ORIGINAL ARTICLE

Biomonitoring an albumin adduct of the cooked meat carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in humans

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Abstract

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is formed in cooked meats and may be linked to dietary-associated colorectal, prostate and mammary cancers. Genotoxic N-oxidized metabolites of PhIP react with the Cys³⁴ of albumin (Alb) to form a sulfinamide adduct, a biomarker of the biologically effective dose. We examined the kinetics of PhIP-Alb adduct formation in plasma of volunteers on a 4-week semicontrolled diet of cooked meat containing known quantities of PhIP. The adduct was below the limit of detection (LOD) (10 femtograms PhIP/mg Alb) in most subjects before the meat feeding but increased by up to 560-fold at week 4 in subjects who ate meat containing 8.0 to 11.7 µg of PhIP per 150–200 g serving. In contrast, the adduct remained below the LOD in subjects who ingested 1.2 or 3.0 µg PhIP per serving. Correlations were not seen between PhIP-Alb adduct levels and PhIP intake levels ($P = 0.76$), the amount of PhIP accrued in hair ($P = 0.13$), the amounts of N-oxidized urinary metabolites of PhIP ($P = 0.66$) or caffeine CYP1A2 activity ($P = 0.55$), a key enzyme involved in the bioactivation of PhIP. The half-life of the PhIP-Alb adduct was <2 weeks, signifying that the adduct was not stable. PhIP-Alb adduct formation is direct evidence of bioactivation of PhIP *in vivo*. However, the PhIP hair biomarker is a longer lived and more sensitive biomarker to assess exposure to this potential human carcinogen.

Introduction

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is one of the most abundant heterocyclic aromatic amines (HAAs) formed in well-done cooked red meat and poultry (1,2). The carcinogenicity of PhIP in the colon, prostate and mammary glands of rodents is well documented (1). PhIP is classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans (Group 2B carcinogen) (3). Moreover, IARC classified processed meats and red meat, two important sources of PhIP in the Western diet, as carcinogenic (Group 1) and probably carcinogenic (Group 2A) to humans,

respectively (4). Some epidemiologic studies that have investigated dietary consumption of well-done cooked meat in relation to various tumor sites reported a positive association between cancer risk and well-done meat consumption (5–7), although other studies have shown no associations between well-done cooked meat and cancer risk (4,8,9). Therefore, the evidence about HAAs and cancer risk remains controversial. A major limiting factor in most epidemiological studies is the uncertainty in quantitative estimates of chronic exposure to HAAs, and thus, the association of HAAs formed in

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Abbreviations

Alb	albumin
CI	confidence interval
HAAs	heterocyclic aromatic amines
HONH-PhIP	2-hydroxyamino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
LOD	limit of detection
LOQ	limit of quantification
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
SPE	solid phase extraction.

cooked meat and cancer risk has been difficult to establish. There is a critical need to establish long-term biomarkers of HAAs exposure that can be implemented in molecular epidemiology studies (10). We previously established the hair biomarker to measure PhIP intake (11,12). However, a long-term, non-invasive biomarker of the biologically effective internal dose of PhIP is sought.

PhIP undergoes metabolic activation, by human cytochrome CYP1A2, to form 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (HONH-PhIP) (13), a genotoxic metabolite capable of reacting with DNA and proteins (10). The biomonitoring of DNA adducts is the most definitive biomarker to assess the biologically effective dose and genotoxic potential of a chemical (14,15). However, the measurement of DNA adducts in humans is often difficult because of the paucity of tissue samples. The employment of circulating blood protein adducts is an alternative approach to assess exposure to some hazardous chemicals (16,17). Moreover, stable covalent protein adducts are expected to accumulate during the

kinetics of the lifetime of the protein and facilitate chemical analyses (18,19).

Two studies used accelerator mass spectrometry to measure [^{14}C]-PhIP blood protein adduct formation in human volunteers undergoing colorectal cancer surgery (20,21). Subjects were given a capsule containing a dietary equivalent of PhIP, and the levels of PhIP bound to hemoglobin and albumin (Alb) were measured 24 h after dosing. Both studies reported that PhIP bound poorly to hemoglobin, but ~0.2 to 1.0% of the dose bound to Alb. We recently showed that HONH-PhIP and its nitroso intermediate bind to human Alb to form a sulfinamide adduct at the Cys³⁴ residue *in vitro* (22–24). Acid hydrolysis of this PhIP-Alb adduct occurs under conditions sufficiently mild that the Alb is not degraded, and the released PhIP can be readily separated from the protein and assayed by sensitive MS-based methods. We showed that a portion of the [^{14}C]-PhIP bound to Alb in the group of volunteers from the accelerator mass spectrometry study conducted by Dingley et al. (21) was labile to mild acid treatment or pronase digestion, which regenerated PhIP (25). These findings suggest that a portion of the PhIP bound to Alb *in vivo* probably occurs as the sulfinamide adduct at the Cys³⁴ residue.

Further validation of the PhIP-Alb adduct is required before this biomarker can be implemented in epidemiologic studies designed to address the role of cooked meat containing PhIP in cancer risk. In this study, we have examined the kinetics of formation of the presumed PhIP-Alb sulfinamide adduct and its rate of removal in plasma of healthy volunteers on a 4-week semicontrolled diet of cooked meat containing known quantities of PhIP (12). The findings of the study also provide important information about the relationship between PhIP-Alb adduct formation and dietary intake of PhIP. The associations between

Figure 1. Chemical structures of PhIP biomarkers and biospecimens employed for human biomonitoring in this feeding study. The PhIP-Alb sulfinamide adduct undergoes hydrolysis *in vitro* to produce PhIP and the Cys³⁴ sulfinic acid of Alb. Caffeine in coffee was used to metabolically phenotype CYP1A2 activity.

PhIP-Alb adduct levels and CYP1A2 activity—a key enzyme involved in PhIP bioactivation (26,27)—, N-oxidized urinary metabolites of PhIP and the level of PhIP accrued in hair are also reported (Figure 1) (12,28). The feasibility of employment of the PhIP-Alb adduct as a biomarker to assess PhIP exposure is discussed.

Materials and methods

Study subjects

The study was conducted at the University of Hawaii, Manoa campus, Honolulu, HI. Subjects were drawn from our previously reported study (12). Thirty-eight healthy, non-smoking volunteers (82% males, 18% females, and 45% white, 44% Asian/Pacific Islander, 9% African American, 2% Hispanic non-white) were recruited among University of Hawaii students and staff. Inclusion criteria included regular beef eater, age >18, no use of hair dyes, taking no prescribed or over-the-counter medication except an occasional analgesic, no history of gastrointestinal tract disorders, having a weight not <90% or >130% of 1983 Metropolitan Life Insurance desirable weights, no weight change of >10 pounds in the past 12 months, no special diet (e.g. vegetarian, macrobiotic, weight loss, diabetic, etc.), fruit and vegetable intake ≤ 7 servings/day, fiber intake ≤ 2 g/day, alcohol intake not >2 drinks/day and caffeine intake not >2 caffeinated drinks/day.

Feeding study

The study was divided into three phases. In phase I, a 3-week prefeeding period, subjects refrained from eating pan-fried, grilled or oven-broiled meats, poultry or fish, cooked well-done. After that, in phase II, subjects ate dinner, 5 days/week, for 4 weeks at a study site at the University of Hawaii at Manoa campus. As part of this meal, subjects were fed a portion of ground beef (150 or 200 g) grilled well-done. The rest of the meal was varied on a 5-day rotating basis but was low in dietary fiber and included a starch (rice, potato or pasta), a vegetable, a fruit, dessert and a drink. For the remainder of their daily meals and on Saturdays and Sundays, the subjects followed their normal diet, except that they were asked to avoid eating any well-done meats, poultry or fish. After this 4-week feeding period, in phase III, the subjects went back to their regular diets but refrained from eating meats, poultry or fish that were grilled well-done for 4 weeks. This study was run in groups of 10 to 20 subjects. The amounts of PhIP ingested per serving by the subjects were on average (μg PhIP/serving): 1.2 (Group 1), 3.0 (Group 2), 11.7 (Group 3) and 8.0 (Group 4). All subjects provided informed consent, and the study was approved by the Institutional Review Board at the University of Hawaii.

Biological specimens

Non-fasting blood samples were drawn at a clinical laboratory during phase I (days 14 and 21), phase II (days 35 and 49) and phase III (days 63 and 77). Components were separated through centrifugation and then stored at -80°C . Urine was collected for the 12 h period immediately after dinner time until rising the following morning and kept on blue ice in a cooler until aliquoting and freezing. Subjects collected urine samples during phase II on days 23, 35 and 49 (2, 14 and 28 days following the start of consumption of well-done meat) and at days 63 and 77 (the end of the fourth week of cessation of consumption of well-done meat). Urine was stored at -80°C until assayed. Newly grown hair (50–200 mg) from the nape of the neck was collected by study staff once during phase I and at the end of phases II and III. Samples were stored in sealed envelopes at 4°C until assayed.

Chemicals

PhIP and 2-amino-1- $[\text{H}_3\text{C}]$ -methyl-6-phenylimidazo[4,5-b]pyridine ($[\text{H}_3\text{C}]$ -PhIP, 99% isotopic purity) were purchased from Toronto Research Chemicals. The metabolites of PhIP and its internal standard $[\text{H}_3\text{C}]$ -PhIP, N^2 -(β -1-glucosiduronyl)-2-(hydroxyamino)-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP-HO- N^2 -Gluc) and O-(β -D-glucosiduronyl)-2-(hydroxyamino)-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP-HN 2 -O-Gluc) were prepared

as reported (29). SOLA SCX SPE cartridges (10 mg/ml) were from Thermo Scientific (Bellefonte, PA).

Preparation of cooked meat and analysis of PhIP

Ground beef (15% fat) was cooked as half-pound patties on a commercial flat top griddle for an average of 10 min/side. The surface temperature of the griddle ranged from 440 to 600°F. After cooking, the meat was minced in a food processor, thoroughly mixed and frozen until meal preparation. Several samples were analyzed for PhIP to estimate the dose provided to each study group. PhIP was isolated from meat, and quantitative mass spectrometric measurements were done as described (12,30).

PhIP biomarkers

PhIP hair and urinary biomarkers were quantified using stable isotopically labeled internal standards. The within-day and between-day percentage coefficients of variation were $\leq 10\%$ (12,31,32).

PhIP in hair

The levels of PhIP in hair were measured by ultra-performance liquid chromatography—triple quadrupole mass spectrometry following digestion of hair with 1 N NaOH and clean-up by tandem solvent/solid phase extraction (SPE) (12,31).

Melanin in hair

The melanin content was estimated by visible spectrophotometry following digestion of hair with Soluene 350. The absorbance at 500 nm was used to estimate the total amount of melanin (12,33).

Urinary levels of PhIP and Glucuronide (Gluc) conjugates of HONH-PhIP

PhIP, PhIP-HO- N^2 -Gluc and PhIP-HN 2 -O-Gluc were isolated by SPE and analyzed by ultra-performance liquid chromatography/electrospray ionization/tandem mass spectrometry (32,34).

Caffeine phenotyping for cytochrome CYP1A2 activity

Subjects fasted for 10 h, then consumed two cups of coffee (100 mg of caffeine) upon rising, maintained fasting for another 2 h, abstained from other caffeine consumption and collected their urine during the fifth hour after dosage. Caffeine metabolites (17U: 1,7-dimethyluric acid; 17X: 1,7-dimethylxanthine and 137X: caffeine) were analyzed by high-performance liquid chromatography with a photodiode array detector to assess metabolic phenotypes (35). The ratio (17U + 17X)/caffeine was taken as a measure of CYP2A2 activity on days 14 and 21 (prefeeding phase I), days 35 and 49 (feeding phase II) and days 70 and 77 (postfeeding phase III).

Isolation of Alb from plasma

Alb was purified from plasma using the Pierce Alb Depletion Kit (36). Isolated Alb (10 mg) was spiked with $[\text{H}_3\text{C}]$ -PhIP (100 femtograms/mg Alb) and hydrolyzed in 0.5 ml of 0.1 N HCl at 37°C for 3 h, followed by extraction with ethyl acetate (25). After that, the solution was made alkaline with 1 N NaOH, and the liberated PhIP was extracted with ethyl acetate and processed by SPE except that the SOLA SCX SPE was used for purification (25,37). The method was validated using commercial plasma of healthy subjects from Bioivt (Westbury, NY), which served as the negative control and for determination of the limit of detection (LOD) and limit of quantification (LOQ). The validation and performance of the method were recently reported except that the Velos Pro ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) was used in this current study (25).

Quantification of acid-labile PhIP-Alb by UPLC-ESI/MS n

PhIP was quantified by ultra-performance liquid chromatography-electrospray ionization-multistage mass spectrometry (UPLC-ESI/MS n) employing a Dionex Ultimate 3000 LC with a Velos Pro ion trap MS and an Advance CaptiveSpray (Michrom Bioresource, Auburn, CA). Details on MS

acquisition parameters and performance of the method are reported in [Supplementary Material](#), available at *Carcinogenesis* Online.

Data and statistical analyses

The levels of PhIP-Alb adduct were measured in duplicate on days 14 and 21 in prefeeding phase I, days 23, 35 and 49 in feeding phase II and days 63 and 77 in postfeeding phase III. The duplicate values averaged within 10% and assigned as the PhIP-Alb adduct level for each subject for each time point. The value, 10 femtograms/mg Alb, was assigned when PhIP-Alb adduct levels occurred below the LOD.

Spearman rank correlation coefficients (ρ) were used to assess associations between levels of PhIP-Alb adduct and levels of PhIP intake and other PhIP biomarkers measured in hair and urine at the end of the feeding period (day 49). Partial Spearman correlations were also calculated, adjusting for sex, race, age at screening and body mass index. Fold changes from day 49 to prefeeding days 14 and 21 and from day 49 to postfeeding day 77 were estimated as ratios of means across all subjects, and differences in the distribution of PhIP-Alb adduct levels were assessed using the Wilcoxon rank sum test. The LOD value (10 femtograms/mg Alb) was used in calculating fold changes in the adduct levels.

Results

Identification and quantification of PhIP-Alb adducts in human plasma

The LOD and LOQ values for the acid-labile PhIP adduct are 10 and 23 femtograms/mg Alb, respectively, and defined,

respectively, as 3.3σ and 10σ SD units above the background level in commercial plasma (25). The reconstructed ion chromatograms of acid-labile PhIP adduct recovered from the plasma of volunteer 56 ($11.7 \mu\text{g}$ PhIP/serving) prefeeding, during feeding and postfeeding of consumption of well-done cooked meats are shown in [Figure 2A](#). The product ion spectra of PhIP and $[\text{H}_3\text{C}]$ -PhIP acquired at the MS³ scan stage confirmed their identities ([Figure 2B](#)) (25). The level of PhIP-Alb adduct was estimated at 3715 femtograms/mg Alb for this volunteer at day 49

Kinetics of PhIP-Alb adduct formation and removal in subjects on a semicontrolled meat-feeding study

The kinetics of PhIP-Alb adduct formation and removal are presented in [Figure 3](#) for the 20 volunteers in Groups 3 and 4 (13 and 7 subjects, respectively). Of the 13 volunteers in Group 3 who consumed $11.7 \mu\text{g}$ PhIP per serving, one subject had PhIP-Alb adduct levels below the LOD at all time points and a second subject had levels below the LOD at four time points, including the feeding phase II, and these levels were assigned to 10 femtograms/mg Alb. The remaining 11 volunteers had levels of PhIP-Alb adduct above the LOD (10 subjects had levels above the LOQ) after 4 weeks of cooked meat consumption (day 49). All seven volunteers in Group 4 who consumed $8.0 \mu\text{g}$ PhIP per serving had levels above the LOQ values after 4 weeks on the cooked meat diet. The 20 subjects in Groups 3 and 4 comprised the samples used for the primary analysis. The amount of PhIP-Alb

Figure 2. Quantification of acid-labile PhIP-Alb adduct by UPLC-ESI/MS³. (A) Reconstructed ion chromatograms of PhIP and its internal standard $[\text{H}_3\text{C}]$ -PhIP of Alb from blood obtained from Biovit (negative control), (B) PhIP and $[\text{H}_3\text{C}]$ -PhIP in plasma of subject 56 prefeeding, during feeding ($11.7 \mu\text{g}$ PhIP/serving) and post feeding. The product ion spectra of PhIP and $[\text{H}_3\text{C}]$ -PhIP and their corresponding mechanism of fragmentation are shown.

sulfonamide sharply increased between weeks 2 and 4 (days 35 and 49) of the semicontrolled cooked meat diet. At 4 weeks of meat consumption (day 49), the levels of PhIP-Alb adduct ranged from 10 to 5609 femtograms/mg Alb for Group 3, and 192 to 4833 femtograms/mg Alb for Group 4 (Figure 3). The mean level of PhIP-Alb adduct in the plasma on day 49 for subjects in Group 3 was 37- and 10-fold greater than the mean levels on days 14 and 21 ($P = 0.006$ and $P = 0.01$, respectively) and 30-fold greater than the level on day 77 ($P = 0.007$), when assigning the LOD to any undetectable levels. For subjects of Group 4, the mean level of PhIP-Alb adduct on day 49 was 80 and 127 greater than the mean level on days 14 and 21 ($P = 0.008$ and $P = 0.006$, respectively), and 121-fold greater than the level of day 77 ($P = 0.007$). The levels of PhIP-Alb adducts were not significantly different between Groups 3 and 4 at day 49 ($P = 0.44$). The mean level of the PhIP-Alb adduct declined rapidly in all of the subjects after the feeding of the cooked meat diet had stopped and decreased by 96% two weeks after completion of the meat diet on day 63 (Figure 3). In comparison, the half-life of Alb is 20 days (18). The levels of the PhIP-Alb adduct were below the LOD value at all time points in the 18 subjects assayed in Groups 1 and 2 who ingested, respectively, 1.2 and 3.0 μg PhIP per serving.

Correlation of PhIP-Alb adduct with other PhIP biomarkers and cytochrome CYP1A2 activity

The levels of PhIP-Alb adduct were correlated to levels of PhIP intake and other PhIP biomarkers at the completion of the 4-week phase of feeding of well-done cooked meat among the

20 subjects in Groups 3 and 4 (Table 1). We previously reported that levels of PhIP in hair were strongly correlated to dietary intake of PhIP (11,12); however, the levels of PhIP-Alb adduct were not correlated to the estimated intake of PhIP ($\rho = -0.08$, $P = 0.76$) and only weakly correlated to the amount of PhIP accrued in hair ($\rho = 0.39$, $P = 0.13$) irrespective of whether the PhIP content in hair was normalized for the melanin content ($\rho = 0.20$, $P = 0.46$). The level of PhIP-Alb adduct was not correlated to caffeine CYP1A2 phenotype activity ($\rho = -0.16$, $P = 0.55$)—a key enzyme involved in the bioactivation of PhIP (27,38). Weak, statistically non-significant inverse correlations were observed between PhIP-Alb adduct levels and rapid metabolizers of PhIP for its two major urinary metabolites of PhIP, using the metabolic ratios of HONH-PhIP-Gluc conjugates/PhIP in urine ($\rho = -0.12$, $P = 0.65$ for PhIP-HO-N²-Gluc; $\rho = -0.06$, $P = 0.82$ for PhIP-HN²-O-Gluc).

Discussion

Alb adducts of the aflatoxin B₁ and benzene were successfully used as biomarkers in molecular epidemiology studies designed to address the role of these chemicals in cancer risk (39,40). The benzene-Alb adduct also forms at the Cys³⁴ residue of Alb and occurs as a stable thioether linkage (40). The Cys³⁴ of Alb is a potent scavenger of free radicals and electrophiles of various xenobiotics (17). We have shown that Cys³⁴ of Alb is the major site of adduction of electrophilic N-oxidized metabolites of PhIP, resulting in the formation of an acid-labile sulfonamide linkage *in vitro* (23). Based on our previous chemical characterizations,

Figure 3. Kinetics of formation and removal of acid-labile PhIP-Alb adduct in plasma of subjects on a semicontrolled diet of cooked meat containing known quantities of PhIP. Box and whiskers plot showing the distribution of PhIP-Alb data measured over the entire study, separately for Groups 3 and 4. The volunteers in Group 3 ingested 11.7 μg PhIP/serving ($N = 13$) and volunteers of Group 4 ($N = 7$) ingested 8.0 μg PhIP/serving during the meat-feeding phase (days 22–49).

Table 1. Correlations of PhIP-Alb adduct and other PhIP biomarkers in study groups 3 and 4 at day 49 ($n = 20$)^a

PhIP biomarkers	ρ^b	P value ^b	ρ^c	P value ^c
Hair PhIP (pg/g hair)	0.39	0.09	0.39	0.13
Hair PhIP (ng/g melanin)	0.30	0.21	0.20	0.46
Caffeine CYP1A2	-0.08	0.74	-0.16	0.55
PhIP estimated intake (μg PhIP/serving)	-0.19	0.42	-0.08	0.76
% Dose PhIP in urine	0.18	0.44	-0.02	0.94
% Dose PhIP-HO-N ² -Gluc in urine	0.04	0.88	-0.12	0.66
% Dose PhIP-HN ² -O-Gluc in urine	0.15	0.52	-0.04	0.89
Metabolic ratio of PhIP-HO-N ² -Gluc/PhIP in urine	-0.10	0.68	-0.12	0.65
Metabolic ratio of PhIP-HN ² -O-Gluc/PhIP in urine	-0.03	0.91	-0.06	0.82

^aStatistical analyses included 13 subjects in Group 3 (who consumed 11.7 μg PhIP per serving) and 7 subjects in Group 4 (who consumed 8.0 μg PhIP per serving).

^bUnadjusted.

^cAdjusted for sex, race, age (at screening), body mass index (baseline).

a portion of the PhIP bound to Alb in humans is acid-labile and also probably occurs as the sulfinamide adduct formed at the Cys³⁴ residue (25).

The Airoldi laboratory employed gas chromatography–mass spectrometry or liquid chromatography–tandem mass spectrometry methods to measure acid-labile Alb adducts of PhIP in a cohort from Italy (41). PhIP was detected in ~30% of the subjects. The mean level of Alb adducts of PhIP was significantly higher in meat consumers (6.7 femtomol/mg of Alb = 1500 femtograms PhIP/mg Alb) than in vegetarians (0.7 femtomol/mg of Alb = 156 femtograms/mg Alb) (41). Surprisingly, the mean levels of PhIP-Alb adduct in this Italian cohort on a free-choice diet is comparable with the mean levels PhIP-Alb adduct measured in our group of volunteers under a semicontrolled feeding study with high levels of PhIP intake. The high level of PhIP binding observed in the Italian cohort is unexpected given the instability of the PhIP-Alb adduct. Moreover, the ammonium sulfate precipitation step employed to isolate Alb from plasma (41) resulted in >95% loss of the acid-labile PhIP-Alb adduct, when conducted in our laboratory (M.B., unpublished data). A more recent study conducted with a cohort from the United Kingdom did not replicate the findings obtained from the Italian study and concluded that acid-labile PhIP-Alb adducts were not formed at levels sufficient to be detected by liquid chromatography–tandem mass spectrometry and that the PhIP-Alb adduct was unlikely to be a viable biomarker to assess PhIP exposure (42). Our UPLC–MSⁿ method is >5-fold more sensitive than the liquid chromatography–tandem mass spectrometry methods using triple-quadrupole mass spectrometers (25,42). In our semicontrolled feeding study with cooked meat, PhIP-Alb adduct formation was successfully measured in 90% (18/20) of volunteers who had eaten well-done cooked meat containing 8.0–11.7 µg of PhIP per serving over a period of 4 weeks. However, the adduct was below the LOD (10 femtograms/mg Alb) in all 18 volunteers who had consumed 3 µg or less of PhIP per meat serving. The amounts of PhIP consumed by Groups 3 and 4 are at the high end of the estimated daily exposure levels to PhIP in the general population (43,44). Thus, it is not surprising that PhIP-Alb adduct measurements have not been successfully employed in some human studies.

In some subjects (seven subjects in Groups 3 and 4), the PhIP-Alb adduct was detected after 2 weeks of consumption of well-cooked meat compared with prefeeding levels, and the levels sharply increased at 4 weeks of feeding. The mean level of PhIP-Alb adduct at 4 weeks of feeding (day 49) was 55- and 47-fold higher than the level at 2 weeks of feeding meat (day 35) for Groups 3 and 4, respectively. The sharp increase in PhIP-Alb adduct formation suggests the rate of PhIP N-oxidation increased or there was a decrease in the rate of detoxification of HONH-PhIP during the second 2 weeks of the semicontrolled feeding of meat. However, the urinary metabolic profiles and percentages of the dose of PhIP metabolites eliminated as Gluc conjugates of HONH-PhIP were comparable on day 2 and weeks 2 and 4 of the meat diet (days 23, 35 and 49, respectively) with little evidence for an appreciable change in PhIP metabolism (28). The 95% confidence intervals for PhIP-Alb adduct formation at day 49 were 14- and 277-fold, respectively, for Groups 3 and 4. In contrast, the percent of the dose of PhIP eliminated in urine as its two major metabolites, PhIP-HO-N²-Gluc and PhIP-HN²-O-Gluc, did not substantially change over the 4-week feeding period, and the 95% confidence intervals were about 2.0-fold over the 4-week feeding period (28). Thus, the wide variation in PhIP-Alb adduct formation, which is formed with the genotoxic HONH-PhIP (Figure 1), is not mirrored by the urinary Gluc

metabolites of HONH-PhIP. PhIP-Alb adducts were not detected in Groups 1 and 2. The LOQ value of PhIP-Alb (23 femtograms/mg Alb) is 12- and 28-fold below the median value of PhIP-Alb adducts formed in subjects of Groups 3 and 4 (median values at day 49 for Group 3 = 283 and Group 4 = 653 femtograms PhIP/mg Alb), respectively, at 4 weeks of feeding meat. If adduct formation had occurred in a linear dose-response manner, we would have expected to detect the PhIP-Alb adduct in some of the subjects of Groups 1 and 2, who had consumed, respectively, 1.2 and 3.0 µg PhIP per day. The metabolic profiles and percentages of the dose of PhIP metabolites eliminated in urine as Gluc conjugates of HONH-PhIP were similar for all four Groups over the time course of feeding study (28), indicating the amounts of HONH-PhIP formed *in vivo* were proportional to the dose ingested.

The PhIP-Alb adduct levels were not correlated to the levels of PhIP intake and only weakly correlated to the levels of PhIP accrued in hair ($\rho = 0.39$; $P = 0.13$), less so when normalized for melanin content ($\rho = 0.20$; $P = 0.46$) (Table 1). We previously reported that the PhIP levels in hair were strongly correlated to the levels of PhIP intake ($\rho = 0.53$; $P < 0.001$), and the relationship was strengthened when PhIP hair levels were normalized for the melanin content of hair ($\rho = 0.71$; $P < 0.001$) (12), thereby demonstrating that PhIP accrued in hair is a robust biomarker of exposure to this carcinogen. The poor correlation observed between levels of PhIP accrued in hair and levels of PhIP-Alb adduct may be because the level of PhIP in hair represents a portion of the unmetabolized compound, whereas the PhIP-Alb adduct represents a portion of the bioactivated species. Furthermore, the PhIP-Alb adduct is a less sensitive biomarker than PhIP hair biomarker, and the relationship between these biomarkers could only be examined in Groups 3 and 4, over a narrow range of PhIP exposure (8.0 or 11.7 µg of PhIP per serving). The PhIP-Alb adduct levels were below the LOD in Groups 1 and 2 who ingested ≤ 3.0 µg PhIP per serving, yet the accrual of PhIP in hair is readily measured at this level of PhIP ingested per serving (11). The LOQ of PhIP in hair is 50 pg/g hair, when employing 20 mg of hair for assay, with a between-day coefficient of variation of <10% (12). The baseline geometric mean levels of PhIP in the hair of Groups 1–4 before feeding was 79 pg/g hair [95% confidence interval (CI) = 60–104] or 4.8 ng PhIP/g melanin (95% CI = 3.7–6.3). The nine volunteers in Group 1 who consumed the smallest dose of PhIP (1.2 µg per day) had a mean level of PhIP in hair of 27 ng/g melanin (95% CI = 18–39), and the volunteers in Group 4 who consumed the highest dose of 11.7 µg PhIP per day had a mean level of 133 ng/g melanin (95% CI = 106–165) after 4 weeks of the meat diet (12).

Our findings reveal that PhIP-Alb adduct can be used as a biomarker for subjects who frequently eat well-done cooked meat containing elevated levels of PhIP. However, the adduct levels rapidly declined to near background levels within 2 weeks of the postfeeding phase. *In vitro* kinetic studies conducted on the PhIP-Alb sulfinamide adduct revealed the adduct had a half-life of 50 h (25). Thus, the half-life of the PhIP-Alb adduct *in vivo* is also considerably less than the 20-day half-life of Alb (18), and the PhIP-Alb sulfinamide adduct cannot be used for reliable biomonitoring of PhIP exposure in individuals on free-choice diets.

CYP1A2 is the major cytochrome P450 involved in the metabolism and bioactivation of PhIP (26,27), and it accounts for ~70% of the clearance of PhIP in humans (38). We did not observe a correlation between the rapid caffeine CYP1A2 phenotype activity and the level of PhIP-Alb adducts ($\rho = -0.16$, $P = 0.55$). We also did not observe a statistically significant correlation between the caffeine CYP1A2 phenotype activity and the urinary Gluc

conjugates of HONH-PhIP with this cohort (28). A correlation between the caffeine CYP1A2 phenotype activity and the urinary Gluc conjugates of HONH-PhIP can be obscured by the variable levels of Uridine 5'-diphospho-glucuronosyltransferase activities (45–47). Glutathione S-transferase and NADH-dependent reductase activities that reduce N-oxidized PhIP back to PhIP also may mask rapid CYP1A2 activity (10,48). The Spearman correlations between the PhIP-Alb adduct and the percentage dose of the PhIP-HO-N²-Gluc ($\rho = -0.12$, $P = 0.66$) and percentage dose of the PhIP-HN²-O-Gluc in urine ($\rho = -0.04$, $P = 0.89$) and the metabolic ratios PhIP-HO-N²-Gluc/PhIP ($\rho = -0.12$, $P = 0.65$) and PhIP-HN²-O-Gluc/PhIP ($\rho = -0.06$, $P = 0.82$) in urine showed inverse associations but did not reach statistical significance. The inverse associations imply that individuals with rapid UDP-glucuronosyltransferase activity, who efficiently conjugate HONH-PhIP, have lower levels of the PhIP-Alb adduct and that UDP-glucuronosyltransferase serves as a mechanism of detoxication of the genotoxic HONH-PhIP metabolite. A larger study group would be required to determine whether this relationship reaches statistical significance. Consistent with this premise, the levels of urinary PhIP-HO-N²-Gluc also were reported to be inversely correlated to colon DNA adduct levels in a small human pilot study (49).

The caffeine CYP1A2 phenotype assay varied by >20-fold among the subjects in our controlled feeding study (12), and the range in PhIP-Alb adduct formation varied by >500-fold among the subjects after 4 weeks of the cooked meat diet. We had envisioned a positive correlation would exist between the rapid caffeine CYP1A2 phenotype and the levels of the PhIP-Alb adduct because adduct formation only requires the N-oxidation metabolism pathway and HONH-PhIP or its oxidized nitroso metabolite directly adduct to Alb (23). However, in addition to the multiple metabolic fates described above that impact the levels of HONH-PhIP, there were large intra-individual variations of the CYP1A2 phenotype and inconsistent responses to inducers of CYP1A2 over the 4-week semicontrolled meat-feeding study (12,28). The %CV range for CYP1A2 activity on pre and postfeeding days (days 14, 21, 35, 49) was 2–112% (median 40%), and during the feeding days (days 35, 49), the range was 1–103 (median = 37%) (12). The variable level of CYP1A2 activity over time also contributes to the poor correlation between rapid CYP1A2 phenotype activity and PhIP-Alb adduct formation.

In summary, the genotoxic HONH-PhIP or its nitroso metabolite forms the presumed sulfinamide adduct at the Cys³⁴ of Alb in humans (23). The PhIP-Alb adduct, combined with our recent discovery that PhIP forms a covalent DNA adduct in prostate tissue of cancer patients (50), clearly shows that electrophilic metabolites of PhIP are formed in humans and covalently adduct to DNA and proteins following consumption of cooked meat. The PhIP-Alb adduct can be measured in individuals who frequently eat well-done cooked meat, and it may be a useful biomarker for investigations with subjects on a semicontrolled feeding study, but the PhIP hair biomarker is a more facile, robust and sensitive long-term biomarker to assess exposure to this cooked meat carcinogen in the general population (11,12). However, the PhIP hair biomarker represents a measure of the unmetabolized dose; a long-term, non-invasive biomarker of the biologically effective internal dose of PhIP is still sought for molecular epidemiology studies.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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