The Cooked Meat–Derived Genotoxic Carcinogen 2-Amino-3-Methylimidazo[4,5-*b*]Pyridine Has Potent Hormone-Like Activity: Mechanistic Support for a Role in Breast Cancer

Sandra N. Lauber and Nigel J. Gooderham

Biomolecular Medicine, Faculty of Medicine, Imperial College London, London, United Kingdom

Abstract

The cooked meat–derived heterocyclic amine 2-amino-3-methylimidazo[4,5-*b*]pyridine (PhIP) is activated by CYP1A2 to the *N*-hydroxy metabolite, then esterified by acetyl transferase and sulfur transferase into unstable DNA-reactive products that can lead to mutation. The genotoxicity of PhIP has been implicated in its carcinogenicity. Yet, CYP1A2-null mice are still prone to PhIP-mediated cancer, inferring that alternative mechanisms must be operative in tumor induction. PhIP induces tumors of the breast, prostate, and colon in rats and lymphoma in mice. This profile of carcinogenicity is indicative of hormonal involvement. We recently reported that PhIP has potent estrogenic activity inducing transcription of estrogen (E_2)-regulated genes, proliferation of E_2-dependent cells, up-regulation of progesterone receptor, and stimulation of mitogen-activated protein kinase signaling. In this report, we show for the first time that PhIP at doses as low as of 10^{-11} mol/L has direct effects on a rat pituitary lactotroph model (GH3 cells) and is able to induce cell proliferation and the synthesis and secretion of prolactin. This PhIP-induced pituitary cell proliferation and synthesis and secretion of prolactin can be attenuated by an estrogen receptor (ER) inhibitor, implying that PhIP effects on lactotroph responses are ERα mediated. In view of the strong association between estrogen, progesterone, prolactin, and breast cancer, the PhIP repertoire of hormone-like activities provides further mechanistic support for the tissue-specific carcinogenicity of the chemical. Furthermore, the recent epidemiology studies that report an association between consumption of cooked red meat and premenopausal and postmenopausal human breast cancer are consonant with these observations. [Cancer Res 2007;67(19):9597–602]

Introduction

The major group of heterocyclic amines found in cooked meats have been identified as compounds with a quinoline, quinoxaline, or pyridine moiety. An important member of this group, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), is the most abundant mutagen in cooked beef by mass and is reported to be responsible for ~20% of the total mutagenicity found in fried beef (1). PhIP has been detected in a variety of cooked meat and fish products (1, 2). Thus, humans are exposed to PhIP frequently through diet and lifestyle, and daily intake of PhIP among Americans has been estimated to be around 280 to 460 ng/d per person (3).

PhIP is mutagenic in a variety of bacterial and mammalian toxicity assays (4) and is carcinogenic in the rat colon, mammary gland, and prostate (5, 6). PhIP also induces a high incidence of lymphoma in mice (7). Additionally, transplacental and *trans*-breast milk exposure to PhIP has also been shown to increase the risk of mammary tumors (8).

Whereas the genotoxicity of high-dose activated PhIP has been characterized comprehensively, the mechanisms of its actions are not as well understood at a cellular level, and little is known about the effects exerted at concentrations of the compound that are systemic after consumption of a cooked meat meal. It is widely accepted that the formation of PhIP-DNA adducts, which lead to mutations in critical genes, is central to the role of PhIP as a carcinogen (9). The mechanistic basis of this is thought to be the CYP1-mediated oxidation to the *N*-hydroxy derivative (primarily catalyzed by CYP1A2; ref. 10) and then the esterification resulting in an unstable product that generates a nitrenium ion that attacks and adducts to guanine in DNA (11, 12). Yet treatment of CYP1A2-null mice with PhIP still results in carcinogenesis (13), probably via CYP1A1 and CYP1B1 oxidative activation of the compound (14). Clearly, factors other than just CYP1A2-mediated DNA adduction are important in explaining the tumorigenicity of PhIP (12, 13). Indeed, PhIP-DNA adducts are widely distributed across various tissues and organs, but adduct levels do not correlate with outcome in terms of carcinogenic response.

PhIP has several other effects on the mammary gland, in addition to DNA adduct formation, which may be relevant to its mechanisms of carcinogenesis. These include effects on mammary gland development and proliferation, alterations in levels of circulating hormones, and changes in cell signaling and gene expression (15). These properties indicate that PhIP might act as a tumor promoter in the mammary gland. In support of this, we have recently reported that PhIP has potent estrogenic activity mediated through the estrogen receptor α (ERα; ref. 16). From a mechanistic viewpoint, clarification of the promotional potential of PhIP is important for assessing risk to humans. Given the unique target-tissue profile of PhIP and the fact that organ specificity is thought to be largely dependent on promotional effects (17), it is probable that, like E_2, PhIP may be a promoter of cancer as well as an initiator.

The pituitary lactotroph is a well-established target for estrogens. The primary function of the cell is to synthesize and secrete prolactin (PRL), a hormone that exerts a wide range of physiologic effects in mammals, including stimulation of mammary gland development and lactation, hair maturation, synergism with androgen in male sex accessory growth, and maintenance and secretion of the corpus luteum. The potential role of PRL in human

Requests for reprints: Nigel J. Gooderham, Biomolecular Medicine, Imperial College London, Sir Alexander Fleming Building, London SW7 2AZ, United Kingdom. Phone: 44-20-7594-3188; E-mail: n.gooderham@imperial.ac.uk.

doi:10.1158/0008-5472.CAN-07-1661

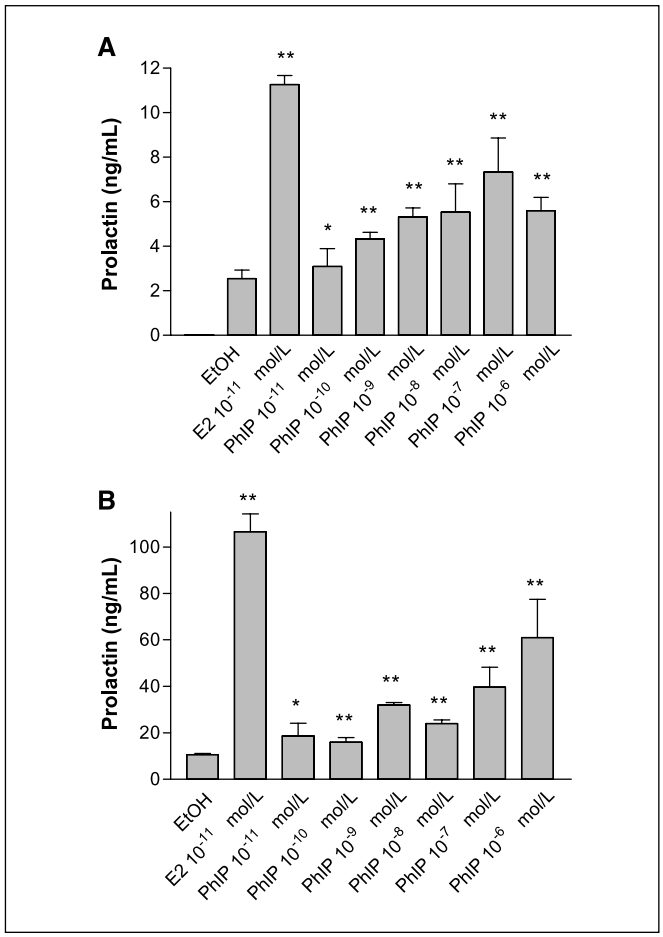

Figure 1. Stimulation of PRL release from GH3 rat pituitary cells by PhIP. Cells were treated with 10^{-8} mol/L E_2 or 10^{-6} to 10^{-11} mol/L PhIP for 24 h (*A*) or 72 h (*B*). PRL secreted into the tissue culture medium was measured by a rat prolactin immunoassay. *Columns,* mean ($n = 3$); *bars,* SD. *, $P < 0.05$; **, $P < 0.01$, versus control.

breast cancer has recently been reviewed by Harvey (18), who reported that hyperprolactinaemia is consistently associated with human breast cancer growth, development, and poor prognosis, and agents that induce PRL-associated mammary carcinogenesis may pose a risk to humans. Studies have shown that E_2 controls the expression of PRL in pituitary lactotrophs primarily by a transcription-dependent mechanism mediated through the ER (19).

Whereas great efforts have been expended to characterize the effects of xenoestrogens in recent years, many studies neglect the potential effect of these compounds on the neuroendocrine axis. In this investigation, we have examined the effects of PhIP on PRL release using a rat somatolactotroph cell line to understand the range of biological responses attributable to exposure to PhIP.

Materials and Methods

GH3 rat pituitary lactotroph cells were obtained from the European Collection of Cell Cultures. Ham's F10 Nutrient Mixture, 17β-estradiol, and PD98059 were purchased from Sigma. Horse serum, FCS, L-glutamine, and penicillin-streptomycin were obtained from Invitrogen. Phenol red–free Ham's F-10 Nutrient Mixture was purchased from Autogen Bioclear. PhIP was purchased from Toronto Chemicals, Inc. ICI 182,780 was obtained from Astra Zeneca. Poly-D-lysine–coated tissue culture flasks and plates were purchased from Becton Dickinson. SuperSignal West Pico chemiluminescent substrate was purchased from Pierce Biotechnology. Goat polyclonal anti-PRL antibody (C-17) was purchased from Santa Cruz Biotechnology. Dextran-coated charcoal-stripped fetal bovine serum (FBS) was prepared as previously described (16). Hybond enhanced chemiluminescence nitrocellulose membrane and rat PRL (rPRL) enzyme immunoassay system were purchased from Amersham Pharmacia Biotech. All other reagents were purchased from Merck-BDH and were of AnaLar grade.

GH3 cell culture. Cells were maintained in culture in Ham's F10 Nutrient Mixture supplemented with 15% horse serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The flasks used to propagate the cells were poly-D-lysine–coated 75-cm^2 flasks. For routine maintenance, cells were seeded at $4 \times 10^4/cm^2$.

For experiments, subconfluent GH3 cells were trypsinized and seeded at a density of 7.5×10^5 per well in poly-D-lysine–coated six-well plates and allowed to adhere overnight. The following day, cells were washed twice with PBS and the growth medium was replaced with 2 mL of experimental medium consisting of phenol red–free Ham's F-10 Nutrient Mixture supplemented with 5% dextran–coated charcoal-stripped FBS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were treated with 10^{-7} mol/L ICI 182,780 for 3 days to eliminate endogenous estrogenic activity present in the serum used to propagate the cell line. After this period, cells were washed twice with PBS and replaced with fresh experimental medium for 1 day before treatment with compounds.

Cell treatment. Stock solutions of E_2, PhIP, ICI 182,780, and PD98059 (2′-amino-3′-methoxyflavone) were prepared in ethanol. PD98059 is a selective and cell-permeable inhibitor of mitogen-activated protein kinase (MAPK) kinase (MEK) that acts by inhibiting the activation of MAPK and subsequent phosphorylation of MAPK substrates. All compounds were diluted 1:1,000 in experimental medium to give the desired final concentration. Ethanol was added to control wells to produce the same final solvent concentration (0.2%) in all wells.

Cell cultures were treated in triplicate with 10^{-8} mol/L E_2 and concentrations of PhIP ranging from 10^{-11} to 10^{-6} mol/L, in the presence or absence of ICI 182,780 or PD98059 for 24 h. Seventy-two hours later, the culture medium was collected and centrifuged to pellet cellular debris. The supernatant was then aliquoted into tubes and stored at −20°C until analysis.

Immunoblotting. Lysates were prepared from GH3 cells treated for 24 h with radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 1% Triton X-100, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA (pH 7.4), 1 mmol/L sodium fluoride, and 50 μg/mL leupeptin] and incubated at 4°C for 15 min, and 15 μg of each protein sample were separated on a 10% SDS-polyacrylamide gel. The proteins in the gel were electrotransferred onto nitrocellulose membranes in transfer buffer [192 mmol/L glycine, 25 mmol/L Tris base, and 20% (v/v) methanol, pH 8.3]. Nonspecific binding sites on the membranes were blocked by

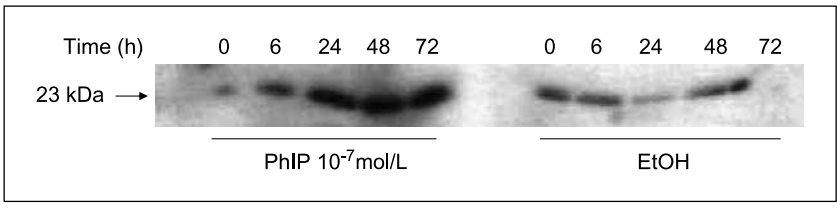

Figure 2. PhIP-induced PRL secretion in GH3 cells. Cells were grown in the presence of 10^{-7} mol/L PhIP or ethanol (*EtOH*) vehicle. Equal aliquots of the medium were removed from the culture plates at the times indicated and analyzed by immunoblot for PRL.

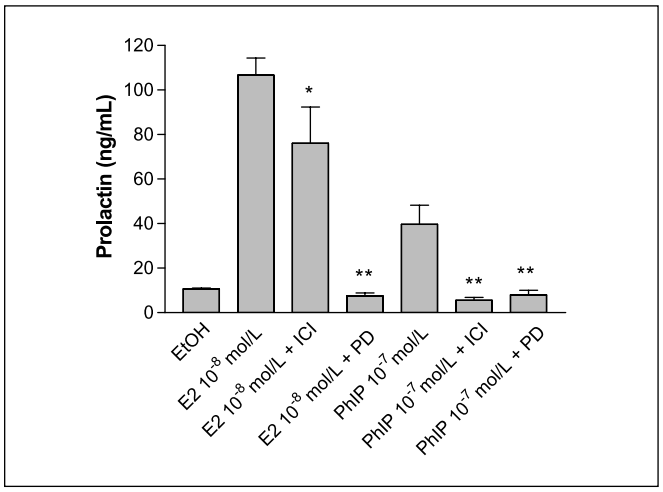

Figure 3. Effects of the antiestrogen ICI 182,780 and the MEK inhibitor PD98059 on E_2- and PhIP-induced PRL secretion. GH3 cells were treated with 10^{-8} mol/L E_2 or 10^{-7} mol/L PhIP in the presence of the antiestrogen ICI 182,780 (10^{-7} mol/L) or the MEK inhibitor PD98059 (5×10^{-6} mol/L) for 72 h. The amount of PRL secreted into the medium was measured using a rPRL immunoassay. *Columns,* mean ($n = 3$); *bars,* SD. *, $P < 0.05$, versus E_2 alone; **, $P < 0.01$, versus PhIP alone.

incubation of the membranes in blocking buffer (5% nonfat powdered milk, 0.05% Tween 20 in PBS) for 30 min at room temperature. Membranes were incubated with goat polyclonal anti-PRL antibody (dilution, 1:500) overnight at 4°C in blocking buffer. The following day, unbound antibody was removed by washing the membrane in PBST (PBS containing 0.5% Tween 20) for 3 × 10 min. After washing, membranes were incubated in horseradish peroxidase–conjugated antigoat antibody (1:10,000 dilution in blocking buffer) for 1 h at room temperature. Membranes were washed again and target protein bands were visualized using SuperSignal chemiluminescent reagent.

rPRL enzyme immunoassay. Secreted rPRL was analyzed in GH3 culture medium with a rPRL enzyme immunoassay system. This assay is based on the competition between unlabeled rPRL and a fixed quantity of biotin-labeled rPRL for a limited amount of rPRL-specific antibody. The labeled ligand that is bound to the antibody is immobilized on precoated microtitre wells. After washing, Amdex amplification reagent is added. Briefly, 50 μL of each sample and 50 μL of goat anti-rPRL were pipetted into wells of a microtitre plate precoated with donkey anti-goat immunoglobulin G. The plate was left to incubate at room temperature for 3 h and then 50 μL of rPRL conjugate were added to each well. The plate was incubated at room temperature for 30 min, after which the wells were washed four times with wash buffer [0.01 mol/L PBS (pH 7.4) containing 0.2% Tween 20 and 0.01% thiomerosal]. The wells were then filled with 100 μL of Amdex amplification reagent and allowed to incubate at room temperature for 30 min. Amdex reagent is a conjugate based on chemistry that uses a hydrophilic straight-chain dextran backbone to which many hundreds of horseradish peroxidase molecules are covalently coupled with 10 streptavidin molecules. The washing steps were then repeated and 100 μL of TMB substrate [3,3′,5,5′-tetramethylbenzidine/hydrogen peroxide solution in 20% (v/v) dimethylformamide] were added to each well. The plate was incubated at room temperature for 30 min, after which 100 μL of 1 mol/L sulfuric acid were pipetted into the wells to terminate the reaction. The absorbance of the wells was then determined by measuring the absorbance at 450 nm on a microplate reader. ANOVA was used to determine the statistical difference in PRL secreted for each treatment versus vehicle-treated control.

Cell proliferation assay. GH3 cells were plated into 24-well plates at a density of 5×10^4 per well and grown for 3 days in medium containing 10^{-7} mol/L ICI 182,780 to eliminate estrogenic activity present in the serum. Cells were then washed twice with PBS and cultured in fresh experimental medium for 1 day before treatment with E_2 (10^{-9} or 10^{-11} mol/L) or PhIP (10^{-6}–10^{-11} mol/L) in the presence or absence of ICI 182,780 (10^{-7} mol/L). Control cells were incubated with medium containing an equivalent amount of ethanol vehicle (0.2%). After 48 h, the compounds were washed off and fresh medium was added; the cells were cultured for a further 5 days, after which the relative cell number was estimated with the resazurin reduction assay as previously described (16).

Results

Effect of PhIP on PRL release from GH3 cells. When GH3 cells are maintained under conditions of low estrogen concentrations, exogenous estrogens increase the synthesis of PRL and its release, and therefore these cells are widely used as an *in vitro* lactotroph model. E_2 has been shown to regulate pituitary function by increasing the synthesis and secretion of PRL. To examine the effect of PhIP on the secretion of PRL, a rPRL immunoassay was done on medium obtained from GH3 cells incubated with various concentrations of PhIP (10^{-11}–10^{-6} mol/L). Treatment with PhIP for 24 or 72 h resulted in a dose-dependent increase in PRL secretion into the medium (Fig. 1). After 72 h, maximal stimulation was achieved at 10^{-6} mol/L PhIP, which gave 57% of the response elicited by E_2 at 10^{-8} mol/L. PhIP significantly stimulated PRL secretion over the entire dose range examined (10^{-11}–10^{-6} mol/L). The temporal effect of PhIP treatment on PRL release was also examined by immunoblot of aliquots of conditioned medium removed from treated GH3 cultures. Maximal induction of PRL was observed at 48 h posttreatment (Fig. 2).

Effects of ICI 182,780 and PD98059 on PhIP-stimulated PRL release. To determine if the increase in PRL secretion was mediated by PhIP/ER binding, PRL secretion was measured in the presence of the antiestrogen ICI 182,780. Treatment of GH3 cells with 10^{-7} mol/L PhIP in the presence of an equimolar concentration of

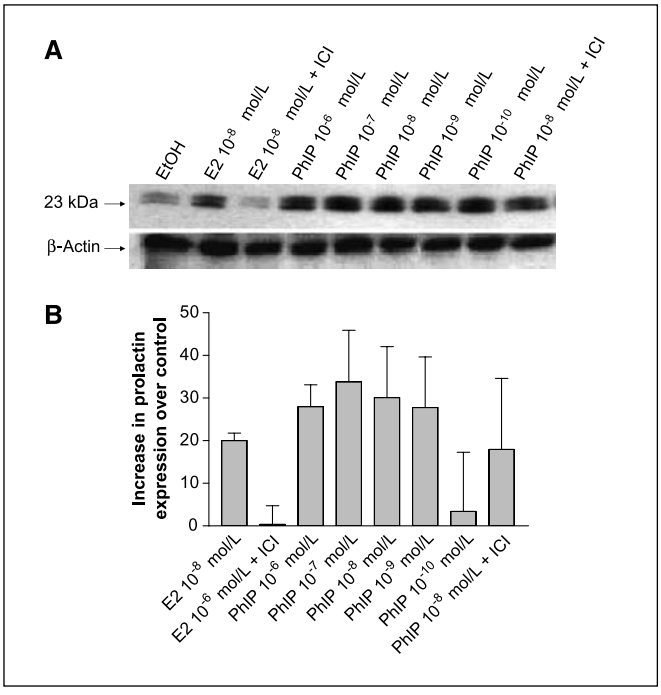

Figure 4. Effects of E_2 and PhIP on PRL synthesis in GH3 cells in the presence or absence of ICI 182,780 (10^{-7} mol/L). *A,* cell extract proteins (15 μg) from GH3 cells were subjected to SDS-PAGE on 10% gels and intracellular PRL was measured with the use of anti-PRL polyclonal antibody. A representative blot is shown. *B,* induction of PRL protein expressed as a percent increase over ethanol-treated control cells (where ethanol = 0 arbitrary units). *Columns,* mean of three independent determinations; *bars,* SD.

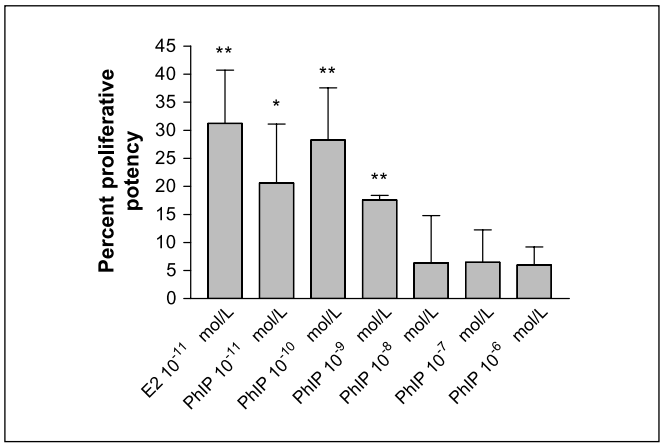

Figure 5. Effect of PhIP on GH3 cell proliferation. GH3 cells were treated with 10^{-11} mol/L E_2 or various concentrations of PhIP for 48 h, after which they were washed with PBS and left to grow for a further 5 d. *Columns,* mean of triplicate wells; *bars,* SD. *, $P < 0.05$, versus vehicle control; **, $P < 0.01$, versus vehicle control.

ICI 182,780 resulted in a total inhibition of PhIP-induced PRL secretion (Fig. 3). The release of PRL stimulated by E_2 was also significantly inhibited by concurrent treatment with ICI 182,780 albeit to a lesser extent than PhIP, implying a role for ER in the observed activity.

Watters et al. (20) reported that E_2-induced PRL expression requires an intact MAPK signal transduction pathway in GH3 cells. For this reason, the effect of the MEK inhibitor PD98059 on the activity of PhIP was also investigated. Treatment of cells with PD98059 completely abrogated the PRL-stimulating effects of both E_2 and PhIP (Fig. 3).

Induction of intracellular PRL expression by PhIP. Intracellular PRL was analyzed by immunoblotting of lysates of GH3 cells treated for 24 h. Both E_2 and PhIP increased the intracellular levels of PRL (Fig. 4). PRL resolved as two bands at ~23 and 25 kDa. The lower band on the blot is unmodified PRL, whereas the upper band represents the phosphorylated form of the hormone. Unmodified and phosphorylated PRL together constitute 90% to 98% of the PRL produced by the rodent pituitary.

Administration of ICI 182,780 reduced PRL accumulation in the intracellular pools, indicating that the effects of the antiestrogen are not solely on PhIP-induced PRL secretion in these cells.

Effect of PhIP on GH3 cell proliferation. Both E_2 and PhIP stimulated the proliferation of GH3 cells grown in low-estrogen conditions (Fig. 5). E_2 (10^{-11} mol/L) had a 30% proliferative potency over vehicle treatment after the 5-day growth period, showing the estrogen responsiveness of this cell line. PhIP induced a proliferative response in GH3 cells only at the lower doses used (i.e., at 10^{-9}–10^{-11} mol/L). PhIP exhibited a maximal proliferative response at 10^{-10} mol/L, suggesting that the proliferative response of GH3 cells is more sensitive to PhIP than the PRL secretion response observed at the higher doses.

Discussion

The role of PRL in breast cancer. The data reported here show for the first time that PhIP is able to directly stimulate the proliferation of a rat pituitary lactotroph model (GH3 cells) and to induce the synthesis and secretion of the hormone PRL. PRL plays a key role in the normal growth, differentiation, and development of the mammary gland. There is evidence to suggest that development of the alveolar cells in the breast requires not only E_2 and progesterone but also PRL. Indeed, mice in which the *PRL* gene has been deleted have impaired mammary gland development and fail to lactate after their first pregnancy (21). Rodent model systems have also illustrated a direct role for PRL in the development of breast cancer (22–24).

The role of PRL in the etiology of human breast cancer is less clear. Biswas and Vonderhaar (25) have shown that more than 80% of human breast cancer cell lines express the PRL receptor, bind PRL with high affinity, and proliferate in response to PRL. Furthermore, serum PRL levels are significantly elevated in women at risk for familial breast cancer, and hyperprolactinaemia is considered to be an indicator of unfavorable prognosis in breast cancer patients and has been associated with metastases, early disease relapse, and poor overall survival (26).

PRL expression. PRL expression is subject to a complex and multihormonal regulation, which includes the stimulatory effects of estradiol (E_2), thyrotropin-releasing hormone, epidermal growth factor, and the inhibitory influence of dopamine. By a transcription-dependent mechanism, E_2 increases *PRL* gene expression, synthesis, storage, and release, as well as stimulates lactotroph proliferation (19, 27). E_2 acts by binding to nuclear ERs that, in turn, interact with specific estrogen response elements present in the promoter sequences of target genes to modulate their expression. The ERs stimulate transcription by recruiting co-activator complexes with histone acetylator and methylase activities (28). In the *PRL* gene, the E_2-ER complex interacts directly with an imperfect palindromic estrogen response element in the 5′ flanking region ~1.8 to 1.5 kb upstream from the transcription initiation site of PRL mRNA (29).

PhIP induces PRL expression. We have previously reported the powerful E_2-like activity of the cooked meat–derived carcinogen PhIP (16), which was subsequently confirmed by others (30). We showed that PhIP binding to ERα leads to transcriptional activation of E_2-responsive genes. PhIP can also mediate proliferation of cells that are E_2 growth dependent and can activate E_2-responsive signal transduction (12, 16). Given the involvement of PRL in breast cancer and the fact that expression of this hormone is under the control of E_2, evaluation of the ability of PhIP to stimulate the secretion of PRL is important. The presence of binding sites for Pit-1, a pituitary-specific transcription factor, is mandatory for E_2 induction of the PRL promoter (31); hence, we used the GH3 rat pituitary cell line (which possesses an endogenous Pit-1 site) in the present investigation. We show here that nanomolar concentrations of PhIP increased both the synthesis and secretion of PRL in GH3 cells in a dose-dependent manner, with a significant effect even at 10^{-11} mol/L. Watters et al. (20) showed that E_2 modulation of *PRL* gene expression requires an intact MAPK pathway in GH3 cells. The results from the present study are in good agreement with this because the effects of both E_2 and PhIP were completely abolished by the administration of the MEK inhibitor PD98059.

Although PhIP has only about a third of the potency of E_2 in terms of PRL-stimulating activity, it is possible that it may act in concert with endogenous hormones or, indeed, other xenoestrogens to exert detrimental effects in the body. For example, combinations of two weak xenoestrogens can be 100 to 1,000 times as potent in eliciting an estrogenic response as each substance alone (32). PhIP and other xenoestrogens may therefore constitute an unsuspected source of compounds capable of altering the natural hormonal balance.

PhIP-DNA adducts have been identified in the brains of both rats and monkeys treated with PhIP (33), confirming that PhIP and/or its metabolites are able to cross the blood-brain barrier and can therefore presumably act directly on pituitary cells to mediate PRL secretion. More significantly, the PRL-stimulating effect of PhIP has previously been reported in an *in vivo* study examining the inhibitory effects of PhIP on mammary gland involution in Sprague-Dawley rats (34). The authors showed that PhIP increased serum levels of PRL ~1.3-fold over that of vehicle-treated control animals. The mechanism involved was suggested to be associated with the inhibitory action of PhIP on 3,4-dihydroxy-L-phenylalanine amino acid decarboxylase, an enzyme involved in the production of dopamine and is a negative regulator of PRL in the hypothalamus. We suggest that an interaction of PhIP with ER offers an alternative explanation.

PhIP induces cell proliferation. GH3 cells are responsive to the mitogenic signal of E_2; in this system, PhIP also induced a proliferative response in GH3 cells, but only at 10^{-9} to 10^{-11} mol/L. The observation that PhIP induces maximal growth at these low concentrations is consistent with the findings of Amara and Dannies (27), who reported that concentrations of E_2 higher than 10^{-11} mol/L decreased cell growth. A maximal proliferative response was seen with PhIP at 10^{-10} mol/L whereas the PRL-stimulating effect increased with dose. This indicates that GH3 cells are differentially responsive to PhIP in terms of growth and PRL expression, an effect also described for E_2 by Chun et al. (35), who found that the proliferative response to E_2 in the PR1 pituitary cell line is much more sensitive than the PRL response. A possible explanation for the observation that PhIP stimulates GH3 cell growth at lower concentrations than it stimulates PRL secretion may be that the ER-mediated proliferation response is not functionally linked to the ER-mediated *PRL* gene expression. Alternatively, perhaps only a small pool of ERs is required for cell proliferation in contrast with the regulation of expression for specific genes.

The mitogenic effects of PRL involve binding to the PRL receptor, receptor dimerization, and phosphorylation of Janus-activated kinase 1 (Jak2), a member of the Janus family of kinases, which is constitutively associated with the receptor. Jak2 reciprocally phosphorylates the PRL receptor and cytoplasmic transcription factors of the signal transducer and activator of transcription (Stat) family (36). Activation of Stat proteins results in translocation to the nucleus, where they bind specific DNA promoter elements to regulate gene transcription. The major Stat protein activated by PRL in the mammary gland, Stat5A, is primarily associated with milk production and cell cycle progression and regulates proteins such as β-casein, β-lactoglobulin, and cyclin D1 (37). Consistent with such an interaction, Shan et al. (38) recently showed that PhIP elevates the level of phosphorylated Stat5 in HC-11 mouse mammary epithelial cells and can alter the expression of genes regulated by PRL in these cells. This activity could be inhibited by the Jak2-specific inhibitor AG490. Activation of the Jak-Stat pathway is associated with the development of breast cancers and lymphomas, among other diseases. Intriguingly, these are the same cancers that have been linked to PhIP, specifically breast cancer in rats (5) and lymphomas in mice (7). Therefore, PhIP stimulation of PRL secretion may be related to its ability to cause cancers at these sites.

In addition to being implicated in the etiology of breast cancer and lymphomas, there is a wide body of evidence to suggest that PRL may be involved in the development of prostate cancer. Studies *in vivo* have consistently shown that hyperprolactinaemia stimulates proliferation in the rat prostate (39). Using a transgenic mouse model, Wennbo et al. (40) showed that mice overexpressing PRL had a 20-fold increase in prostate weight compared with control animals. PRL receptors are expressed in the human prostate, and expression is especially elevated in precancerous lesions (41). Plasma PRL increases at puberty and continues to increase in parallel with the age-related increase seen in the incidence of prostate cancer. The involvement of PRL in prostate cancer is interesting in view of the fact that the prostate is another target site of PhIP carcinogenicity (6).

The metabolism of PhIP in mammals is now well understood and the genotoxic potential of PhIP and its metabolites is well characterized. In assessing the carcinogenic properties of PhIP, metabolic activation into DNA-damaging species has been a significant consideration. The present study does not lessen the importance of metabolism in PhIP carcinogenicity but does emphasize that the additional biological properties of the molecule and its metabolites could influence the site specificity of its carcinogenicity.

In summary, it has been shown that concentrations of PhIP that approximate those expected to be circulating after consumption of a cooked meat meal (12) are able to stimulate the growth of pituitary cells and the synthesis and secretion of PRL in those cells. These effects are consistent with the reported stimulation of PRL by PhIP *in vivo* in rats (34). Together with previous reports that describe the potent estrogenicity of PhIP and its ability to influence progesterone receptor expression, c-Myc expression, and MAPK signal transduction pathway (16, 30), the present findings provide clues about the mechanisms involved in the tissue-specific carcinogenicity of PhIP and, in particular, mammary carcinogenesis. In connection with this, it is pertinent to note the very recent epidemiology studies that report an association between consumption of cooked red meat and both premenopausal and postmenopausal human breast cancer (42–44).

Acknowledgments

Received 5/4/2007; revised 6/15/2007; accepted 7/17/2007.

Grant support: Department of Health and the Food Standards Agency, United Kingdom.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Felton JS, Knize MG, Shen NH, et al. The isolation and identification of a new mutagen from fried ground beef: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Carcinogenesis 1986;7:1081–6.
2. Murray S, Lynch AM, Knize MG, Gooderham NJ. Quantification of the carcinogens 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in food using a combined assay based on gas chromatography-negative ion mass spectrometry. J Chromatogr 1993;616:211–9.
3. Byrne C, Sinha R, Platz EA, et al. Predictors of dietary heterocyclic amine intake in three prospective cohorts. Cancer Epidemiol Biomarkers Prev 1998;7:523–9.
4. Felton JS, Knize MG. Occurrence, identification, and bacterial mutagenicity of heterocyclic amines in cooked food. Mutat Res 1991;259:205–17.
5. Ito N, Hasegawa R, Sano M, et al. A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Carcinogenesis 1991;12:1503–6.
6. Shirai T, Sano M, Tamano S, et al. The prostate: a

target for carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) derived from cooked foods. Cancer Res 1997;57:195–8.
7. Esumi H, Ohgaki H, Kohzen E, Takayama S, Sugimura T. Induction of lymphoma in CDF1 mice by the food mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. Jpn J Cancer Res 1989;80:1176–8.
8. Hasegawa R, Kimura J, Yaono M, et al. Increased risk of mammary carcinoma development following transplacental and *trans*-breast milk exposure to a food-derived carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), in Sprague-Dawley rats. Cancer Res 1995;55:4333–8.
9. Schut HA, Snyderwine EG. DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis. Carcinogenesis 1999; 20:353–68.
10. Zhao K, Murray S, Davies DS, Boobis AR, Gooderham NJ. Metabolism of the food derived mutagen and carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) by human liver microsomes. Carcinogenesis 1994;15:1285–8.
11. Rindgen D, Turesky RJ, Vouros P. Determination of *in vitro* formed DNA adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine using capillary liquid chromatography/electrospray ionization/tandem mass spectrometry. Chem Res Toxicol 1995;8:1005–13.
12. Gooderham NJ, Creton S, Lauber SN, Zhu H. Mechanisms of action of the carcinogenic heterocyclic amine PhIP. Toxicol Lett 2007;168:269–77.
13. Kimura S, Kawabe M, Yu A, et al. Carcinogenesis of the food mutagen PhIP in mice is independent of CYP1A2. Carcinogenesis 2003;24:583–7.
14. Crofts FG, Sutter TR and Strickland PT. Metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by human cytochrome P4501A1, P4501A2 and P4501B1. Carcinogenesis 1998;19:1969–73.
15. Snyderwine EG, Venugopal M, Yu M. Mammary gland carcinogenesis by food-derived heterocyclic amines and studies on the mechanisms of carcinogenesis of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Mutat Res 2002;506/507:145–52.
16. Lauber S, Ali S, Gooderham NJ. The cooked food derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine is a potent oestrogen: a mechanistic basis for its tissue-specific carcinogenicity. Carcinogenesis 2004;*25*:2509–17.
17. Shirai T, Kato K, Futakuchi M, et al. Organ differences in the enhancing potential of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine on carcinogenicity in the prostate, colon and pancreas. Mutat Res 2002;506–507: 129–36.
18. Harvey PW. Human relevance of rodent prolactin-induced non-genotoxic mammary carcinogenesis: prolactin involvement in human breast cancer and significance for toxicology risk assessments. J Appl Toxicol 2005;25:179–83.
19. Maurer RA. Estrogen regulates the transcription of the prolactin gene. J Biol Chem 1982;257:2133–6.
20. Watters JJ, Chun T, Kim Y, Bertics J, Gorski J. Estrogen modulation of prolactin gene expression requires an intact mitogen-activated protein kinase signal transduction pathway in cultured rat pituitary cells. Mol Endocrinol 2000;14:1872–81.
21. Ormandy CJ, Camus A, Barra J, et al. Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes Dev 1997;11:167–78.
22. Boot LM, Muhlbock O, Ropcke G, van Ebbenhorst T. Further investigations on induction of mammary cancer in mice by isografts of hypophyseal tissue. Cancer Res 1962;22:713–27.
23. Boyns AR, Buchan R, Cole EN, Forrest AP, Griffiths K. Basal prolactin blood levels in three strains of rat with differing incidence of 7,12-dimethylbenz(*a*)anthracene induced mammary tumours. Eur J Cancer 1973;9:169–71.
24. McManus MJ, Welsch CW. The effect of estrogen, progesterone, thyroxine, and human placental lactogen on DNA synthesis of human breast ductal epithelium maintained in athymic nude mice. Cancer 1984;54:1920–7.
25. Biswas R, Vonderhaar BK. Role of serum in the prolactin responsiveness of MCF-7 human breast cancer cells in long-term tissue culture. Cancer Res 1987;47: 3509–14.
26. Bhatavdekar JM, Patel DD, Shah NG, et al. Prognostic value of insulin-like growth factor-1 receptors in patients with colon/rectal cancer: correlation with plasma prolactin. Eur J Surg Oncol 1995;21:23–6.
27. Amara J, Dannies P. 17β-Estradiol has a biphasic effect on GH cell growth. Endocrinology 1983;112:1141–3.
28. Chen D, Ma H, Hong H, et al. Regulation of transcription by a protein methyltransferase. Science 1999;284:2174–7.
29. Maurer RA, Notides AC. Identification of an estrogen-responsive element from the 5′-flanking region of the rat prolactin gene. Mol Cell Biol 1987;7:4247–54.
30. Bennion BJ, Cosman M, Lightstone FC, et al. PhIP carcinogenicity in breast cancer: computational and experimental evidence for competitive interactions with human estrogen receptor. Chem Res Toxicol 2005;18: 1528–36.
31. Day RN, Koike S, Sakai M, Muramatsu M, Maurer RA. Both Pit-1 and the estrogen receptor are required for estrogen responsiveness of the rat prolactin gene. Mol Endocrinol 1990;4:1964–71.
32. Arnold SF, Klotz DM, Collins BM, Vonier PM, Guilette LJ, McLachlan JA. Synergistic activation of estrogen receptor with combinations of environmental chemicals. Science 1996;272:1489–92.
33. Snyderwine EG, Schut HAJ, Sugimura T, Nagao M, Adamson RH. DNA adduct levels of 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in tissues of cynomolgus monkeys after single or multiple dosing. Carcinogenesis 1994;15:2757–61.
34. Venugopal M, Callaway A, Snyderwine EG. 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) retards mammary gland involution in lactating Sprague-Dawley rats. Carcinogenesis 1999;20:1309–14.
35. Chun T, Gregg D, Sarkar DK, Gorski J. Differential regulation by estrogens of growth and prolactin synthesis in pituitary cells suggests that only a small pool of estrogen receptors is required for growth. Proc Natl Acad Sci USA 1998;95:2325–30.
36. DaSilva L, Rui H, Erwin RA, et al. Prolactin recruits STAT1, STAT3 and STAT5 independent of conserved receptor tyrosines TYR402, TYR479, TYR515 and TYR580. Mol Cell Endocrinol 1996;117:131–40.
37. Hennighausen L, Robinson GW, Wagner KU, Liu W. Prolactin signaling in mammary gland development. J Biol Chem 1997;272:7567–9.
38. Shan L, Rouhani SA, Schut HA, Snyderwine EG. 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) modulates lactogenic hormone-mediated differentiation and gene expression in HC11 mouse mammary epithelial cells. Cell Growth Differ 2001;12:649–56.
39. Negro-Vilar A, Saad WA, McCann SM. Evidence for a role of prolactin in prostate and seminal vesicle growth in immature male rats. Endocrinology 1977;100:729–37.
40. Wennbo H, Kindblom J, Isaksson OG, Tornell J. Transgenic mice overexpressing the prolactin gene develop dramatic enlargement of the prostate gland. Endocrinology 1997;138:4410–5.
41. Leav I, Merk FB, Lee KF, Loda M, Mandoki M, McNeal JE. Prolactin receptor expression in the developing human prostate and in hyperplastic dysplasia, and neoplastic lesions. Am J Pathol 1999;154:863–70.
42. Cho E, Chen WY, Hunter DJ, et al. Red meat intake and risk of breast cancer among premenopausal women. Arch Intern Med 2006;166:2253–9.
43. Taylor EF, Burley VJ, Greenwood DC, Cade JE. Meat consumption and risk of breast cancer in the UK Women's Cohort Study. Br J Cancer 2007;96:1139–46.
44. Steck SE, Gaudet MM, Eng SM, et al. Cooked meat and risk of breast cancer—lifetime versus recent dietary intake. Epidemiology 2007;18:373–82.