose-dependent manner. Note the difference in the dose-response curve of lung lesions compared to colon ACF.

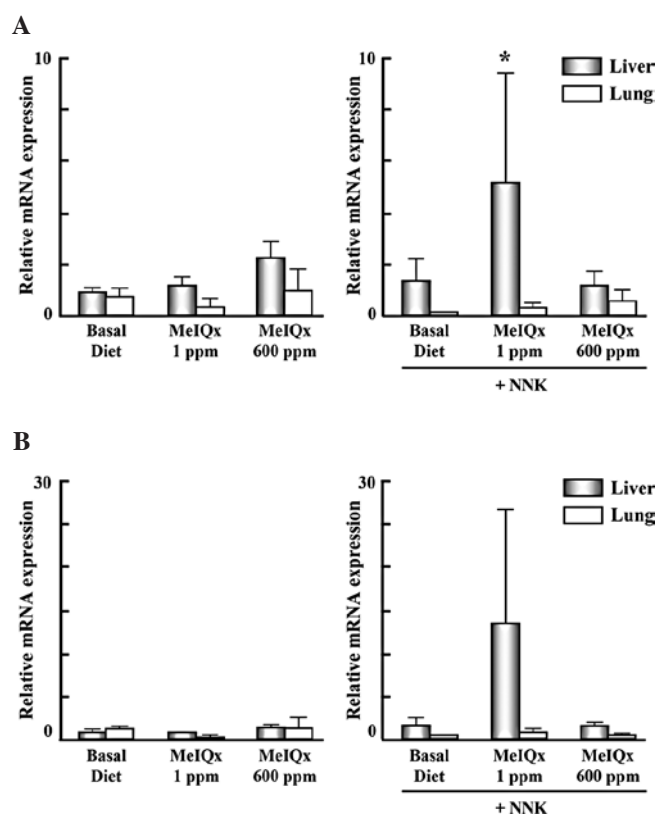


Figure 3. Relative mRNA expression of CYP2A5 and MGMT in the liver and lungs. (A) Significant increase in mRNA expression of liver CYP2A5 (*P=0.05) in group 3 [MeIQx (1 ppm) with NNK]. (B) Increase in mRNA expression of liver MGMT in group 3 [MeIQx (1 ppm) with NNK].

No significant changes were observed in the mRNA expression of CYP1A2 and CYP2A5 in the liver and lungs.

Experiment 2-2. Data regarding the expression of CYP1A2, CYP2A5 and MGMT are shown in Fig. 3. A significant increase in CYP2A5 (P=0.05) as well as an increase in MGMT in the liver, but not in the lungs, was observed in the NNK + MeIQx (1 ppm) group. However, expression of CYP2A5 and MGMT in the liver of the NNK + MeIQx (600 ppm) group was similar to that in the NNK alone group.

Discussion

In the present study, in Experiment 1, macroscopic and microscopic lung lesions were not induced in the treated mice receiving MeIQx at ≥ 100 ppm for 32 weeks. However, our previous study using the same protocol demonstrated that a dose of 600 ppm significantly increased lung neoplastic lesions (16). Therefore, the NOEL was set at 100 ppm for lung tumorigenesis. By contrast, the incidence and multiplicity of ACF in the colon significantly increased in the ≥ 10 ppm groups. Therefore, the NOEL for colon ACF formation was considered to be 1 ppm in Experiment 1 of the present study. In our previous experiments, treatment with MeIQx alone did not promote colon tumors (14); the formation of ACF may mainly reflect the initiation activity of MeIQx. Initially, Experiment 2 of the present study aimed to investigate the NOEL for a combination treatment of two genotoxic carcinogens, NNK and MeIQx. However, the dose-response curve of the lung lesions did not display so-called 'hockey stick type' (22,23), so it was not possible to determine a value for lung tumorigenesis.

The incidence and multiplicity of colon ACF showed a dose-dependent increase, and the NOEL for the additional formation of colon ACF multiplicity by MeIQx was altered at 100 ppm. However, the incidence and multiplicity of colon ACF were not markedly altered by NNK pre-treatment, so that the formation of colon ACF was considered to not have been greatly affected by the initiation activity of MeIQx.

The formation of lung lesions with the administration of MeIQx alone was less than two lesions per mouse on average in terms of multiplicity at 600 ppm (16). By contrast, with NNK alone, on average more than six lesions per mouse were found (Experiment 2-1, basal diet with NNK group, summarized in Table III). Thus, the lung carcinogenicity of MeIQx alone proved to be very low compared with that of NNK, and any interaction was effectively concealed. We therefore investigated the expression of CYP2A5 and CYP1A2, major metabolic enzymes for NNK and MeIQx, in the liver and lungs, respectively. No significant change was observed in these CYPs at 32 weeks. Next, we investigated CYP and MGMT expression with a combination dose of NNK and MeIQx during the early stage of initiation (Experiment 2-2). Increased expression of CYP2A5, a major metabolic enzyme of NNK, was observed in the NNK + MeIQx (1 ppm) group (Experiment 2-1, Table III). The importance of CYP is underlined by reports on the inhibitory effect of CYP inhibitors on lung tumorigenesis (24-27), and by the widely accepted relationship between metabolism and tumorigenesis (9,28,29).

MGMT is a DNA repair protein that removes mutagenic adducts, and can therefore be indirectly used as a marker for the detection of DNA adducts (30). The data regarding MGMT were quite similar to those of CYP2A5 (Fig. 3). DNA adduct formation is considered to be an important factor in tumorigenesis, caused by heterocyclic amines and other genotoxic carcinogens. The increased DNA adduct formation observed with NNK treatment may be due to the induction of CYP2A5.

In conclusion, the present study showed clear differences in dose thresholds for MeIQx in lung and colon carcinogenesis, and demonstrated that additional exposure to NNK

primarily affects the lungs, masking the weaker carcinogenicity of the heterocyclic amine. Recently, the existence of a NOEL was reported for several genotoxic carcinogens. The NOEL for these carcinogens alone is clearly important for investigating virtual thresholds in risk assessment. However, it may also be necessary to investigate drug interactions in cases of combination treatment. Furthermore, effects should be considered separately for each organ. The present results thus provide fundamental and useful information for human risk assessment.

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