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The effects of coffee on enzymes involved in metabolism of the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine in rats

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Abstract

The effects of coffee on the metabolism and genotoxicity of the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) were investigated. Coffee diminished the bacterial mutagenicity of PhIP in the Ames reversion assay through inhibition of cytochrome P450 1A2 (CYP1A2), a key enzyme involved in the metabolic activation of PhIP. When given as part of the diet (0, 1 or 5% w/w) to male Fischer-344 rats for 2 weeks, coffee affected the expression of hepatic enzymes involved in PhIP metabolism. Coffee increased the expression of CYP1A2 by 16-fold in the 5% coffee-treated group, and approximately half of this inductive effect was attributed to caffeine. Coffee also increased the expression of enzymes involved in the detoxication of PhIP. A 2-fold increase in expression of glutathione *S*-transferase alpha was observed, UDP-glucuronosyl transferase (UGTs) activities of *p*-nitrophenol increased 2-fold, while N^2-and *N*3-glucuronidation of the genotoxic metabolite 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (HONH-PhIP) increased by 1.3-fold in the 5% coffee-treated over the control group. The amount of PhIP (0.75 mg/kg, 24 h) eliminated in urine as the N^2-and *N*3-glucuronide conjugates of HONH-PhIP increased by 1.8- and 2.5-fold, respectively, in the 5% coffee-treated group over control rats, suggesting either increased rates of *N*-oxidation of PhIP or *N*-glucuronidation of HONH-PhIP. Despite the strong induction of CYP1A2, there was no increase in PhIP-DNA adduct formation in colon and pancreas while liver adducts decreased by 50% over control animals. These data suggest that the effect of coffee on inhibition of PhIP *N*-oxidation and ensuing DNA damage is more important in vivo than its effect on induction of PhIP *N*-hydroxylation.

Keywords: Heterocyclic aromatic amines; Coffee; Caffeine; Metabolism; Chemoprotection

1. Introduction

There has been considerable interest on the beneficial effects that antioxidants in foods, bev-

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erages, and ingredients may have on human health [1]. Because of the occurrence of numerous naturally occurring polyphenolic antioxidants in tea and coffee, these beverages have attracted attention as a possible safe means to protect against reactive oxygen species and ensuing oxidative damage that may lead to development of diseases including cancer [2]. Moreover, constituents in these beverages may also exert chemoprotective effects against genotoxins through modulation of phase I and phase II enzymes that influence toxicity and carcinogenic potency [1,3,4]. A number of foods and beverages contain isothiocyanates, polyphenols, diterpenes, and other components that have been reported to induce glutathione *S*-transferases (GSTs), enzymes that detoxify a wide range of dietary and environmental contaminants [3–7]. Some of these dietary constituents also may inhibit phase I enzymes involved in the activation of chemicals to genotoxins [1]. However, other dietary constituents may induce the expression of phase I enzymes, which could enhance the genotoxic potential of procarcinogens. For example, consumption of grilled meats has been reported to increase the metabolism of some xenobiotics [8] through the increased expression of cytochrome P450 1A2 (CYP1A2) [9], a key enzyme involved in the metabolic activation of carcinogenic aromatic amines and heterocyclic aromatic amines (HAAs) [10,11]. Caffeine, an important constituent of coffee and tea [12], also has been reported to induce CYP1A2 levels in experimental animals [13–15], and concerns have been raised about the possible co-carcinogenic effects of this alkaloid with chemicals that are activated by CYP1A2. Indeed, co-administration of caffeine with the HAA 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) led to increased levels of colon tumors in rats [16]. However, there are also components in coffee, including caffeine, melanoidins, and phenolics, that inhibit the metabolic activation of PhIP and other HAAs to bacterial mutagens [17–19]. Potent antioxidants in coffee also abolish the genotoxicity of radicals derived from *t*-butylhydroperoxide [20].

Several studies have shown that green tea, black tea, and polyphenolic constituents provide protection against chemically- and radiation-induced cancer in animal models [21–23]. Coffee and its constituents also display strong antioxidant and chemoprotective effects in vitro and in experimental animal models [19,20,24]. Because of the wide popularity of coffee, numerous studies have been conducted on the safety of this beverage [12]. Several long-term animal carcinogen bioassays have been conducted in mice and rats receiving coffee at amounts in far excess to human consumption and have shown no evidence for increase in tumorigenesis [25,26]. In fact, coffee resulted in a significant reduction of spontaneous tumors at multiple sites in both species. In humans, the vast amount of data from epidemiological studies reveals little evidence for the increase in cancer associated with coffee consumption at the major organ sites [27], and there is much epidemiological data showing that coffee may exert chemoprotective effects against colo-rectal cancer [28–30] (and references within).

The significance of the anti- and pro-mutagenic effects of coffee on human health risk/benefit is uncertain. Because of the potent inhibition of HAAs mutagenicity by coffee in bacterial studies, we speculated as to whether the protective effects of coffee against colo-rectal cancer reported in epidemiology studies could be attributed to anti-mutagenic effects of coffee against carcinogenic HAAs such as PhIP, which may contribute to the development of human colo-rectal cancer [31,32].

PhIP is structurally representative of a series of HAAs formed in grilled meats, fish, and poultry [31]. The metabolic activation of PhIP by rodents and humans occurs principally by CYP1A2 mediated *N*-oxidation of the exocyclic amine group to form HONH-PhIP, which then may undergo further esterification reactions by phase II enzymes to produce reactive species that bind to DNA [33]. In this study, we investigated the anti-mutagenic effects of coffee against PhIP in bacterial mutagenesis assays and the potential protective effects of coffee in vivo in rats through modulation of enzymes involved in PhIP metabolism and DNA adduct formation. The urinary N^2-and $N3$-glucuronide conjugates of the genotoxic metabolite, HONH-PhIP, and PhIP-DNA adduct formation were used as biomarkers of *N*-oxidation

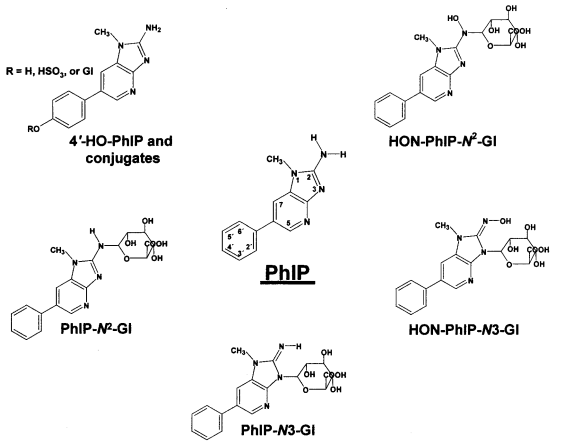

Fig. 1. Chemical structures of PhIP and urinary metabolites.

activity in vivo (Fig. 1) and were correlated to the modulation of several enzymes involved in the activation and detoxication of this dietary carcinogen. These investigations also enabled us to assess whether the co-genotoxic effects of caffeine observed with PhIP in colon of rats [16] also occurs by coffee treatment using PhIP-DNA adduct formation as an index of genotoxic damage.

2. Materials and methods

2.1. Chemicals

PhIP and [2-^{14}C]PhIP (10 mCi/mmol) were purchased from Toronto Research Chemicals (Downsview, Ontario, Canada). Resorufin was purchased from Pierce (Rockford, IL) and 7-methoxyresorufin was purchased from Molecular Probes Europe BV (Leiden, Holland). Caffeine, UDPGA, *p*-nitrophenol, acetic anhydride, 2-aminofluroene (2-AF), 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) were obtained from Aldrich (Buchs, Switzerland). HONH-PhIP was synthesized as previously reported [34], and 2-amino-4′hydroxyl-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (4′HO-PhIP) was prepared biosynthetically from liver microsomes of rats pretreated with polychlorinated biphenyl (PCB) [11]. The 4′sulfate, 4′glucuronide conjugates of 4′HO-PhIP, and the *N*-and *N*3-glucuronide conjugates of PhIP and HONH-PhIP were prepared as previously reported [11,35]. Rat liver S-9 from animals pretreated with PCBs was purchased from Molecular Toxicology, Inc. (Boone, NC). Pooled human liver microsomes, rat liver microsomes from untreated and PCB-pretreated rats were purchased from Xenotech, LLC, Lenexa, Kansas. 2-Acetamidofluorene was prepared by reaction of 2-AF (1 mg) in 0.9 ml of pyridine with 0.1 ml of acetic anhydride and heating at 70 °C for 1 h. The identity of the structure was confirmed by mass spectrometry. Tritium labeled AF was kindly provided by Dr F. Kadlubar, NCTR, AR and diluted with unlabeled AF to a final specific activity of 100 mCi/mmol.

2.2. Animals and treatment

Male Fischer-344 rats were purchased from Iffa Credo (L'Abresle, France) and allowed to acclimatized 5–7 days in standard cages. Animals were provided rat chow Nalfag 890 (Nähr-und Futtermittel AG; Gossau, Switzerland) and water ad libitum. A 'home-brew' coffee extract was prepared by roasting a blend of Guatemala (Arabica): Togo (Robusta) (50:50), under typical conditions that are used for the preparation of commercial instant coffee. The roasted grounds were then diluted with water (15 volumes of water per gram coffee), and filtered through commercial coffee filter paper to remove particulates. This filtration procedure effectively eliminated 99% of the cafestol and kawheol to levels that are present in commercial drip-filtered and instant coffees [36]. Thus, the amounts of these diterpenoids remaining in coffee could not contribute to the induction of GSTs [5,6,37]. The coffee extract was then lyophilized. The caffeine content was estimated at 6.72 g/100 g powder (personal communication, Dr R. Liardon, Labior, Nestec Ltd). The freeze-dried powder was mixed with the rat chow powder (0, 1 or 5% w/w), which was dissolved in water and further mixed. The material was then pelleted and dried for 24 h at 70 °C, followed by pasteurization for 30 mm at 90 °C.

Animals of 8 weeks of age ($N = 4$ or 5 per group) were given rat chow that was premixed

with the lyophilized coffee (0, 1, or 5% w/w) or comparable amounts of caffeine (0.068 and 0.34% caffeine as part of the diet) and water ad libitum for 2 weeks. After 11 days of treatment, animals were placed in metabolic cages for adaptation for 3 days. Initially, the animals consuming the rat chow containing the highest coffee dose (5% w/w) ate less food but after several days of adaptation food consumption was comparable to the control diet. At the end of the 2-week feeding period, animal body weights in the high coffee treatment were 10% lower than in the untreated and 1% treatment group, although the growth rate gains after the initial 3 days were identical in all groups. The percent liver weight/total body weight were 4.10 ± 0.21, 4.26 ± 0.15 and 4.41 ± 0.20, respectively for the 0, 1 and 5% coffee-treated groups, and not significantly different (1-way ANOVA, $P = 0.12$).

On day 14, animals were dosed by gavage with a 1 ml solution of 2-^{14}C-PhIP (10 mCi/mmol, 0.75 mg/kg body weight, 8.4 μCi/per animal) in phosphate buffered saline (pH 7.4). The animals were sacrificed 24 h after treatment by treatment with pentobarbital (60 mg/kg, i.p. injection) followed by bleeding of the abdominal artery. Urine and feces were collected during this 24-h period for radioactive mass balance studies. The urine receptacle contained 2 ml of isopropanol as a preservative. Radioactivity in urine and feces was determined by liquid scintillation counting as previously described [38].

2.3. Metabolites and analysis

Metabolites were analyzed by HPLC with a Hewlett-Packard 1090M HPLC and photodiode array detector system. The analysis of PhIP metabolites was conducted with a Supelco C18 dB column (25 cm × 4.6 mm i.d., 5-μm particle size). The A buffer was 20 mM diethylamine acidified to pH 5.0 with acetic acid and the B eluent was methanol [39]. The flow rate was 1 ml/min using a gradient of 20–70% B over 40 min, followed by an increase to 100% B at 50 mm. Product formation was determined by radioactive measurements on-line with a Berthold LB 506 C-1 radioactivity monitor.

2.4. Mutation tests

Mutagenicity was assessed in the Ames reversion assay with tester strain TA98, which was kindly provided by Prof. B.N. Ames, University of California, Berkeley, using a preincubation assay at 37 °C for 30 min [11]. Varying concentrations of PhIP and HONH-PhIP were assessed for activity in the presence of either coffee or caffeine as described in the respective figure legends.

2.5. Immunoquantitation of CYP1A1, CYP1A2 and glutathione S-transferases

Liver microsomes and cytosolic fractions were prepared by differential centrifugation as previously reported [34] and stored in 10 mM Tris–HCl buffer (pH 7.4) containing 0.1 mM EDTA and 20% glycerol (v/v). Protein content was estimated with Coomassie Brilliant Blue G reagent (Pierce, Rockford, IL) using bovine serum albumin as a reference standard. Immunoquantitation of rat liver microsomal CYP1A1 and CYP1A2 was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 25–50 μg of microsomal protein) [11] and precasted Novex 10 or 12% Tris–glycine gels (San Diego, CA). Proteins were visualized by enhanced chemiluminescent detection as described by Amersham specifications using polyclonal goat anti-rabbit immunoglobulin G:horseradish peroxidase complex as the secondary antibody (10 000-fold dilution). Liver microsomes from 3-MC-pretreated rats were purchased from Gentest (Worcester, MA), and the amount of CYP1A1 was provided by the supplier. Purified rat CYP1A2 was kindly provided by Prof. F.P. Guengerich, Vanderbilt University School of Medicine. Tris–glycine gels from Novex (4–20%) were used for analysis of GSTs. Cytosolic protein (600 ng) was assayed with antisera of rabbit anti GST-yc or GST-ya (Biotrin International, Dublin, Ireland) and diluted 1000-fold. Anti-rabbit immunoglobulin G:horseradish peroxidase complex was used as the secondary antibody (10 000-fold dilution).

2.6. Enzyme assays

Glucuronidation assays were conducted with rat liver microsomes of untreated and coffee-treated rats. The *O*-glucuronidation of *p*-nitrophenol was measured by the decrease in absorbance at 405 nm as previously reported, except that the substrate concentration was 1 mM [40]. The glucuronidation of [2-^{14}C]HONH-PhIP (1 mCi/mmol) was done as described by Nowell et al. [41] using 1 mg of microsomal protein and alamethicin (60 μg/mg protein), except that saccharolactone was not employed in the assay. Analyses were conducted by HPLC employing the conditions as described above with an on-line radioactive monitor and UV diode array detector, which permitted the quantitation and identification of both isomeric N^2- and $N3$-glucuronide conjugates of HONH-PhIP. The rates of *N*-acetylation of AF were determined as previously reported [34] and product formation measured by HPLC on-line with a radioactive monitor. Methoxyresorufin *O*-demethylase activity was conducted with rat and human liver microsomes as previously reported [11]. Inhibition studies were done by precincubating microsomes containing 1 mM NADPH with varying amounts of coffee for 3 min prior to the addition of methoxyresorufin.

2.7. Accelerator mass spectrometry analysis

Liver, colon and pancreas DNA were purified using Qiagen DNA extraction columns, as previously described [42]. The DNA was purified a second time with the columns to remove any unbound [^{14}C]-PhIP and metabolites. The DNA pellets were then washed with 70% ethanol and reconstituted in ultrapure water. The concentration of DNA was calculated by measuring the absorbance at 260 nm, assuming an absorbance value of 1.0 is equal to a DNA concentration of 50 μg/ml. DNA purity was determined from the A260 nm/A280 nm ratio and all DNA used in this study had a ratio of 1.6–1.8. The carbon-14/carbon-13 ratios of purified DNA samples (84–655 μg aliquots) was determined by AMS, as previously described [43,44]. Prior to analysis, 2 mg of tributyrin was added to each DNA sample to provide the carbon content necessary for efficient graphitization. The carbon-14/carbon-13 ratios were converted to DNA adduct levels following the subtraction of the carbon-14 contribution from any added tributyrin and control samples.

2.8. Statistical analysis

The data (0, 1 and 5% coffee-treated groups) were analyzed with a one-way ANOVA followed by Dunnett's or Bonferroni's multiple comparison test using GRAPHPAD PRISM™ software package (San Diego, CA).

3. Results

There was a concentration-dependent decrease in bacterial mutagenicity when PhIP (5 μg) was incubated with varying amounts of lyophilized, filtered coffee in the presence of liver S-9 of rats pretreated with PCBs (Fig. 2A). Caffeine inhibited some of the mutagenicity of PhIP, probably through competitive reactions with CYP1A2 (Fig. 2B) [45]. However, the amount of caffeine present in the roasted coffee extract (6.7% w/w) only accounts for a minor portion of this effect. The mutagenic activity of HONH-PhIP, a direct-acting mutagen, was not decreased to an appreciable extent in the presence of coffee when liver S-9 was omitted (Fig. 2A), indicating that coffee constituents do not react with HONH-PhIP, compete with mammalian or bacterial mediated esterification of HONH-PhIP, or scavenge reactive esterified metabolites of HONH-PhIP. Preincubation of liver microsomes of rats (untreated and PCB-pretreated) or pooled human liver microsomes with coffee resulted in a concentration-dependent decrease in the MROD activity (Fig. 3), which is carried out principally by CYP1A2 [11]. The efficacy of coffee to inhibit MROD was similar in all 3 microsomal preparations even though there is more than a 100-fold difference in the expression of CYP1A2 in these microsomal samples [11], suggesting that some of the protective effects of coffee against PhIP mutagenesis in vitro are through non-competitive inhibition of the CYP1A2-mediated *N*-oxidation.

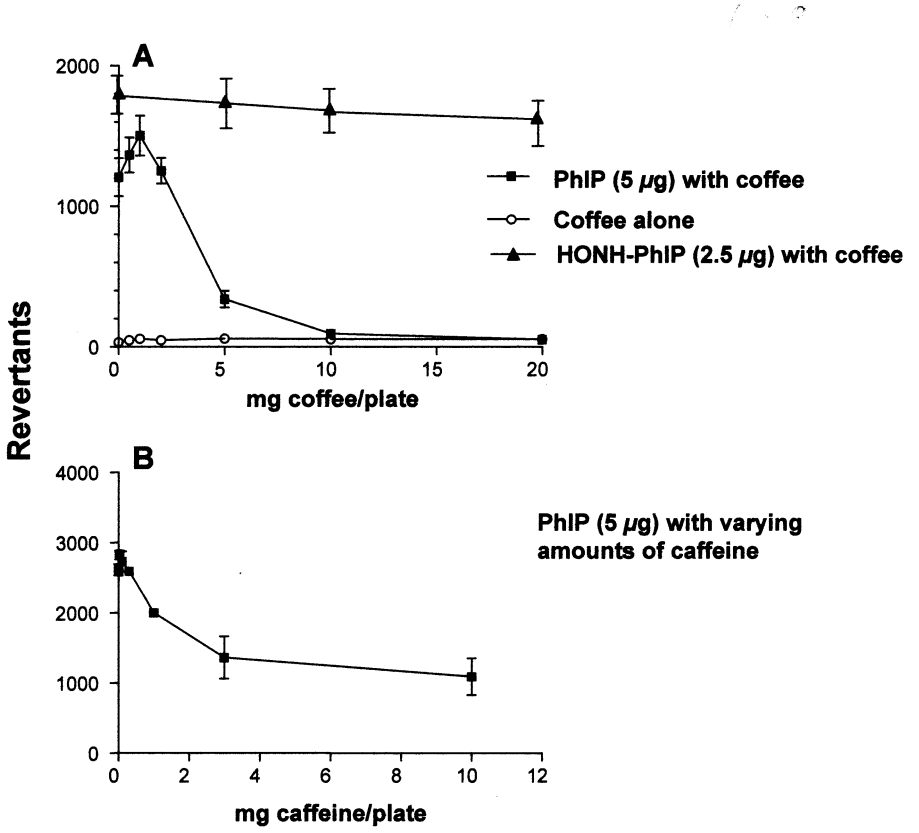

Fig. 2. (A) Mutagenicity of coffee, PhIP (5 μg) in the presence or absence of coffee with liver S-9 homogenate from rats pre-treated with PCBs; HONH-PhIP (2.5 μg) in the presence of coffee; and coffee alone. (B) The inhibitory effect of caffeine towards PhIP in the presence of S-9 homogenate from rats pre-treated with PCBs. The amount of caffeine assayed was in great excess to that present in coffee (6.7% w/w).

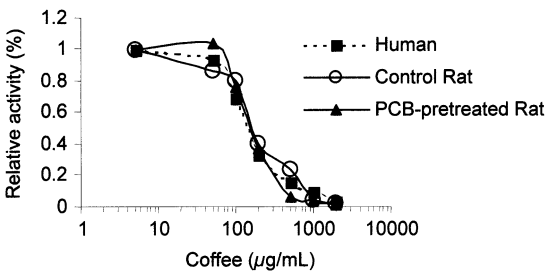

Fig. 3. The inhibitory effect of coffee towards MROD activity with control, PCB-pretreated rat liver microsomes, and a pooled ($N = 10$) human liver microsome sample. Coffee was preincubated at 37 °C for 3 min with microsomal protein and 1 mM NADPH prior to addition of methoxyresorufin (2.5 μM). Incubations were then carried out for 2 min.

Studies on the chemoprotective effects of coffee were extended to investigations in rats to determine whether coffee may modulate enzyme activities involved in PhIP metabolism in vivo and alter the levels of DNA adduct formation. The influence of coffee on the expression of phase I and II hepatic enzymes involved in PhIP metabolism was assessed in rats given coffee as part of the diet (0, 1, or 5% w/w) for 2 weeks prior to exposure to 2-[^{14}C]-PhIP.

Oxidation of PhIP is carried out primarily by CYP1A1 and CYP1A2 [11,33]. We observed that coffee treatment resulted in a strong, concentration-dependent induction of CYP1A2 in liver (Fig. 4). In contrast to CYP1A2, the induction of hepatic CYP1A1 was weak and only detected in

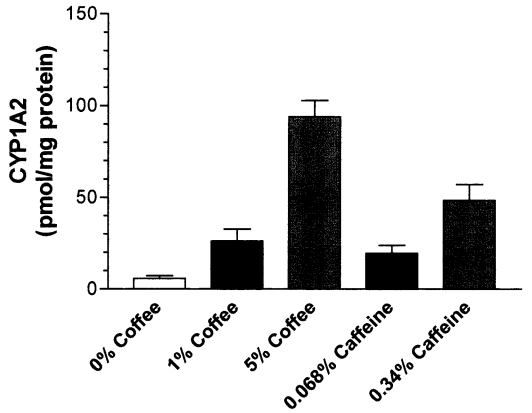

Fig. 4. The effect of coffee and caffeine on the induction of CYP1A2 in rat liver. The amount of caffeine (0.068 and 0.34%) given to rats was equivalent to the amount present in the 1 and 5% coffee-treated animals. ANOVA $P < 0.0001$; Dunnett's test, $P < 0.01$ for 0% vs. all treatment groups.

rats treated with 5% coffee and the protein level was vastly lower than CYP1A2 (1.4 ± 0.4 pmol CYP1A1/mg microsomal protein vs. 94 ± 9 pmol CYP1A2/mg microsomal protein). Caffeine accounted for approximately half of the inductive effect of CYP1A2 observed with coffee, whereas CYP1A1 expression (< 0.5 pmol/mg protein) was not detected in the caffeine-treated groups.

Enzyme induction of CYP1A2 as a function of coffee and caffeine treatment was assessed in vitro by measuring hepatic microsomal ring- and *N*-oxidation of PhIP, and MROD activities. In the highest treatment group with coffee (5%) where CYP1A2 protein expression increased by 16-fold (Fig. 3), MROD activity was increased by 11-fold while PhIP-*N*-oxidation and PhIP-C4′ oxidation rates increased by 3.5-fold over the control rats when expressed as nmol product formed/mg protein/mm (Table 1). However, when the oxidation rates were expressed as nmol product formed/nmol CYP1A2/min, the rates of HONH-PhIP and 4′HO-PhIP formation in the 5% coffee-treated group were decreased to approximately 22% of the control group (0% coffee), whereas the MROD activity was not significantly decreased from the control group ($P > 0.05$). Liver microsomes of rats pretreated with caffeine also displayed diminished PhIP-mediated oxidation activities when expressed as nmol product formed/nmol CYP1A2/min,

Table 1
Liver microsomal CYP1A2-mediated oxidation activity as a function of coffee and caffeine treatment

	4′HO-PhIP		HONH-PhIP		MROD	
Coffee (%)	nmol/min/mg protein[a,b]	nmol/min/nmol CYP1A2[a,b]	nmol/min/mg protein[a]	nmol/min/nmol CYP1A2[a,b]	nmol/min/mg protein[b]	nmol/min/nmol CYP1A2
0	0.09 ± 0.01	15.5 ± 2.43	0.23 ± 0.01	39.6 ± 2.4	0.05 ± 0.02	9.22 ± 3.97
1	0.15 ± 0.01	5.73 ± 0.54	0.36 ± 0.06	14.5 ± 2.16	0.13 ± 0.06	5.77 ± 1.61
5	0.31 ± 0.07	3.30 ± 0.75	0.84 ± 0.00	8.94 ± 0.00	0.54 ± 0.13	5.70 ± 1.38
Caffeine (%)	nmol/min/mg protein[c]	nmol/min/nmol CYP1A2[d,e]	nmol/min/mg protein[b]	nmol/min/nmol CYP1A2[d,e]	nmol/min/mg protein[b]	nmol/min/nmol CYP1A2
0.07	0.15 ± 0.06	8.33 ± 3.33	0.36 ± 0.06	20.00 ± 3.33	Not assayed	Not assayed
0.34	0.29 ± 0.03	5.80 ± 0.60	0.78 ± 0.11	16.20 ± 2.29	0.41 ± 0.07	8.5 ± 1.40

Values are presented as the mean ± SD ($N = 5$).
[a] ANOVA $P < 0.01$; Dunnett's test, $P < 0.01$ for 0 vs. 1% coffee treatment.
[b] ANOVA $P < 0.001$; Dunnett's test, $P < 0.01$ for 0 vs. 5% coffee treatment and vs. 0.34% caffeine.
[c] ANOVA $P < 0.01$; Dunnett's test, $P < 0.01$ for 0 vs. 0.34% caffeine treatment.
[d] ANOVA $P < 0.01$; Dunnett's test, $P < 0.01$ for 0 vs. 0.07% caffeine treatment.
[e] ANOVA $P < 0.001$; Dunnett's test, $P < 0.01$ for 0 vs. 0.34% caffeine treatment.

although the inhibitory effect was less pronounced than that observed for coffee treatment. Moreover, MROD activity in the liver microsomes of rats pretreated with caffeine remained unchanged from the control rats when expressed as nmol product formed/nmol CYP1A2/min. The K_m values of PhIP *N*-oxidation and C4′oxidation by CYP1A2 are 5.5 and 15 μM, respectively, while the K_m value of CYP1A2 mediated MROD is 0.21 μM [11]. The large differences between the K_m values of CYP1A2-mediated oxidation of PhIP and methoxyresorufin may result in preferential competitive inhibition of PhIP oxidation by coffee constituents, including caffeine and/or its metabolites residing in the microsomal fraction and explain the discrepancy in the turnover (nmol product/nmol CYP1A2/min) of these two substrates as a function of coffee or caffeine treatment.

The influence of coffee and caffeine on several phase II enzymes involved in the detoxication of PhIP was also assessed. The alpha-class GST enzymes rGSTA1 and rGSTA3 have been reported to detoxify HONH-PhIP and its reactive *N*-acetoxy derivative [46] and various UDP-glucuronosyl transferases (UGTs) are involved in conjugation of glucuronic acid to HONH-PhIP, which may be viewed as a means of detoxication [35,41,47]. Coffee treatment (5% w/w) resulted in increased hepatic cytosol GST activities, some of which are associated with the alpha and mu-class isoforms [48]. In contrast to coffee, caffeine treatment did not increase these enzyme activities. The data in Table 2 summarizes the effect of coffee on GST-mediated reduction of cumene hydroperoxide (CuOOH), and glutathione conjugation to DCNB and CDNB. The increases in activities are consistent with the immunoquantitation data, which revealed an increase in the expression of the rGSTA1 and rGSTA3 isoforms, respectively, by 1.4- and 2.6-fold (Fig. 5).

Changes in hepatic UGTs activities as a function of coffee were also observed. At the highest coffee treatment, there was a 2-fold induction of UGT activity, based upon glucuronidation of *p*-nitrophenol. There were modest increases in the rates of N^2- and *N*3-glucuronidation of HNOH-PhIP as a function of coffee treatment; however, these changes in activities did not reach statistical significance ($P < 0.08$). The *N*-acetylation rates of 2-AF also remained unchanged as a function of coffee treatment (Table 2).

The influence of coffee on the metabolism and biodisposition of PhIP was assessed in vivo by analysis of urinary metabolites including 4′HO-PhIP derivatives that are detoxication products of PhIP, and glucuronide conjugates of the genotoxic HONH-PhIP metabolite (markers of CYP1A2 activity) 24 h post-treatment when maximal PhIP absorption and urinary elimination has occurred [33] (Figs. 1 and 6). PhIP-DNA adduct formation was also measured in liver, colon, and pancreas to determine if the alteration of xenobiotic enzyme activities by coffee resulted in altered levels of PhIP-DNA adducts. The amount of the PhIP dose eliminated 24 h after treatment was similar in control and coffee-treated groups. However, there was an increased elimination of the PhIP metabolites in urine of rats treated with 5% coffee (Table 3). HPLC analysis of the urine samples revealed the presence of five principal metabolites: 4′HO-PhIP, 4′*O*-glucuronide and 4′-sulfate conjugates of 4′HO-PhIP, and the N^2-and *N*3-glucuronide conjugates of HONH-PhIP. These metabolites and unmetabolized PhIP accounted for approximately 70–80% of the total radioactivity eliminated in urine 24 h following treatment with PhIP. At the highest coffee concentration, there was a significant increase in the amounts of the N^2-and *N*3-glucuronide conjugates of HONH-PhIP, and 4′HO-PhIP eliminated in urine and indicative of either increased CYP1A2-mediated oxidation of PhIP or *N*-glucuronidation of HONH-PhIP.

PhIP-DNA adduct formation was measured by AMS in liver, colon and pancreas tissues 24 h after exposure to PhIP (Fig. 7). There was no differences amongst PhIP-DNA adduct levels in colon in the 0 vs. 1 or 0 vs. 5% coffee treatment. There was a slight increase in pancreas PhIP-DNA adduct levels between the 1 vs. 5% coffee-treated groups, but not in 0 vs. 1% or 0 vs. 5% (ANOVA $P < 0.007$, Bonferroni's Multiple Comparison Test $P < 0.01$). In the case of the liver, there was a 50% decrease in adduct levels in the 5% coffee treatment vs. the 0% treated group (ANOVA $P < 0.01$, Dunnett's test 0 vs. 5% coffee treatment $P < 0.01$).

Table 2
The effect of coffee on hepatic phase II enzyme activities

Glutathione S-transferase activities (μmol/min/mg cytosolic protein) *substrates*				
Coffee (%)	H_2O_2	CuOOH[a]	CDNB[b]	(DCNB)[b] × 10^3
0	1.41 ± 0.42	1.47 ± 0.39	1.22 ± 0.12	59.5 ± 2.6
1	1.51 ± 0.19	1.58 ± 0.34	1.30 ± 0.28	56.2 ± 2.8
5	1.93 ± 0.69	2.13 ± 0.40	1.95 ± 0.14	84.5 ± 6.0
Caffeine (%)	H_2O_2	CUOOH	CDNB	(DCNB) × 10^3
0.07	1.89 ± 0.48	1.99 ± 0.58	1.09 ± 0.33	44.7 ± 8.2
0.34	1.52 ± 0.35	1.41 ± 0.20	1.29 ± 0.14	51.7 ± 5.6
UDP glucuronyl transferase activities (nmol/min/mg microsomal protein) *substrates*				
Coffee (%)	NO_2–phenol[a]	HONH-PhIP (HON-PhIP-N^2-GI) × 10^3	HONH-PhIP (HON-PhIP-N3-GI) × 10^3	
0	40.3 ± 10.5	7.4 ± 1.1	64.4 ± 12.1	
1	52.7 ± 9.7	7.8 ± 1.4	60.2 ± 7.1	
5	79.3 ± 22.7	10.0 ± 2.0	78.8 ± 16.1	
Caffeine (%)	NO_2–phenol[c]	HONH-PhIP		
0.07	73 ± 21	Not assayed		
0.34	109 ± 47	Not assayed		
N-acetyltransferase activity (nmol/min/mg cytosolic protein)				
Coffee (%)	2-AF			
0	1.13 ± 0.23			
1	1.18 ± 0.05			
5	1.17 ± 0.07			

Values are presented as the mean ± SD ($N = 5$).
[a] ANOVA $P < 0.05$, Dunnett's test, $P < 0.05$ for 0 vs. 5% coffee treatment; 0 vs. 0.34% caffeine treatment.
[b] ANOVA $P < 0.0001$; Dunnett's test, $P < 0.01$ for 0 vs. 5% coffee treatment.
[c] ANOVA $P < 0.05$; Dunnett's test, $P < 0.05$ for 0 vs. 0.34% caffeine treatment.

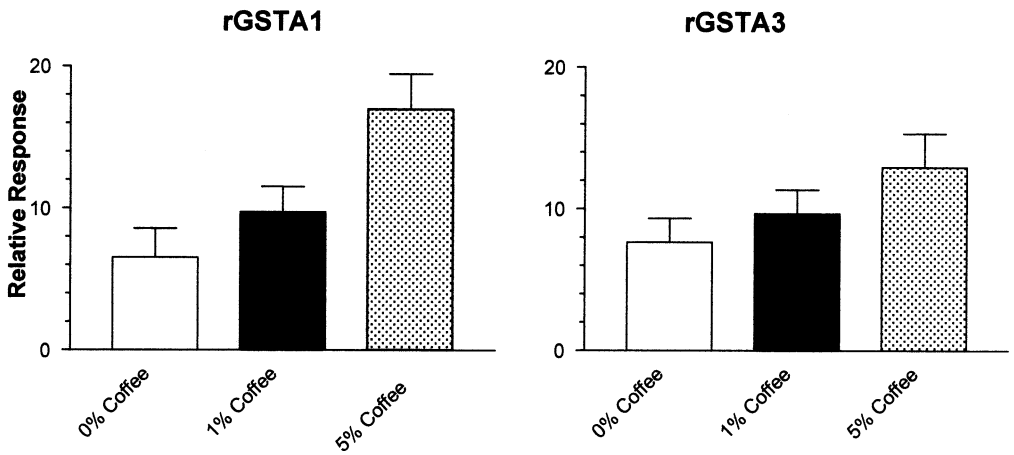

Fig. 5. The effect of coffee on the induction of rGSTA1 and rGSTA3 isoforms in rat liver cytosol fractions. For rGSTA1 ANOVA $P < 0.0001$; Dunnett's test, $P < 0.05$ 0 vs. 1% coffee treatment, and $P < 0.01$ for 0 vs. 5% coffee treatment. For rGSTA2 ANOVA $P < 0.0002$; Dunnett's test, $P < 0.01$ for 0 vs. 5% coffee treatment.

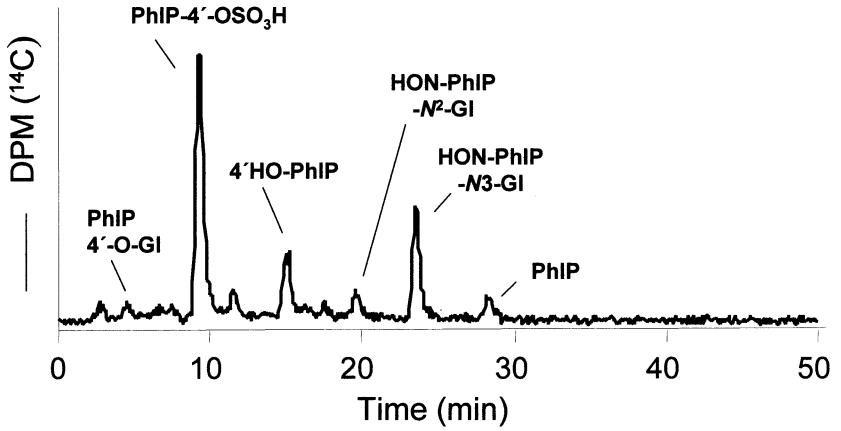

Fig. 6. HPLC analysis and radiochromatogram of urinary metabolites of PhIP in the animals on the 5% coffee treatment.

4. Discussion

The effects of coffee on the metabolism and gentoxicity of PhIP were examined in vitro and in vivo in the rat, an experimental animal model which develops colon tumors following long-term feeding with PhIP [32]. Coffee constituents exert significant antimutagenic activity towards PhIP (Fig. 2) and consistent with the findings of a previous study where coffee constituents displayed protective effects against several other HAAs in bacterial mutation assays [18]. Caffeine, which is metabolized by CYP1A2, contributes to this antimutagenic effect probably through competitive enzyme inhibition, resulting in decreased rates of PhIP-*N*-oxidation [45]. Other components in coffee, including melanoidins have been reported to inhibit the mutagenicity of several HAAs [18], and in combination with heterocyclic and phenolic compounds in coffee also contribute to this inhibitory effect. Nevertheless, PhIP is extensively metabolized by CYP1A2 in rats pretreated with coffee, suggesting that some coffee constituents are not well absorbed from the gastro-intestinal tract, or that the effects of coffee constituents on CYP1A2 induction acts to counter-balance some of these inhibitory effects. The data in vitro with liver microsomal preparations of rats on a coffee

Table 3
Biodisposition of [^{14}C]-PhIP and percent of total PhIP dose excreted in urine over 24 h as a function of coffee treatment

Biodisposition of [^{14}C]-PhIP in urine and feces over 24 h		
Coffee treatment (%)	Urine[a]	Feces
0	17.0±3.5	30.1±3.0
1	18.4±2.0	29.2±2.6
5	25.1±0.9	26.6±4.3

Percent of total PhIP dose excreted in urine over 24 h						
Coffee treatment (%)	4′-HO-PhIP glucuronide	4′-HO-PhIP sulfate[b]	4′-HO-PhIP[b]	HON-PhIP-N^2-glucuronide[c]	HON-PhIP-*N*3-glucuronide[c]	PhIP
0	0.4±0.1	6.9±1.2	1.3±0.2	0.5±0.1	1.3±0.3	1.2±0.4
1	0.4±0.1	7.9±0.3	1.4±0.3	0.5±0.1	1.5±0.2	1.0±0.2
5	0.5±0.0	9.6±0.9	2.3±0.2	0.9±0.2	3.2±0.2	0.9±0.2

Values are presented as the mean±SD ($N=4$).
[a] ANOVA $P<0.002$; Dunnett's test, $P<0.01$ for 0 vs. 5% coffee treatment.
[b] ANOVA $P<0.006$; Dunnett's test, $P<0.01$ for 0 vs. 5% coffee treatment.
[c] ANOVA $P<0.004$; Dunnett's test, $P<0.01$ for 0 vs. 5% coffee treatment.

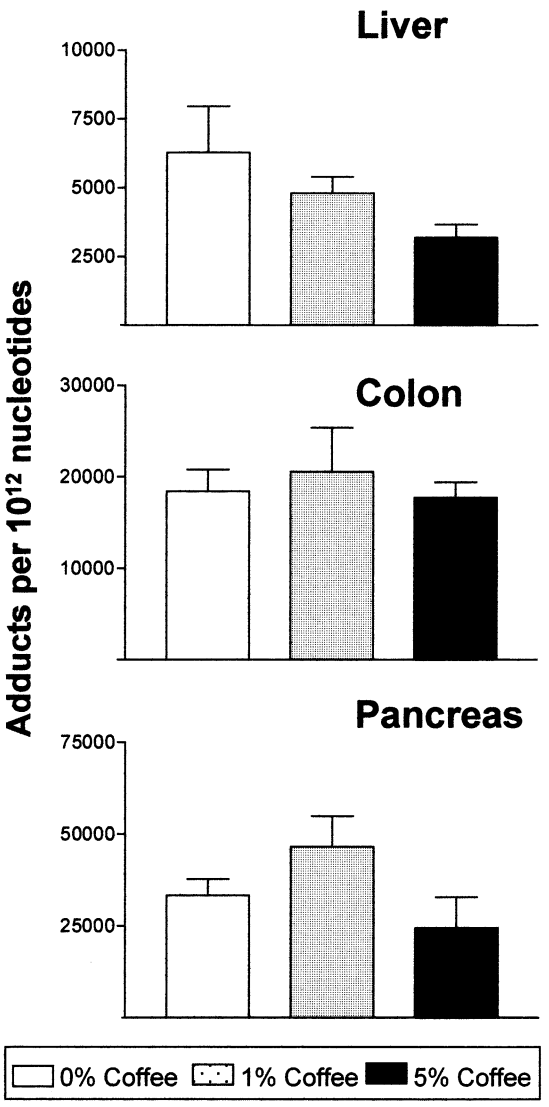

Fig. 7. The effect of coffee on PhIP-DNA adduct formation in liver, colon and pancreas. ANOVA $P < 0.01$ for liver adducts; Dunnett's test, $P < 0.01$ for 0 vs. 5% coffee treatment. ANOVA $P < 0.01$ for pancreas adducts; Bonferroni's multiple comparison test $P < 0.05$ for 1 vs. 5% coffee treatment.

diet (Table 1) suggest that there is inhibition of CYP1A2 activity towards PhIP ring- and *N*-oxidation but not MROD activity as a function of coffee treatment when expressed as nmol product formed/min/nmol CYP1A2. Approximately half of this inhibitory effect appears to be attributed to caffeine and/or its metabolites (Table 1). These findings are consistent with a previous report on dietary caffeine administration reducing the genotoxicity of MeIQx in the host-mediated mutation assay in mice [49], where caffeine consumption (0.01%) led to a 47% reduction in the number of mutants induced by MeIQx. In the same study, the capacity of liver S-9 fractions from caffeine-fed mice to activate MeIQx to a bacterial mutagen in vitro was also significantly reduced.

Coffee is a potent inducer of CYP1A2 and approximately half of this inductive effect is attributed to caffeine, which was previously reported to induce CYP1A2 levels in studies in rats given either caffeine or tea [14,15,50,51]. The mechanism of caffeine-mediated induction of CYP1A2 is not known; however, transcriptional activation of the AH receptor was reported not to occur. CYP1A2 induction may occur through interaction with other related receptors or through other molecular mechanisms such as mRNA or protein stabilization [51]. In contrast to CYP1A2, the induction of hepatic CYP1A1 was very low. Health concerns have been raised over the effects of caffeine consumption and CYP1A2 induction [14] because several different classes of carcinogens, including aromatic and HAAs, are activated to genotoxins by this enzyme. In fact, the incidence of colon tumors increased in rats given PhIP with caffeine, possibly through the selective induction of CYP1A2. However, in the same study, caffeine was observed to decrease the tumorigenic potency of mammary tumors induced by PhIP [16]. In another study, caffeine was shown to strongly reduce the incidence of lung tumors of rats treated with the tobacco carcinogen 4-(-methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK), but did not affect the incidence of nasal tumorigenesis [21].

The effects of coffee constituents on xenobiotic enzymes involved in PhIP metabolism were readily discerned in vivo. The 16-fold increase in hepatic CYP1A2 content in rats resulted in an increase in *N*-oxidation of PhIP with increased amounts of the N^2- and *N*3-glucuronide conjugates of HONH-PhIP excreted in urine. However, despite the elevated expression of CYP1A2, DNA adduct formation was not increased in colon or pancreas as a function of coffee treatment. Moreover, a 50%

decrease in adduct formation was observed in the liver of rats on the 5% coffee diet vs. the control group. An induction of glutathione *S*-transferases (α-class) rGSTA1 and rGSTA3, which are involved in the detoxication of HONH-PhIP and its reactive *N*-acetoxy intermediate [46] may contribute to this protective effect in liver. The protective effects of coffee appear to be restricted to liver and not extrahepatic tissues, based upon PhIP-DNA adduct formation. In this study, the exposure to PhIP was short-term (24 h) and the effect that coffee may exert on PhIP-DNA adduct formation with chronic treatment of PhIP at low, physiologic doses merits investigation.

The effects of tea on DNA adduct formation of PhIP in rats were also reported [52]. Although the study design was somewhat different with respect to the tea regimen, the protective effects of tea against PhIP-DNA adduct formation were most evident in the liver. The metabolism of another HAA, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and the effects of tea were also investigated in the rat, where elevated levels of the N^2-glucuronide conjugate of the carcinogenic *N*-hydroxy-IQ metabolite were reported to be excreted in urine of rats pretreated with tea [17]. The increased formation of this metabolite was ascribed to be a mechanism of detoxication of IQ, although increased levels of *N*-oxidation may have occurred. IQ-DNA adduct formation was not measured in that study.

There is a considerable amount of epidemiological data on coffee consumption and risk of colon cancer. Much of the reported data supports the hypothesis that coffee is a protective factor against colon cancer [27–30] (and references within). Coffee treatment in the rat results in the induction of UGTs and GSTs, which may detoxify various classes of genotoxins and could help to explain some of these protective effects. Moreover, several antioxidants have been reported to decrease the number of HAA-induced tumors in rats [16] and coffee, which is rich in antioxidant polyphenolics, including caffeic acid derivatives, also may modulate the promotion, proliferation, and progression steps of tumorigenesis [48,53,54].

This study shows that coffee contains many components, including caffeine, affects the expression of various xenobiotic enzyme systems involved in the metabolism and biological potency of PhIP. The influence of coffee constituents on the modulation of xenobiotic enzymes is complex and the implications on the genotoxic potential of procarcinogens such as PhIP are difficult to predict. These data reveal that coffee constituents, including caffeine, induce CYP1A2 expression in vivo but also inhibit CYP1A2 activity that may lead to decreased rates of *N*-oxidation and act as a mechanism of chemoprotection against PhIP and other HAAs.

The doses of coffee and caffeine used in this experimental animal model study were elevated relative to daily human consumption. The estimated caffeine intake in these animals was about 88 and 425 mg/kg per day, for the 1 and 5% coffee treatment, respectively. In contrast, the estimated daily intake of caffeine in humans from drinking 2–4 cups of coffee is about 4 mg caffeine/kg body weight and for heavy coffee drinkers the amount may approach 15 mg/kg [55]. Caffeine is also ingested from many other sources including tea, soft drinks and some medicinal preparations. It is uncertain whether the amounts of caffeine in the diet may influence metabolism of HAAs in humans. The analysis of urinary metabolites of PhIP and other HAAs in humans may serve as a means to assess the effects of beverages and dietary constituents on enzyme expression, metabolism and possible chemoprotective effects.

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