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Inhibition of Cell Death in Human Mammary Epithelial Cells by the Cooked Meat-Derived Carcinogen 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

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2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a mammary gland carcinogen present in the human diet in cooked meat. To examine if PhIP and its reactive metabolite *N*-hydroxy-PhIP inhibit apoptosis in human mammary epithelial MCF-10A cells, confluent cultures deprived of serum and growth factors were incubated for 24 h with either compound. The percentages of dead cells (mean ± SEM, $n = 3$) as measured by trypan blue exclusion were 5.7 ± 0.6, 3.4 ± 0.3, 2.7 ± 0.3, and 0.2 ± 0.003%, in control, 1 μM *N*-hydroxy-PhIP-, 5 μM *N*-hydroxy-PhIP-, and 100 μM PhIP-treated dishes, respectively. The expression of Bcl-2 and Bcl-x_L as quantitated by Western blotting was 1.2- to 1.9-fold higher in the treated groups. PhIP–DNA adducts induced by *N*-hydroxy-PhIP in MCF-10A cells measured by the ^{32}P-postlabeling assay were low ($<1 \times 10^7$, relative adduct labeling). No adducts were detected after incubation with PhIP. Western blot analysis indicated that PhIP increased ERK2 phosphorylation concomitant with Bcl-2. The results suggest that the inhibition of cell death in mammary epithelial cells by PhIP occurs independently of PhIP–DNA adducts and may involve enhanced signaling through the MAP kinase pathways.

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a member of a family of carcinogenic/mutagenic heterocyclic amines (HCAs) formed during the cooking of muscle meats including beef, chicken, and fish (1–5). PhIP is currently recognized as the major carcinogenic HCA found in the human diet (2, 3). Several studies have shown that PhIP is a mammary gland carcinogen in rats (4–10). In addition, epidemiological studies have raised the possibility that HCAs in cooked meat play role in etiology of human breast cancer (5 for review, 11).

PhIP is a procarcinogen that requires metabolic activation via cytochrome P450-mediated N-hydroxylation to *N*-hydroxy-PhIP (4, 5, 12). *N*-Hydroxy-PhIP subsequently undergoes phase II esterification to reactive ester derivatives that form DNA adducts (4, 5, 13, 14). The formation of PhIP-DNA adducts appears to be critical for the mammary gland carcinogenicity of PhIP (15). In addition, PhIP exposure in rats is associated with an increased proliferation in terminal end buds of the mammary gland, an inhibition of mammary gland differentiation, and an elevation of serum prolactin, effects that also appear to play a role in the targeting of PhIP to the mammary gland (16–18).

It is generally recognized that apoptosis is an important factor in the regulation of tissue development, differentation and homeostasis (19). Dysregulation of apoptosis has been shown to contribute to carcinogenesis (19–21). Genes belonging to the Bcl-2 family are now known to be critical regulators of the apoptotic process (19, 22). Several Bcl-2 family proteins are expressed in the mammary gland including the anti-apoptotic proteins Bcl-2 and Bcl-x_L (23, 24). Involution of the postlactational mammary gland involves elimination of the secretory mammary epithelial cells largely through apoptosis (25, 26). We recently reported that PhIP exposure retards mammary gland

Abbreviations used: HCA, heterocyclic amine; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; *N*-hydroxy-PhIP, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; SFM, serum free medium; D-SFM, serum free medium with 0.007% dimethyl sulfoxide.

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involution in rats (18). These findings supported the possibility that PhIP inhibits mammary gland apoptosis. The previous study also showed that PhIP exposure increased serum prolactin, a finding which may partly explain the retardation of involution (18). However, this study left open for question whether PhIP might have a direct inhibitory action on mammary gland apoptosis. Recently, an immortalized human mammary epithelial cell line, MCF-10A has been shown to undergo apoptosis when deprived of growth factors and serum after reaching confluency (27, 28). In the current study, we use this *in vitro* model to test the hypothesis that PhIP and its metabolite *N*-hydroxy-PhIP contribute directly to the inhibition of cell death in the mammary gland.

MATERIALS AND METHODS

Cell line. MCF-10A cells were provided by Dr. David Solomon, NCI. MCF-10A cells are a spontaneously immortalized human mammary epithelial cell line that is not transformed (29). Cells were maintained in 1:1 DMEM (Quality Biological, Inc., Gaithersburg, MD)/Ham's F12 (GIBCO BRL, Gaithersburg, MD) growth medium supplemented with 10% fetal horse serum (GIBCO BRL, Gaithersburg, MD), 2 mM L-glutamine (GIBCO BRL, Gaithersburg, MD), 0.01 mg/ml insulin (GIBCO BRL, Gaithersburg, MD), 20 ng/ml EGF (Collaborative Biomedical Products, Bedford, MA), 100 ng/ml cholera toxin (GIBCO BRL, Gaithersburg, MD), 500 ng/ml hydrocortisone (Clonetics, San Diego, CA), and 10 μg/ml gentamicin sulfate (GIBCO BRL, Gaithersburg, MD). Serum-free medium (SFM) was prepared from 1:1 DMEM/Ham's F12 supplemented with 2 mM L-glutamine, 100 ng/ml cholera toxin, 500 ng/ml hydrocortisone, and 10 μg/ml gentamicin sulfate, and was devoid of the growth factor supplements.

Preparation of N-hydroxy-PhIP and PhIP solutions. Stock solutions of PhIP (Toronto Research Chemicals, North York, Ontario, Canada) and *N*-hydroxy-PhIP (custom synthesized by SRI International, Menlo Park, CA) were prepared in dimethyl sulfoxide (DMSO) and working solutions were prepared by diluting the stock solutions in SFM. The final concentration of DMSO in working solutions was 0.007%. SFM containing 0.007% DMSO (D-SFM) served as control throughout these studies.

Treatment of MCF-10A cells with N-hydroxy-PhIP and PhIP. MCF-10A cells were allowed to grow to confluency in 60 mm tissue culture dishes. The growth medium was then removed, and the cells were washed twice with phosphate-buffered saline (PBS). Cells were incubated with 1 and 5 μM of *N*-hydroxy-PhIP, 100 μM PhIP or D-SFM (control) at 37°C in a humidified atmosphere of 5% CO_2 for 24 h prior to analysis of cell death. In studies examining the effect of PhIP on MAP kinase and Bcl-2 expression at early time periods (up to 180 min), cells were 75% confluent before being placed in SFM (also devoid of antibiotic and other supplements) for 24 h. PhIP (100 μM) was then added for the indicated time. In preliminary studies using the MTS cell viability assay (Promega, Madison WI) we determined that incubation of MCF-10A cells in SFM for at least 24 h with 100 μM PhIP or 5 μM *N*-hydroxy-PhIP was not toxic to the cells.

Quantitation of cell death. The percentage of dead cells was quantitated by the trypan blue dye-exclusion method of Pastorina *et al.* (30). Floating and adherent cells were collected from the dishes. Adherent cells were detached from the plates by trypsinization. Cells were spun at 1000 rpm for 10 min, and the cell pellet was resuspended in SFM. Subsequently, 100 μl of a 0.5% trypan blue solution was mixed with 100 μl of the cell suspension. Viable and nonviable cells were counted on a hemocytometer with a minimum of 500 total cells counted.

Detection of apoptotic cells. Apoptotic cells were visualized by propidium iodide staining and fluorescence microscopy (31). MCF-10A cells were seeded on polystyrene 2-well chamber slides, grown to confluency, and treated as described above. The cells were fixed for 30 min in a mixture of methanol-and acetic acid mixed in a ratio of 3:1 and stained with a solution of propidium iodide.

Western blotting. Antibodies were used in Western blotting to detect and quantitate the expression of Bcl-2 (N-19, Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-x_L (B22630, Transduction Laboratories, Lexington, KY), the phosphorylated plus unphosphorylated forms of ERK-2 (D-2, Santa Cruz Biotechnology, Santa Cruz, CA), and the phosphorylated forms of ERK 1 (p44) and 2 (p42) (phospho-ERK, E-4, Santa Cruz Biotechnology, Santa Cruz, CA). Western blotting was carried out by methods recommended by Santa Cruz Biotechnology (Santa Cruz, CA). Briefly, cells were rinsed twice with ice-cold PBS and lysed on ice in buffer containing PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 10 mg/ml phenylmethylsulfonyl fluoride, 30 μg/ml aprotinin and 100 mM sodium orthovanadate. Nuclei and unlysed cellular debris were removed by centrifugation at 15,000*g* at 4°C for 20 min. Protein content in the lysate was determined by the Bradford method (32). Proteins (25 μg) were fractionated on 10% or 10-20% gradient Tris-Glycine gels (Novex, San Diego, CA), transferred to a nitrocellulose membrane, blocked in 10 mM Tris–HCl, pH 8.0; 150 mM NaCl containing 0.05% Tween 20 and 5% nonfat dry milk at room temperature for 1 h, washed three times with the same solution and immunoreacted with antibody. The membranes were then washed again three times and incubated with the secondary antibody for 2 h at room temperature. Protein bands were visualized by the enhanced chemiluminescence detection system (Amersham Life Sciences, Buckinghamshire, England) and band intensities were quantitated using ImageQuant software (version 3.3). Expression was routinely normalized to actin (Ab-1, Oncogene Research Products, Cambridge, MA).

^{32}P-postlabeling analysis. DNA was isolated from control and carcinogen treated MCF-10A cells by a standard phenol extraction method. ^{32}P-postlabeling analysis was performed by the intensification (ATP-deficient) method as previously described (33).

Statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA). All statistical analyses were performed by using SigmaStat software program (version 2.0, Jandel Scientific Software, San Rafael, CA).

RESULTS

Consistent with previous studies describing the induction of apoptosis with serum and growth factor deprivation of confluent MCF-10A cells (27, 28), confluent MCF-10A cells exhibited characteristic cytoarchitectural features indicative of apoptotic cells after 24 h in SFM. Propidium iodide staining and fluorescence microscopy verified the presence of apoptotic nuclei (Fig. 1A). The percentage of dead cells was quantitated by trypan blue exclusion (Fig. 1B). At the end of the 24 h incubation in SFM, significantly fewer dead cells were found in dishes treated with PhIP or *N*-hydroxy-PhIP than in the controls (ANOVA, $P < 0.05$).

To further examine whether PhIP and *N*-hydroxy-PhIP inhibited apoptosis, the expression of the anti-apoptotic genes Bcl-2 and Bcl-x_L was quantitated by Western blotting (Fig. 2). The expression of these

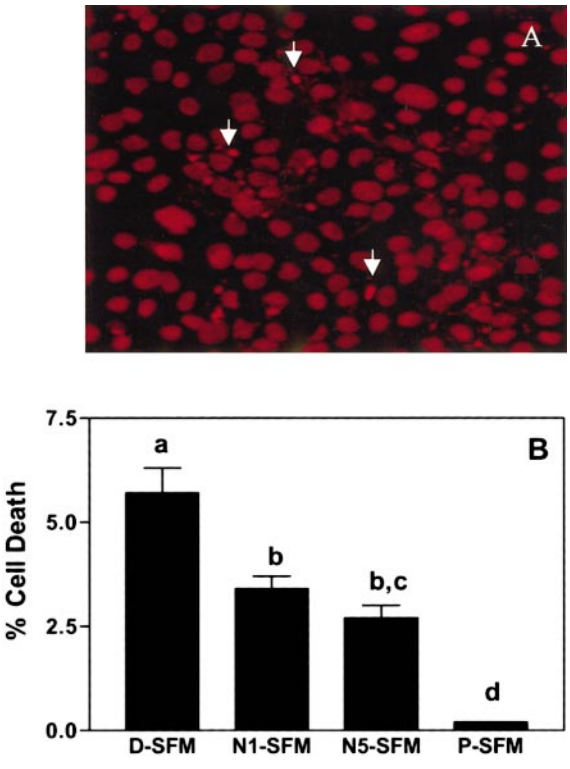

FIG. 1. Cell death in MCF-10A cells. (A) Apoptotic nuclei in MCF-10A cells observed by propidium iodide staining and fluorescence microscopy. Confluent MCF-10A cells were grown for 24 h in SFM prior to detection. (B) Affect of *N*-hydroxy-PhIP and PhIP on cell death in MCF-10A cells as quantitated by the trypan blue exclusion. Confluent MCF-10A cells were grown for 24 h in D-SFM (SFM containing 0.007% DMSO), N1-SFM (1 μM *N*-hydroxy-PhIP), N5-SFM (5 μM *N*-hydroxy-PhIP), P-SFM (100 μM PhIP) as described under Materials and Methods. Values represent the means ± standard error of three separate determinations. Bars with different letters are statistically different (ANOVA, $P < 0.05$).

genes was statistically higher in cells treated with either concentration of *N*-hydroxy-PhIP or with PhIP (ANOVA, $P < 0.05$). In comparison to the control group (D-SFM), the expression of Bcl-x_L was 1.4-, 1.6-, and 1.9-fold higher in 100 μM PhIP, 5 μM *N*-hydroxy-PhIP, and 1 μM *N*-hydroxy-PhIP, respectively. Bcl-2 expression was 1.2- to 1.3-fold higher in the PhIP and *N*-hydroxy-PhIP treated cells than in the control group.

N-Hydroxy-PhIP formed DNA adducts in MCF-10A cells after a 24-h incubation in SFM at the 5 μM concentration, however, adducts were not detected at 1 μM (Table 1). PhIP did not produce detectable adduct levels even at 100 μM. PhIP-DNA adduct formation by *N*-hydroxy-PhIP was considerably lower in MCF-10A cells incubated in SFM than in cell incubated in regular medium. For example, at 5μM *N*-hydroxy-PhIP, adduct levels were 6.5-fold lower in cells incubated SFM than in those incubated in regular medium. Whether phase II esterification in the cells is reduced

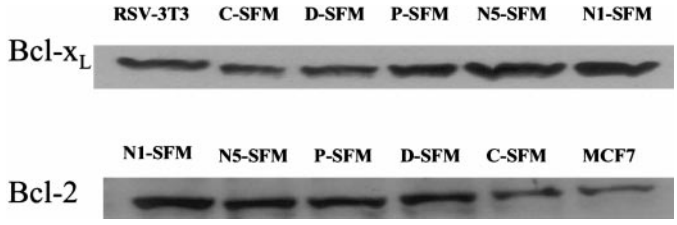

FIG. 2. Bcl-2 and Bcl-x_L expression in MCF-10A cells detected by Western blotting. MCF-10A cells were treated with *N*-hydroxy-PhIP or PhIP in SFM for 24 h as described under Materials and Methods. Lanes are designated N1-SFM (1 μM *N*-hydroxy-PhIP), N5-SFM (5 μM *N*-hydroxy-PhIP), P-SFM (100 μM PhIP), and D-SFM (SFM with 0.007% DMSO). Also included are cells incubated in SFM without 0.007% DMSO (C-SFM). Lysates from RSV-3T3 and MCF-7 cells were used as markers for the Bcl-X_L and Bcl-2 proteins, respectively. Band intensities were quantitated from three gels. For either gene, statistically significant differences were observed in the mean expression between D-SFM (carcinogen control) and cells treated with carcinogen (ANOVA, $P < 0.05$). A statistically significant difference between C-SFM and D-SFM was observed with Bcl-2 but not Bcl-x_L.

under conditions of serum and growth factor deprivation may require further study.

Western blot with the phospho-specific antibody to MAP kinase which detects only the active phosphorylated isoforms of ERK1 (p42) and ERK2 (p44) detected increased levels of phospho-ERK2 after PhIP exposure of MCF-10A cells. The level of phosphorylated ERK2 in MCF-10A cells as shown by Western blot analysis increased with time being maximal at 120 and 150 min after incubation with 100 μM PhIP (Fig. 3). The total level of ERK2 (phosphorylated plus unphosphorylated) was unchanged during this time period and effectively served as a loading standard. Concomitant with the increase in phosphorylated ERK2, there was a parallel increase in the expression of Bcl-2 over the 150 min time period after the addition of PhIP to the cultures.

TABLE 1

PhIP–DNA Adduct Levels in MCF-10A Cells

Carcinogen	Concentration (μM)	Serum and growth factors	Adducts (RAL × 10^7) (mean ± SD, $n = 2$)
N-Hydroxy-PhIP	1	no	ND
	1	yes	0.03 ± 0.001
N-Hydroxy-PhIP	5	no	0.92 ± 0.08
	5	yes	6.00 ± 0.15
PhIP	100	no	ND
	100	yes	ND

Note. Cells were incubated with *N*-hydroxy-PhIP or PhIP for 24 h in either complete medium or medium without serum and growth factors (SFM). DNA adducts were measured by the ^{32}P-postlabeling assay using intensification conditions. ND, not detectable. RAL, relative adduct labeling. Limit of detection, 1.5–3 adducts per 10^9 nucleotides. Adduct profiles were identical to those reported previously (12, 31).

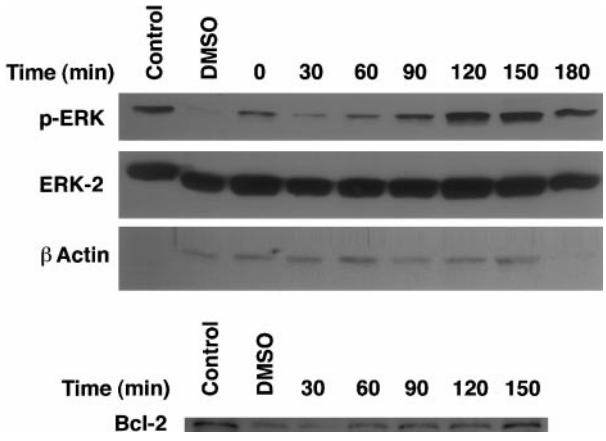

FIG. 3. Phospho-ERK, ERK-2, and Bcl-2 expression in MCF-10A cells incubated with 100 μM PhIP for the indicated times in minutes. DMSO, 0.007% DMSO (carcinogen control) for the maximum time period shown. Control, designates the positive control for the proteins.

DISCUSSION

Our previous studies showed that PhIP exposure can retard mammary gland involution in rats apparently by inhibiting apoptosis (18). The death of MCF-10A cells under conditions of serum and growth factor deprivation has been described previously as providing an *in vitro* model for apoptosis in the involuting mammary gland (27, 28). In both the *in vitro* model and the involuting mammary gland, disruption of cell-cell and cell-matrix interactions are associated with apoptosis (25–28). In addition, both processes are p53-independent (25–28). The similarity of the *in vitro* model to involution made this model useful to further explore whether PhIP and *N*-hydroxy-PhIP could directly inhibit apoptosis in the mammary gland. Our results show that both PhIP and *N*-hydroxy-PhIP decreased the percentage of dead cells in cultures of MCF-10A cells starved of serum and growth factors. Propidium iodide staining confirmed the presence of apoptotic nuclei after starvation. Both PhIP and *N*-hydroxy-PhIP increased the levels of Bcl-2 and Bcl-x_L, antiapoptotic proteins, further supporting that PhIP and *N*-hydroxy-PhIP were associated with an inhibition of apoptosis. The inhibitory effects of PhIP exposure on the mammary gland involution *in vivo* was previously explained as being partially due to an indirect action of PhIP on increasing serum prolactin levels (18). The results from the current study are consistent with the notion that PhIP and *N*-hydroxy-PhIP have a direct effect on inhibiting apoptosis in human mammary epithelial cells that may also explain the inhibition of apoptosis *in vivo*.

Incubation of MCF-10A cells with *N*-hydroxy-PhIP, but not PhIP, produced DNA adducts. PhIP-DNA adduct formation with *N*-hydroxy-PhIP was relatively low under serum-free conditions, a finding that may also explain the lack of apparent toxicity. With other chemicals, DNA adduct formation has been shown to induce p53 which can lead to G1 arrest or an induction of apoptosis rather than an inhibition (34). At the concentrations used here, *N*-hydroxy-PhIP, but not PhIP, did cause an induction of p53 in MCF-10A cells as detected by Western blotting (data not shown). Since apoptosis in the *in vitro* model is p53 independent, it is plausible that the effect of PhIP-DNA adduct formation on p53 induction did not impact the effect of *N*-hydroxy-PhIP on apoptosis seen in this model. Indeed, irrespective of DNA adduct formation, PhIP and *N*-hydroxy-PhIP were both associated with the inhibition of apoptosis in MCF-10A cells.

To address the possible mechanisms by which PhIP might inhibit apoptosis MCF-10A cells, we looked at components of the MAP kinase pathway. This pathway is a collection of cytoplasmic protein kinases involved in the transduction of a variety of extracellular signals, such as growth factor signals, to regulatory molecules in the cytoplasm and nucleus (35). The MAP kinases including ERK1 and ERK2 are involved in the regulation of various cellular processes including proliferation, differentiation, and development (35). Recent studies have also demonstrated the involvement of the MAP kinase cascade in apoptosis (36–40). Studies carried out with a variety of cell lines have indicated that ERK phosphorylation is associated with the inhibition of apoptosis (36–40). In the current study, we demonstrate that PhIP increased the expression of the activated phosphorylated form of ERK2. A time course study revealed that the increased expression of phosphorylated ERK2 occurred concomitantly with the increased expression of the antiapoptotic protein Bcl-2 (Fig. 3). Similar to the findings reported here, activated ERK2 kinase has been shown to increase the expression of Bcl-2 apparently accounting for the inhibitory effects of nicotine on apoptosis in human lung cells (39). The results from our study are consistent with the notion that PhIP inhibits apoptosis via an increased activity of ERK2.

The mechanisms involved in the increased level of phosphorylated ERK2 by PhIP is not entirely known. To date, a diverse group of xenobiotics has been shown to increase the activity of MAP kinases including DDT, asbestos, nicotine, and tumor promoters (38, 39, 41, 42). A growing body of evidence indicates that certain environmentally prevalent compounds may act as xenomitogens by mimicking or modulating the activity of growth factor receptors including c-erbB2, c-met, epidermal growth factor receptor, and the insulin-like growth factor receptor and subsequently their signaling pathways (41–43). For example, benzo[*a*]pyrene and 2,3,7,8- tetrachlorodibenzo-*p*-dioxin alter tyrosine phosphorylation and insulin-like growth factor signaling pathways in MCF-10A cells (43). Therefore, the

question of whether the locus of action of PhIP is at the level of modulation of growth factor signals upstream of the MAP kinases requires further study.

A common property shared by known and suspected tumor promoters is their ability to block the process of apoptosis, and components of the ERK signaling cascade appear to provide important molecular targets for tumor promoters (20, 38, 39, 44). The inhibition of apoptosis in human mammary epithelial cells by PhIP is a hitherto unrecognized action of this compound which implicates PhIP as a factor in tumor promotion and progression. The findings from this study raise the possibility that the parent amine itself might have properties that could potentially influence mammary carcinogenesis. Further studies are warranted to test this hypothesis.

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