Validation in rats of two biomarkers of exposure to the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP): PhIP–DNA adducts and urinary PhIP

Marlin D. Friesen 4, David A. Cummings 1, Liliane Garren, Ross Butler 2, Helmut Bartsch 3 and Herman A. J. Schut 1

International Agency for Research on Cancer, 69372 Lyon, France.
1Department of Pathology, Medical College of Ohio, Toledo, OH 43614, USA and 2Women’s and Children’s Hospital, North Adelaide, Australia

& Present address: German Cancer Research Center, Division of Toxicology and Cancer Risk Factors, D-69120 Heidelberg, Germany

& To whom correspondence should be addressed

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP): PhIP–DNA adducts in white blood cells and tissues and unmetabolized PhIP in urine were validated as biomarkers of exposure in male Fischer-344 rats treated with daily PhIP doses ranging from 1 to 0.0001 mg/kg. At the end of the 23 day treatment period all rats were killed and their blood and 10 tissues were collected for isolation of DNA and analysis of PhIP–DNA adducts by 32P-postlabeling and alkaline hydrolysis with GC/MS. PhIP–DNA adducts could be detected only in animals receiving 1 or 0.1 mg/kg/day, with highest adduct levels in the pancreas, heart and kidneys. There was a good correlation (r = 0.77, P < 0.005) between the two methods of analysis, with average adduct levels determined by 32P-postlabeling ~ 1.4 times higher than those determined by alkaline hydrolysis with GC/MS. PhIP–DNA adducts accumulated in most tissues, especially in the liver, kidneys, heart and pancreas, with lower levels in the white blood cells, small intestine, stomach, colon and cecum. Using GC/MS levels of unmetabolized PhIP were measurable in four weekly 24 h urine samples even at 0.0001 mg/kg/day, a dose comparable with reported human dietary exposure. A linear dose–response was obtained for excretion of unmetabolized PhIP across the range of doses, with ~1.8% of the dose excreted daily, largely independent of the number of doses. No PhIP was detected in the urine of untreated rats. If it can be shown that a constant percentage of PhIP is excreted unchanged in human urine, irrespective of dose, as has been found with the rat, measurement of urinary PhIP could be used as an accurate measure of dietary exposure to this amine in man.

Introduction

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP*) is one of several heterocyclic aromatic amines formed at ng/g levels when foods rich in animal protein (meat, fish and poultry) are heated at high temperature (1,2). PhIP produces adenocarcinomas of the large intestine in male Fischer-344 rats and mammary carcinomas in female Fischer-344 rats (3,4). Unmetabolized PhIP has been detected in the urine of human volunteers eating a normal diet (5,6), indicating that humans are continuously exposed to PhIP in the diet. A number of epidemiological studies in humans have found weak associations between colon cancer and the eating of well-cooked meat (7–9).

Results from in vitro studies have shown that PhIP is activated to its ultimate carcinogenic form by N-hydroxylation (10–14), a reaction catalyzed by cytochrome P450, principally by isoenzymes IA1 and IA2 (11,13,14). Further esterification of N-hydroxy-PhIP by sulfotransferase or O-acetyltransferase followed by deesterification (15,16) yields the ultimate reactive form, probably the nitrenium ion (17), which reacts with DNA to form specific adducts. In vitro the major adduct formed between N-hydroxy-PhIP and DNA is N2-(2′-deoxyguanosin-8-yl)-PhIP (16). Several studies have demonstrated the formation of PhIP–DNA adducts in animal tissues after treatment with PhIP (15,18–20). Recently we have reported the presence of PhIP–DNA adducts in human tissues (21). Levels of PhIP–DNA adducts in surgical tissue samples from pancreas, urinary bladder and colon were measured by blind analysis using two different analytical methods: alkaline hydrolysis with GC/MS and 32P-postlabeling. Both methods provided evidence for PhIP–DNA adducts in two of six colon samples, but not in 12 samples from human pancreas or six samples from urinary bladder.

In the light of these positive results in humans with both urinary PhIP and PhIP–DNA adducts, we now report on the further validation of two quantitative biomarkers of exposure to PhIP for possible application in human epidemiological studies. The approach of a previous study on PhIP–DNA adducts in rats (21), comparing alkaline hydrolysis with GC/MS with 32P-postlabeling, has been extended from a single large oral dose of PhIP to multiple oral doses over a wide range of lower dose levels. In addition, results are presented for a method to measure unmetabolized PhIP in the urine which can quantitatively determine recent exposure to PhIP at the level to which humans are exposed, based on the same GC/MS technique used to determine PhIP after alkaline hydrolysis of DNA.

Materials and methods

Materials

PhIP was obtained from Toronto Research Chemicals (Downsvicw, Ontario, Canada). PhIP-d5, used as the internal standard for GC/MS, was prepared as reported previously (21). Pentafluorobenzyl (PFB) bromide and N,N-dimethylformamide were obtained from Aldrich Chemical Company (Milwaukee, WI) and were used without further purification.

Treatment of animals

Twenty-four 7-week-old male Fischer-344 rats (average weight 186–196 g) were divided into six groups (A–F) of four animals each. Animals were housed four per cage and were given free access to tap water and regular laboratory chow. Beginning on day 1 and through day 22 rats were treated by gavage with a solution of PhIP, dissolved in a mixture containing 0.5 ml H2O, 15 μl dimethylsulfoxide and 0.2 μl 0.1 N HCl to aid in solubility of the compound. Group A (control group) received only the solvent mixture. Groups B–F received doses of 0.1, 1, 10, 100 and 1000 μg/kg/day (~0.02, 0.2, 2, 20
and 200 μg PhIP/day) respectively or total accumulated doses of 0.52, 4.8, 48, 486 or 4864 ng respectively over the 23 day period.

To compare the levels of PhIP-DNA adducts formed in the 24 h following a single dose with levels formed over a period of 23 days of daily dosing one male Fischer-344 rat was treated with a single oral dose of 25 mg/kg PhIP (corresponding to a total dose of 5 μg), using the procedure outlined above.

Collection of urine, white blood cells (WBCs) and tissues

On days 2, 9, 16 and 23 after beginning treatment animals were placed in individual metabolism cages. Twenty-four hour urine samples were collected for each animal and stored at -20°C until analysis for PhIP levels. On day 24, 24 h after the last treatment, rats were sacrificed and blood and tissue samples were collected as described previously (18). Fifteen minutes after administration of a heparin solution (5000 U/kg i.p.) rats were killed by injection of sodium pentobarbital (180 mg/kg i.p.). After complete anesthesia (~5 min) the abdomen was opened and blood was collected from abdominal veins (5-7 ml) into lithium heparin tubes. The rat receiving a single dose of 5 mg PhIP was sacrificed 24 h after treatment, as outlined above.

Tissue samples were collected for the following organs: liver, lungs, stomach, small intestine, colon, cecum, kidneys, heart, spleen and pancreas. The stomach and intestines were removed, cut open and rinsed with phosphate-buffered saline containing 1 mM EDTA to remove their contents. The epithelial layer was scraped off from the stomach, small intestine, cecum and colon. The tissues were quick-frozen in liquid nitrogen and stored at -20°C until isolation of DNA.

Extraction of PhIP from urine

Just prior to analysis urine samples were allowed to thaw at room temperature and were centrifuged at low speed to remove sediment. For animals from groups A (control), B (0.1 μg/kg) and C (1 μg/kg) urine samples were analysed without dilution. To avoid injection of large amounts of PhIP into the mass spectrometer urine samples from the other three treatment groups were diluted as follows: group D (10 μg/kg), 10-fold dilution; group E (100 μg/kg), 100-fold dilution; group F (1000 μg/kg), 1000-fold dilution.

To a Teflon-stoppered, disposable glass tube (13x100 mm; Corning Inc., Corning, NY) were added 320 μg PhIP-di for internal standard dissolved in 11 μl methanol, 100 μl NaCl (5% w/v) and 100 μl water (HPLC grade, BDH Laboratory Supplies, Poole, UK). After mixing 200 μl 1 M NaClO3 were added, the mixture was extracted with 2 ml ethyl acetate (HPLC grade, Merck, Darmstadt, Germany) and centrifuged at 2700 g for 10 min. The upper organic layer was transferred into a 2 ml flame-sealable glass vial (Wheaton, Millville, NJ) and the extraction repeated with 1 ml ethyl acetate. The combined extracts were then evaporated to dryness at 55°C under a stream of nitrogen.

Isolation of DNA from tissues and WBCs

Aliquots of 0.5 g tissue were used for isolation of DNA after suspension in 5 ml lysis buffer. For WBC DNA the entire blood sample was mixed with 45 ml lysis buffer (0.83% ammonium chloride, 0.1% potassium bicarbonate, 0.04% tetrasodium EDTA, pH 7.3) and the mixture left at room temperature for 8 min. WBCs were isolated by centrifugation at 3000 g for 8 min. The supernatant was discarded and the pellet was resuspended in 2 ml lysis buffer (10 mM Tris-HCl, 400 mM sodium chloride, 2 mM tetrasodium EDTA, pH 8.2) (18).

DNA was then isolated from tissues and WBCs by a direct salt precipitation procedure as described by Miller et al. (22). After the final DNA pellet was redisolved in 1 ml 1.5 M sodium chloride, 0.5 M sodium citrate (0.1X SSC) and then incubated with RNase A (100 μg/ml) and RNase T1 (50 U/ml) at 37°C in a shaking bath for 1 h. After addition of 0.01X SSC and 3 ml phenol/chloroform/isooamy alcohol (25:24:1) the mixture was vortexed, centrifuged at 4°C for 10 min at 3000 g and the aqueous (top) layer extracted once more with chloroform/isooamy alcohol (24:1). DNA was precipitated from the aqueous (top) layer as described (22) and its concentration measured spectrophotometrically using its absorbance at 260 nm and a value of 20 A260/absorbance units/mg DNA (18).

Alkaline hydrolysis of PhIP from DNA

DNA (80-1000 μg) which had been isolated from WBCs or tissues was transferred to a Teflon-stoppered glass tube (13x100 mm; Corning Inc., Corning, NY) and 100 μl 1 N NaOH and 200-250 pg d1 labeled PhIP internal standard was added. The samples were then heated overnight at 100°C to release PhIP. PhIP was extracted from the hydrolysate using a method based on modifications of a procedure for extraction of 2-amino-3,8-dimethylimidazo[4,5-f]quinoline from urine (23). The solution resulting from hydrolysis was made basic with 100 μl 0.1 N NaOH and was then extracted with three 1 ml aliquots of ethyl acetate. The organic extracts were combined in a 2 ml flame-sealable glass vial (Wheaton, Millville, NJ) and brought to dryness in a vacuum centrifugal evaporator at room temperature.

Analysis of PhIP from urine or DNA by GC/MS

PhIP which had either been extracted from urine or hydrolyzed from DNA was then reacted at 55°C for 30 min with PFB bromide to form a volatile, electron-capturing bis(pentafluorobenzyl) (BPFB)-derivative, as reported previously (21). GC/MS was carried out on a non-polar Hewlett Packard HP-1 fused silica capillary column (25 m x 0.2 mm i.d.) coupled directly to the mass spectrometer ion source (280°C) through a heated interface (300°C). The mass spectrometer (Hewlett Packard 5988A) was operated in the negative ion mode with methane as reagent gas. The MS was tuned to monitor negative ions at m/z 403.1 ([BPFB-PhIP-d5]-) and m/z 408.1 ([BPFB-PhIP-d2]-) representing loss of single PFB groups from the molecular anions (21).

Identification of a chromatographic peak as PhIP-d5 was dependent on its retention time being 0.02 min greater than that of the PhIP-d5 internal standard. Amounts of PhIP in the sample were calculated from a plot of the ratio of the integrated peak areas of m/z 403.1 and m/z 408.1 on the y-axis versus the amount of PhIP-d5 in the sample on the x-axis. A linear calibration curve was obtained over the range 2-500 pg/sample. The detection limit for PhIP was <1 pg PhIP/injection (21).

Analysis of PhIP-DNA adducts by 32P-postlabeling

PhIP-DNA adducts were isolated and quantified as described previously (18,24) using the intensification version of the 32P-postlabeling assay (25) with continuous transfer of adducts after chromatography in the first dimension (26). Polyethyleneimine (PEI)-cellulose plates were prepared in the laboratory (27) using recently described modifications (28) [γ-32P]ATP (sp. act ~4000 Ci/mmol) was prepared fresh for each 32P-postlabeling assay (27).

After enzymatic digestion and 32P-postlabeling (18,24) the [5',32P]deoxyribo- nucleoside 3',5'-biphosphates of PhIP-DNA adducts were isolated and separated on PEI-cellulose thin layer plates as described previously (18,24). The solvents used for chromatography were: D1: 2.3 M sodium phosphate, pH 5.8; D3: 2.81 M lithium formate, 6.63 M urea, pH 3.5; D4: 0.8 M Tris-HCl, 7.4 M urea, pH 8.0; D5: 1.0 M magnesium chloride. 32P-Labeled adducts were located on chromatograms by autoradiography. Adducts were cut out and radioactivity was then quantified by Cerenkov counting. The adduct levels obtained under intensification conditions, expressed as relative adduct labeling (<RAL>) values, were corrected to actual RAL values using the previously determined intensification factors for each adduct (18).

Results

Urinary excretion of unmetabolized PhIP

Using GC/MS levels of unmetabolized PhIP were measured weekly over 23 days in 24 h urine samples from six groups of four male Fischer-344 rats treated by gavage with daily

<table>
<thead>
<tr>
<th>Group</th>
<th>Daily dose* (μg/kg)</th>
<th>Percent of dose excreted as PhIP (mean ± SD, n =4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
<td>Day 9</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
<td>1.8 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Day 16</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>B</td>
<td>0.1</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Day 23</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>C</td>
<td>1.0</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Day 23</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Day 23</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>E</td>
<td>1000</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>F</td>
<td>2.0 ± 0.7</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

*Doses were adjusted weekly, based on animal weight gain.
doses of 1000, 100, 10, 1, 0.1 and 0 μg PhIP/kg. Results over the 23 day treatment period are summarized by treatment group in Table I. Unmetabolized PhIP could be measured in the urine of all of the five treated groups, even at the lowest dose level of 0.1 μg/kg. PhIP levels were below the limit of detection for the control group of rats not receiving PhIP. Using an aliquot of 0.1 ml of each 24 h urine sample for analysis the detection limit of the method was determined to be 80–200 pg PhIP excreted/24 h, dependent on the volume of urine excreted daily, which ranged from 3.8 to 10.5 ml/24 h (7.3 ± 1.6 ml/24 h).

Mean PhIP excretion in the urine by group was relatively constant from 1.6 to 2.1% of the dose over the 23 day period (Table I). There was no significant accumulation of unmetabolized PhIP in the urine. A linear dose–response (r = 0.94) was obtained for urinary excretion of unmetabolized PhIP over the 23 day period, across the range of doses (Figure 1).

**PhIP-DNA adducts in WBCs and tissues**

On day 24 all rats were killed and their blood and tissues were collected for isolation of DNA. Using \(^{32}\)P-postlabeling and alkaline hydrolysis with GC/MS levels of PhIP-DNA adducts were measured in WBCs and 10 tissues: liver, lung, stomach, small intestine, colon, cecum, kidney, heart, spleen and pancreas (Figure 2). PhIP-DNA adduct levels could be detected by both \(^{32}\)P-postlabeling and alkaline hydrolysis with GC/MS in all tissues from animals receiving PhIP at the highest dose level, 1000 μg/kg/day (Group F). For animals receiving 100 μg PhIP/kg/day (Group E) PhIP-DNA adducts were detectable in all tissues except the stomach by \(^{32}\)P-postlabeling and only in the kidney, heart and pancreas by alkaline hydrolysis with GC/MS. Ratios of PhIP-DNA adduct levels in Group F versus Group E averaged 8.0 ± 3.3 for \(^{32}\)P-postlabeling (n = 10 tissues) and 8.5 ± 1.4 for alkaline hydrolysis with GC/MS (n = 3 tissues), indicating a nearly 10-fold increase in adduct level for a 10-fold increase in dose. Levels of PhIP-DNA adducts were highest in the pancreas, heart, kidney and WBCs and lowest in the stomach, cecum, colon and liver (Figure 2).

There was a good correlation (r = 0.77, P < 0.005) between levels of PhIP-DNA adducts determined by the two methods (Figure 3). A slope of 0.70 for the correlation curve indicates that, on average, adduct levels determined by alkaline hydrolysis with GC/MS were ~70% of adduct levels determined by \(^{32}\)P-postlabeling. Exceptions are the kidney, WBCs, liver and cecum, where higher adduct levels were determined by alkaline hydrolysis with GC/MS.

**Comparison of adduct levels after multiple and single doses**

Levels of PhIP-DNA adducts were also determined by alkaline hydrolysis with GC/MS in the WBCs and tissues of one rat 24 h after treatment with a single oral dose of 5 mg PhIP. PhIP-DNA adduct levels ranged from 11 to 350 adducts/10\(^8\) normal nucleotides (Table II) and were highest in the WBCs and pancreas. Adduct levels in this study were consistent with those obtained for rats treated with a higher dose in a previous study (21) which used the same analytical method. In Table II results for a single dose of 5 mg are compared with results for the animals in Group F, which received an accumulated dose of 4.9 mg (0.200–0.238 mg/day over 23 days). The ratio of PhIP-DNA adduct levels for the accumulated dose over the single dose ranged from 0.02 for WBCs to 0.48 for the liver. Ratios >0.04 (the ratio of the two daily doses of 0.2 and 5 mg/day) indicate an accumulation of adducts with multiple dosing. Accumulation of PhIP adducts was highest in the liver, kidney, heart and pancreas and lowest in WBCs and the small intestine.
In applying the excretion constant (1.8 ± 0.7% of the dose) can be estimated to within ~±40% by measuring the amount excreted in the urine, which are precursors for PhIP formation during the past 24 h. There is a slight increase in the rate of unmetabolized PhIP excretion with time over a wide range of doses, including doses comparable with levels to which humans are commonly exposed. PhIP has been reported to occur in heavily fried meat and pan scrapings at levels ranging from 15 to 140 ng/g (1,29-31). For a 70 kg human a daily diet containing 200 g meat prepared in this way would result in a daily exposure of from 3 to 30 μg PhIP or a dose of 0.04–0.4 μg/kg/day. The rats in Group B of our experiment received 0.1 μg/kg/day.

There is a clear linear dose–response relationship over four orders of magnitude between daily oral exposure to PhIP and daily excretion of PhIP in the urine (Figure 1). The rate of PhIP excretion is not a function of the dose, but remains constant at ~1.8% of the dose over the range of doses studied. This value agrees well with levels of unmetabolized PhIP excretion in rats reported earlier (32,33). There is a slight increase in the rate of unmetabolized PhIP excretion with time (Table I), apparently related to the accumulated dose of PhIP, however, the excretion rate has not doubled even after 23 days. It is also important to note that even though a slightly different protocol for 32P-postlabeling was employed for this study, the correlation between alkaline hydrolysis with GC/MS and the two 32P-postlabeling procedures is similar.

Table II. Formation of PhIP-DNA adducts after an accumulated or single dose of 5 mg PhIP

<table>
<thead>
<tr>
<th>Organ</th>
<th>Adducts/10^8 nucleotides</th>
<th>Ratio of adduct levels (accumulated/single dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Daily dose 0.2 mg^a</td>
<td>Single dose 5 mg^b</td>
</tr>
<tr>
<td>WBCs</td>
<td>8</td>
<td>350</td>
</tr>
<tr>
<td>Small intestine</td>
<td>2</td>
<td>43</td>
</tr>
<tr>
<td>Stomach</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Colon</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>Cecum</td>
<td>4</td>
<td>34</td>
</tr>
<tr>
<td>Spleen</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>Lungs</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Pancreas</td>
<td>17</td>
<td>70</td>
</tr>
<tr>
<td>Heart</td>
<td>7</td>
<td>29</td>
</tr>
<tr>
<td>Kidneys</td>
<td>12</td>
<td>39</td>
</tr>
<tr>
<td>Liver</td>
<td>5</td>
<td>11</td>
</tr>
</tbody>
</table>

Adduct levels determined by alkaline hydrolysis with GC/MS.
^aDose 1 mg/kg/day for 23 days, average from four rats; dose accumulated over 23 days 4.9 mg.
^bSingle dose of 25 mg/kg; one rat only.

Discussion

In this study in rats we have tried to determine whether two biological parameters, urinary levels of unmetabolized PhIP (a measure of dietary exposure) and tissue levels of PhIP-DNA adducts (a measure of activation of the parent amine), can be useful as biomarkers of exposure to the suspected human carcinogen PhIP. The validation has been carried out over a wide range of doses, including doses comparable with levels to which humans are commonly exposed. PhIP has been reported to occur in heavily fried meat and pan scrapings at levels ranging from 15 to 140 ng/g (1,29-31). For a 70 kg human a daily diet containing 200 g meat prepared in this way would result in a daily exposure of from 3 to 30 μg PhIP or a dose of 0.04–0.4 μg/kg/day. The rats in Group B of our experiment received 0.1 μg/kg/day.

There is a clear linear dose–response relationship over four orders of magnitude between daily oral exposure to PhIP and daily excretion of PhIP in the urine (Figure 1). The rate of PhIP excretion is not a function of the dose, but remains constant at ~1.8% of the dose over the range of doses studied. This value agrees well with levels of unmetabolized PhIP excretion in rats reported earlier (32,33). There is a slight increase in the rate of unmetabolized PhIP excretion with time (Table I), apparently related to the accumulated dose of PhIP, however, the excretion rate has not doubled even after 23 days. It is also important to note that even though a slightly different protocol for 32P-postlabeling was employed for this study, the correlation between alkaline hydrolysis with GC/MS and the two 32P-postlabeling procedures is similar.

For the tissue and WBC DNA samples analyzed in this study the overall correlation between the alkaline hydrolysis with GC/MS method and 32P-postlabeling was very good (r = 0.77; Figure 3). Inter-assay variability for both methods (< 18% for 32P-postlabeling and <16% for alkaline hydrolysis with GC/MS) was comparable with that found previously (23). Accordingly, the majority of tissues had higher PhIP-DNA adduct levels by 32P-postlabeling than by alkaline hydrolysis with GC/MS (Figure 2). In some cases, however, e.g. the cecum, liver, WBCs and kidney, PhIP–DNA adduct levels measured by alkaline hydrolysis with GC/MS were higher than those measured by 32P-postlabeling. Although further investigation of these tissue-dependent differences is clearly needed, several explanations are possible. For example, the relative abundance of the three major adducts observed by 32P-postlabeling can vary as a function of tissue type (20). The extent of hydrolysis of PhIP from DNA before GC/MS analysis for these different adducts may also vary. Tissue-specific levels of substances which affect the efficiency of labeling of the 3'-monophosphates or the completeness of enzymatic hydrolysis of DNA should also be studied.

One of the most interesting results of this study is shown in Table II: a daily dose of ~200 μg/day over 23 days (4.9 mg accumulated over 23 days) gave up to half the level of PhIP-DNA adducts in some tissues as a single dose 25 times larger (5 mg). This result, indicating accumulation of PhIP-DNA adducts in some tissues, is supported by a comparison with a previous study (18), where no PhIP adducts were detected in seven organs of rats treated with a single dose of ~100 μg/day (0.5 mg/kg). In this study, in the same laboratory using the same 32P-postlabeling protocol, adducts were detected in six of the seven organs of rats treated with a daily dose of 20 μg/day over 23 days (cumulative dose 466 μg). This implies that PhIP-DNA adducts accumulate with repeated daily doses, especially in tissues like the liver, kidney, heart and pancreas.

The high levels of PhIP–DNA adducts in non-target organs for tumors are of interest because they confirm earlier findings of PhIP and other heterocyclic amines in the Fischer-344 rat (36,37) and in the monkey (20) after prolonged daily exposure. Because of the prevalence of heart disease and the presence of heterocyclic amines in the human diet, these compounds
merit consideration as potential etiologic agents in cardiovascular disease. Indeed, both PhIP and 2-amino-3-methylimidazo[4,5-f]quinoxine (IQ), a related heterocyclic amine and food mutagen, have been shown to cause myocardial degeneration in rats (38) and IQ-DNA adducts have been implicated in myocyte degeneration in monkeys (39). It is likely, however, that mechanisms other than cardiac DNA adduct formation contribute to the cardiotoxicity of reactive metabolites of PhIP or IQ. To date the high levels of adducts in the pancreas and kidney, which are seen in both the rat (36) (Table II) and monkey (20), have not been associated with any pathological condition. Clearly the organ distribution of adducts depends on the manner in which PhIP is administered, since a single high dose of PhIP results in the rat colon having the highest or nearly the highest DNA adduct levels (18,24). PhIP–DNA adducts in the colon, target organ of PhIP in the male Fischer-344 rat (3,4), are probably formed after local esterification of N-hydroxy-PhIP, which is formed principally in the liver and reaches the colon via the circulation (40). The presence of N-hydroxy-PhIP in the circulation may be inferred from the relatively high PhIP–DNA adduct levels in WBCs (18; Table II and Figure 2).

An important limitation in the use of DNA adducts as markers of exposure is the difficulty in obtaining human tissue samples of the cancer target organ under study. We have thus looked at the possibility of estimating PhIP adduct levels in WBCs as a surrogate marker of adduct levels in other tissues. While unchanged PhIP in the urine is easily detectable, even after a dose of 0.1 μg/kg/day (Table I), PhIP–DNA adducts in rat tissues and WBCs are not detectable at doses of 10 μg/kg/day or less (Figure 2). Thus, based on the rat data, the possibility of using human WBC adducts as a marker of exposure to PhIP appears limited, as human dietary intake of PhIP from total cooked proteinaceous foods is estimated to be <17 ng/kg/day (41). In the human, however, it has been calculated that 70% of ingested PhIP is eliminated by N-hydroxylation (42), catalyzed by hepatic microsomal cytochrome P450 IA2 (43), while in rodents and monkeys a number of additional products are formed (12-14,19,44). Thus the probability of detecting PhIP–DNA adducts in human WBCs at low doses of exposure is higher than that in rats. These differences between animals and humans may also explain why PhIP–DNA adducts have been detected in human colon (21). Such studies in the human, however, have not been conducted in a systematic manner. In contrast, urinary PhIP, which is generally considered a marker of recent exposure, is readily detectable in healthy human volunteers on a normal diet (5) or following consumption of a fried beef meal (6).

Therefore, while a direct estimate of DNA damage from PhIP in man may not be possible, the finding that in rats a constant percentage of unmetabolized PhIP is excreted in the urine irrespective of oral dose is important. If this can be shown to apply also in man, urinary assay for PhIP has the potential to give an accurate and sensitive measure of individual human exposure to the amine in the diet.

Acknowledgement

This study was supported by a Fogarty Senior International Fellowship for HAJS at the International Agency for Research on Cancer.

References

21. Friesen, M.D., Kaderlik, K., Lin, D., Garren, L., Bartsch, H., Lang, N.P. and...


