DNA adduct formation, cell proliferation and aberrant crypt focus formation induced by PhIP in male and female rat colon with relevance to carcinogenesis

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2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) induces colon tumors in male, but not female, F344 rats. We investigated the mechanisms leading to this difference by measuring the level of PhIP-DNA adducts, the enhancement of cell proliferation and aberrant crypt focus (ACF) formation in colon mucosa. PhIP was administered in the diet at a level of 0.04% to both male and female F344 rats for 1-8 weeks. The level of DNA adducts in the colon mucosa was measured using the 32P-postlabeling method. Four major PhIP-DNA adducts were detected in fairly constant proportions in all the animals examined. The level of PhIP-DNA adducts in male and female rats was the same, indicating no direct correlation between adduct levels and carcinogenesis. Labeling indices (LIs) were determined by measuring BrdU incorporation in rats after feeding with a PhIP diet for 4, 8 and 12 weeks. After 8 weeks administration the LI had increased 1.5-fold in the colon of the male rats, but no increase was observed in the female rats. ACF formation was examined after feeding with a PhIP diet for 14 weeks. The number of aberrant crypt foci was 6.6 ± 1.5 per rat in males and 1.9 ± 0.5 per rat in females. Thus differences in colon tumor development in male and female rats takes place at an early stage(s). Our results suggest that, in addition to DNA adduct formation, enhanced proliferation contributes to the formation of ACFs, which are pre-malignant lesions of the colon.

Introduction

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP*) was first detected in fried beef by Felton et al. (1). Of the known carcinogenic heterocyclic amines (HCAs), PhIP is the most abundant in various heated foods at levels of 0.6-70 ng/g (1-4). PhIP is genotoxic, inducing base pair and frame-shift mutations in Salmonella typhimurium (1) and 6-thioguanine-resistant- (5) and diphtheria toxin-resistant (unpublished result) and minisatellite recombination mutations in mammalian cells (6).

Unlike other HCAs, PhIP does not induce liver tumors in mice or rats. It induces predominantly colon tumors in male F344 rats with an incidence of 55% on addition at 0.04% to the diet for 52 weeks (7). In female rats, however, it induced predominantly mammary adenocarcinomas with an incidence of 47% after 52 weeks (7). The incidence of colon carcinomas was only 7%, which was not statistically significant (7). These striking differences in the target organs of PhIP in different sexes prompted us to examine the cause of the differences in the incidence of colon carcinogenesis in male and female F344 strain rats.

PhIP is metabolized to the proximate form 2-hydroxy-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (N-OH-PhIP), mainly by cytochrome P4501A2, and is then converted to the ultimate form N-acetoxy-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (N-OAc-PhIP), by acetyltransferase in the presence of acetyl CoA (8), and O-sulfonylesters of N-OH-PhIP, by sulfotransferase (9), although some differences in the features of this metabolism have been observed among different species. The ultimate form binds covalently mainly to guanine residues of DNA, forming N2-(guanin-8-yl)-PhIP (10). One possible reason for the difference in colon carcinogenesis in male and female rats is a difference in their capacity for metabolic activation of PhIP to form DNA adducts. Another possibility is a difference in their capacity to repair DNA adducts. Therefore, in this study we measured the levels of PhIP-DNA adducts after chronic administration of PhIP to male and female rats using the 32P-postlabeling method, which represent the sum of their capacities for metabolic activation and repair.

As reported previously, PhIP forms DNA adducts in various organs of rats fed a diet containing PhIP (11) at levels comparable with that in the colon. This suggests that the DNA adduct level alone is not sufficient to explain differences in carcinogenicity. It was considered that cell proliferation, which is enhanced by complete carcinogens and tumor promoters, might be involved in the organ specificity of PhIP carcinogenesis. As cells in the proliferative compartment (12) of the colon mucosa proliferate constitutively, it was supposed that their proliferation might be enhanced by PhIP, as has been observed with another colon carcinogen, dimethylhydrazine (13). Therefore, we also examined the effect of PhIP on cell proliferation in the colon of male and female rats to determine whether it enhances proliferation in the colon mucosa specifically in males.

We further analyzed the formation of aberrant crypt foci (ACF) to determine whether the sex differences in colon carcinogenesis are due to differences in the processes before ACF formation, which are widely recognized as resulting in early pre-malignant lesions of the colon (14,15).

Materials and methods

Animals

Five-week-old male and female F344 rats were obtained from Charles River Japan (Atsugi, Japan) and given CE-2 basal diet (CLEA Japan, Tokyo, Japan). From 6 weeks of age the animals were given a diet containing 0.04% PhIP...
(Nard Institute, Osaka, Japan) for varying periods. The PhIP lot number was the same as that in previous experiments (7,11).

**32P-Postlabeling**

After varying amounts of time four to six animals were killed and their colon mucosa scraped off with a glass slide, frozen in liquid nitrogen and stored at -80°C until DNA extraction. DNA was extracted as reported previously (11) and subjected to 32P-postlabeling. The levels of PhIP-DNA adducts were analyzed by the intensification method reported previously (11), except for the use of 2.4 μM [γ-32P]ATP with a lower specific activity (650 Ci/mmol) and a Bio-imaging analyzer (BAS 2000, Fuji Photo Film Co., Tokyo, Japan), as opposed to X-ray film and a liquid scintillation counter. In order to determine the relative adduct levels the intensification factors for each spot were calculated from the results obtained under standard conditions using the lungs of a rat fed a diet containing 0.05% PhIP for 4 weeks. Under the standard conditions the DNA digest was 32P-labeled with T4 polynucleotide kinase (Takara Shuzo, Kyoto, Japan) and 58 μM [γ-32P]ATP (92 Ci/mmol; ICN Biomedicals, Irvine, CA).

**Labeling index**

Bromodeoxyuridine (BrdU) dissolved in saline was injected i.p. at a dose of 100 mg/kg body wt. One hour later the animals were killed and their colons promptly removed. The colon was opened, flattened on a paper towel, fixed in ice-cold acetone and cut into six segments of equal length. The labeling index (LI) of about five pit columns for each segment was then examined as described below.

**Immunohistochemical staining**

The acetone-fixed organs were processed by the AMeX method (16), embedded in paraffin and cut into 5 μm thick sections. The sections were then deparaffinized, digested with 0.02% actinase E (Kaken Co., Tokyo, Japan) and treated sequentially with normal porcine serum, mouse anti-BrdU antibody (diluted 1:100; DAKO PATTs a/s, Denmark), biotin-labeled goat anti-mouse IgG and ABC according to a previously reported method (17). In the colon mucosa the number of labeled and unlabeled cells in ~30 pit columns throughout the colon was counted for calculation of the LI. Six rats were used for analysis at each time point.

**Detection of ACF**

Seven male and seven female rats administered a PhIP diet for 14 weeks from 6 weeks of age and five male and five female rats administered a basal diet were examined for formation of ACFs. After sacrifice their colons were removed and flushed with buffered formalin. They were then cut open along the longitudinal median axis and fixed flat between filter papers overnight. Following the procedure described by Bird (14), the colons were stained with 0.2% methylene blue in phosphate-buffered saline for 10-20 min. The number, size and location of ACFs and the multiplicity of aberrant crypts (ACs) per ACF were examined at 40 or 100X magnification using a light microscope.

**Results**

**PhIP-DNA adducts**

In the colon mucosa of a male rat fed a PhIP diet for 2 weeks four major spots were detected by the 32P-postlabeling method under conditions of intensification (Figure 1A). This TLC pattern was the same as that reported previously (11). The pattern of PhIP-DNA adducts in the colon mucosa of a female rat fed a PhIP diet for 2 weeks (Figure 1B) was the same as that of the male rat. The TLC patterns of PhIP-DNA adducts remained the same throughout the experimental period in all the animals examined (data not shown).

The changes in PhIP-DNA adduct levels over time in the colon mucosa of a male rat fed a PhIP diet for 2 weeks four major spots were detected by the 32P-postlabeling method under conditions of intensification (Figure 1A). This TLC pattern was the same as that reported previously (11). The pattern of PhIP-DNA adducts in the colon mucosa of a female rat fed a PhIP diet for 2 weeks (Figure 1B) was the same as that of the male rat. The TLC patterns of PhIP-DNA adducts remained the same throughout the experimental period in all the animals examined (data not shown).

The changes in PhIP-DNA adduct levels over time in the colon mucosa of male and female rats during the period of PhIP administration are shown in Figure 2. The levels of PhIP-DNA adducts in male rats were all ~4/100 nucleotides after 1, 2 and 4 weeks of treatment, but appreciably lower after 6 and 8 weeks. The adduct levels in female rats were essentially the same as those in males.

**Enhancement of cell proliferation**

The effect of PhIP administration in the diet on cell proliferation in the colon was examined in male rats. The LI in the colon had not increased at 4 weeks, but had increased after 8 weeks administration of the PhIP diet (Table I). This increase was only 42%, but was statistically significant. No increase in cell numbers in the crypts was observed (data not shown). Cells located in the lower two-thirds of the crypts were labeled in groups, with and without PhIP treatment. The distribution of labeled cells in the control and experimental groups of rats is illustrated in Figure 3. In contrast, no significant increase in LI was observed after treatment periods of 4 and 12 weeks in males.

![Figure 1](image1.png)

**Fig. 1.** TLC pattern of PhIP-DNA adducts detected with a bio-imaging analyzer under conditions of intensification. The imaging plate was exposed for 6 h. DNA was from the colon mucosa of animals fed 0.04% PhIP in their diet for 2 weeks. (A) Male rat; (B) female rat.

![Figure 2](image2.png)

**Fig. 2.** Changes in PhIP-DNA adduct levels over time during PhIP administration. Animals were fed a diet containing 0.04% PhIP continuously. Each point indicates the average value for four to six animals and the bar indicates the standard deviation. □, Male rats; ○, female rats.

| Table 1. The effects of PhIP on LI in the colon mucosa of male and female rats. |
|-----------------------------------|----------|-----------|-----------|
| Sex     | Treatment | 4 weeks   | 8 weeks   | 12 weeks  |
| Male    | PhIP      | 8.8±0.4   | 10.5±0.4b | 9.3±0.7   |
| None    | None      | 8.1±0.2   | 7.4±0.3   | 8.4±0.4   |
| Female  | PhIP      | 5.9±0.3   | 7.9±0.6   | 7.2±0.7   |
| None    | None      | 7.5±0.4   |           |           |

*Average of six rats.

bSignificantly different from untreated 8 week group at P < 0.001.
The LI in the colon of the female rats did not increase after 8 or 12 weeks of PhIP administration (Table I).

**ACF formation**

The average number of ACFs in male and female rats was 6.6 ± 1.5 and 1.9 ± 0.9 respectively after administration of a PhIP diet for 14 weeks. The average number of ACPs per ACF was almost the same in the male and female rats, being 1.6 and 1.5 respectively. These ACFs developed mainly in the distal colon, with some in the proximal colon, and there was no difference in the ACF distribution between males and females. No ACFs were induced in the control rats. Thus, induction of ACFs in males was much more efficient than in females.

**Discussion**

We reported previously that the levels of PhIP–DNA adducts in the rat were relatively high in the heart, pancreas and lung (11). N-OH-PhIP and N-OAc-PhIP, the proximate and ultimate forms of PhIP respectively, are produced in the liver. However, in the liver adduct levels are low, probably because of preventive effects of reduced glutathione and an α-class glutathione S-transferase on N-OAc-PhIP (18,19) and glucuronidation of N-OH-PhIP (18). N-OH-PhIP and N-OAc-PhIP are relatively stable and are thought to be transported via the circulation to various organs, including the colon mucosa. N-OH-PhIP transported from the liver to the colon would be converted to N-OAc-PhIP in the colon and may form DNA adducts, because the α-class glutathione S-transferase level is much lower in this organ than in the liver (19,20). In this study we demonstrate that adduct levels in the colon mucosa of male and female rats were the same. 4-Aminobiphenyl has been reported to induce tumors preferentially in the liver of female mice and in the bladder of male mice (21). In this case, however, tissue- and sex-specific differences in carcinogenicity coincided well with differences in DNA adduct levels (22).

Four major PhIP–DNA adducts were detected in the colon mucosa of all animals examined by the 32P-postlabeling method under conditions of intensification and their TLC patterns were the same. One of the adducts detected in vivo was N2-(deoxyguanosin-8-yl)-PhIP 3',5'-diphosphate (3',5'-pdGp-C8-PhIP) (23–25). The other three adducts were found to be dinucleotides or oligonucleotides derived from 3',5'-pdGp-C8-PhIP and the major PhIP–DNA adduct formed in vivo was identified as being 3'-guanine-8-yl-PhIP (26).

Table II. The effects of PhIP on ACF formation in male and female rats

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of rats</th>
<th>Treatment</th>
<th>ACF/rat</th>
<th>ACS/ACF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>7</td>
<td>PhIP</td>
<td>6.6±1.5</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>PhIP</td>
<td>1.9±0.5</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Significantly different from the values obtained from the corresponding females at P <0.001.

The LI in the colon of the female rats did not increase after 8 or 12 weeks of PhIP administration (Table I).

As well as inducing colon cancers in the long-term experiments (31), ACFs, which are considered to be precursors of colon cancer, were induced dose-dependently in male F344 rats by PhIP administration for 8 weeks (31). In this study we compared the effects of PhIP on ACF formation in male rats with that in female rats and found a remarkable difference in the total number of ACFs in each group. Since the number of ACPs per ACF was almost the same in both sexes, formation of AC seems to be repressed in females. Since the adduct levels are the same in both sexes, the reason for this difference may be a difference in the levels of enhanced cell proliferation. However, enhanced cell proliferation was observed throughout the colon and was not limited to any specific region, such as the precursors of ACs. Thus it is suspected that under these conditions the initiated cells can grow, being released from the growth control of surrounding normal cells, and some initiated cells might result in the formation of ACFs. The mechanisms involved in the enhancement of cell proliferation remain to be solved.

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**References**


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