

## Enhancement by Cigarette Smoke Exposure of 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline-induced Rat Hepatocarcinogenesis in Close Association with Elevation of Hepatic CYP1A2

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The modifying effects of cigarette smoke (CS) exposure on a heterocyclic amine (HCA) 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx)-induced carcinogenesis were investigated in male F344 rats. Groups 1 and 2 were fed MeIQx at a dose of 300 ppm, and simultaneously received CS and sham smoke (SS) for 16 weeks, respectively. Groups 3–5 were given the MeIQx diet for 4 weeks, and simultaneously exposed to CS for 4 weeks (group 3), exposed to CS for 12 weeks after the MeIQx treatment (group 4) or received SS for 16 weeks (group 5). Groups 6 and 7 were fed basal diet and respectively received CS and SS for 16 weeks. In terms of the mean number or area, the development of glutathione *S*-transferase placental form-positive (GST-P<sup>+</sup>) liver cell foci was significantly ( $P < 0.01$ ) greater in group 1 than in group 2. The mean number of colonic aberrant crypt foci (ACFs) per animal was increased by continuous CS exposure regardless of MeIQx feeding, the differences between groups 4 and 5 ( $P < 0.05$ ), and between groups 6 and 7 ( $P < 0.05$ ) being significant. Immunoblot analysis confirmed that the hepatic CYP1A2 level in group 6 was remarkably increased as compared to that in group 7. In addition, liver S9 from rats in group 6 consistently increased the mutagenic activities of six HCAs including MeIQx as compared to those in group 7. Thus, our results clearly indicate that CS enhances hepatocarcinogenesis when given in the initiation phase via increasing intensity of metabolic activation for MeIQx and possibly colon carcinogenesis when given in the post-initiation phase in rats induced by MeIQx.

Key words: Cigarette smoke — MeIQx — Liver — Metabolic activation — Colon

Epidemiological studies have suggested that cigarette smoking is closely associated with increased risk of cancers in various organs such as the lung, oropharynx, pancreas, stomach, liver and colon.<sup>1,2</sup> It has been shown that even a single dose of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a potentially carcinogenic and tobacco-specific nitrosamine, can induce respiratory tract tumors in hamsters, but smoking inhalation does not result in an increase in respiratory tract tumor incidence in NNK-initiated animals.<sup>3</sup> However, it was recently reported that long-term exposure to cigarette smoke (CS) increases the incidence of spontaneous lung tumors in A/J mice.<sup>4</sup> We also found that cigarette smoking promotes upper respiratory tract tumorigenesis in hamsters initiated with diethylnitrosamine.<sup>5</sup> Nevertheless, the influences of CS on experimental tumorigenesis in organs other than the respiratory tract remain to be elucidated.

Previously, we have reported that CS exposure for 2 weeks induces hepatic CYP1A isozyme, especially CYP1A2, in both rats and hamsters,<sup>6</sup> and in fact S9 fraction from the livers of animals exposed to CS specifically increases the mutagenicity of various heterocyclic amines

(HCAs) in Ames assay.<sup>6,7</sup> It is well known that HCAs are principally activated by CYP1A2 to proximate carcinogens<sup>8</sup> and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), one of the major HCAs contained in cooked foods,<sup>9</sup> is a liver and possible colon carcinogen in rodents.<sup>10</sup> Therefore, in the present study, the modifying effects of CS exposure on MeIQx-induced liver and colon carcinogenesis in rats and on HCA-induced mutagenesis and hepatic CYP (cytochrome P450 superfamily) levels were investigated, in addition to those on a detoxification activity.

### MATERIALS AND METHODS

**Animals and chemicals** A total of 105 male 3-week-old, specific-pathogen-free F344/DuCrj rats purchased from Charles River Japan, Inc. (Kanagawa) were housed five animals per polycarbonate cage and maintained under standard laboratory conditions: room temperature, 23 ± 2°C; relative humidity, 60 ± 5%; a 12h/12h light/dark cycle. After a 1-week acclimation period, the animals were used. 2-Amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP),

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3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), 4-nitrophenol, benzo[*a*]pyrene (BP) and *N*-nitrosodimethylamine (DMN) were obtained from Wako Pure Chemicals (Osaka). Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) and MeIQx were obtained from Makor Chemicals, Ltd. (Jerusalem, Israel) and Toronto Research Chemicals (Ontario, Canada), respectively. Non-filter cigarettes were purchased from Japan Tobacco Co., Ltd. (Tokyo). *N*-Nitrosobis(2-hydroxypropyl)amine (BHP) was synthesized in our laboratory as described previously.<sup>11)</sup>

**Experimental design** As illustrated in Fig.1, animals in groups 1 and 2, each consisting of 15 rats, were fed the basal diet Oriental MF (Oriental Yeast Co., Tokyo) supplemented with MeIQx at a concentration of 300 ppm, and simultaneously received CS exposure and sham smoking (SS) for 16 weeks, respectively. Group 3–5 rats were fed MeIQx at a dose of 300 ppm for 4 weeks, and simultaneously exposed to CS for 4 weeks (group 3), exposed to CS for 12 weeks after the MeIQx treatment (group 4) or received SS for 16 weeks (group 5). Group 6 and 7 rats were fed basal diet and respectively received CS and SS for 16 weeks. CS was transnasally administered 7 days a week by using a Hamburg type II smoking machine (Borgwaldt, Hamburg, Germany) under the following conditions: exposure period, 6 min/day; doses, 30 cigarettes/time; inhalation volume, 35 ml; inhalation flow, 17.5 ml/s; dilution of cigarette smoke, 1/7.<sup>6)</sup> As SS treatment, animals were restricted in a chamber of the smoking machine under the same conditions as for CS treatment, but without real smoking. Body weights were recorded once every week. At the 16th week of the experiment, all surviving animals were sacrificed under ether anesthesia. At autopsy, the liver, lung and colon tissues were removed carefully and the weights of the liver and lungs were measured. After fixation in 10% buffered formalin solution, the liver and lung were examined histopathologically. Lesions of glutathione *S*-transferase placental form-positive liver cell foci (GST-P<sup>+</sup> foci) larger than 0.2 mm in diameter were quantified by an image analyzer IPAP (Sumika Technos Corp., Osaka), which measured the numbers and areas. Colon aberrant crypt foci (ACFs) comprising of more than one crypt were counted by the methods described previously.<sup>12)</sup>

**Mutation assay** The influence of liver S9 from rats exposed to CS on mutagenic activation of various carcinogens was investigated by the method reported previously.<sup>13)</sup> BHP and DMN were dissolved in water (100 μl) and other carcinogens in 50 μl of dimethyl sulfoxide. The mutagenic response to MeIQ (0.03 μg/plate), Glu-P-1, Trp-P-2, IQ and MeIQx (all 0.3 μg), BP and PhIP (5 μg), MeAαC (10 μg), AFB<sub>1</sub> (1 μg) and *N*-nitrosamines (10 mg) was checked in the presence of liver S9 from either con-

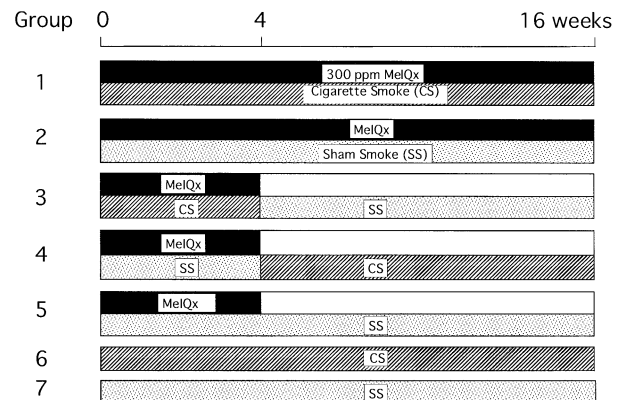


Fig. 1. Experimental design.

trol (group 7) or CS-exposed (group 6) rats according to our previous procedure using *Salmonella typhimurium* TA98 or TA100.<sup>6)</sup> Livers were perfused *in situ* with ice-cold sterile 1.15% KCl and hepatic S9 was prepared from the 25% homogenates using established conditions.<sup>11)</sup>

**Immunoblot analysis** The liver microsomal proteins from group 6 and 7 rats were separated according to the method of Laemmli.<sup>14)</sup> Western blotting and subsequent quantitative analysis were performed with goat anti-rat polyclonal antibodies for CYP1A1/1A2, CYP2B1/2B2, CYP2E1 and CYP3A2 (Daiichi Pure Chemicals, Tokyo) as described previously.<sup>6)</sup>

**Assay of UDP-glucuronyl transferase (UDPGT) activity** UDPGT1A6 activity in liver microsomes from group 6 and 7 animals was determined using 4-nitrophenol as a substrate according to the method described by Isselbacher *et al.*<sup>15)</sup>

**Statistical evaluation** Statistical significance of intergroup differences was evaluated using Fisher's exact probability test and Student's *t* test.

## RESULTS

**Survivals, body and organ weights** Only three animals accidentally died of asphyxia during CS exposure. As shown in Fig. 2, body weights were decreased by CS exposure, the final body weights being significantly ( $P < 0.05$ ) lower in the 16-week CS-exposed groups than in the corresponding 16-week SS groups regardless MeIQx feeding (Table I). Relative liver weights were somewhat increased by the MeIQx treatment in an administration period-dependent manner, albeit without statistical significance, but were not influenced by the CS exposure (Table I). Relative lung weights were significantly ( $P < 0.05$ ) increased by the 16-week CS exposure regardless of the MeIQx treatments (Table I).

**Effects on GST-P<sup>+</sup> foci** As summarized in Table II, the mean number of GST-P<sup>+</sup> liver cell foci per square centi-

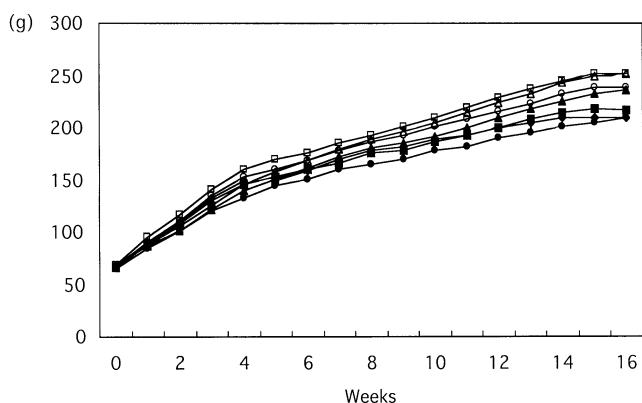


Fig. 2. Body weight curves for rats receiving MeIQx and/or cigarette smoke. ● group 1, ○ group 2, ▲ group 3, ◆ group 4, △ group 5, ■ group 6, □ group 7.

meter was respectively 91.2, 52.3, 1.0, 0.2, 0.7, 0 and 0 in groups 1–7, the differences between groups 1 and 2 ( $P < 0.01$ ) being statistically significant. The mean area of GST-P<sup>+</sup> foci (mm<sup>2</sup>/cm<sup>2</sup>) was respectively 12.2, 6.8, 0.08, 0.01, 0.05, 0 and 0 in groups 1–7, the difference between groups 1 and 2 being again statistically significant ( $P < 0.01$ ). Thus, the development of GST-P<sup>+</sup> foci was significantly increased by simultaneous 16-week CS exposure with MeIQx feeding.

**Effects on ACFs** As shown in Table II, the mean number of colonic ACFs was respectively 5.8, 4.9, 1.2, 3.7, 1.8, 0.8 and 0.1 in groups 1–7. The development of ACFs was increased by 12-week or 16-week CS exposure regardless of MeIQx treatment, the differences between groups 4 and 5 ( $P < 0.05$ ), and groups 6 and 7 ( $P < 0.05$ ) being significant.

**Effects on mutagenicity** Liver S9 from group 6 rats exposed to CS for 16 weeks increased the mutagenic activities of MeIQ, MeIQx, IQ, Glu-P-1, Trp-P-2 and PhIP by 1.8–3.3 times more than liver S9 from group 7 rats

Table II. Effects of Cigarette Smoke (CS) Exposure on MeIQx-induced GST-P<sup>+</sup> Liver Cell Foci and Colonic ACFs in Rats

Group	GST-P <sup>+</sup> foci		No. of ACFs
	No./cm <sup>2</sup>	Area (mm <sup>2</sup> )/cm <sup>2</sup>	
1. MeIQx 16W+CS 16W	91±16 <sup>a)</sup>	12.2±4.8 <sup>a)</sup>	5.8±2.1
2. MeIQx 16W+SS 16W	52±26	6.8±4.5	4.9±1.9
3. MeIQx 4W+CS 4W →SS 12W	1.0±1.3 <sup>b)</sup>	0.08±0.12 <sup>b)</sup>	1.2±0.9
4. MeIQx 4W+SS 4W →CS 12W	0.2±0.5	0.01±0.02	3.7±2.2 <sup>c)</sup>
5. MeIQx 4W+SS 16W	0.7±1.0	0.05±0.07	1.8±1.6
6. CS 16W	0	0	0.8±0.9 <sup>d)</sup>
7. SS 16W	0	0	0.1±0.2

Values are mean±SD.

a)  $P < 0.01$  vs. group 2.

b)  $P < 0.05$  vs. group 4.

c)  $P < 0.05$  vs. group 5.

d)  $P < 0.05$  vs. group 7.

receiving SS for 16 weeks (Fig. 3), whereas no significant alteration of mutagenicity was observed with MeAαC, BP, AFB<sub>1</sub>, DMN or BHP.

**CYPs and UDPGT** Immunoblot analysis revealed that in a comparison of group 6 with group 7, hepatic CYP1A1 was clearly induced, but at less than half of the constitutive CYP1A2 level. Hepatic CYP1A2 was significantly (2.5-fold,  $P < 0.01$ ) increased by CS while other CYPs such as CYP2B1, 2B2, 2E1 and 3A2 were not affected (Fig. 4). On the other hand, liver microsomes from group 6 rats also showed increased UDPGT1A6 activity toward 4-nitrophenol by 1.9-fold above the SS control group (group 7) as shown in Fig. 5.

**Histopathology** It was observed that CS exposure for 12 weeks or longer induced slight to moderate thickening of pulmonary alveolar septa, especially in the peripheral area close to the visceral pleura, and focal aggregation of macrophages, regardless of MeIQx treatment. MeIQx feeding

Table I. Body and Relative Organ Weights

Group	No. of rats	Body (g)	Liver (g%)	Lungs (g%)
1. MeIQx 16W+CS 16W	15	210±11 <sup>a)</sup>	3.40±0.20	0.44±0.19 <sup>a)</sup>
2. MeIQx 16W+SS 16W	15	293±11	3.41±0.23	0.38±0.16
3. MeIQx 4W+CS 4W→SS 12W	15	236±8	2.88±0.13	0.39±0.23
4. MeIQx 4W+SS 4W→CS 12W	14	208±11	2.72±0.13	0.44±0.21
5. MeIQx 4W+SS 16W	15	249±15	2.66±0.14	0.37±0.14
6. CS 16W	14	217±14 <sup>b)</sup>	2.50±0.08	0.45±0.23 <sup>b)</sup>
7. SS 16W	14	251±13	2.48±0.11	0.36±0.33

Values are mean±SD.

a)  $P < 0.05$  vs. group 2.

b)  $P < 0.05$  vs. group 7.

Fig. 3. Mutagenic activation of various carcinogens by rat liver S9 in groups 6 (■) and 7 (□). Each test was carried out in duplicate (4–8 plates) with S9 fractions from the pooled livers of five rats, and spontaneous revertant colonies (TA98, 19; <sup>a</sup>TA100, 134) were subtracted. \*  $P < 0.01$ , compared to group 7 (Student's  $t$  test).

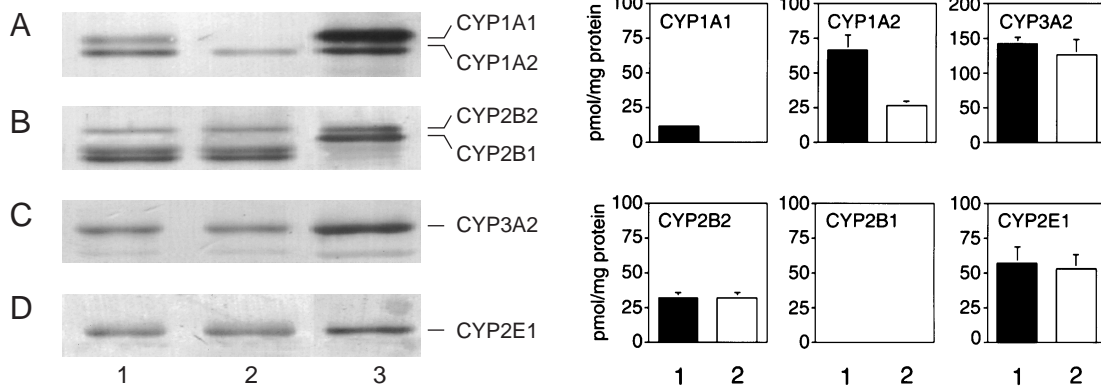
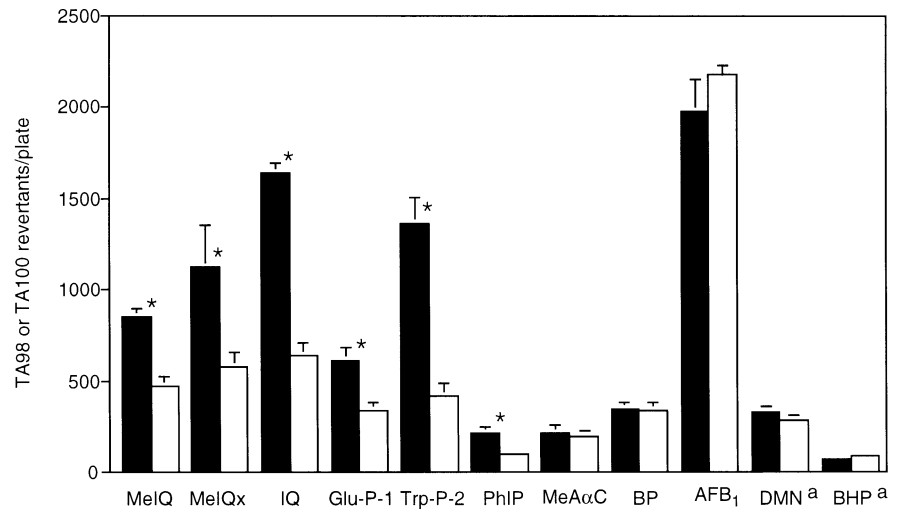


Fig. 4. Western blots (left) and densitometric analyses (right) of CYP isozymes in liver microsomes pooled from five rats in groups 6 (lane 1) and 7 (lane 2). Each lane contains 1.0  $\mu\text{g}$  of microsomal protein in panels A and B, and 0.4  $\mu\text{g}$  in panels C and D. Lane 3 contains CYP standards (0.4  $\mu\text{g}$  of microsomal protein) from rats treated with 3-methylcholanthrene (A), phenobarbital (B and C) and acetone (D). The values in densitometric analyses are expressed as mean  $\pm$  SD of four to seven experiments.

induced various altered liver cell foci such as eosinophilic, clear cell and basophilic types in an exposure duration-dependent manner with an increasing tendency in the CS groups.

## DISCUSSION

A case-control multicenter study on lung cancer patients has revealed a pronounced effect of CS on the metabolism of xenobiotics.<sup>16</sup> Also, in experimental studies, alterations in drug metabolic enzymes by CS have been demonstrated in the lung, liver and kidney of rodents.<sup>6, 17, 18</sup> It has therefore been suggested that such alterations in xenobiotic metabolism could affect the carcinogenicity of various

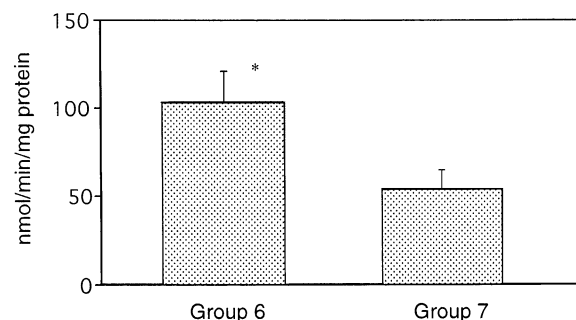


Fig. 5. Effect of cigarette smoke on UDPGT1A6 activity in rat liver microsomes. Each bar represents the mean  $\pm$  SD of six to eight experiments of liver microsomes pooled from five rats. \*  $P < 0.01$ , compared to group 7 (Student's  $t$  test).

compounds, i.e., procarcinogens, that are metabolically activated. The results of the present study in fact clearly demonstrated that CS exposure enhances the induction by MeIQx of putative preneoplastic liver lesion when given in the initiation phase. Our results are in line with human data showing a positive correlation between CS exposure and liver cancer development.<sup>19)</sup>

The selective enhancing effect of CS on the mutagenic activities of six HCAs except for MeA $\alpha$ C indicates the possible induction of CYP1A isozymes, which was confirmed by western blotting analyses (Fig. 4). It has been reported that hepatic CYP1A1 and 1A2 are differently involved in mutagenic activation of eight HCAs and BP in rats.<sup>20)</sup> The mutagenic activity of MeA $\alpha$ C, BP and Trp-P-2 was induced 3.8-, 4.8-, and 80.6-fold by 3-methylcholanthrene (MC), respectively, while MC induced hepatic CYP1A1 up to 2.9-fold above CYP1A2 in rats.<sup>21)</sup> However, in the present study, the induction of CYP1A1 in liver microsomes from CS-treated rats was 1/6 of the CYP1A2 level, and mutagenic activity of Trp-P-2 was induced only 3.3-fold by CS. Furthermore, hepatic BP hydroxylase in rats is known not to be inducible by CS.<sup>17)</sup> Therefore, it is reasonable that liver S9 from CS-treated rats could not activate MeA $\alpha$ C and BP to mutagens. There are conflicting reports on the involvement of rat CYP isozymes in the mutagenic activation of AFB<sub>1</sub>; (a) activated by CYP1A2, CYP2B and CYP2C11<sup>22)</sup>; (b) activated by CYP3A2 and CYP2C11, but not by CYP1A2.<sup>23)</sup> The latter finding is supported by the present result that CS did not affect the activation at all, despite the marked induction of CYP1A2. Since the mutagenicities of BHP and DMN are known to be activated by CYP2B and 2E1,<sup>11)</sup> it also seems reasonable that CS had no effect on the mutagenic activations of BHP and DMN.

It has been well documented that all the carcinogenic HCAs including MeIQx are metabolically activated by hepatic CYP isozymes, especially CYP1A2.<sup>8, 24-26)</sup> Because CYP1A2 was consistently increased in the liver of rats exposed to CS for 16 weeks in the present study and for 2 weeks in our previous experiment,<sup>6)</sup> such increase of hepatic CYP1A2 by CS could be principally involved in the enhancement of the initiation phase of MeIQx-induced hepatocarcinogenesis found in the present study. Importantly, CS is also associated with enhanced hepatic CYP1A2 levels in humans.<sup>27, 28)</sup> It has been reported that CYP1A2 is 75% identical in amino acid sequence in rats and humans<sup>29)</sup> and thus CYP1A2 is more or less commonly active in metabolizing MeIQx to reactive intermediates in both rodents and man.<sup>24-26)</sup> However, in addition to the considerable differences between human and rat CYPs in activation and detoxication of HCAs,<sup>30)</sup> it was recently reported that the catalytic efficiencies of P450-mediated *N*-oxidation of MeIQx by human CYP1A2 are 10-19-fold higher than by rat CYP1A2.<sup>25)</sup> These facts

suggest that such interspecies differences in CYP expression and catalytic activities may, to some extent, mislead to underestimation of the data regarding co-initiation effects of CS on MeIQx-carcinogenicity found in the present study as well as carcinogenicity *per se* of HCAs.<sup>30)</sup>

It has also been reported that genetic polymorphism of *CYP* and *GST* genes, alone or in combination, acts as a risk modifier of tobacco-related cancers.<sup>31, 32)</sup> Therefore, besides interspecies differences, polymorphisms of CYP1A2, frequent in humans, could be associated with the extent of CS effects. CYP1A2, a member of the CYPs, is involved in the metabolic activation of several carcinogens including HCAs and aromatic amines, nitroaromatic compounds, mycotoxins and estrogens.<sup>33)</sup> Individual differences in CYP1A2 activity may thus influence individual susceptibility to a wide spectrum of cancer development. In humans, CYP1A2 has been detected only in the liver, where it seems to be regulated by at least two mechanisms, one controlling constitutive levels of expression and another regulating inducibility.<sup>33)</sup> Two genetic variants have been identified which seem to be associated with CYP1A2 inducibility only.<sup>29)</sup> Because polymorphisms of other genes such as CYP1A1 and GSTM1 may affect the function of CYP1A2,<sup>34)</sup> gene-gene interactions among these genes could be important confounders in the interpretation of our results. In fact, CS has consistently induced hepatic CYP1A1 in rats in our present and previous studies.<sup>6)</sup>

It is speculated that CS contains hundreds of constituents that may play a role in carcinogenesis,<sup>35)</sup> and therefore, the chemicals responsible for co-initiating activity of CS with MeIQx as well as increase of hepatic CYP1A2 remain to be elucidated. However, some constituents of CS such as BP, nicotine and HCAs are known to induce CYP1A1/1A2.<sup>36)</sup> In addition to MeIQx, some other HCAs such as Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, PhIP, IQ, A $\alpha$ C and MeA $\alpha$ C have been identified in main-stream smoke condensates.<sup>9)</sup> It is well known that in liver microsomes from rats treated with these HCAs, CYP1A2 expression is much greater than CYP1A1 expression.<sup>37)</sup> The characteristics of CS induction (Fig. 4) are similar to those of HCAs, but not to those of BP<sup>38)</sup> and nicotine,<sup>39)</sup> suggesting that HCAs in CS may be mainly due to CYP1A induction and thereby the co-initiating activity. Because BP, AFB<sub>1</sub> and most HCAs are carcinogens in rodents and metabolically activated by CYP1A1 and/or 1A2,<sup>8, 40)</sup> CS may enhance possible CS-related carcinogenesis by itself.

In the present study, CS also induced MC-inducible UDPGT activity in rat liver microsomes,<sup>41)</sup> and this means that CS is a bi-functional inducer in rats, in line with previous finding in smokers<sup>28)</sup> and mice.<sup>18)</sup> *N*-Glucuronidation of HCAs activated by CYP1A2 is regarded as an important detoxication reaction.<sup>42)</sup> However, *N*-hydroxy-

MeIQx is a very poor substrate for human and rat liver UDPGT compared to other *N*-hydroxy-HCAs such as Glu-P-1, PhIP and IQ,<sup>43,44</sup> and in fact, the sum of *N*-hydroxy-MeIQx-glucuronide in urine and bile represents less than 1% of the administered dose in rats.<sup>45</sup> In conjunction with the finding of marked induction of CYP1A2 level, it is suggested that CS may have almost no influence on the detoxication of MeIQx, or rather results in further bioactivation such as acetylation.<sup>43</sup>

In the present experiment, rats were transnasally exposed to CS for 6 min per day for 4, 12 or 16 consecutive weeks. In comparison with previous reports, the daily exposure time in the present study was relatively short<sup>4)</sup> or long,<sup>6,7,17)</sup> but could be sufficient to observe CS effects in terms of increased lung weights and hepatic CYP1A2, induced hepatic CYP1A1 and consequent enhancement of MeIQx-induced tumorigenesis. In addition, aggregation of alveolar macrophages is in good agreement with the pulmonary histopathology characteristic of CS exposure.<sup>46)</sup>

Although it is hypothesized that carcinogenic compounds formed by high temperature cooking techniques,

such as HCAs, may contribute to the human risk of developing colorectal cancers,<sup>47)</sup> only PhIP but not MeIQx induced colon tumors in rats, the latter inducing only ACFs.<sup>48)</sup> Therefore, it remains unknown whether MeIQx is capable of inducing colon cancer. However, the results of the present study suggest that CS may promote colon tumorigenesis regardless of induction by MeIQx. In conclusion, our results clearly indicate that CS exposure enhances hepatocarcinogenesis when given in the initiation phase and possibly colon tumorigenesis when given during the post-initiation phase. Further studies, including the matter of species differences, are needed.

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