Introduction

It is generally recognized that diet and lifestyle influence human breast cancer risk (1). Breast cancer rates are relatively high in the Western world and appear to be partly associated with Western-style dietary habits (see ref. 2 for review). The possibility that the Western diet harbors specific carcinogens that are causative agents in human breast cancer has been raised (3–9). Cooked meat, a staple of the Western diet, contains potent mutagens and rodent carcinogens belonging to the heterocyclic amine (HCA) family of compounds (4,10–16). One of the HCAs, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridazine (PhIP), has been shown to be a mammary gland carcinogen in rats under a variety of dosage and experimental conditions (17–22). This rat mammary gland carcinogen is currently acknowledged as the principle carcinogenic HCA found in the Western diet (13,15). It is produced in meat during cooking by the reaction of an amino acid with creatine, precursors found in muscle meats (12,14). Originally isolated from fried hamburger, PhIP has now been found in a variety of fried, broiled or barbecued meats including beef, pork and chicken (12,14–16).

Studies in our laboratory reported that PhIP rapidly induces mammary gland tumors in female Sprague–Dawley rats when administered orally during mammary gland development according to a modified Huggins’ method for rat mammary tumor induction (19,23). In addition, the incidence of PhIP-induced rat mammary gland carcinomas is increased when rats were given a high fat diet after dosing with PhIP (19). Using the potent experimental mammary gland carcinogen 7,12-dimethylbenz(a)anthracene (DMBA), previous studies have shown that the rat mammary gland is most susceptible to chemical carcinogenesis during the period of active post-natal growth and development that occurs roughly between 30 and 55 days of age, with susceptibility declining with maturation (23–28). The sensitivity of the rat mammary gland to chemical carcinogenesis has been shown to correlate with the presence of mammary gland terminal end buds (TEBs) (25,26). During mammmary gland development, TEBs are actively growing ductal structures that differentiate to alveolar buds (ABs) and then to lobules (28–31). Some TEBs also regress to terminal ducts (TDs) that remain until further differentiation during pregnancy and lactation (29). During the early stages of DMBA-induced mammary gland carcinogenesis, Russo and Russo found that the differentiation of TEBs to ABs is inhibited

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*Abbreviations: AB, alveolar bud; DAB, diaminobenzidine; DMBA, 7,12-dimethylbenz[a]anthracene; HCA, heterocyclic amine; IDP, intraductal proliferation; Ig, immunoglobulin; PCNA, proliferating cell nuclear antigen; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; TD, terminal duct; TEB, terminal end bud.

Proliferation, development and DNA adduct levels in the mammary gland of rats given 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and a high fat diet

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is a heterocyclic amine derived from cooked meat that is a mammary gland carcinogen in rats. A carcinogenic dose-regimen of PhIP (75 mg/kg, p.o., 10 doses, once per day) was administered to 43-day old female Sprague–Dawley rats, and the rats were then placed on a defined high fat diet. The mammary glands from rats on a high fat diet also showed a statistically higher density of TEBs compared with rats on a low fat diet (2.08 ± 0.20% versus 0.34 ± 0.2% (n = 99) versus 4.2 ± 0.6% (n = 127), respectively). The mammary glands of PhIP-treated rats showed a significantly lower density of alveolar buds (ABs) and a higher density of TEBs than control rats, which suggests that PhIP exposure partially inhibited the normal glandular differentiation of TEBs to ABs. After 6 weeks on the diet, proliferation in TEBs was statistically higher in PhIP-treated rats than in control rats receiving vehicle only (7.5 ± 0.9% (n = 99) versus 4.2 ± 0.6% (n = 127), respectively). The mammary glands of PhIP-treated rats showed a significantly lower density of alveolar buds (ABs) and a higher density of TEBs than control rats, which suggests that PhIP exposure partially inhibited the normal glandular differentiation of TEBs to ABs. After 6 weeks on the diet, proliferation in TEBs was statistically higher in rats given PhIP plus a high fat diet than in rats given vehicle plus a low fat diet. The mammary glands from rats on a high fat diet also showed a statistically higher density of TEBs than rats on a low fat diet [2.08 ± 0.34% versus 1.04 ± 0.20%, respectively (n = 6)]. PhIP–DNA adduct levels were relatively high in mammary epithelial cells of treated rats. At 3 h after the last dose of PhIP, DNA adduct levels [relative adduct labeling (RAL) × 107, mean ± SE] were 10.5 ± 1.7 (n = 8) and 0.9 ± 0.2 (n = 7) in epithelial cells isolated from mammary gland and in the liver, respectively. DNA adduct removal rates from the mammary gland were not different between rats on the high fat and low fat diets. Adducts were still detected after 6 weeks on either diet. Thus, events that occurred prior to neoplasia in the mammary glands of PhIP-treated rats include formation of PhIP–DNA adducts at relatively high levels, and enhanced proliferation in TEBs (putative sites of origin of mammary gland carcinomas) and partial inhibition of TEB differentiation. The high fat diet, a promoter of PhIP-induced mammary gland carcinogenesis, appeared to sustain the proliferative effect of PhIP in mammary gland TEBs at a time when PhIP–DNA adducts are still detectable. These early events may contribute to the targeting and carcinogenicity of PhIP to the mammary gland of rats.
Materials and methods

Animals and treatment

Female Sprague-Dawley rats, 35 days old, were obtained from the NIH animal supply (Animal Production Area, FCRDC, Frederick, MD) and maintained on NIH Lab Chow. Throughout the study, rats were given food and water ad libitum and housed in a NIH animal facility on a 12 h light–dark cycle. At 43 days of age, rats received 10 oral doses of PhIP (75 mg/kg body wt and 5 ml corn oil/kg body wt) once per day as described (19). Control rats were given an identical treatment with the corn oil vehicle only. After the final dose of PhIP or corn oil, rats were either killed after 3 h or placed on a defined high fat diet (23.5% corn oil) or defined low fat diet (5% corn oil) for various times up to 6 weeks before being killed. The diets were deparaffinized and rehydrated prior to incubation in periodic acid to inhibit endogenous peroxide activity. Non-specific binding of immunoglobulin G (IgG) was suppressed by incubating the sections in 1% horse serum. Next, the sections were incubated with PCNA antibody (PC10; Dako, Copenhagen, Denmark) and then with biotinylated anti-mouse IgG (Vectastain ABC kit, Vector Lab, Burlingame, CA). This was followed sequentially by a 30-min incubation with Vectastain ABC reagent, staining with diaminobenzidine (DAB Chromagen tablets; Dako) and counterstaining with hematoxylin. The PCNA-stained epithelial cells and the total number of epithelial cells were counted in each TEB identified in the mammary gland section. The number of stained cells per total cells (multiplied by 100) represented the percentage of proliferating cells or PCNA index in each TEB. TEBs were identified according to the criteria of Russo et al. as glandular structures displaying at least three epithelial cell layers around a central lumen (30).

Mammary gland whole mounting and analysis of structural development

The whole mount preparation of the rat mammary gland was carried out according to the method of Russo et al. (24). Briefly, the skin pelt with attached mammary glands was removed from the rat, stretched and pinned onto a board, and fixed in 10% buffered formalin. The fourth mammary gland was dissected from the skin, defatted in acetone, hydrated in ethanol, and stained with toluidine blue. After washing in distilled water and alcohol, the stain was fixed in 4% ammonium molybdate and then washed in distilled water. Next, the gland was dehydrated in increasing concentrations of ethanol and stored in xylene until microdissection and mounting. Surrounding muscle and connective tissue was removed from the gland by microdissection under a Zeiss Steini 2000 stereomicroscope (Carl Zeiss, Thornwood, NY), and the gland was mounted on a glass slide. Mammary gland structures including TEBs, terminal ducts (TDs), ABs, and lobules identified by size and shape according to the criteria of Russo et al. (25,26,30), were counted in the outer 2 mm of zone C of the mammary gland. Identification and counting of the structures was aided by an image analysis system that consisted of M1 DC-330 3CCD color camera (DAGE-MTI, Michigan City, IN) attached to the stereomicroscope and a Pentium computer equipped with Zeiss Image (version 3.0) software. All measurements were carried out at ×32 after calibration with a micrometer.

Statistical analysis

Data were analyzed by Student’s t-test. Mann–Whitney rank sum test, one-way ANOVA or two-way ANOVA using the statistical package SigmaStat (version 2.01, Jandel Scientific Software, San Rafael, CA).

Results

At 3 h after the rats received the 10th oral dose of PhIP, PhIP–DNA adduct levels (mean RAL×107 ± SE) in mammary gland and liver were 10.5 ± 1.7 (8 mammary epithelial cell preparations each from 2–3 rats) and 0.9 ± 0.2 (7 livers). Adduct levels were also examined in PhIP-treated rats placed on the high and low fat diets for 2, 7, 14 and 42 days after dosing (Figure 1A and B). In rats on either diet, PhIP–DNA adduct levels in the mammary gland were not statistically different at 2 days from those at 3 h after dosing. Irrespective of diet, PhIP–DNA adduct levels in the mammary gland epithelial cells declined rapidly from 2 to 7 days on the diet and more slowly from 7 to 14 days (Figure 1A). Very little additional decline in PhIP–DNA adduct levels in the mammary gland was observed from 14 to 42 days on either diet. At 42 days, PhIP–DNA adduct levels were 0.7–0.8 (RAL×107) in the mammary gland epithelial cells isolated from rats on either diet. At each time point examined, no statistical difference in PhIP–DNA adduct levels was found between the high and low fat diet groups.

Adduct levels in the liver of rats on the high fat diet
decreased steadily from 2 to 14 days after dosing (Figure 1B). At 7 days after dosing, hepatic PhIP–DNA adduct levels were significantly higher in rats on the low fat diet (Student’s t-test, \( P < 0.05 \)), which suggests that DNA adduct removal from the liver was faster in rats on the high fat diet. After 42 days on diet, however, hepatic PhIP–DNA adduct levels were similar in both diet groups. Comparing adduct levels between liver and mammary gland indicates that PhIP–DNA adduct levels were 12- to 15-fold higher in mammary gland than liver at 3 h and 2 days after dosing, and 4- to 6-fold higher in the mammary gland at 7, 14 or 42 days after dosing, irrespective of diet. The PhIP–DNA adduct profiles were identical in both organs (data not shown). The 32P-post-labeling profile for PhIP–DNA adducts in the mammary gland of PhIP-treated rats have been reported previously (32). Three guanine adducts, one identified as the C8-guanine adduct, were found in both tissues. In either tissue and diet, there did not appear to be a selective removal of any of the three guanine adduct spots.

As TEBs are considered to be a putative site of origin of rat mammary gland carcinomas (24–31), the effect of PhIP on the percentage of proliferating cells in TEBs was assessed by PCNA immunohistochemistry. On average, the percentage of proliferating cells in the TEBs of mammary glands from rats exposed to PhIP was nearly 2-fold higher than that observed in rats given vehicle only (\( P < 0.05 \), one-way ANOVA on ranks) (Figure 2). A greater percentage of TEBs were more proliferative in mammary glands from PhIP-treated rats than in mammary gland from control rats, and highly proliferative TEBs were relatively rare in the mammary gland of vehicle control rats. Specifically, whereas just 13% of the TEBs in glands from vehicle control rats had a PCNA index > 10%, 36% of the TEBs in glands from PhIP-treated rats had a PCNA index > 10%.

To evaluate the effect of diet on TEB proliferation, proliferation was measured in TEBs from PhIP-treated and vehicle control rats maintained on the high fat and low fat diets for 42 days (6 weeks) after dosing (Figure 3). A two-way ANOVA revealed a statistically significant effect of the high fat diet on TEB proliferation (\( P < 0.05 \)). The percentage of proliferating cells was 1.5-fold higher in PhIP-treated rats on the high fat diet compared with PhIP-treated rats on a low fat diet, and it was 1.75-fold higher in control rats on a high fat diet compared with vehicle control rats on a low fat diet. The highest percentage of proliferating cells was found in vehicle control rats on a high fat diet and the lowest was found in vehicle control rats on a low fat diet, which suggests that PhIP combined with dietary fat provided the greatest stimulus for proliferation (\( P < 0.05 \), one-way ANOVA on ranks). It is notable that the PCNA index in TEBs was 66% lower in rats on the diet for 6 weeks than in rats killed before the diet (compare control with control on low fat diet, Figures 2 and 3). The ages of the animals before and after diet are 54 and 96 days in Figures 2 and 3. The ages of the animals before and after diet are 54 and 96 days in Figures 2 and 3, respectively. A previous study showed that the rate of proliferation in TEBs declines as rats mature (26), and the
decline observed in our study is therefore likely to be associated with normal maturation of the gland.

Whole mount analysis was carried out to examine the effect of PhIP and diet on the architecture and development of the rat mammary gland. A view of the various structures observed by whole mounting the mammary glands of PhIP-treated rats is provided in Figure 4A–D. Mammary glands from rats killed 3 h after PhIP or vehicle (54-day-old rats) consisted primarily of TEBs, TDs and ABs. Very little lobular development was found in animals of this age group. Differences, however, were observed between the glands isolated from PhIP-treated and control rats. The density of TEBs (number of TEBs/mm²) was 1.5-fold higher in PhIP-treated rats than in vehicle control rats \((P < 0.05, \text{Student’s } t\text{-test})\) (Figure 5). In addition, the density of ABs in the mammary gland was 2-fold lower in PhIP-treated rats than in the vehicle controls \((P < 0.05, \text{Student’s } t\text{-test})\). Lobular development was low to non-detectable in the mammary glands from vehicle control and PhIP-treated rats.

After 6 weeks on either the high fat or low fat diet, lobular development was similar in PhIP-treated and control rats and ranged from 0.63 to 1.01 structures/mm² (Figure 6). Statistical differences in AB levels were not observed at this time point \((P > 0.05, \text{two-way ANOVA})\). In addition, there were no statistical differences in the densities of TDs between any of the four groups. There was, however, a statistically significant effect of the high fat diet on the density of TEBs irrespective of PhIP treatment (two-way ANOVA, \(P < 0.05\)). The mean density of TEBs \(\pm SE\) was 2.08 \(\pm\) 0.34 \((n = 6)\) and 1.04 \(\pm\) 0.20 \((n = 6)\) in all rats (PhIP plus control) on the high fat diet and low fat diet, respectively.

**Discussion**

PhIP is a well-recognized rat mammary gland carcinogen among the family of HCAs. However, the mechanisms of mammary gland carcinogenicity of PhIP are still poorly understood. In the current study, several alterations in the mammary glands of PhIP-treated rats were observed that may be germane to the targeting of PhIP to the mammary gland. First, relatively
high DNA adduct levels are sustained in the mammary epithelial cells of the gland. Indeed, the adduct levels are upwards of 15-fold higher than those found in the liver, an organ that is not a target site of PhIP carcinogenesis (Figure 1). These adducts were also detected in the mammary gland epithelial cells of rats for 6 weeks after dosing, irrespective of diet, suggesting that there might be ample opportunity for these adducts to induce mutations. In addition to high DNA adduct levels, PhIP exposure is associated with an enhanced proliferation in the TEBs (Figures 2 and 3), which are structures in the developing mammary gland that appear to be the site of origin of carcinomas (25,26). Enhanced proliferation in TEBs is also observed after exposure to the potent mammary gland carcinogens N-nitroso-N-methylurea and DMBA (24,42), which further supports the notion that elevated proliferation in these glandular structures is relevant for mammary gland carcinogenesis. Hypothetically, the increase in proliferation in TEBs may facilitate the fixation of mutations from PhIP–DNA adducts and enhance the likelihood of initiating carcinogenesis.

Another effect of PhIP on the rat mammary gland is an inhibition of TEB differentiation. The inhibition of differentiation in PhIP-treated rats is reflected in a statistically higher level of TEBs and accordingly, a statistically lower level of the more differentiated AB structures (Figure 5). Since TEBs are more sensitive than ABs to chemical-induced mammary gland carcinogenesis (28), extending the duration of TEBs in the mammary gland may increase the opportunity for induction of neoplasia by PhIP. PhIP–DNA adduct levels are also relatively high at the time when the inhibition of mammary gland differentiation was observed (i.e. immediately after dosing with PhIP). The concordance of these two events, high adduct levels and inhibition of differentiation, may further enhance the likelihood of initiation by PhIP. The mechanisms involved in the inhibition of TEB differentiation by PhIP are not known. It is tempting to speculate that exposure to the carcinogenic-dose regimen of PhIP results in the stimulation of stem-cell like epithelial cells harbored in TEBs that have qualities of preneoplastic cells.

The effect of PhIP on mammary gland structure and differentiation was less severe and much less prolonged than the effect reported previously with DMBA (25). Russo and Russo (25) reported a marked inhibition of TEB differentiation to ABs, as well as to lobules and TDs after DMBA exposure. In addition, the inhibitory effects on differentiated structures and elevated levels of TEBs were observed for at least 6 weeks after DMBA treatment. In contrast, 6 weeks after PhIP exposure there were no differences in the densities of TEBs, TDs, AB or lobules within each diet group (Figure 6). (We did, however, observe TEB levels to be affected by dietary fat, as discussed further below.) The difference between the effects of the two carcinogens on the mammary gland structure may relate to the difference in their carcinogenic potencies in rats. A single 10 or 15 mg dose of DMBA (roughly 50–100 mg/kg, p.o.) is sufficient to induce a 100% incidence of mammary gland tumors, whereas a cumulative dose of 750 mg PhIP/kg induces an ~50% tumor incidence in rats on a high fat diet (19,23,43, E.G.Snyderwine et al., submitted for publication). The differences between the two carcinogens further supports the notion raised previously (24,28,29) that specific structural alterations in the mammary gland are relevant for carcinogenesis and targeting of carcinogens to the gland. Thus, it appears that the more potent a mammary gland carcinogen, the greater is its inhibitory effect on TEB differentiation.

High fat diets rich in linoleic acid are well recognized to promote rat mammary gland chemical carcinogenesis (43–46), and in our rat model, a linoleic acid-rich high fat diet enhances PhIP-induced rat mammary gland carcinogenesis (19, E.G.Snyderwine et al., submitted for publication). The current study shows that consumption of this high fat diet was associated with specific preneoplastic alterations in the mammary glands of PhIP-treated rats. After 6 weeks on defined high fat or low fat diet, epithelial cell proliferation in TEBs of the mammary gland was higher in rats on a high fat diet than in rats on a low fat diet (Figure 3). These findings are in agreement with studies carried out in mice that reported that certain high fat diets increase the proliferation in ductal epithelial cells of the mammary gland (47–50). Unique to our study was the finding that a high fat diet acts in concert with a chemical carcinogen to sustain proliferation in the TEB epithelial cells of the rat mammary gland. This was shown by the result that epithelial cell proliferation was ~2-fold higher in TEBs from PhIP-treated rats on a high fat diet than in TEBs from vehicle control rats on a low fat diet (Figure 3).

Dietary fat intake has been reported to affect the development of the mammary gland when rats are weaned onto diet (38). In addition, maternal dietary fat intake has also been reported to alter mammary gland development in female offspring (37). Therefore, we examined the effect of diet on the mammary gland development in PhIP-treated rats. In our model, placing the rats on a high fat diet for 6 weeks after dosing with PhIP or vehicle increased the level of TEBs relative to the level observed in the mammary gland of rats on a low fat diet. Thus, the consequence of placing rats on a high fat diet is to increase both the density and proliferation of TEBs during the period of time when PhIP–DNA adducts are present in the mammary epithelial cells. It is interesting that the high fat diet did not alter PhIP–DNA adduct removal rates from the epithelial cells of the mammary gland (Figure 1). Thus the enhanced proliferation and effects on mammary gland differentiation may be potentially more relevant for the mechanism of dietary fat promotion than is the effect of diet on DNA adduct levels. Speculatively, the promotional effect of a high fat diet may in part involve a further fixation of PhIP–DNA adduct-induced mutations through an enhanced proliferative stimulus rather than by an inhibition of DNA adduct repair processes.

The HCA PhIP is a genotoxic carcinogen that is well recognized for its ability to form DNA adducts in target and non-target tissues of experimental animals (34,40,51). This report is the first to indicate that PhIP has an effect on mammary

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gland development and proliferation in ductal epithelial cells. Therefore in addition to DNA adduct formation, PhIP has several effects on the mammary gland that may be relevant for carcinogenesis, It is not yet known if the proliferative and developmental effects of PhIP on the gland are associated with the genotoxic effects of PhIP–DNA adducts or to other properties of the carcinogen that facilitate targeting to the gland. Further studies are still required to address this issue.

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References

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