Cruciferous vegetable consumption alters the metabolism of the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in humans

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Consumption of red meat is associated with an increased risk of colorectal cancer, whereas cruciferous vegetable consumption reduces cancer risk. While the mechanisms remain to be determined, cruciferous vegetables may act by altering the metabolism of carcinogens present in cooked food, such as the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). The aim of this study was to evaluate the effect of cruciferous vegetable consumption on the metabolism of PhIP in 20 non-smoking Caucasian male subjects. The study consisted of three 12-day phases, namely two periods of avoidance of cruciferous vegetables (phases 1 and 3) and a high cruciferous vegetable diet period (phase 2), when subjects ingested 250 g each of Brussels sprouts and broccoli per day. At the end of each study phase, the subjects consumed a cooked meat meal containing 4.90 μg PhIP and urine samples were collected for up to 48 h. Cruciferous vegetable consumption significantly increased hepatic CYP1A2, as demonstrated by changes in saliva caffeine kinetics. Samples of N2-hydroxy-PhIP-N3-glucuronide (the major urinary metabolite of PhIP in humans), N2-hydroxy-PhIP-N3-glucuronide and their tri-deuterated derivatives (to serve as internal standards) were synthesized and a liquid chromatography-mass spectrometry-mass spectrometry method developed for their analysis. In phases 1 and 3, the excretion of N2-hydroxy-PhIP-N3-glucuronide in 0-48 h urine samples was six times that of N2-hydroxy-PhIP-N3-glucuronide. Cruciferous vegetable consumption significantly increased the urinary excretion of N2-hydroxy-PhIP-N3-glucuronide in 0-48 h urine samples to 127 and 136% of levels observed in phases 1 and 3, respectively. In contrast, the urinary excretion of N2-hydroxy-PhIP-N3-glucuronide was unchanged. While the urinary excretion of both PhIP metabolites accounted for ~39% of the PhIP dose in phases 1 and 3, they accounted for ~49% of the dose in phase 2. This study demonstrates that cruciferous vegetable consumption can induce both the phase I and II metabolism of PhIP in humans.

Introduction

Diet is a major risk factor in human cancer (1). Epidemiological studies indicate that the consumption of cooked meat and meat products predisposes individuals to neoplastic disease, particularly of the colon (2,3). Dietary factors, which may be important in the aetiology of human cancer include heterocyclic amines, which are formed during the cooking of meat and fish (4–7). The most abundant food-derived heterocyclic amines (4,8) include 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx).

Experimentally, PhIP is a potent mutagen and genotoxin and has been shown to produce mammary gland, prostate and large intestine tumours in the rat (5–7). PhIP is only carcinogenic after metabolic activation. This involves an initial cytochrome P450 (CYP) catalysed N-hydroxylation step, to form N2-hydroxy-PhIP (Figure 1), which is followed by subsequent esterification by sulphotransferase and N-acetylation enzymes to form highly reactive N-O-sulphophenyl- and -acetyl esters, which can form covalent adducts with DNA and proteins (8–11). PhIP is extensively metabolized in humans, largely by N-hydroxylation, the major CYP form involved being hepatic CYP1A2 (8,12–14). In one study, the CYP1A2-catalysed N-hydroxylation pathway was shown to account for ~70% of the overall elimination of a dose of PhIP ingested by human volunteers as a cooked meat meal (14). PhIP may also be metabolized by other CYP forms, including CYP1A1 and CYP1B1 in extrahepatic tissues (8,10,15). Studies with human hepatocytes have demonstrated that PhIP is metabolized to a number of products including glucuronides of both PhIP and N2-hydroxy-PhIP (16). Investigations with human liver microsomes and with cDNA-expressed human UDPglucuronosyltransferase (UGT) forms have demonstrated the formation of various glucuronide conjugates including PhIP-N2-glucuronide, PhIP-N3-glucuronide, N2-hydroxy-PhIP-N2-glucuronide and N2-hydroxy-PhIP-N3-glucuronide (17–19).

The metabolism of PhIP has been studied in human volunteers given either 70–84 μg of [2-14C]PhIP or a well done chicken meal containing known amounts (9–27 μg) of PhIP (20–22). Various urinary PhIP metabolites were detected including N2-hydroxy-PhIP-N2-glucuronide, N2-hydroxy-PhIP-N3-glucuronide, PhIP-N2-glucuronide and 4'-hydroxy-PhIP-N3-glucuronide (structures shown in Figure 1). Other studies have also reported the presence of unchanged PhIP and phase I and II PhIP metabolites in urine samples.

Abbreviations: AUC0–∞, area under the salivary concentration/time curve; CYP, cytochrome P450; HPLC, high performance liquid chromatography; LC-MS-MS, liquid chromatography-mass-spectrometry-mass spectrometry; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline; MRM, multiple reaction monitoring; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; UGT, UDPglucuronosyltransferase.
from human volunteers after the ingestion of a cooked meat meal (23–25).

In contrast to the ingestion of cooked meat, a number of epidemiological studies have demonstrated a negative association between the consumption of fruit and vegetables (including cruciferous vegetables) and human cancer risk, including cancers of the digestive tract (26–28). While the mechanisms for the protective effects are not yet known, an obvious possibility is that dietary components such as cruciferous vegetables can alter the metabolism of genotoxins in the diet such as PhIP. Recently, we reported the effect of a high cruciferous vegetable diet on the urinary excretion of PhIP and MeIQx in 20 non-smoking Caucasian male subjects after the ingestion of a cooked meat meal (29). The study consisted of three 12-day phases, with cruciferous vegetables being excluded in phases 1 and 3. In phase 2 the subjects received a high cruciferous vegetable diet (250 g of each of Brussels sprouts and broccoli per day). At the end of each study phase, the subjects ingested a cooked meat meal containing known quantities of PhIP and MeIQx. Compared with phase 1, consumption of cruciferous vegetables in phase 2 reduced the urinary excretion of unmetabolized PhIP and MeIQx by 21 and 23%, respectively, suggesting that cruciferous vegetable consumption increased the metabolism of both heterocyclic amines (29).

The objective of the present study was to investigate the effect of cruciferous vegetable consumption on PhIP metabolism in the 20 non-smoking Caucasian male subjects from the above study. Samples of N²-hydroxy-PhIP-N²-glucuronide, and N²-hydroxy-PhIP-N³-glucuronide, together with their trideuterated analogues were synthesized and a liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS) method developed to quantify these two PhIP metabolites in human urine. N²-Hydroxy-PhIP-N²-glucuronide was selected for quantification in this study, as this has been shown previously to be the major metabolite of PhIP in human urine (20–22).

**Materials and methods**

**Chemicals**

PhIP and its trideuterated analogue [2-amino-1-(trideuteromethyl)-6-phenylimidazo[4,5-b]pyridine; [2H₃]PhIP] were obtained from Toronto Research Chemicals (Downsview, Ontario, Canada). N²-Hydroxy-PhIP and [2H₃]N²-hydroxy-PhIP were synthesized by established methods (13). Microsomes from baculovirus-infected insect cells containing cDNA-expressed human UGT1A1 (specific activity 683 pmol/min/mg protein with octyl gallate as substrate) were obtained from PanVera Corporation (Madison, WI) and alamethicin and uridine 5'-diphosphoglucuronic acid (UDPGA) from Sigma-Aldrich Company (Poole, Dorset).

**Synthesis of N²-hydroxy-PhIP-N²-glucuronide, N²-hydroxy-PhIP-N³-glucuronide and their trideuterated derivatives**

All glucuronides were synthesized employing cDNA-expressed human UGT1A1 (PanVera Corporation) using procedures modified from previous studies (21). Briefly, incubation mixtures contained 0.4 mM of either N²-hydroxy-PhIP or [2H₃]N²-hydroxy-PhIP, 5 μg/ml baculovirus-infected insect cell microsomal protein, 5 mM MgCl₂, 0.5 mM EDTA, 6.0 mM UDPGA, 60 μg/mg protein alamethicin and 0.1 mM Tris-HCl buffer pH 7.8 in a final volume of 0.5 ml. Samples were incubated for 6 h at 37°C, before terminating the reactions by the addition of 1 vol of ice-cold methanol and centrifuging to remove precipitated proteins. The N²-hydroxy-PhIP-N²-glucuronide, N²-hydroxy-PhIP-N³-glucuronide (co-formed in the reaction) and their trideuterated derivatives were purified by high performance liquid chromatography (HPLC). Chromatography was performed with a 220 × 4.6 mm column of TSK-Gel ODS-80TM (TosoHaas, Montgomeryville, PA) with a flow rate of 1.0 ml/min using a gradient of 30% (v/v) methanol and 0.1% (v/v) triethylamine pH 6.0, changing to 55% (v/v) methanol and 0.1% (v/v) triethylamine pH 6.0 over 8 min, which was then held from 8 to 20 min. The eluant was monitored at 315 nm. The identities of the two N²-hydroxy-PhIP-N-glucuronides were confirmed by co-elution with authentic samples (20) and by subsequent mass spectrometry (see below). The peaks corresponding to N²-hydroxy-PhIP-N²-glucuronide, N²-hydroxy-PhIP-N³-glucuronide and their trideuterated derivatives were
collected and evaporated to dryness under nitrogen gas. Purity of all synthesized glucuronides was >95%.

Dietary intervention study
The design and conduct of this study with 20 non-smoking Caucasian male subjects has been described in detail elsewhere (29). Phases 1 and 3 consisted of 12-day periods of consumption of their normal diet excluding cruciferous vegetables and other known modifiers of xenobiotic metabolism, such as allulose. During phase 2 the subjects consumed 250 g of either Brussels sprouts or broccoli as a soup at breakfast and 250 g of the other vegetable at dinner for 12 days. The preparation of the Brussels sprouts and broccoli meals has been described previously (29). At the end of each study phase and following an overnight fast the subjects consumed a meal of 275 g of well-cooked lean minced steak shown to contain 4.90 g of caffeine. Urine was collected for 0–10, 10–24 and 24–48 h periods after the cooked meat meal, the weight of each sample recorded and aliquots stored at −80°C, prior to analysis. Unstimulated salivary samples were collected for up to 2 h after the caffeine dose and stored at −80°C prior to analysis of caffeine levels (29).

Analysis of $N^2$-hydroxy-PhIP-$N^2$-glucuronide and $N^2$-hydroxy-PhIP-$N^3$-glucuronide in human urine
$N^2$-Hydroxy-PhIP-$N^2$-glucuronide and $N^2$-hydroxy-PhIP-$N^3$-glucuronide in human urine were determined by gradient HPLC with tandem mass spectrometric detection (LC-MS-MS), using their trideuterated analogues as internal standards. Separation of 20 μl injections of urine was achieved on a 250 × 3.0 mm YMC ODS-A, 120 Å, S-5 μm column (YMC Europe GmbH, Schermbeck, Germany), at a temperature of 40°C and flow rate of 0.2 ml/min, with a binary gradient. Solvent A was water:methanol:acetic acid, 97:2:1 (v/v/v), Solvent B was methanol:water:acetic acid, 95:4:1 (v/v/v) and the gradient was held for 1 min at 5% B, increasing linearly to 25% B at 5 min and then to 100% B at 30 min, held to 35 min before returning to initial conditions over 5 min and equilibrating for a further 20 min. Mass spectrometric detection (Quattro LC, Micromass, Manchester, UK) was by Z-sprayTM positive electrospray ionization and multiple reaction monitoring (MRM) of two molecular weight transitions, m/z 416.80—225.13 for both $N$-hydroxy-PhIP-$N^2$-glucuronides and m/z 419.90—228.01 for both deuterated internal standards, using argon as the collision gas. The dwell time was 1 s, cone voltage 40 V and collision energy 20 V, with desolvation and source temperatures of 175 and 100°C, respectively.

Stock solutions of $N^2$-hydroxy-PhIP-$N^2$-glucuronide, $N^2$-hydroxy-PhIP-$N^3$-glucuronide and their deuterated analogues were prepared, from the synthesized standards, by dissolving in 50% (v/v) methanol:water and determining the concentration from their UV absorption maxima (320 ± 8 nm) in 1 cm cells, using a molar extinction coefficient for PhIP of 19 545 mol/l (30). Urine samples were filtered prior to analysis (Anotop 25 plus, 0.2 μm, Whatman, UK) and 1.0 ml aliquots treated with 25 μl of an internal standard mixture containing 200 nM each of the trideuterated analogues of $N^2$-hydroxy-PhIP-$N^2$-glucuronide and $N^2$-hydroxy-PhIP-$N^3$-glucuronide. These were vortex mixed and centrifuged at 3500 g for 20 min at 4°C before injection of 20 μl aliquots. For each subject and urine collection period the samples for all three study phases were analysed together. Control urine obtained from a long-term male vegetarian (i.e. an individual not exposed to meat-derived heterocyclic amines) was filtered as above and used to prepare calibration standards by spiking 0.975 ml aliquots with 25 μl of standard mixtures containing 40, 160 and 400 nM each of $N^2$-hydroxy-PhIP-$N^2$-glucuronide and $N^2$-hydroxy-PhIP-$N^3$-glucuronide, prior to internal standard addition and centrifugation as above. These were prepared and run in duplicate on each day of analysis, using the same set of standard solutions throughout, to generate calibration curves from the ratios of integrated peak areas of each analyte to its deuterated analogue. These curves were then used to calculate urinary concentrations of each analyte in unknown samples, which multiplied by the urine weights collected, permitted the determination of the total excretion of that analyte in that collection period. The specific signal intensity of $N^2$-hydroxy-PhIP-$N^3$-glucuronide was around an order of magnitude greater than that of $N^2$-hydroxy-PhIP-$N^2$-glucuronide.

Statistical analysis
Urinary PhIP metabolite data were analysed using a two-sided paired t-test.

Results

Method development
Using a previously reported HPLC procedure (21,22), the synthesized samples of $N^2$-hydroxy-PhIP-$N^2$-glucuronide, $N^2$-hydroxy-PhIP-$N^3$-glucuronide and their deuterated derivatives were examined by LC-MS-MS, using positive electrospray ionization and MRM (Figures 2–5). For $N^2$-hydroxy-PhIP-$N^3$-glucuronide and its deuterated derivative, the primary molecular weight transitions (i.e. parent to primary daughter ion)

![Fig. 2. Parent and daughter ion mass spectra for $N^2$-hydroxy-PhIP-$N^2$-glucuronide. Lower trace shows spectrum for daughters of m/z 416.80.](https://academic.oup.com/carcin/article-abstract/25/9/1659/2475976)
were $m/z$ 416.80$\rightarrow$225.13 and $m/z$ 419.94$\rightarrow$228.01, respectively (Figures 3 and 5). The primary molecular weight transitions for $N^2$-hydroxy-PhIP-$N^3$-glucuronide and its deuterated derivative were $m/z$ 416.80$\rightarrow$241.06 and $m/z$ 419.87$\rightarrow$244.07, respectively (Figures 2 and 4). However, while the above MRM functions for $N^2$-hydroxy-PhIP-$N^3$-glucuronide and its trideuterated derivative could be used for the analysis of these compounds, the primary transitions for $N^2$-hydroxy-PhIP-$N^2$-glucuronide and its trideuterated derivative were adversely affected by urinary interference. During method development it was noted that unknown endogenous components of human urine underwent the same molecular weight transition, leading
to interfering peaks in the chromatograms and hence reducing sensitivity for the components of interest. In order to be able to determine N2-hydroxy-PhIP-N3-glucuronide and its deuterated derivative in human urine samples, secondary molecular weight transitions of m/z 416.80—225.07 and m/z 419.87—228.08, respectively, were used (Figures 2 and 4). These transitions comprised the second most intense daughter ion, with an abundance of ~40% of the largest daughter ion. The analysis of the two N-hydroxy-PhIP-N-glucuronides and their trideuterated internal standards could then be performed with just two sets of combined molecular weight transitions, m/z 416.80—225.13 and m/z 419.90—228.01, leading to improved overall sensitivity by use of increased dwell times.

Studies were then performed to develop a method for the analysis of both N-hydroxy-PhIP-N-glucuronides and, if possible, other known PhIP metabolites in human urine.

To help develop and optimize the analytical method, use was made of a concentrated sample of urine from mice treated with high levels of PhIP (21,22). This sample was spiked with deuterated N2-hydroxy-PhIP-N2-glucuronide as internal standard. Employing positive electrospray ionization and MRM, a number of PhIP metabolites were detected in this sample (Figure 6). Based on the observed parent to daughter transitions, the presence of both N2-hydroxy-PhIP-N2-glucuronide and N2-hydroxy-PhIP-N3-glucuronide were positively identified (Figure 6). Other PhIP metabolites tentatively identified in this sample of concentrated mouse urine, using the extra MRM functions shown in Figure 6, were PhIP-N2-glucuronide, PhIP-N3-glucuronide, 4'-hydroxy-PhIP-sulphate and possibly 4'-hydroxy-PhIP-glucuronide.

During the method development a number of urine purification and concentration procedures were investigated. These included a previously reported solid phase urine extraction and concentration scheme for analysis of PhIP metabolites in human urine (21,22). None of these procedures were found to give any better precision or sensitivity over the simple direct injection approach adopted, sensitivity being achieved by the use of a triple quadrupole mass spectrometer instead of an ion trap instrument used in previous studies (21,22).

**Effect of cruciferous vegetable consumption on levels of N2-hydroxy-PhIP-N2-glucuronide and N2-hydroxy-PhIP-N3-glucuronide in human urine**

As reported previously (29) cruciferous vegetable consumption significantly increased the metabolism of caffeine as demonstrated by changes in caffeine kinetics in saliva. Mean ± SEM (n = 20) values for caffeine clearance in saliva were 39.0 ± 3.1, 45.1 ± 3.6 and 39.2 ± 3.5 ml/h/kg for study phases 1, 2 and 3, respectively. Corresponding values for caffeine half-life in saliva were 4.83 ± 0.45, 4.32 ± 0.36 and 4.90 ± 0.46 h, respectively, and for area under the salivary concentration/time curve (AUC0–∞) were 0.722 ± 0.071, 0.619 ± 0.057 and 0.728 ± 0.068 µg/ml/h/kg, respectively. For all parameters of caffeine metabolism, phase 2 values after cruciferous vegetable consumption were significantly different (P < 0.001 for clearance and AUC0–∞; P < 0.05 for half-life) from the phases 1 and 3 washout periods. In contrast, no significant differences in caffeine metabolism were observed between phases 1 and 3.

Levels of N2-hydroxy-PhIP-N2-glucuronide and N2-hydroxy-PhIP-N3-glucuronide were determined in 0–10, 10–24 and 24–48 h urine samples from all subjects during phases 1, 2 and 3 of the cruciferous vegetable intervention study. A typical LC-MS-MS chromatogram is shown in Figure 7. Levels of both compounds were expressed as microgram glucuronide excreted in the total urine sample for each time point (i.e. 0–10, 10–24, 24–28 and 0–48 h).

The urinary excretion of both N2-hydroxy-PhIP-N2-glucuronide (Figure 8) and N2-hydroxy-PhIP-N3-glucuronide...
(Figure 9) was greatest during the first 10 h after ingestion of the cooked meat meal. While levels of $N^2$-hydroxy-PhIP-$N^2$-glucuronide were greater in the 10–24 h than in the 24–48 h urines, levels of $N^2$-hydroxy-PhIP-$N^3$-glucuronide excretion were similar in both the 10–24 and 24–48 h urines. In the washout periods (i.e., phases 1 and 3), the mean urinary 0–48 h excretion of $N^2$-hydroxy-PhIP-$N^2$-glucuronide and $N^2$-hydroxy-PhIP-$N^3$-glucuronide were ~5.0 and 0.5 µg glucuronide/0–48 h urine, respectively. Thus, the excretion of $N^2$-hydroxy-PhIP-$N^2$-glucuronide was some six times greater than the excretion of $N^2$-hydroxy-PhIP-$N^3$-glucuronide.

Compared with the washout periods (i.e., phases 1 and 3), treatment with cruciferous vegetables significantly increased the urinary excretion of $N^2$-hydroxy-PhIP-$N^2$-glucuronide in 0–48 h urine samples (Figure 8). Mean levels of $N^2$-hydroxy-PhIP-$N^2$-glucuronide in phase 2 (0–48 h) urine samples were increased to 127 and 136% of those in phases 1 and 3 (0–48 h) urine samples, respectively. Levels of $N^2$-hydroxy-PhIP-$N^3$-glucuronide were also significantly increased in the 0–10 h phase 2 urine samples compared with either phases 1 or 3 (0–10 h) urine samples. The excretion $N^2$-hydroxy-PhIP-$N^2$-glucuronide was also

![Fig. 6. Analysis of PhIP metabolites in a concentrated sample of urine from a PhIP treated mouse. Chromatogram A is the MRM of m/z 419.87→244.07 detecting [1H$_3$] $N^2$-hydroxy-PhIP-$N^2$-glucuronide. Chromatogram B is the MRM of m/z 416.80→241.06 detecting $N^2$-hydroxy-PhIP-$N^2$-glucuronide. Chromatogram C is the MRM of m/z 416.80→225.13 detecting $N^2$-hydroxy-PhIP-$N^3$-glucuronide. Chromatogram D is the MRM of m/z 401.00→225.00 detecting PhIP-$N$-glucuronides with no hydroxy groups. Chromatogram E is the MRM of m/z 321.00→241.00 detecting 4′-hydroxy-PhIP-sulphate. For chromatograms D and E, m/z were calculated as no samples of the authentic compounds were available. With the MRM functions employed, peaks present in both chromatograms B and C were tentatively assigned to 4′-hydroxy-PhIP glucuronide.]
significantly increased in the 10–24 h phase 2 urine samples compared with phase 3, but not phase 1, urine samples. In contrast, no statistically significant differences were observed in $N^2$-hydroxy-PhIP-$N^2$-glucuronide excretion in 24–48 h phase 2 urine samples, compared with either phase 1 or 3 urine samples (Figure 8). From the data shown in Figure 8, the half-life for $N^2$-hydroxy-PhIP-$N^2$-glucuronide excretion in human urine was calculated to be $\sim 14.5$ h and this did not appear to be affected by cruciferous vegetable consumption.

While the urinary excretion of $N^2$-hydroxy-PhIP-$N^2$-glucuronide was significantly affected by cruciferous vegetable consumption, no statistically significant differences were observed in the urinary excretion of $N^2$-hydroxy-PhIP-$N^3$-glucuronide in 0–10, 10–24, 24–48 and 0–48 h urine samples (Figure 9). Moreover, the urinary excretion of both
Discussion

There is considerable interest in the possibility of chemoprevention, the ability to reduce cancer risk by the prophylactic consumption of compounds or foodstuffs on a regular basis. The observation in the majority of studies performed that consumption of a diet rich in cruciferous vegetables reduces cancer risk (26,27), has provided the impetus for developing an effective chemoprevention strategy involving these foods. However, a serious limitation at present is the lack of knowledge of the mechanisms involved. One possibility, for which there is some evidence (26,31-39), is the induction of detoxification enzymes involved in the elimination of genotoxic carcinogens present in the diet. Amongst common diet-associated cancers, colon cancer is linked to the consumption of red meat. There is some evidence that this might, in part, be due to the formation of carcinogenic compounds such as PhIP during the cooking process. Hence, PhIP (administered as a PhIP-containing cooked meat meal) can serve as a suitable model compound with which to investigate the effects of cruciferous vegetable consumption on the disposition of a dietary genotoxin in human volunteers.

PhIP and other food-derived carcinogenic heterocyclic amines are metabolized by both phase I and II xenobiotic metabolizing enzymes. The major route of phase I metabolism of PhIP in humans is the formation of N₂-hydroxy-PhIP, which is primarily catalysed by hepatic CYP1A2 and to a lesser extent by CYP1A1 and CYP1B1 in extrahepatic tissues (8,10,12-15). Both PhIP and N₂-hydroxy-PhIP can be glucuronidated by human hepatic UGT forms in the presence of UDPGA at both the N₂- and N³-positions (17-19). Investigations with cDNA-expressed human UGT forms have shown that PhIP was metabolized to a variety of products. The major urinary metabolite was N₂-hydroxy-PhIP-α-glucuronide, together with smaller amounts of N₂-hydroxy-PhIP-β-glucuronide, PhIP-glucuronides, 4′-hydroxy-PhIP-sulphate

N₂-hydroxy-PhIP-N₂-glucuronide and N₂-hydroxy-PhIP-N³-glucuronide were not statistically significantly different between the two washout periods (i.e. phases 1 and 3) in 0-10, 10-24, 24-48 and 0-48 h urine samples (Figures 8 and 9).

For all subjects the mean ± SEM (n = 20) combined excretion of N₂-hydroxy-PhIP-N₂-glucuronide and N₂-hydroxy-PhIP-N³-glucuronide in the 0-48 h urines from phases 1, 2 and 3 of the study were 3.60 ± 0.18, 4.46 ± 0.24 and 3.40 ± 0.24 μg, respectively. The values for excretion of both N₂-hydroxy-PhIP-N₂-glucuronides of 3.50 (mean of phases 1 and 3) and 4.46 (phase 2) μg glucuronides per 0-48 h urine, are equivalent to 1.89 and 2.40 μg of PhIP per 0-48 h urine, respectively. In this study each subject received 4.90 μg of PhIP in the cooked meat meal at the end of each phase (29). Hence the formation of both N₂-hydroxy-PhIP-N₂-glucuronides accounted for ~39% of the dose of PhIP administered in phases 1 and 3 of the study and for ~49% of the dose in phase 2.

Urinary levels of N₂-hydroxy-PhIP-N₂-glucuronide were compared with the previously reported (29) data for levels of unchanged PhIP and saliva caffeine kinetic parameters (clearance, half-life and AUC₀₋₄₈) using correlation analysis. Data were analysed for each study phase separately and for all three study phases combined. Overall, no significant correlations were observed between levels of N₂-hydroxy-PhIP-N₂-glucuronide and the other parameters examined. For example, for all three study phases combined, urinary levels of N₂-hydroxy-PhIP-N₂-glucuronide showed no correlation with either levels of unchanged PhIP (r² = 0.067) or with saliva caffeine kinetics (r² values with clearance, half-life and AUC₀₋₄₈ all <0.006). In contrast, good correlations (r² = 0.643-0.945) were found between the parameters of caffeine saliva kinetics studied.

Discussion

There is considerable interest in the possibility of chemoprevention, the ability to reduce cancer risk by the prophylactic consumption of compounds or foodstuffs on a regular basis. The observation in the majority of studies performed that consumption of a diet rich in cruciferous vegetables reduces cancer risk (26,27), has provided the impetus for developing an effective chemoprevention strategy involving these foods. However, a serious limitation at present is the lack of knowledge of the mechanisms involved. One possibility, for which there is some evidence (26,31-39), is the induction of detoxification enzymes involved in the elimination of genotoxic carcinogens present in the diet. Amongst common diet-associated cancers, colon cancer is linked to the consumption of red meat. There is some evidence that this might, in part, be due to the formation of carcinogenic compounds such as PhIP during the cooking process. Hence, PhIP (administered as a PhIP-containing cooked meat meal) can serve as a suitable model compound with which to investigate the effects of cruciferous vegetable consumption on the disposition of a dietary genotoxin in human volunteers.

PhIP and other food-derived carcinogenic heterocyclic amines are metabolized by both phase I and II xenobiotic metabolizing enzymes. The major route of phase I metabolism of PhIP in humans is the formation of N₂-hydroxy-PhIP-N₂-glucuronide, which is primarily catalysed by hepatic CYP1A2 and to a lesser extent by CYP1A1 and CYP1B1 in extrahepatic tissues (8,10,12-15). Both PhIP and N₂-hydroxy-PhIP can be glucuronidated by human hepatic UGT forms in the presence of UDPGA at both the N₂- and N³-positions (17-19). Investigations with cDNA-expressed human UGT forms have shown that UGT1A1, UGT1A4 and UGT1A9, but not UGT1A6, catalyse the glucuronidation of both PhIP and N₂-hydroxy-PhIP (19).

In a study where [2-¹⁴C]PhIP was administered orally to human volunteers, Malfatti and coworkers (20) demonstrated that PhIP was metabolized to a variety of products. The major urinary metabolite was N₂-hydroxy-PhIP-N₂-glucuronide, together with smaller amounts of N₂-hydroxy-PhIP-N³-glucuronide, PhIP-glucuronides, 4′-hydroxy-PhIP-sulphate
and other unknown metabolites. Two subsequent volunteer studies were performed, where PhIP was administered to human volunteers as a well-done chicken meal (21,22). In both studies four PhIP metabolites were quantified by LC-MS-MS. The major metabolite of the four PhIP metabolites determined was N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide, followed by PhIP-N\textsuperscript{2}-glucuronide, with smaller quantities of N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide and 4'-hydroxy-PhIP-sulphate being formed (21,22).

The aim of the present study was to evaluate the effect of cruciferous vegetable consumption on the metabolism of PhIP in healthy non-smoking Caucasian male volunteers. We demonstrated previously (29) that cruciferous vegetable consumption increased saliva caffeine clearance, which is an accepted measure of hepatic CYP1A2 activity (40). Saliva caffeine clearance was increased by 15% in phase 2 compared with the mean of the phase 1 and 3 values, whereas saliva half-life and AUC\textsubscript{0}–\textsubscript{∞} of these results are in agreement with other studies where cruciferous vegetable consumption has been reported to induce hepatic CYP1A2 activity (26,33–36). In most of these studies, the effect on caffeine metabolism was assessed by determination of caffeine urinary metabolite ratios, with a reduction in plasma caffeine half-life being reported in one study (33).

The change in caffeine elimination following cruciferous vegetable consumption observed in our previous study (29) was accompanied by a decrease in the amount of unchanged PhIP excreted in the urine, most likely a consequence of increased hepatic CYP1A2-catalysed N\textsuperscript{2}-hydroxylation (14). The present study was therefore undertaken to determine the effect of cruciferous vegetable consumption on the further metabolism of N\textsuperscript{2}-hydroxy-PhIP. For this purpose an analytical procedure was devised to quantify PhIP metabolites in human urine. Particular attention was paid to N\textsuperscript{2}-hydroxy-PhIP-glucuronides as these represent detoxification pathways of N\textsuperscript{2}-hydroxy-PhIP (Figure 1), with N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide being known to be the major metabolite of PhIP in human urine (20–22). Examination of urine samples after ingestion of the cooked meat meals revealed a number of PhIP metabolites. With the availability of synthesized trideuterated standards, levels of both N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide and N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide could be quantified in 0–48 h urine samples. Moreover, both compounds could be analysed directly in human urine samples, without the need for urine extraction and concentration procedures employed prior to LC-MS-MS analysis in other studies (21,22).

From the urinary profiles obtained, it is evident that excretion of both N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide and N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{2}-glucuronide is essentially complete within 48 h after ingestion of a dose of PhIP. In the two washout periods, levels of N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide in 0–48 h urine samples were some six times those of N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide. The observation that N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide is a more abundant metabolite of PhIP than N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide is in agreement with previous studies (20–22), with a very similar ratio being reported for the 24 h excretion of these two PhIP metabolites by three subjects given an oral dose of [2\textsuperscript{14}C]PhIP (20).

In this study, cruciferous vegetable consumption was found to significantly increase the urinary excretion of N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide by ~32% compared with the mean of study phases 1 and 3, but had no effect on the urinary excretion of N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide. While the excretion of both PhIP metabolites accounted for ~39% of the PhIP dose in phases 1 and 3, they accounted for ~49% of the PhIP dose in phase 2 after treatment with cruciferous vegetables.

Apart from inducing CYP-dependent enzyme activities, cruciferous vegetables are known to induce phase II xenobiotic metabolizing enzymes in humans. For example, cruciferous vegetable consumption has been reported to increase GSH S-transferase activities and also the glucronidation of paracetamol (26,32,37–39). The N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide PhIP metabolite is derived from CYP1A2-catalysed N\textsuperscript{2}-hydroxylation and subsequent UGT-catalysed glucuronidation (Figure 1). From the urinary excretion data, the half-life for N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide excretion was estimated to be ~14.5 h and this did not appear to be affected by cruciferous vegetable consumption. Given that the half-life of the parent amine is <5 h, due largely to CYP1A2-dependent metabolism (14), this suggests that the elimination of the N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide is not formation limited. Thus, the increase in the formation of this glucuronide following consumption of cruciferous vegetables is most likely a consequence of induction of phase II metabolism, consistent with previous reports that such diets can induce phase II xenobiotic metabolizing enzymes in humans (26,32,37–39). Although cruciferous vegetable consumption increased urinary levels of N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide, there was no change in the amount of N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide formed. This is presumably attributable to these two N\textsuperscript{2}-hydroxy-PhIP metabolites being formed by different UGT enzymes, which is consistent with the differential activities reported for formation of these two N\textsuperscript{2}-glucuronides by human UGT1A1, UGT1A4 and UGT1A9 (19). If the increased excretion of the N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide were merely due to increased formation of N\textsuperscript{2}-hydroxy-PhIP by hepatic CYP1A2, then a similar increase in the formation of the N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide would have been expected. Moreover, no correlations were observed between the urinary excretion of N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide and CYP1A2 activity, as determined by saliva caffeine kinetics. Rather these results are most readily interpreted as the induction of the N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide pathway, such that there is competition for the uninduced N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide pathway. It has been reported that UGT1A1 and UGT1A9 are differentially induced via the constitutive androstane receptor and the aryl hydrocarbon receptor, respectively (41). Recent studies have also implicated the aryl hydrocarbon receptor in the induction of UGT1A1 (42), whereas there is little information on the inducibility of UGT1A4. Hence, the specificity and mechanisms of induction of these UGTs by constituents of cruciferous vegetables remains to be determined. A possible alternative explanation for the current data would be that the UGT forms catalysing N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide formation have a higher affinity for N\textsuperscript{2}-hydroxy-PhIP than the UGT forms catalysing N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide formation.

In summary, this study demonstrates that consumption of cruciferous vegetables modulates the metabolism of PhIP in humans. Although cruciferous vegetables induce hepatic CYP1A2, the detoxification of the pro-carcinogenic N\textsuperscript{2}-hydroxy-PhIP formed is enhanced by the increased formation of N\textsuperscript{2}-hydroxy-PhIP-N\textsuperscript{3}-glucuronide, which may result in an overall reduction in the formation of genotoxic metabolites of PhIP. While additional studies are required to elucidate the effect of cruciferous vegetable consumption on other activation and deactivation pathways of the metabolism of PhIP and

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other carcinogenic heterocyclic amines in humans, these data provide a possible rationale, at least in part, for the anticancer effects of such diets.

Acknowledgement

The authors are grateful to the UK Food Standards Agency (Contract Number T01009) for financial support of these studies.

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Received January 12, 2004; revised March 8, 2004; accepted April 3, 2004.