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Exposure to Heterocyclic Amines

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Many mutagenic heterocyclic amines (HAs) have been isolated from cooked foods and pyrolysates of amino acids and proteins, and the carcinogenicity of 10 of these HAs in rodents and of 1 in monkeys has been reported. Quantification of these carcinogenic HAs in various kinds of cooked foods indicated that the level of 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) was highest (0.56–69.2 ng/g), that of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) was second highest (0.64–6.44 ng/g), and those of other HAs were 0.03–2.50 ng/g. Heterocyclic amines were found in urine samples of 10 healthy volunteers consuming a normal diet, but HAs were not detectable in urine samples of three patients receiving parenteral alimentation. These results strongly suggest that humans are continuously exposed to HAs derived from food in the normal diet. Based on quantitative data on the levels of HAs in cooked foods and urine samples, the daily exposures to PhIP and MeIQx were estimated to be 0.1–13.8 μg and 0.2–2.6 μg per person, respectively. These levels of carcinogenic HAs are in the same range as those of other carcinogens such as *N*-nitrosodimethylamine and benzo[*a*]pyrene to which humans are exposed.

Introduction

A series of heterocyclic amines (HAs) in cooked meat and fish and in pyrolysates of amino acids and proteins has been identified as mutagenic (*1–4*). These HAs are classified into two groups, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)-type HAs and non-IQ-type HAs. IQ-type HAs have a 2-aminoimidazole moiety as a common structure and include aminoimidazoquinoline, aminoimidazoquinoxaline, and aminoimidazopyridine compounds. The aminoimidazole moiety is derived from creatinine in raw meat and fish, and other parts of IQ-type HAs are from amino acids and sugars or from amino acids themselves (*5–7*). Non-IQ-type HAs, which include aminopyridoindole and aminodipyridoimidazole compounds, contain a 2-aminopyridine moiety as a common structure. Aminopyridoindoles are produced by heating tryptophan, and aminodipyridoimidazoles are produced by heating glutamic acid. In addition to IQ- and non-IQ type HAs, two mutagenic HAs containing oxygen atoms in their structure were recently discovered. These are 4-amino-1,6-dimethyl-2-methylamino-IH,6H-pyrrolo[3,4-*f*]benzimidazole-5,7-dione (Cre-P-1), isolated from a creatine pyrolysate (*8*), and 2-amino-(1 or 3),6-dimethylfuro[2,3(or 3,2)-*e*]imidazo[4,5-*b*]pyridine (MeIFP), isolated from a creatine-supplemented fried-meat product (*9*). The structure of MeIFP has not yet been fully determined. Cre-P-1 and MeIFP induced 19,000 revertants/μg of *Salmonella typhimurium* TA98 and 10,000 revertants/μg of *S. typhimurium* TA1538, respectively, with a metabolic activation system (S9 mix).

Of the 19 mutagenic HAs known, 10 have been shown to be carcinogenic in rats and/or mice when administered in the diet at concentrations of 100–800 ppm (*1–3,10–12*). The structures of these carcinogenic HAs are shown in Figure 1. All the HAs except 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) induced tumors in the livers of rats and mice. Tumors were also induced in extrahepatic tissues such as the small and large intestine, Zymbals gland, clitoral gland, skin, oral cavity, and mammary gland in rats, and the forestomach, lung, hematopoietic system, and blood vessels in mice. PhIP, the most abundant HA by weight in cooked food, induced tumors of the colon and mammary gland in rats (*11*) and lymphomas in mice (*12*). Furthermore, IQ was found to induce hepatocellular carcinomas in monkeys when given by nasal–gastric intubation 5 times/week at 10 or 20 mg/kg (*13*).

The capacities of liver enzymes of rats, monkeys, and humans to activate carcinogenic HAs are similar (*14,15*). Moreover, the same DNA adducts have been found in various organs, including the liver of rats and monkeys treated with IQ (*16*). Thus, HA-DNA adducts are probably also formed in human tissue.

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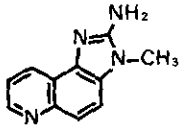
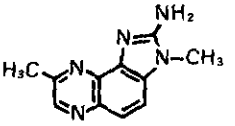
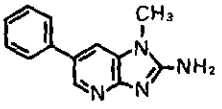


2-Amino-3-methylimidazo-[4,5-*f*]quinoline (IQ)

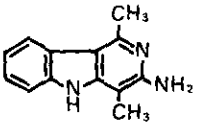
2-Amino-3,4-dimethyl-imidazo[4,5-*f*]quinoline (MeIQ)



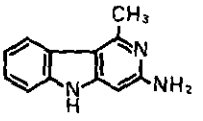
2-Amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx)



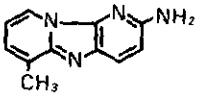
2-Amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP)



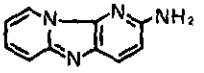
3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1)



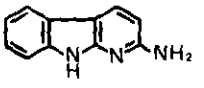
3-Amino-1-methyl-5*H*-pyrido-[4,3-*b*]indole (Trp-P-2)



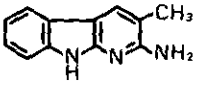
2-Amino-6-methyldipyrido-[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1)



2-Aminodipyrido[1,2-*a*:3',2'-*d*]-imidazole (Glu-P-2)



2-Amino-9*H*-pyrido[2,3-*b*]-indole (AαC)



2-Amino-3-methyl-9*H*-pyrido-[2,3-*b*]indole (MeAαC)

FIGURE 1. Structures of carcinogenic heterocyclic amines.

To evaluate the risk of HAs in cancer development in humans, the levels of exposure of humans to HAs must be measured. In this paper, we report the quantification of HAs in cooked foods and human urine samples and discuss the levels of exposure of humans to HAs and other environmental carcinogens.

Quantification of Heterocyclic Amines in Cooked Foods

Samples of 25 g of cooked meat were homogenized in 250 mL of 0.1 N HCl three times, and the extract was mixed with trichloroacetic acid at a final concentration of 5% and centrifuged to remove protein. The supernatant was adjusted to a volume of 1000 mL at pH 6–7 with a dilute solution of sodium hydroxide. For the study of a food-grade beef extract, a sample of 25 g was dissolved in 1000 mL of water.

The solutions obtained were purified by blue cotton treatment, 0.1 N HCl-methylene chloride partitioning, and chromatography in a SEP-PAK silica cartridge (*17,18*). Heterocyclic amines in the partially purified samples were analyzed by HPLC using a combination of octadecyl silane (ODS) and cation exchange columns. The following five compounds were detected by fluorometry: 3-amino-1,4-dimethyl-5*H*- pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole(MeAαC), and PhIP. As aminoimidazoquinoline and aminoimidazoquinoxaline compounds have no fluorescence, they were detected with an electrochemical detector.

Using these methods, eight HAs were detected in four kinds of cooked meat (broiled beef, fried ground beef, broiled chicken, broiled mutton) and in food-grade beef extract. The recoveries of each of the HAs during the separation process were estimated by spiking with an equivalent level of authentic HA to that detected in cooked food and were found to be 51–72%. The original levels of HAs in cooked food were estimated by correcting the amount of HAs detected by HPLC for their recoveries and are shown in Table 1. Under the conditions used in these experiments, the minimum amounts of HAs detectable in 1 g of cooked food were as follows: 0.03 ng for IQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), and 2-amino-

Table 1. Amounts of heterocyclic amines in cooked foods.

Sample	Amount, ng/g cooked food								
	IQ	MeIQ	MeIQx	4,8-DiMeIQx	PhIP	Trp-P-1	Trp-P-2	AαC	MeAαC
Broiled beef	0.19		2.11	Di	15.7	0.21	0.25	1.20	
Fried ground beef			0.64	0.12	0.56	0.19	0.21		
Broiled chicken			2.33	0.81	38.1	0.12	0.18	0.21	
Broiled mutton			1.01	0.67	42.5		0.15	2.50	0.19
Food-grade beef extract			3.10		3.62				
Fried codfish	0.16	0.03	6.44	0.10	69.2				

Abbreviations: IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo [4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole; MeAαC, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole.

Table 2. Amounts of heterocyclic amines in urine.

Subject[a]	Age, years	Sex	Amount, ng/24-hr urine			
			MeIQx	PhIP	Trp-P-1	Trp-P-2
1	13	F	11	0.28	0.06	0.05
2	32	F	41	1.19	0.26	0.17
3	33	M	19	0.19	0.39	0.45
4	35	M	33	0.43	1.43	0.15
5	35	M	33	0.34	0.04	0.05
6	41	F	47	1.97	0.06	0.03
7	42	M	44	0.38	0.71	0.68
8	49	F	12	0.28	0.09	0.06
9	67	F	34	0.12	0.06	0.03
10	80	M	16	0.14	0.09	0.07
11	39	M	<1	<0.01	<0.01	<0.01
12	65	M	<1	<0.01	<0.01	<0.01
13	66	F	<1	<0.01	<0.01	<0.01

Abbreviations: MeIQx, 2-amino-3,8-dimethy-limidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2,3-amino-1-methyl-5*H*-pynido[4,3-*b*]indole.

[a]Subjects 1–10 are healthy volunteers; subjects 3, 7, and 10 are smokers; subjects 11–13 are patients.

3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx); 0.05 ng for 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx); 0.01 ng for PhIP, Trp-P-1 and Trp-P-2, and 0.02 ng for AαC and MeAαc (*3,17,18*). In addition to cooked meat and beef extract, HAs in cooked codfish were analyzed by a similar method to that used for cooked meat and beef extract, and the results are shown in Table 1 (*19*).

PhIP is the most abundant HA in cooked food, being present at levels of 0.56–69.2 ng/g. The level of MeIQx is the second highest (0.64–6.44 ng/g). Other HAs were detected at levels of 0.03–2.50 ng/g.

Detection of Heterocyclic Amines in Human Urine

Carcinogenic HAs are present in ordinary cooked meat and fish, and so humans should be exposed to them continuously in normal, daily life. Urine is convenient for monitoring the degree of human exposure to environmental carcinogens, so we next examined the levels of four HAs, MeIQx, PhIP, Trp-P-1 and Trp-P-2, in human urine.

Samples of 24-hr urine were obtained from 10 healthy volunteers, 5 males (33–80 years old) and 5 females (13–67 years old), eating a normal diet. Heterocyclic amines in urine samples were adsorbed on blue cotton and then extracted with methanol–ammonia water solution. The extract was applied to a cation exchange TIN-100 H05E fiber column. The column was washed with methanol and developed with methanol–ammoni a water solution. The eluate was further purified by HPLC on a semi-preparative ODS column. The HAs in the partially purified samples obtained were analyzed by HPLC on three kinds of analytical columns. MeIQx was detected by its UV absorbance at 275 nm, and the other three HAs were detected by their fluorescence with excitation and emission wavelengths of 345 and 395 nm for PhIP, and of 265 and 410 nm for Trp-P-1 and Trp-P-2, respectively.

The age and sex of the 10 healthy volunteers are given in Table 2. Figure 2*A* shows the HPLC elution pattern of MeIQx in a partially purified sample from subject 2 on an ODS column. The retention time of 13 min coincided with that of authentic MeIQx. This peak fraction was collected and further anlyzed on a CAPCELL PAK C-18 column. A single peak was detected at the same retention time as that of authentic MeIQx (Fig. 2*B*). The amounts of MeIQx detected on the ODS and CAPCELL PAK C-18 column were almost the same, indicating that the materials

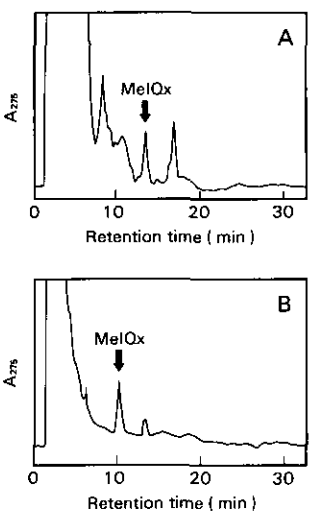


FIGURE 2. Detection of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in a sample of urine from subject 2 by HPLC. A partially purified fraction containing MeIQx was applied to an analytical YMC A303 ODS column (*A*) and material was eluted with 12% CH_3CN in 25 mM H_3PO_4/Na_2HPO_4 (pH 2.0) at a flow rate of 0.8 mL/min and monitored as UV absorbance at 275 nm. The fraction with the same retention time as authentic MeIQx was collected and purified further on a CAPCELL PAK C-18 column (*B*) developed with 15% CH_3CN in 25 mM Na_2HPO_4/NaH_2PO_4 (pH 8.0) at a flow rate of 0.8 mL/min.

Table 3. Levels of exposure of humans to carcinogens.

Compound	Source of expousre	Daily intake, μg/person	Reference
MeIQx	Food	0.2–2.6	Ushiyama et al., 1991 (*20*)
PhIP	Food	0.1–13.8	Wakabayashi et al., 1992 (*3*)
N-Nitrosodimethylamine	Food	0.5–1.8	Satoh et al., 1984 (*23*)
	Cigarette smoking	0.1	National Academy of Sciences, 1981 (*24*), Scanlan, 1983 (*25*)
Benzo[*a*]pyrene	Food	0.01–1.4	Ministry of Health and Welfare, 1989 (*26*)
	Cigarette smoking	0.1–1.6	IARC, 1983 (*27*)
NNK	Cigarette smoking	3.0	National Academy of Sciences, 1981 (*24*), Scanlan, 1983 (*25*)

Abbreviations: MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

in the peaks corresponding to the retention time of authentic MeIQx on the two columns were homogeneous. By this method, MeIQx was found in all the urine samples from healthy volunteers examined. The amounts of MeIQx in the samples of subjects 1–10 were estimated from a standard curve made with various concentrations of authentic MeIQx: the values were 6–26 ng/24-hr urine. The recovery of MeIQx in a urine sample during the purification process was determined to be 55.0% by spiking with a similar level of authentic compound to that detected in the sample. By correcting the amounts of MeIQx detected by HPLC for the recovery, the original levels of MeIQx in urine samples from the 10 healthy volunteers were calculated to be 11–47 ng/24-hr urine (Table 2) (*20*).

Similarly, PhIP, Trp-P-1, and Trp-P-2 were detected in all the urine samples from healthy subjects. The original levels of these three HAs in 24-hr urine samples were calculated by correcting the amounts of HAs detected by HPLC for their recoveries, which were 55.2% for PhIP, 61.5% for Trp-P-1, and 50.8% for Trp-P-2. In this way, the original levels of PhIP, Trp-P-1, and Trp-P-2 in 24-hr urine samples from subjects 1–10 were estimated as 0.12–1.97 ng, 0.04–1.43 ng, and 0.03–0.68 ng, respectively (Table 2) (*20*).

To confirm the structures of the compounds in the peak fractions with the same retention times as the respective authentic HAs on the final HPLC column, the HPLC procedures were repeated several times, and the collected peak fractions were analyzed with a photodiode array detector and a fluorometric detector. The UV-absorption pattern of MeIQx isolated from urine samples was the same as that of authentic MeIQx. Moreover, the fluorescence emission spectra of the compounds corresponding to PhIP, Trp-P-1, and Trp-P-2 were identical to those of the respective authentic HAs. Thus, the compounds detected by HPLC were concluded to be MeIQx, PhIP, Trp-P-1 and Trp-P-2.

We also analyzed the four HAs in urine samples from three patients who received parenteral alimentation for 1 day before and 2–4 days after surgery. None of the four HAs could be detected in any of the urine samples. Thus, judging from the minimum level of MeIQx detectable by UV absorbance, its excretion by these patients must be less than 1 ng/24-hr urine (Table 2). Similary, the excretion of PhIP, Trp-P-1, and Trp-P-2, if any, was calculated to be less than 0.01 ng/24-hr urine (Table 2) (*20*).

The finding that all four HAs were present in all the urine samples from healthy volunteers eating a normal diet, but were not detectable in urine from patients receiving parenteral alimentation, strongly suggests that HAs present in the urine of healthy volunteers are derived from the diet. Of the 10 healthy subjects, three were smokers: subject 3 smoked 40–45 cigarettes/day, and subjects 7 and 10 smoked 15–20 cigarettes/day. No significant effects of smoking on the urinary levels of the four HAs were observed, although the number of smokers was limited.

Murray et al. (*21*) reported that humans excrete 1.8–4.9% of an oral dose of MeIQx unchanged in the urine. Based on this observation, the daily intake of MeIQx was estimated to be 0.2–2.6 μg/person. As shown above, MeIQx is present in cooked foods at levels of 0.64–6.44 ng/g. Assuming that humans ingest 200 g of cooked meat or fish per day, a probable estimate in many cases in Japan (*22*), we calculated the daily intake of MeIQx to be 0.1–1.3 μg/person. Thus, the daily levels of exposure to MeIQx estimated from the levels in the urine and in cooked meat and fish were comparable. The ratios of PhIP, Trp-P-1, and Trp-P-2 excreted unchanged in human urine have not yet been determined, so the daily intakes of these three HAs were calculated assuming that humans ingest 200 g of cooked meat or fish per day. In this way, the daily exposures to PhIP, Trp-P-1, and Trp-P-2 per person were estimated to be 0.1–13.8 μg, 0.02–0.04 μg, and 0.03–0.05 μg, respectively.

Discussion

It is evident that humans are continuously exposed to carcinogenic HAs in normal, daily life, although the levels of exposure are small. For comparison of the levels of human exposure to MeIQx and PhIP with those to other typical carcinogens, the daily intakes of the compounds from foods and cigarette smoking are summarized in Table 3 (*23–27*). The daily level of exposure to MeIQx is calculated from level of MeIQx excreted unchanged in human urine, and the levels of other carcinogens are based on their amounts in food or cigarette smoke. As shown in Table 3, the daily levels of exposure to MeIQx, PhIP, *N*-nitrosodimethylamine, benzo[a]pyrene, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are within similar ranges. The TD_{50} values in CDF_1 mice and F344 rats, respectively, of MeIQx are 11.0 and 0.7 mg/kg/day and those of PhIP are 31.3 and 0.9 mg/kg/day. Thus, the daily levels of exposure to MeIQx and PhIP are less than 1000 times their TD_{50} values. The same may be true for the levels of exposure to the other carcinogens listed in Table 3. Thus, the levels of none of these carcinogens alone seem sufficient to explain the development of human cancer.

On the other hand, a linear relation was demonstrated between the levels of DNA adducts in the liver and doses of MeIQx in the

range of 0.4–400 ppm fed to rats and of 500 ng to 5 mg/kg body weight administered by stomach intubation to mice (*28,29*). These results suggest that HAs can form DNA adducts in the human body, even when present as low as the daily exposure levels. Furthermore, we recently found that five HAs in combination had additive or synergistic effects on the development of glutathione *S*-transferase placental form (GST-P)-positive foci, a marker of preneoplastic lesions of the liver in rats, when given at concentrations of 1/5 or 1/25 of the doses used in the carcinogenicity test of the rat (*30*). We also found that MeIQx at doses of 1/10 and 1/100 of the dose used in the carcinogenesis experiment (400 ppm) induced GST-P positive foci in the livers of rats treatd with carbon tetrachloride and given a choline-deficient diet, respectively (H. Sone et al., unpublished data). Thus, even at low levels, HAs may be involved in the development of cancer in the presence of other carcinogens, tumor promoters, and cancer progression factors. In fact, short-term administration of IQ combined with partial hepatectomy followed by long-term treatment with phenobarbital was found to induce liver cancers in rats, although short-term administration of IQ alone did not induce liver cancers (*31*).

The environment contains a variety of naturally occurring and man-made mutagenic and carcinogenic compounds to which humans are exposed during normal daily life. Humans are also exposed to mutagens and carcinogens formed endogenously. In addition to mutagens and carcinogens, many other factors are involved in the development of human cancer. Many genetic alterations are involved in the development of human cancer (*32,33*), and many factors are related to its multistep process. Further extensive studies are required on the involvement of HAs in human cancer, including the detection of HA–DNA adducts in human tissues.

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