Abstract

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is a mammary gland carcinogen in cooked meat. Using the HC11 mouse mammary epithelial cell line, a well-characterized model for hormone-mediated differentiation, we examined whether PhIP altered the expression of genes regulated by lactogenic hormones dexamethasone, insulin, and prolactin (DIP). When HC11-Lux cells (stably transfected with a β-casein promoter luciferase construct) were cultured in DIP-containing medium, PhIP (100 μM) enhanced luciferase activity 11-fold over that observed in DIP medium alone. The effect of PhIP on augmenting luciferase activity was observed only when lactogenic hormones were included in the medium. Expression of the endogenous β-casein gene was also higher in HC11 cells treated with PhIP in hormone-enriched medium. With the increased expression of β-casein gene, the level of phospho-signal transducer and activator of transcription 5A (phospho-STAT5A), the transcription factor regulating β-casein gene expression, was elevated in PhIP-exposed HC11 cells. AG490, a Janus kinase 2 (JAK2)-specific inhibitor, blocked the effect of PhIP on β-casein gene expression. PhIP-treated cells also showed higher expression of Bcl-2 and lower expression of Bax, consistent with a possible antiapoptotic action of PhIP. The findings indicate that PhIP modulates lactogenic hormone-mediated gene expression in mammary epithelial cells, apparently via enhanced phosphorylation of STAT5A. The findings have implications for a novel mechanism of action of the mammary gland carcinogen PhIP.

Introduction

Cultured HC11 cells, derived from midpregnant BALB/c mouse mammary gland tissue, provide a well-established in vitro model system for studying signal transduction pathways and hormonal/growth factor regulation of mammary epithelial cell differentiation (1–9). HC11 cells have retained the characteristics of normal mammary epithelial stem cells. After growth to confluence in the presence of insulin and epidermal growth factor, HC11 cells respond to stimulation by the lactogenic hormones DIP2 undergoing differentiation with a synthesis of milk proteins such as β-casein, a marker of mammary gland terminal differentiation (1, 2, 5, 6). Several transcription factors regulate milk protein gene expression; among them, is the STAT5A (5, 10–14). STAT5A is activated by a variety of cytokines, growth factors, and hormones (reviewed in 15). It is the principal transcription factor mediating the action of prolactin on β-casein gene expression (5, 11, 16–18). The binding of prolactin to its receptor induces receptor dimerization, transphosphorylation and activation of JAK2. JAK2, in turn, phosphorylates STAT5A, which, on dimerization and translocation to the nucleus, binds to specific sequences of target gene promoters activating gene transcription. In addition to regulating β-casein transcription, STAT5A is recognized as an important transcription factor influencing the expression of genes involved in a variety of critical cellular processes in the mammary gland, including proliferation, apoptosis, and oncogenesis (19–22).

PhIP is currently recognized as the principal carcinogenic heterocyclic amine present in the human diet, especially in well-done cooked meat (23, 24). PhIP is a mammary gland carcinogen in rats, and recent population studies support the possibility that PhIP may be an etiological factor in human breast cancer (25–30). Previous studies carried out in our laboratory have suggested that PhIP might alter signal transduction in mammary epithelial cells as a mode of action in mammary carcinogenesis (31). We recently observed that lactating rats given PhIP on weaning show retardation of mammary gland involution (32). PhIP was shown to facilitate the maintenance of the differentiated lobular alveolar structures and to inhibit involution-associated apoptosis, an effect that was akin to a “prolactin-like” action of PhIP on the mammary gland (32). PhIP was also found to inhibit serum starvation-induced apoptosis in cultured human mammary
induction of luciferase activity in HC11-Lux cells treated with various concentrations of PhIP (25, 50, 100, and 200 μM) in DM was compared with the expression observed in control cells in DM (Fig. 1B). This concentration of PhIP was chosen because it was not toxic to the cells, as indicated by proliferation assays (data not shown), and it induced a high level of reporter-gene activity. A significant elevation in luciferase expression with PhIP exposure was observed as early as 6 h and continued to increase over 48 h in DM. At 48 h, cells treated with PhIP in DM showed a more than 11-fold higher reporter-gene activity than control cells in DM.

To further confirm that PhIP exposure influenced hormone-mediated gene expression, we also examined the expression of the endogenous β-casein gene by Northern blot analysis. β-Casein gene expression is a well-recognized marker of mammary epithelial cell differentiation induced by lactogenic hormones (1, 18). After 6, 24, and 48 h of exposure, competent cells treated with 100 μM of PhIP in DM showed elevated levels of β-casein RNA in comparison to control cells in DM. The expression of β-casein in the presence of PhIP showed a time-dependent increase from 6 to 48 h (Fig. 1, C and D). The induction of β-casein expression paralleled the induction of luciferase activity driven by the β-casein promoter (compare Fig. 1C with Fig. 1C).

Specific Hormones Are Required for PhIP-induced Gene Expression. The lactogenic hormones DIP are known to induce β-casein gene expression in HC11 cells (1, 3). We analyzed the influence of these hormones on the ability of PhIP to augment β-casein gene expression in HC11 cells using the β-casein gene promoter-luciferase reporter assay in HC11-Lux cells (4). Competent cells were treated for 48 h with or without PhIP (100 μM) with different combinations of lactogenic hormones in defined medium without serum to examine the effects of specific hormones (Fig. 2). Luciferase activity was exceptionally low in the absence of insulin, irrespective of the presence of PhIP or other hormones. In insulin-only medium, PhIP did not augment luciferase activity over that observed in the absence of PhIP. Interestingly, when either prolactin or dexamethasone was added with insulin, PhIP significantly enhanced luciferase activity above that observed in the absence of PhIP by about 1.9-fold (prolactin) and 1.2-fold (dexamethasone), respectively (Student’s t test, both, P < 0.01). The greatest increase in reporter gene expression with PhIP, however, was observed when HC11-Lux cells were incubated with DIP. Under these hormone conditions, PhIP increased luciferase activity by 3.3-fold, a difference that was statistically significant (Student’s t test, P < 0.01). The addition of serum to the DIP hormone combination even further augmented the induction of gene expression by PhIP to more than 11-fold above control (compare Fig. 1A with Fig. 2).

PhIP Exposure and STAT5A Expression and Activity. Prolactin-induced β-casein gene expression in HC11 cells is mediated by tyrosine phosphorylation of STAT5 (5, 7, 9). Two STAT5 genes exist, STAT5A and STAT5B, which show a 96% homology (34). Studies in HC11 cells have shown that lactogenic hormone treatment leads to tyrosine phosphorylation of STAT5A, a process generally regarded as an in vitro model for involution (33). To better understand how PhIP might inhibit involution and might help to maintain the lobular alveoli and differentiated epithelium, the present study used the HC11 cell model to address whether PhIP modulates hormone-mediated differentiation, including phosphorylation of STAT5A. These studies were also undertaken to gain insight into possible mechanisms relevant to mammary gland carcinogenesis of PhIP.

Results

Induction of β-Casein Gene Expression by PhIP. When competent HC11-Lux cells harboring the β-casein-promoter luciferase construct were treated for 48 h with various concentrations of PhIP (25, 50, 100, and 200 μM) in DM, luciferase activity increased in a concentration-dependent manner (Fig. 1A). At all of the concentrations examined, the presence of PhIP enhanced the luciferase activity over that observed in control cells in DM. The induction of luciferase activity was also analyzed at 1, 3, 6, 24, and 48 h after treatment of HC11-Lux cells with 100 μM PhIP in DM and compared with the expression observed in control cells in DM (Fig. 1B). This concentration of PhIP was chosen because it was not toxic to the cells, as indicated by proliferation assays (data not shown), and it induced a high level of reporter-gene activity. A significant elevation in luciferase expression with PhIP exposure was observed as early as 6 h and continued to increase over 48 h in DM. At 48 h, cells treated with PhIP in DM showed a more than 11-fold higher reporter-gene activity than control cells in DM.
ation of both proteins (7). Of the two isoforms, STAT5A appears to be the predominant one regulating mammmopoi-
esis (12, 35). Studies in deficient mice have shown that
STAT5A is mandatory for mammmopoiesis and lactogenesis,
whereas STAT5B-deficient mice show relatively mild defects
(12, 35). Therefore, the present study focused on the level of
phospho-STAT5A.

Because β-casein expression and promoter activity was
increased by the addition of PhIP to DIP-containing DM, we
examined whether the effect of PhIP was achieved by in-
creasing the levels of phospho-STAT5A. Western blot anal-
ysis indicated that the level of phospho-STAT5A was 4-fold
higher in cells that were treated with 100 μM PhIP for 48 h
than in control cells in DM (Fig. 3A). Although the level of
phospho-STAT5A was elevated, the total level of STAT5A
was not significantly altered by the addition of PhIP (Fig. 3, B
and D). Consistent with a concentration-dependent effect of
PhIP on β-casein promoter activity (Fig. 1A), the level of
phospho-STAT5A also increased with increasing concen-
trations of PhIP in the medium (Fig. 3C). The intensity of the
phospho-STAT5A band increased 60% from 50 μM to 100
μM PhIP and increased 12% from the 100 μM to 200 μM
concentration. The data indicate that enhanced PhIP-
induced β-casein gene expression in the presence of lactor-
genic hormones is mediated via enhanced phosphorylation
of STAT5A.

Possible Roles of JAK2 and the Raf-1/MEK/ERK Path-
ways in PhIP-enhanced β-Casein Promoter Activity.
JAK2 mediates phosphorylation and hence activation of
STAT5A (16, 17), whereas the Ras/Raf-1/MEK/MAPK kinase
pathway has been shown to negatively regulate STAT5A in
HC11 cells and, hence, negatively regulate β-casein expres-
sion (7–9). Therefore, we examined whether the effect of
PhIP on β-casein promoter activity, as a measure of phos-
pho-STAT5A levels, might potentially involve alterations in
JAK2 and the MAPK kinase pathway. Specific inhibitors
AG490 and PD98059 for JAK2 and MEK, respectively, were
used in HC11-Lux cells, and β-casein promoter activity was
measured by the luciferase assay. At concentrations of ≥25
μM, AG490, an inhibitor of JAK2, inhibited luciferase activity
in PhIP-treated HC11 cells (Fig. 4A). A >50% inhibition of
luciferase activity was observed at 25 μM AG490 in PhIP-
treated cells. At inhibitor concentrations of ≥50 μM, the level
of expression in PhIP-treated cells was approximately the
same as that observed in HC11 cells in DM without inhibitor,
and only basal levels of luciferase expression were detected
in PhIP-treated cells. The inhibitor, at concentrations of ≥25
μM, also reduced DIP-mediated luciferase expression in con-
control cells to near basal levels.

Use of the MEK-specific inhibitor PD98059 increased the
β-casein promoter activity in cells treated with or without
PhIP in DIP medium (Fig. 4A). Notably, the highest luciferase
expression was observed in HC11-Lux cells treated with
PhIP (100 μM) plus PD98059 inhibitor (10–50 μM). Indicative
of a synergic interaction, luciferase expression in PhIP-
treated cells containing ≥0 μM of inhibitor was ~5-fold
higher than that observed in the cells treated only with PhIP.
At a concentration above 10 μM, the inhibitor also aug-
mented luciferase activity observed in control cells (without
PhIP). By Western blot analysis, the level of raf-1 and phos-
pho-ERK were lower in cells that were treated with 100 μM
PhIP for 48 h than in control cells (Fig. 4C).

Bcl-2 and Bax Protein Expression in HC11 Cells Ex-
posed to PhIP. There is recent evidence supporting the
hypothesis that phospho-STAT5A regulates cell survival and
apoptosis (20, 36). The expression of the Bcl-2 and Bax was
examined in HC11 cells incubated in DM with and without
PhIP (100 μM) for 48 h. By immunocytochemistry, both Bcl-2
and Bax proteins were localized in the cytoplasm of the
HC11 cells (Fig. 5A–D). The mean number of Bcl-2 expres-

Fig. 3. Western blot analysis of phospho-STAT5A and STAT5A expression in competent HC11 cells treated with PhIP in DM that contained lactogenic hormones, DIP, A, levels of phospho-STAT5A in cells treated with 100 μM PhIP or DMSO (control). Two representative incubations are shown. B, levels of total STAT5A in cells treated with 100 μM PhIP or DMSO (control). C, levels of phospho-STAT5A in cells treated with different concentrations of PhIP. D, level of total STAT5A protein in cells treated with different concentrations of PhIP. All incubations with PhIP or DMSO were carried out for 48 h in DM containing DIP.

Fig. 2. Effect of specific lactogenic hormones on the induction of β-
casein promoter activity by PhIP. Competent cultures of HC11-Lux cells
were treated by different combinations of lactogenic hormones with and
without 100 μM of PhIP as indicated, and the β-casein promoter activity
was analyzed using luciferase assay. No serum was added to the medium
in this analysis. Values are the means ± SE of four determinations.
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specific inhibitor PD98059. Concentrations of either the JAK2-selective inhibitor AG490 or the MEK-100 Western blot analysis of raf-1 and phospho-ERK levels in cells exposed to means/H11006/sion cells detected per /H9252/sion of the apoptotic protein Bax in PhIP-treated and control

Fig. 4. Possible role of the MAPK and JAK/STAT pathways in the augmentation of β-casein gene promoter activity by PhIP. Competent HC11 cells were treated with (+) or without (−) PhIP (100μM) and indicated concentrations of either the JAK2-selective inhibitor AG490 or the MEK-specific inhibitor PD98059. β-Casein promoter activity was assayed after 48 h. A, effect of AG490 and/or PhIP on β-casein promoter activity. B, effect of PD98059 and/or PhIP on β-casein promoter activity. C and D, Western blot analysis of raf-1 and phospho-ERK levels in cells exposed to 100 μM PhIP (or DMSO control) for 48 h. In A and B, values are the means ± SE of four determinations.

Discussion

The present study shows that PhIP affects hormone-mediated cell signaling that augments the effect of lactogenic hormones on β-casein promoter activity and gene expression in HC11 mammary epithelial cells (Fig. 1). The effect of PhIP on β-casein promoter activity was observed only in combination with specific lactogenic hormones, requiring, at a minimum, insulin plus prolactin or dexamethasone to significantly enhance β-casein promoter activity (Fig. 2). With the addition of all three lactogenic hormones, or DIP, PhIP further increased in β-casein promoter activity, and the greatest synergism was observed when PhIP was combined with the three hormones plus serum (Fig. 1). Serum apparently contains additional factors that facilitate β-casein gene transcription. It is well recognized that multiple regulatory elements on the β-casein promoter bind factors that have both positive and negative effects on transcription, and it is known that multiple signaling pathways converge in the activation of the β-casein gene promoter (2, 6–10, 14). The results from this study show that PhIP participates in this regulation and activation in conjunction with specific hormones.

There are potentially multiple mechanisms involved in the modulation of hormone-mediated gene expression by PhIP. Findings from the current study indicate two possible mechanisms by which PhIP augments lactogenic-hormone driven β-casein gene expression. First, the action of PhIP may be, in part, mediated via a down-regulation of the Ras/raf/MEK/MAPK pathway, a pathway that is previously shown to negatively regulate β-casein gene expression in HC11 cells (7–9). Indeed, as was reported previously (7), we found that the MEK inhibitor PD98059 significantly increased β-casein gene activity when added to HC11 cell medium containing lactogenic hormones (Fig. 4). The possible role of the MAPK pathway in mediating the PhIP effect is suggested by the observation that PhIP-treated HC11 have a lower expression of raf-1 and the phosphorylated forms of ERK1 and ERK2. Thus, the partial relief of the negative-regulation imparted by the MAPK pathway may be one mechanism by which PhIP enhanced β-casein gene expression. However, because β-casein promoter activity was at least 2-fold higher in HC11
cells treated with PhIP plus MEK inhibitor PD98059 than MEK inhibitor alone (10–50 μM; Fig. 4), the data further suggest that an additional pathway contributes to the effect of PhIP on β-casein gene transcription.

A second, and perhaps predominant, route by which PhIP enhances β-casein promoter activity and gene expression appears to be via phosphorylation of the transcription factor STAT5A. Western blotting showed that phospho-STAT5A was higher in HC11 cells treated with PhIP than in control cells (Fig. 3). In addition, the increase in phospho-STAT5A with PhIP exposure was concentration-dependent, and it paralleled the concentration-dependent increase in β-casein gene transcription. STAT5A is phosphorylated via the JAK-STAT5A pathway, a major route for prolactin-mediated β-casein gene transcription (16–18). The use of the JAK2 inhibitor AG490 completely attenuated the effect of PhIP on β-casein promoter activity. These data support the notion that the action of PhIP on modulating lactogenic hormone gene expression is largely mediated via the JAK/STAT5A pathway.

STAT5A is a critical transcription factor involved in mammary gland development and lactogenesis (12, 17). STAT5A phosphorylation markedly declines within 12 h after weaning, which suggests a role for STAT5A in the onset of involution (22, 37). PhIP has been shown to retard involution of the rat mammary gland (32). Although this has been attributed, at least in part, to an increase in serum prolactin in rats treated with PhIP (32), the findings from the present study support the possibility of a direct action of PhIP on levels of phospho-STAT5A in vivo as a possible mechanism for the inhibitory effect on involution. In light of multiple genes regulating involution (36, 37), additional studies are needed to clarify the locus of action of PhIP on the inhibition of involution in the rat mammary gland. It is notable that treatment with PhIP during mammary gland involution induces mammary tumors, and, although the tumors are largely adenomas, it is tempting to speculate that the alterations induced by PhIP in cell signaling, including STAT5A phosphorylation status, might play a role in PhIP carcinogenesis.

Recent studies have indicated that STAT5A is a survival factor in the mammary gland (22). Therefore, the ability of PhIP to enhance phosphorylation of STAT5A suggests that PhIP also potentially functions as a survival factor in mammary epithelial cells via increased phospho-STAT5A. To further examine this possibility, the expression of Bax and Bcl-2 was examined in HC11 cells exposed to PhIP. We detected a higher expression of the antiapoptotic protein Bcl-2 and a lower expression of the proapoptotic protein Bax in HC11 cells exposed to PhIP (Fig. 5), consistent with a possible survival function of PhIP in these cells. These present findings also concur with previous data indicating that PhIP...
inhibits serum-starvation-induced apoptosis in MCF10A cells, a human mammary epithelial cell line (31). However, unlike the situation in MCF10A cells, PhIP produces DNA adducts in HC11 cells; nevertheless, we observed no toxicity in HC11 cells at the concentrations of PhIP examined. Although carcinogen-DNA adducts have been implicated in inducing apoptosis via induction of p53 (38), the p53-null background in HC11 cells (33) may, in part, explain why the formation of PhIP-DNA adducts did not trigger this response. The formation of PhIP-DNA adducts in HC11 cells indicates that PhIP is metabolically activated via N-hydroxylation and Phase II esterification (39). Because induction of luciferase activity with increasing concentrations of PhIP appears to parallel the concentration-dependent formation of PhIP-DNA adducts (compare Fig. 1A with Fig. 6), the data suggest that a reactive metabolite may account for the effects of PhIP on cell signaling. However, additional studies are required to confirm whether a specific metabolite is responsible for the alterations observed in gene expression in HC11 cells.

A growing body of evidence indicates that environmental contaminants may act as xenobiotics by mimicking or modulating the activity of various cell signaling pathways (31, 40–42). For example, the pesticide 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; dichlorodiphenyltrichloroethane (DDT) has been shown to stimulate STAT1 tyrosine phosphorylation in human breast epithelial cells (42). Our report is the first to show that PhIP alters hormone-mediated responses in mammary epithelial cells, increasing the levels of phospho-STAT5A, a critical transcription factor regulating mammary gland development. Speculatively, these aberrant responses that are triggered in the presence of PhIP could facilitate abnormal gene regulation that under certain circumstances might facilitate mammary carcinogenesis. For example, the ability of PhIP to induce phospho-STAT5A and facilitate cell survival concomitant with PhIP-DNA adduct formation, as observed in HC11 cells, might be expected to increase the likelihood of malignant transformation. Additional studies are needed to better understand the effects of PhIP on hormone-mediated signal transduction and the possible implications for mammary carcinogenesis.

Materials and Methods

Materials. PhIP was purchased from Toronto Research Chemicals (North York, Ontario, Canada). JAK2-selective inhibitor AG490 and MEK inhibitor PD98059 were obtained from Calbiochem (San Diego, CA) and New England Biolabs (Beverly, MA), respectively. Protein G plus-Sepharose beads and the following antibodies: STAT5A (L-20); ERK-1 (C-16); phospho-ERK (E-4); ras-f1 (E-10); Bax (N-20); Bcl-2 (N-19); and rabbit secondary antibody, were all purchased from Santa Cruz Biotechnology (St. Louis, MO). Antiphosphotyrosine antibody (P-Tyr-100) was obtained from Cell Signaling Technology (Beverly, MA). Mouse secondary antibody was obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell Culture and PhIP Treatment. HC11 mammary epithelial cell line (a subclone of COMMA-1D cells) and HC11-Lux cells [HC11 cells stably transfected with a β-casein promoter luciferase construct (p-344/−1βc-Lux; Ref. 4)] were kindly provided by Dr. David S. Salomon (National Cancer Institute, Bethesda MD) and Dr. Nancy E. Hynes (Friedrich Miescher Institute, Basel, Switzerland). Both cell lines were routinely maintained in growth medium, which consisted of RPMI 1640 (Life Technologies, Inc., Rockville, MD), 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.), 5 μg/ml bovine insulin (Life Technologies, Inc.), 10 ng/ml epidermal growth factor (Life Technologies, Inc.) and 50 μg/ml gentamicin (Life Technologies, Inc.). Cells were grown to 100% confluence and maintained in growth medium for 1 to 2 days. At this stage, the cells were competent to respond to lactogenic hormones. Competent cells were cultured in DM in the presence of PhIP (or DMSO for control). The DM contained RPMI 1640, 10% heat-inactivated fetal bovine serum, 5 μg/ml bovine insulin, 1 μM dexamethasone (Sigma Chemical Co., St. Louis, MO), 5 μg/ml bovine prolactin (Sigma Chemical Co.) and 50 μg/ml gentamicin. Inhibitors and PhIP were used at concentrations which were not toxic to the cells.

β-casein Gene Promoter Activity Assay. HC11-Lux cells were cultured in 2-cm-diameter dish and harvested at various times after PhIP treatment. The β-casein gene promoter activity was assayed on triplicate samples using the luciferase assay system (Promega, Madison, WI) according to the manufacturer’s protocol. The light emission (expressed as light units) was measured on a luminometer. The β-casein promoter activity was expressed as light units/μg protein.

Western Blot and Immunoprecipitation Analysis. Cells were washed twice with PBS and collected in protein lysis buffer containing 1% NP40, 50 mM Tris-HCl (pH 7.5), 140 mM NaCl, 2 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 0.2 units/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4. Cells were lysed on ice for 30 min. The supernatant from the whole cell lysate was used for Western blot analysis and immunoprecipitation. Protein concentration was determined using bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). For Western blot analysis, equal amounts of protein (20–100 μg) were separated in 8% or 12% precasted Tris-glycine gel and transferred to polyvinylidene difluoride membranes (Novex, San Diego, CA). The membranes were blocked using 5% nonfat dry milk for 1 h and were hybridized for 2 h at room temperature or overnight at 4°C with primary antibodies. After washing with TBS-T buffer [20 mM Tris (pH 8.0), 150 mM NaCl, and 0.1% Tween 20], the membrane was rehybridized with mouse or rabbit secondary antibody for 1 h. Specific protein signals were detected using an enhanced chemiluminescence kit (ECL; Amersham). For immunoprecipitation analysis, equal amounts of protein (600–900 μg) were first incubated with 0.25 μg of the control rabbit IgG and 20 μl of protein G plus-agarose conjugate at 4°C for 30 min. The agarose beads were pelleted and the supernatants were then incubated with anti-STAT5A antibody at 4°C overnight. The protein was collected with agarose and dissolved in 15 μl of protein loading buffer by boiling for 3 min. Ten μl were loaded onto 8% Tris-glycine gel and transferred to polyvinylidene difluoride membrane. The membranes were first used to detect phosphorylation status of STAT5A using antibody against phosphotyrosine. Then the membranes
were stripped and reprobed with STAT5A antibody. Density of the bands were quantified using Molecular Dynamics ImageQuant software (version 5.1).

**Immunocytochemistry.** The expression of the apoptosis-related proteins Bcl-2 and Bax was detected using a direct immunocytochemical method. Briefly, PhIP-treated and control cells were fixed in 10% neutralized formalin for 10 min and then rinsed in PBS buffer with 0.1% Triton X-100 and 1% horse serum for 30 min. The cells were incubated sequentially with primary (Bcl-2 and Bax; 1:100 dilution) and horseradish peroxidase-labeled secondary antibody (1:2000 dilution) for 1 h each. Sections were developed using diaminobenzidine (DAB)-Tris solution with 0.3% hydrogen peroxide and counterstained with 1% methyl green. At least three replicate slides for both PhIP-treated and control cells were stained. Positive cells were counted in at least 10 x20 magnification fields and Bcl-2 and Bax levels were expressed as the mean (± SE) of the positive-stained cells per field.

**Northern Blot Analysis.** Total RNA from the PhIP-treated and control HC11 cells was isolated using TRIZol reagent (Life Technologies, Inc., Gaithersburg, MD). Twenty-five μg of total RNA were separated on a 1% agarose-formaldehyde gel. Loading of the RNA was evaluated after ethidium bromide staining. The RNA was transferred to nylon membranes (Micron Separations, Westboro, MA) and hybridized overnight to a β-casein probe. The probe was an 800-bp β-casein gene cDNA insert in the pFlag vector, provided by Dr. Nancy E. Hynes. The probe was 33P-labeled using the HotPrime DNA labeling kit (GenHunter, Nashville, TN).

**DNA Adduct Analysis.** Genomic DNA was extracted from PhIP-treated and control HC11 cells using phenol/chloroform. PhIP-DNA adducts were analyzed using 32P-postlabeling method as described previously (43). The intensification (ATP-deficient) method was used to resolve PhIP-DNA adducts as [32P]ATP-labeled bis-phosphonucleotide adduct “fingerprints” on autoradiograms after chromatography. Adduct levels were quantified by Cerenkov counting. DNA adducts were expressed as relative adduct labeling.

**Statistical Analysis.** The statistical significance between treated cells and controls were analyzed using SigmaStat 2.0 statistical software.

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References


