

Concentration of 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) in urine and alkali-hydrolyzed urine after consumption of charbroiled beef

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Abstract

Heterocyclic amines (HAs) and polycyclic aromatic hydrocarbons are carcinogenic products formed during the cooking of meat at moderate to high temperatures. We have previously shown that the urinary concentration of 1-hydroxypyrene-glucuronide, a metabolite of pyrene, increased significantly in ten subjects who had ingested charbroiled ground beef. We now report the time course and interindividual variation of 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) concentration in the urine samples from these ten subjects. PhIP concentration was determined in both untreated and alkali-hydrolyzed urine to obtain estimates of the proportion of conjugated PhIP metabolites in each subject. PhIP was measured by gas chromatography-negative ion chemical ionization-mass spectrometry after derivatization with pentafluorobenzyl bromide. Ten healthy non-smoking males consumed identical amounts of broiled beef on five consecutive days. The morning after the first day of broiled beef consumption, urinary concentration of PhIP increased 14–38 fold above mean pre-feed concentration of PhIP in individual alkali-hydrolyzed urine samples. Following cessation of broiled beef consumption, urinary PhIP concentration declined to near pre-feed levels within 48–72 hrs. The ratio of total alkali-labile PhIP metabolites to unmetabolized PhIP varied by about 2.7-fold among subjects, ranging from 18:1 to 48:1, suggesting that interindividual differences in PhIP metabolism occur and can be detected by this method. This study of urinary PhIP following ingestion of meat cooked by charbroiling, that contains both HAs and polycyclic aromatic hydrocarbons, extends previous studies of ingestion of pan-fried meat that contains primarily HAs. The results indicate that significant amounts of PhIP are bioavailable from ingestion of charbroiled ground beef and that measurement of proportions of alkali-labile PhIP metabolites and parent PhIP in human urine may yield information on individual metabolism of ingested PhIP. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Heterocyclic aromatic amine; Broiled meat; Carcinogen; PhIP; Urine metabolites

1. Introduction

The risk of colorectal cancer is associated with

consumption of red meat [1], however, the biochemical etiology of this association is unclear. It remains to be determined whether the increased risk is due to inherent constituents of red meat or to compounds produced during cooking processes [2–5]. Associations have been found between certain meat cooking practices, such as grilling or cooking until meat is

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well-done, and colon cancer in some [6–8], but not all [9,10], epidemiological studies.

Polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic amines (HAAs) are carcinogenic products formed during the cooking of meat at moderate to high temperatures [2–5]. Highly mutagenic HAAs are formed during broiling or frying due to pyrolysis of amino acids and proteins [2]. More than a dozen HAAs have been identified [2,3] and 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) is the most common HAA, being formed at concentrations of 0.1–100 µg/kg cooked meat [11,12].

The carcinogenic characteristics of PhIP and the enzymes involved in its metabolism have been reported. Male rats fed PhIP develop colon adenocarcinomas, whereas female rats primarily develop mammary adenocarcinomas [13]. PhIP is activated to the mutagenic metabolite 2-hydroxyamino-1-methyl-6-phenylimidazo(4,5-b)pyridine (N²-OH-PhIP) by cytochromes P450 (CYP) 1A2, 1A1, and 1B1; or metabolized to an inactive form, 4'-hydroxy-2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (4'-OH-PhIP), by CYP 1A1 and 1B1 [14–16]. These metabolites can subsequently form conjugates with glucuronide, glutathione or sulfate [17,18]. However, in human liver microsomes, glucuronide conjugation with PhIP has also been shown to occur at the N³ position of PhIP in the absence of hydroxylation [19].

Several HAAs, including PhIP, have been detected in the urine of healthy subjects eating normal diets [20,21]. In controlled feeding studies, urinary HAAs have been used to examine urinary clearance rates [22], the usefulness of conjugated and unconjugated HAAs as biomarkers of dietary exposure [23], and the effect of systemic CYP1A2 and *N*-acetyltransferase activities on HAA excretion [24,25]. Reistad et al. [23] examined the ratio of hydrolyzable to parent PhIP in urine from eight subjects following ingestion of a fried meat meal and found considerable variation between subjects ranging from 1.4- to 5.8-fold. These results suggested that individual differences in metabolic capacity or induction may influence the relative excretion of PhIP in conjugated and unconjugated forms.

In the present study, we characterized the time course and interindividual variation of PhIP concentration in the urine of ten subjects who had ingested charbroiled (CB) ground beef. PhIP concentration was

determined in both untreated and alkali-hydrolyzed urine to obtain estimates of the proportion of conjugated PhIP metabolites in urines collected at multiple time points. We have previously shown that the urinary concentration of 1-hydroxypyrene-glucuronide, a metabolite of pyrene, increased significantly in the urine of these subjects after CB meat ingestion [26].

2. Materials and methods

2.1. Chemicals

PhIP was obtained from Toronto Research Chemicals (Downsview, Ontario, Canada). PhIP-*d*5, used as the internal standard for GC/MS, was prepared as reported previously [27]. Pentafluoro-benzyl bromide and *N,N*-diisopropylethylamine were obtained from Aldrich Chemical Company (Milwaukee, WI) and were used without further purification. All solvents used, including water, were HPLC grade.

2.2. Feeding protocol

The protocol was approved by the Committee on Human Research of the Johns Hopkins School of Hygiene and Public Health. A detailed description of the feeding protocol has been published previously [26]. Informed consent was obtained from ten male non-smoking subjects aged 25–45 who had no known occupational or medicinal exposure to PAHs. During the first 2 weeks of the study, the subjects refrained from eating any CB, smoked, or fried meat. During the third week, 9 ounces (252 g) of CB beef was consumed on the first day, and 6 ounces (168 g) was consumed on each of the next 4 days. The CB beef was centrally prepared by cooking ground beef patties over charcoal briquettes on an outdoor grill until well done. The broiled patties were then homogenized in a blender to a granular consistency and portions weighed (cooked weight) and labelled. The study subjects met daily during the 5 day feeding period and ate the prepared CB beef between 12:00 and 14:00 h. The subjects again refrained from eating any CB, smoked, or fried meat for 10 days after the CB beef feeding period.

The mean PhIP content of five daily CB beef samples was 30.7 ng/g beef (range 25–39 ng/g) determined by the method of Felton et al. as previously

Table 1
Amounts of charbroiled beef, PhIP, and pyrene ingested daily during the feeding study

Day	Beef g	PhIP ^a µg	Pyrene ^a µg
1	252	7.7	1.7
2–5	168	5.2	1.1

^a Calculated from mean concentration measured in pooled CB-beef samples.

described [27]. This corresponds to a daily intake of 7.7 µg on day 1 of feeding and 5.2 µg on days 2–5 (Table 1). First morning voided urine samples (100 mls) were collected before (2 days), during (4 days), and after (4 days) the CB beef feeding period. Urine samples were frozen at –70°C within 2 h of collection.

2.3. Extraction of PhIP from urine

Just prior to analysis, urine samples were allowed to thaw at room temperature. After thorough mixing, a 1 ml aliquot was transferred to a teflon-stoppered, disposable glass tube (13 × 100 mm, Corning Inc., Corning, NY). After addition of 320 pg PhIP-*d5* internal standard dissolved in 25 µl methanol, the urine sample was made alkaline with 2 ml of 0.2 M sodium phosphate buffer, pH 8.0. For alkaline hydrolysis, samples were incubated in 1 N NaOH at 100°C overnight. After vortex mixing, the sample was extracted with 2 × 3 ml ethyl acetate. The combined upper organic layers were then transferred to another disposable glass tube and taken just to dryness under N₂ at 55°C.

2.4. Purification by solid phase extraction

Before purification of the extracted urine sample by solid-phase extraction on Extract-clean C18 cartridges (500 mg/6.0 ml; Alltech Associates, Inc., Deerfield, IL), cartridges were prepared washing with 2 ml methanol, 7 ml water and 5 ml of 0.01 M sodium phosphate buffer, pH 7.0. Elution in all cases was carried out without application of a vacuum. The urine extract was then dissolved in 20 µl of methanol and 5 ml of 0.01 M sodium phosphate buffer, pH 7.0, was added. After mixing, the sample was applied to the C18 solid-phase cartridge and washed with 4 ml methanol/water (45:55; v/v). PhIP was then eluted

with 3.5 ml methanol/water (60:40; v/v) into a 2 ml flame-sealable glass vial (Wheaton, Millville, NJ). This aqueous solution was then taken to dryness overnight in a vacuum centrifugal evaporator at room temperature.

2.5. Derivatization and analysis by GC/MS

For analysis by gas chromatography-negative ion chemical ionization mass spectrometry (GC/MS), the volatile, electron-capturing *bis*(pentafluorobenzyl)derivative (BPFB) of PhIP was formed [28]. To the glass vial were added 20 µl of a 30% (v/v) solution of pentafluorobenzyl bromide (PFB-Br) in ethyl acetate and 20 µl of diisopropylethylamine. The vial was flame-sealed and heated at 55°C for 30 min. The reaction mixture was then taken to dryness under a stream of N₂. After addition of 200 µl of 0.1 N HCl, the solution was washed with 2 × 1 ml of hexane. The hexane was discarded. Then 300 µl of 1 M Na₂CO₃ was added and the PhIP was extracted with 2 × 1 ml of ethyl acetate. The combined organic extracts were then reduced to dryness under N₂ at 55°C and stored at –20°C until analysis by GC/MS.

GC/MS was carried out on a Hewlett Packard 5980A gas chromatograph equipped with a splitless injector (250°C) coupled to a Hewlett Packard 5988A mass spectrometer. The analysis was carried out on a non-polar Hewlett Packard HP1 fused silica capillary column (25 m × 0.2 mm i.d.; film thickness 0.33 µm) coupled directly to the mass spectrometer ion source (250°C) through a heated interface (300°C). Helium was used as carrier gas at a column head pressure of 160 kPa. The temperature of the GC oven was raised from an initial temperature of 200–320°C at 30°C/min, where it was held for 5 min. Under these conditions, the retention time of the BPFB derivative of PhIP was about 9.5 min.

The mass spectrometer was operated in the negative ion mode with methane as reagent gas (~1 Torr). The MS was tuned to monitor negative ions at *m/z* 403.1 [(BPFB-PhIP-*d0*)-(PFB)][–] and *m/z* 408.1 [(BPFB-PhIP-*d5*)-(PFB)][–] representing loss of single PFB-groups from the molecular anions. The detection limit for PhIP was 1 pg PhIP/injection and the inter-assay variability was <16%. Samples with nondetectable PhIP were assigned the value of half the limit of detection, 0.5 pg/ml.

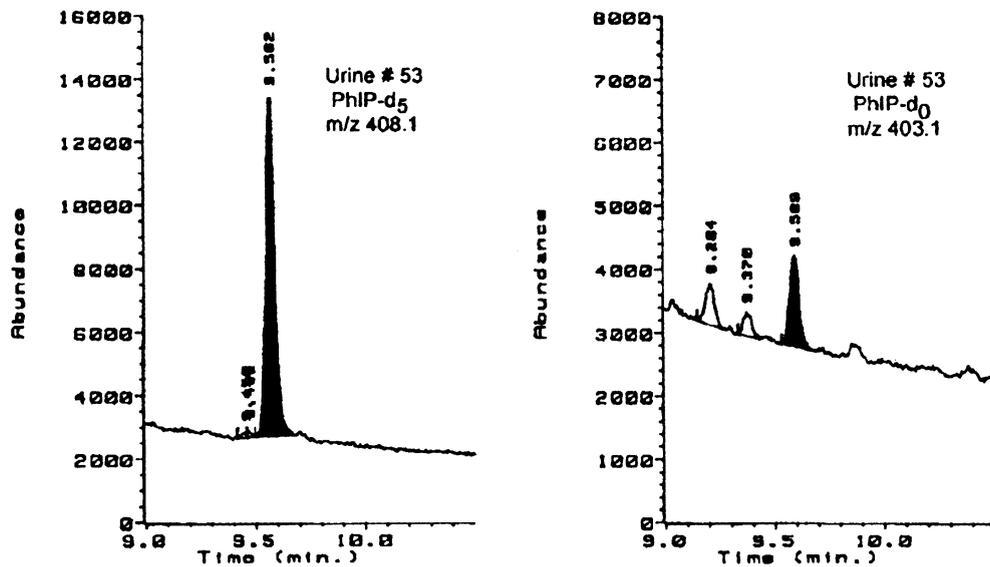


Fig. 1. Selected ion monitoring GC/MS analysis of free PhIP in human urine after alkaline hydrolysis. Left panel shows abundance of PhIP- d_5 internal standard added to urine. Right panel shows abundance of endogenous PhIP- d_0 in urine after alkaline hydrolysis.

3. Results

3.1. PhIP in non-hydrolyzed urine samples

GC/MS analysis of urine samples collected after CB beef ingestion indicated the presence of PhIP at retention time of about 9.5 min in all ten subjects (representative example shown in Fig. 1). This peak co-chromatographed with PhIP standard and demonstrated the expected m/z of 403.1. The baseline concentration of PhIP in unhydrolyzed urine after 2 weeks free of broiled or smoked foods was 0.88 pg/ml urine (range: <1–2 pg/ml, 7 of 20 samples detectable). Mean (\pm SEM) urinary PhIP concentration the morning after the first day of feeding was 26.3 ± 6.6 pg/ml urine (Fig. 2) with a range of 5–75 pg/ml. The concentration of PhIP decreased to near baseline levels by 48–72 h after CB beef consumption ended.

3.2. PhIP in alkali-hydrolyzed samples

The mean (\pm SEM) baseline concentration of PhIP in alkali-hydrolyzed urine after 2 weeks free of broiled or smoked foods was 30.4 ± 5.4 pg/ml urine (range: 1–84 pg/ml). Mean urinary PhIP concentration

the morning after the first day of feeding was 675 ± 97 pg/ml urine (Fig. 3) with a range of 423–1164 pg/ml. This represented an increase of 14 to 38-fold above mean baseline concentration ($P = 0.001$ by Wilcoxon rank sum). The 32% decrease in the

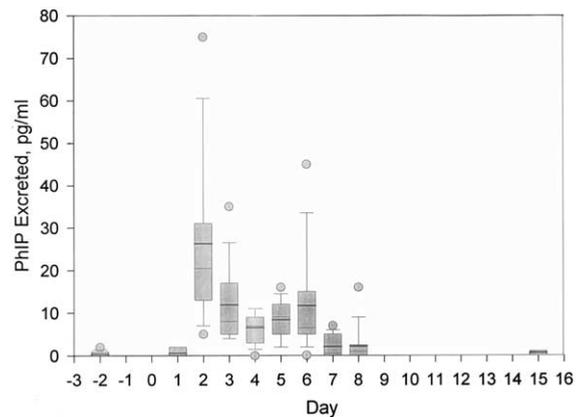


Fig. 2. Concentration of PhIP in unhydrolyzed urine collected before, during and after consumption of CB beef for 5 days. Nine ounces CB beef were consumed on day 1, and 6 ounces were consumed on each of days 2–5. Urine samples were collected in the morning before CB beef consumption at midday. Box plots show mean (heavy bar), median, 10th, 25th, 75th, and 90th percentiles of urinary PhIP concentrations on selected days of study.

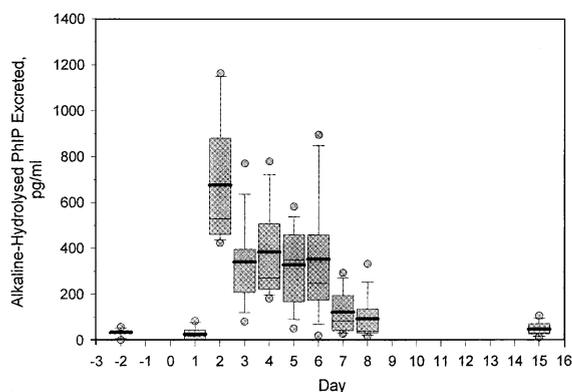


Fig. 3. Concentration of PhIP in alkali-hydrolyzed urine collected before, during and after consumption of CB beef for 5 days. Box plots show mean (heavy bar), median, 10th, 25th, 75th, and 90th percentiles of urinary PhIP concentrations on selected days of study.

amount of PhIP ingested on days 2–5 of the feeding protocol (5.2 μg), compared to day 1 (7.7 μg), was accompanied by a 48% decrease in urinary PhIP concentration on the following mornings. The concentration of PhIP in alkali-hydrolyzed urine

decreased to near baseline levels by 48–72 h after CB beef consumption ended. Although mean urinary PhIP concentration during days 3–6 of the study varied by 2.5-fold among individuals, overall interindividual differences were not significant ($P = 0.20$ by Kruskal–Wallis). After adjustment for urine creatinine, interindividual differences in mean urinary PhIP concentration during days 3–6 of the study were significant ($P = 0.016$ by Kruskal–Wallis). Overall, creatinine adjustment did not change the rank order or time course of individual urine concentrations of PhIP in hydrolyzed or unhydrolyzed urines. Since creatinine adjustment had little effect on the data presented in Figs. 2 and 3, and no effect on the data presented in Fig. 4, the adjustment was not performed on these data.

3.3. Ratio of free PhIP in hydrolyzed to non-hydrolyzed urine

We examined, on an individual basis, the correlation of urinary PhIP concentration in hydrolyzed and unhydrolyzed urines collected at ten time points

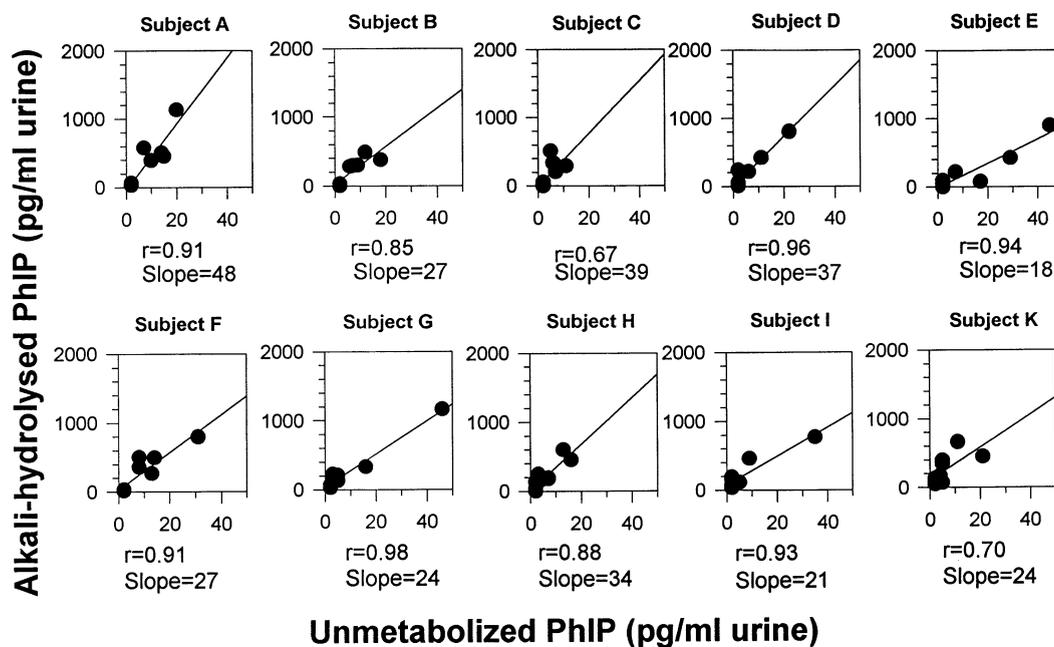


Fig. 4. Individual plots of PhIP concentrations in hydrolyzed urine versus unhydrolyzed urine for each time point of the ten study subjects. Individual plots contain ten time points except plot for Subject I which contains nine time points. The slopes are the ratios of free PhIP in hydrolyzed to nonhydrolyzed urine.

during the feeding study (Fig. 4). Within each subjects' set of ten urines, the correlation was quite good (r coefficient ranged from 0.67 to 0.98). The mean ratios of PhIP in hydrolyzed to unhydrolyzed urine (slopes) varied by 2.7-fold among individuals from a minimum of 18:1 in subject (E) to a maximum of 48:1 in subject (A). Interestingly, the urinary PhIP concentrations measured did not correlate with previously reported [26] levels of 1-OHP-gluc on an individual basis (not shown).

4. Discussion

Colorectal cancer has been associated with the consumption of animal fat and red meat [29], particularly well-done [6,30] or grilled meat [6,31]. Although no clear biochemical etiology for colorectal cancer has been established, evidence suggests that food-borne HAs may play a role [32–34]. PhIP is the most common HA formed in fried, grilled or broiled meat, and is a colon carcinogen in rodents [13]. In open-flame broiled or grilled meats, PAHs also are formed at moderate to high levels.

Biological markers of internal or molecular dose from dietary or environmental carcinogens often show considerable interindividual differences in marker levels after presumed similar exposures. In our controlled feeding study, we rigorously controlled the amount of CB beef ingested in order to examine biological variation in excretion of urinary PhIP in ten study subjects. In addition, we examined the time course of urinary PhIP concentration before, during and after ingestion of CB beef.

Our results show that the concentration of PhIP in either unhydrolyzed or alkali-hydrolyzed first morning urines increased significantly after ingestion of CB meat on the previous day. The 32% decrease in the amount of PhIP ingested on days 2–5 of the feeding protocol, compared to day 1, was accompanied by a 48% decrease in urinary PhIP concentration on the following mornings. As in our previous report on urinary 1-OHP-gluc in these samples [26], significant interindividual differences in PhIP concentration were observed. Some subjects had consistently high concentrations of urinary PhIP on days following CB beef ingestion compared to other subjects with consistently low concentrations. These differences are

presumably due to individual differences in absorption, metabolism and/or excretion of PhIP. Interestingly, a comparison of urinary PhIP and 1-OHP-gluc levels on an individual basis showed no correlation between the two metabolites. That is, subjects with high urinary PhIP levels throughout the study did not necessarily have higher 1-OHP-gluc levels. These findings indicate that interindividual levels of these two metabolites are not mediated by a common factor (e.g., absorption, hydration, physical activity), rather, they appear to be chemical-specific factors.

The obvious candidates for these chemical-specific metabolite modulating factors are the phase I and II metabolic enzymes that are involved in the metabolism of PhIP and pyrene. Previous studies have demonstrated the role of CYP 1A1, 1A2, and 1B1 enzymes in the hydroxylation of both PhIP and pyrene. The CYP1 enzymes are inducible and levels of these enzymes are known to vary significantly between individuals. In addition, the phase II enzyme UDP-glucuronosyl-transferase is inducible by a variety of environmental and dietary compounds. Thus, differences in the metabolic capacity of the test subjects could result in different concentrations of metabolites following ingestion of similar quantities of parent compounds. Also, since both HAs and PAHs can induce and be metabolized by the same enzymes, these compounds may interact to enhance or inhibit their biological effects.

Further evidence for interindividual differences in metabolic modulation of bioconversion of ingested PhIP is seen in the ratio of free PhIP in hydrolyzed to non-hydrolyzed urine at multiple time points. The mean ratio varied from 18:1 to 48:1 in the individuals participating in the current study. This finding indicates that the proportion of unmetabolized PhIP measured in the urine was relatively constant within each subject, and varied 2.7-fold between subjects ingesting the same quantities of charbroiled beef. Whether this variation is related to potential risk of toxicity or adverse health outcome has not been addressed.

Previous studies of urinary PhIP in humans have measured PhIP either in untreated urine [20,22,35] or hydrolyzed urine [21,25], or both [23]. These measures presumably reflect levels of unmetabolized PhIP, in the former case, and metabolized and/or conjugated PhIP in the latter. We have chosen to

measure PhIP in both alkali-hydrolyzed and unhydrolyzed urine in order to estimate the ratio of conjugated to unconjugated PhIP metabolites. The large increase in the amount of PhIP detected in urine following hydrolysis indicates that labile PhIP metabolites represent a major proportion of the PhIP in human urine. Recent studies of metabolism following ingestion of PhIP indicate that PhIP-N2-glucuronide, N2-OH-PhIP-N2-glucuronide, and N2-OH-PhIP-N3-glucuronide are common metabolites in human urine [36,37]. The proportions of these metabolites in our study subjects is under investigation. At the present time we have not definitively identified which metabolites yield PhIP upon alkali-hydrolysis, however we note that N2-OH-PhIP is very unstable, forming 2-OH-PhIP and PhIP under various conditions.

Ushiyama et al. [20] detected and quantitated four different carcinogenic HAs in the urine of ten healthy volunteers eating normal diets, but detected no HAs in the urine of three patients receiving intravenous feeding. Kidd et al. [21] measured PhIP in the acid-hydrolyzed urine from 129 males who consumed an unrestricted normal diet. PhIP was detectable in 51% of African-American subjects, 45% of Asian-American subjects, and only 21% of white subjects. Although the geometric mean levels of urinary PhIP were higher in the Asian-American and African-American subjects than in the white subjects, this difference was not reflected in intake frequencies of cooked meats based on a self-administered dietary questionnaire.

In our study, PhIP was detectable in 35% (7 of 20) of the pre-feed hydrolyzed urine samples collected after the subjects had refrained from eating smoked or broiled foods for 2 weeks. This is similar to the percentages reported for subjects on an unrestricted diet [21], and suggests that the method could be amenable to biomonitoring in the general population.

Controlled feeding studies of meat containing HAs have examined intra- and interindividual variability in internal dose [23,35] and human metabolism by CYP 1A2 [22,24,25] and NAT2 [25]. One of these studies [23] determined the ratio of hydrolyzable to parent PhIP in urine from eight subjects ingesting a fried meat meal. They observed a 1.4- to 5.8-fold increase in measurable PhIP following acid hydrolysis of urine in 1 N HCl at 100°C for 2 h. The magnitude of the increases is much lower than those observed in our

study. This difference may be due to either the different hydrolysis conditions used (strong acid versus alkali) or the different cooking methods (frying versus charbroiling) used to prepare the meals. We have found that different hydrolysis conditions greatly affect the yield of free PhIP from human urine samples [38]. We have also reported [26] that the charbroiled meal consumed in our study contained high levels of PAHs in addition to HAs. Since PAHs are known to induce the CYP1A enzymes, they would be expected to alter the phase I metabolism of HAs. As a result, the urinary HA metabolites might be expected to contain a higher proportion of conjugated HAs relative to unconjugated metabolites.

Since the total volume of urine collected during first morning voids in this study was not recorded, it is difficult to calculate total amounts of PhIP excreted and related percentages of PhIP ingested. A crude estimate could be determined by assuming that the total volume of urine excreted in the first 12 h after feeding (including the first morning void) is about 1 l. Thus the percentage of the PhIP ingested on the first day of feeding (7.7 ug), that was excreted in urine as parent PhIP would be 0.34% (range: 0.06–0.97%) and as alkali-labile PhIP would be 8.8% (range: 5.5–15.1%).

In this report we measured urinary PhIP in untreated or hydrolyzed urine following ingestion of meat cooked by charbroiling, a process that (unlike pan-frying) produces both HAs and PAHs. The results indicate that significant amounts of PhIP are bioavailable from the ingestion of CB beef, and that ratios of conjugated to unconjugated urinary PhIP metabolites may depend on a variety of metabolic, dietary, and hydrolysis factors.

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