Short Communication

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine Induces a Higher Number of Aberrant Crypt Foci in Fischer 344 (Rapid) Than in Wistar Kyoto (Slow) Acetylator Inbred Rats[1]

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Abstract

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most abundant heterocyclic amine carcinogen in the human diet and is a colon carcinogen in the rat. *N*-Acetyltransferase-2 (NAT2) catalyzes the conversion of PhIP and other heterocyclic amines to a DNA-reactive form. NAT2 has a polymorphic distribution in humans and other mammals, including rats. The rapid *NAT2* genotype has been shown to be associated with increased colorectal cancer risk in some, but not all, human epidemiological studies. This investigation was designed to study the role of acetylator genotype in PhIP-induced colon carcinogenesis using aberrant crypt foci (ACF) as an intermediate biomarker. Five-week-old male, rapid-acetylator Fischer 344 (F344) rats and slow-acetylator Wistar-Kyoto (WKY) rats were fed the semipurified AIN76A diet with 0.01% PhIP, 0.04% PhIP, or no PhIP (control) for 8 weeks. PhIP induced ACF in both rapid- and slow-acetylator rats; 0.04% PhIP induced more ACF than 0.01% PhIP. There was no difference in the number of ACF between rapid- and slow-acetylator rats that were fed 0.01% PhIP. However, 0.04% PhIP induced 2-fold higher ACF and a greater dose-dependent increase in PhIP-induced ACF in the rapid-acetylator F344 rats compared with the slow-acetylator WKY rats. The results support human epidemiological studies showing higher risk for colorectal cancer in rapid acetylators who frequently consume meat that is very well done.

Introduction

HCAs[3] are mutagenic and carcinogenic in rats and mice and are putative human carcinogens (1). PhIP is the most abundant carcinogenic HCA found in the North American diet (2). PhIP exposure occurs via dietary intake of well-done meats (2) as well as by exposure to cigarette smoke (3). PhIP has been shown to cause colon tumors in male rats (4, 5). PhIP and other HCAs are not direct-acting carcinogens. To exert their mutagenic and carcinogenic actions, HCAs undergo enzyme-catalyzed activation reactions consisting of *N*-hydroxylation followed by *O*-acetylation (6). Two enzymes responsible for catalyzing these reactions are cytochrome P4501A2 and NAT2. Other enzymes that may play a role in activation include cytochrome P4501A1 and -1B1, *N*-acetyltransferase-1, sulfotransferase(s), and prostaglandin synthase(s) (7).

Humans and other mammals, including rats, display a genetic polymorphism with regard to acetylation (8, 9). Because *O*-acetylation is an important activation step in PhIP metabolism, acetylator genotype is hypothesized to be a determinant of PhIP carcinogenicity. Epidemiological studies of the role of *NAT2* as a determinant of colorectal cancer risk have yielded variable results (reviewed in Ref. 9). However, the combination of rapid acetylator status and high intake of well-done or fried meat has been associated with an increased risk for colon cancer (reviewed in Ref. 9).

This study investigated the effect of acetylator status on PhIP-induced colon carcinogenesis in rats. ACF, the earliest morphologically recognized precursors of colon tumors, were used as an intermediate biomarker for colon carcinogenesis (10). Total ACF are routinely used as a biomarker to study initiation, promotion, and chemoprevention of colorectal cancer in rodents (11). For the classic colon carcinogen, azoxymethane, total ACF number has been shown to accurately predict the results of long-term carcinogenicity assays in an economical and timely manner (12). PhIP has been shown to effectively induce ACF in rat colon, including the F344 rat (13, 14).

Materials and Methods

Animals. Five-week-old male F344 (rapid acetylator) and WKY (slow acetylator) rats were obtained from Charles River Laboratories (Raleigh, NC). The animals were housed in polycarbonate cages in a 12-h light-dark cycle. Animal experimental protocols were approved by the Animal Care and Use Committees of the University of Texas M. D. Anderson Cancer Center and the University of Louisville.

Control and Carcinogenic Diets. The AIN76A diet was procured from Dyets Inc. (Bethlehem, PA). PhIP was purchased from Toronto Research Chemical Inc. (Downsview, Ontario, Canada). The diets were fed in a powder form, and diet jars were replenished daily. The carcinogen was mixed into the

Received 9/30/99; revised 2/2/00; accepted 2/18/00.

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[1] Supported in part by American Institute for Cancer Research Grant 95B029-REV and by USPHS Grant CA-34627 from the National Cancer Institute.

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[3] The abbreviations used are: HCA, heterocyclic amine; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; ACF, aberrant crypt foci; F344, Fischer 344 (rat); WKY, Wistar-Kyoto (rat); NAT1, (*N*-aceyltransferase-1; NAT2, *N*-acetyltransferase-2; PAPS, 3′-phosphoadenosine-5′-phosphosulfate.

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Table 1 PhIP *N*-hydroxylation activity in F344 and WKY liver and colon microsomes[a]

Tissue	F344	WKY
Liver	310 ± 20	230 ± 30
Colon	Not detected[b]	Not detected[b]

[a] Data represent mean ± SE in pmoles/min/mg protein from five animals. Levels were significantly ($P < 0.05$) higher in F344 *versus* WKY liver microsomes.
[b] (<2 pmoles/min/mg).

Table 2 PhIP ACF in F344 and WKY rat colons[a]

PhIP dose	F344 ACF/rat	WKY ACF/rat
0% PhIP (Control)	0	0
0.01% PhIP	0.7 ± 0.4[b]	0.5 ± 0.2[b]
0.04% PhIP	4.9 ± 1.0[c]	2.4 ± 0.4[d]

[a] Data represent mean ± SE from 10 animals.
[b–d] Data not sharing the same superscript are significantly different at $P < 0.05$.

diets at 0.01 and 0.04% (low and high carcinogenic doses, respectively) on a weekly basis and stored at 4°C. The AIN76A diet, with or without the carcinogen, as well as deionized water was provided *ad libitum*. The two doses of PhIP used in this study were based on published PhIP colon carcinogenicity data in rats (4).

Preparation of Liver and Colon Microsomes. Five-week-old male F344 and WKY rats were killed by CO_2 asphyxiation. Microsomal fractions were prepared by sequential centrifugation at 9,000 × *g* and 100,000 × *g*, as described previously (15) with minor modifications. Briefly, livers and colons were homogenized in 250 mM sucrose buffered with 50 mM potassium phosphate (pH 7.4). Homogenates were subjected to centrifugation at 9,000 × *g* for 20 min. Supernatants were removed and subjected to ultracentrifugation at 100,000 × *g* for 60 min. Microsomal pellets were washed with 150 mM potassium chloride buffered with 50 mM potassium phosphate (pH 7.4) and pelleted at 100,000 × *g* for 1 h. Microsomal pellets were resuspended in 250 mM sucrose and 1 mM EDTA buffered with 50 mM potassium phosphate (pH 7.4). Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

PhIP *N*-Oxidation Assays. The *N*-oxidation of PhIP to *N*-hydroxy-PhIP was measured in rat liver and colon microsomes. Assays were carried out at 37°C as reported previously (16) with minor modifications. Microsomes (1 mg/ml) were preincubated for 5 min. at 37°C in a reaction (400 μl) containing 5 mM glucose-6-phosphate, 400 μM $NADP^+$, 500 μM NADPH, 400 μM magnesium chloride, and 1 unit/ml glucose-6-phosphate dehydrogenase (Boehringer Mannheim, Indianapolis, IN). Reactions were initiated by the addition of PhIP to a reaction concentration of 100 μM. The reactions were terminated by the addition of two volumes (800 μl) of ice-cold methanol. Proteins were pelleted by centrifugation at 14,000 × *g* for 10 min. The supernatant was removed and analyzed using a Beckman high-performance liquid chromatography system (Beckman Instruments, Inc., Fullerton, CA) and a Waters μBondapack 300 × 3.9 mm C_{18} column (Waters Corporation, Milford, MA). The metabolites were eluted using a linear gradient of 30% methanol:70% 20 mM diethylamine acetate (pH 4.0) to 76.6% methanol:23.4% 20 mM diethylamine over 20 min. PhIP and *N*-hydroxy-PhIP eluted at 21.4 and 24.1 min, respectively, and were detected by absorbance at 317 nm.

***N*-OH-PhIP *O*-Sulfotransferase Assays.** Tissue cytosols from F-344 (rapid) and WKY (slow) acetylator inbred rats were assayed for *N*-OH-PhIP *O*-sulfotransferase activity by modifying a previously published DNA binding method (17). Briefly, cytosols (<2.5 mg/ml) were incubated at 37°C in a reaction containing *N*-OH-PhIP (100 μM; National Cancer Institute Chemical Carcinogen Reference Standard Repository), PAPS (20 μM; S. S. Singer, University of Dayton, Dayton, OH), and 5 mM 4-nitrophenyl sulfate (Sigma) for 30 min. Control reactions substituted water for PAPS and 4-nitrophenyl sulfate. Reactions were terminated by the addition of 1 M acetic acid. Proteins were precipitated by centrifugation at 13,000 × *g* for 10 min. Reaction supernatants were analyzed using a high-performance liquid chromatography system as described above.

Aberrant Crypt Induction. Rats were placed on the basic AIN76A purified diet for 1 week after arrival. After acclimatization for 1 week, the test groups were switched to the 0.01% or 0.04% PhIP diets. A total of 23 F344 and 23 WKY 5-week-old male rats were randomly assigned to the carcinogen group ($n = 10$, per level of dietary PhIP) or a control group ($n = 3$, no PhIP). The animals were killed by CO_2 asphyxiation after 8 weeks on the PhIP diets. The colons were removed immediately, flushed with PBS, cut longitudinally, flattened on filter paper, and fixed in 70% ethanol. After fixation for at least 24 h, the colons were stained with 0.5% methylene blue for 1–2 min to highlight aberrant crypts. Enumeration of ACF was done with the aid of a dissecting microscope by transilluminating the colon from below under low magnification. Aberrant crypts were distinguished according to the criteria of McLellan and Bird (18). The incidence of aberrant crypts was recorded in terms of the number of crypts forming each incident focus (*e.g.*, as singlets, doublets, triplets, and so forth).

Statistical Analysis. Differences in enzyme activities and ACF between the rapid-acetylator and slow-acetylator rats were analyzed by the two-tailed Student's *t* test and one-way ANOVA, respectively. Differences were considered statistically significant at $P < 0.05$.

Results

PhIP *N*-Hydroxylation by Hepatic and Colon Microsomes. As shown in Table 1, PhIP *N*-hydroxylation activity was slightly higher in inbred F344 *versus* WKY rat liver microsomes. PhIP *N*-hydroxylation activity was not detectable in colon microsomes from the two rat strains.

***N*-Hydroxy-PhIP *O*-Sulfotransferase Activity in Hepatic and Colon Cytosols.** Levels of *N*-hydroxy-PhIP *O*-sulfotransferase activity were below the limit of detection (<2 pmol/min/mg) in hepatic and colon cytosols of F344 and WKY inbred rats.

Effect of Acetylator Status and PhIP Dose on ACF Induction. The effect of acetylator status and PhIP dose on the total number of ACF induced per rat is illustrated in Table 2. ACF were induced in rapid-acetylator F344 and slow-acetylator WKY rats at both of the dose levels of PhIP in the AIN76A diet. No ACF were found in rats fed the AIN76A that did not include any PhIP (the control).

At 0.01% PhIP, there was no difference between strains in the total number of ACF per rat. At 0.04% PhIP, however, the rapid-acetylator F344 rats had double the total ACF per rat compared with the slow-acetylator WKY rats ($P < 0.05$). In both rapid- and slow-acetylator rat strains, more ACF were induced in rats fed the 0.04% PhIP diet compared with the

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Table 3 PhIP aberrant crypt multiplicity in F344 and WKY rat colons[a]

Rat strain and PhIP dose	Singlets (%)	Doublets (%)	Triplets (%)
F344			
0.01%	0.30 ± 0.2[b] (43)	0.20 ± 0.1 (28)	0.20 ± 0.1 (28)
0.04%	3.70 ± 0.9[c] (76)	1.10 ± 0.4 (22)	0.10 ± 0.1 (2)
WKY			
0.01%	0.50 ± 0.2[b] (100)	Not detected	Not detected
0.04%	1.90 ± 0.4[d] (79)	0.50 ± 0.2 (21)	Not detected

[a] Data represent mean ± SE from 10 animals.
[b–d] Data not sharing the same superscript are significantly different at $P < 0.05$.

0.01% PhIP diet. The mean total ACF per F344 rat in the group fed 0.04% PhIP was 7-fold higher than the mean total ACF in the group fed 0.01% PhIP ($P < 0.05$). In the slow acetylator WKY rats, the 0.04% PhIP diet induced twice the total number of ACF per rat compared with the 0.01% PhIP diet ($P < 0.05$).

Effect of Acetylator Status and PhIP Dose on ACF Multiplicity. On the basis of the number of aberrant crypts per aberrant crypt focus (ACF multiplicity), ACF are described as singlets (one aberrant crypt per focus), doublets (two aberrant crypts per focus), triplets (three aberrant crypts per focus), and so forth. The singlet is the earliest form of an aberrant crypt (10). The combined effect of acetylator status and PhIP dose on aberrant crypt multiplicity is shown in Table 3. Both 0.01and 0.04% dose levels of PhIP induced singlets, doublets, as well as triplets in the rapid-acetylator F344 rats. In the slow-acetylator WKY rats, the 0.01% PhIP diet produced only singlets, and the 0.04% PhIP diet produced singlets and doublets but no triplets. As shown in Table 3, ACF multiplicity increased as the dose level of dietary PhIP increased ($P < 0.05$). Although acetylator status affected the number of ACF induced, it had no effect on the percentage of different types of ACF in the high-dose PhIP groups. The main effect of acetylator status was on total ACF numbers induced by PhIP.

Discussion

This study was done to evaluate the role of acetylator status in PhIP-induced colon carcinogenesis. Using ACF as an intermediate biomarker, colon carcinogenesis was studied in two inbred rat strains: the rapid-acetylator F344 strain and the slow-acetylator WKY strain (19). PhIP has been shown to induce ACF in the F344 rat (13, 14), whereas this is the first report of aberrant crypt induction by PhIP in the WKY rat.

The molecular basis of the acetylation polymorphism in the rat was recently reviewed (8, 9). Rats express two *N*-acetyltransferase isozymes, NAT1 and NAT2 (20), analogous to humans and other mammalian species. Although polymorphisms have been identified in both human *NAT1* and *NAT2*, only *NAT2* has been shown to be polymorphic in the rat (20).

Previous studies have shown *N*-acetylation of aromatic amines in colon cytosols of F344 and WKY inbred rat strains to be acetylator genotype-dependent (21). However, PhIP *N*-acetyltransferase activities in rat colon cytosols are undetectable (21). Thus, *N*-acetylation of PhIP is minor in the rat, and the effect of acetylator genotype on the production of ACF after PhIP administration most likely reflects differences in *O*-acetylation of *N*-hydroxy-PhIP. Both NAT1 and NAT2 catalyze the *O*-acetylation of *N*-hydroxy arylamines in hamster colon (22). The *O*-acetylation of *N*-hydroxy-PhIP in rat colon cytosol most likely results from catalysis by both NAT1 and NAT2, because differences between the F344 and WKY inbred strains have not been observed (21). Similar findings on acetylator status and ACF incidence were reported previously in the rapid and slow acetylator congenic Syrian hamster (reviewed in Ref. 9) and the rapid and slow acetylator inbred rat (23) given 3,2-dimethyl-4-aminobiphenyl.

In addition to acetylator genotype, differences in the production of colonic ACF between F344 and WKY inbred rats may reflect, in part, differences in *N*-hydroxylation capacity. A combination of high-activity *N*-acetylation and *N*-hydroxylation phenotypes was shown to increase cancer risk in humans who consume meat very well done (24). Our findings in the rat are consistent with the human studies, because we found that hepatic *N*-hydroxylation of PhIP was slightly (1.3-fold) but significantly ($P < 0.05$) higher in F344 *versus* WKY rats. PhIP-*N*-hydroxylation in colon was not detectable in either strain. Other enzymes may, however, have a modifying role and include sulfotransferases that activate PhIP to a genotoxic metabolite and glutathione-*S*-transferases and glucuronosyltransferases that detoxify *N*-hydroxy-PhIP or *N*-acetoxy-PhIP (25). Although we were unable to detect *N*-OH-PhIP *O*-sulfotransferase activity in hepatic or colon cytosols from the two inbred rat strains, it is premature to conclude that the differences in ACF were attributable solely or even primarily to differences in acetylation capacity.

The interaction of HCA exposure and acetylator status was studied using two carcinogenic dietary levels of PhIP. Hasegawa *et al.* (4) have shown PhIP carcinogenicity to be dose-dependent. In their study, both 0.01% and 0.04% PhIP diets caused colon tumors in male F344 rats, whereas the 0.0025% PhIP diet did not. In our study, no effect of acetylator status was seen at the 0.01% PhIP dose, whereas at the 0.04% PhIP dose, rapid-acetylator status resulted in a doubling of total ACF compared with slow acetylators. Thus, high-dose PhIP and rapid-acetylator genotype yielded more ACF than high-dose PhIP and slow-acetylator genotype. Overall, PhIP exerted a greater carcinogenic effect on the colons of rapid-acetylator F344 rats than on slow-acetylator WKY rats.

Within each acetylator genotype, the PhIP dose response was different. F344 rats exhibited a 7-fold increase in total ACF in response to a 4-fold increase in dietary PhIP, attributable mainly to an increase in the number of singlets induced. Slow-acetylator WKY rats exhibited only a 2-fold increase in total ACF incidence in response to a 4-fold increase in dietary PhIP. The higher dietary PhIP dose induced singlets and doublets, whereas the lower dose induced singlets only. The results of this study suggest that investigation of PhIP tumorigenesis in WKY rats in comparison with F344 rats would shed further light on the role of acetylator status in HCA-induced carcinogenesis.

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