Activation of the Aromatic Hydrocarbon Receptor Pathway Is Not Sufficient for Transcriptional Repression of BRCA-1: Requirements for Metabolism of Benzo[a]pyrene to 7r,8t-Dihydroxy-9t,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene

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ABSTRACT

Reduction of BRCA-1 expression through nonmutational events may be a predisposing event in the onset of sporadic breast cancer. In this study, we investigated the mechanisms through which the environmental carcinogen benzo[a]pyrene (B[a]P) lowered BRCA-1 mRNA levels in breast cancer MCF-7 cells. We report that B[a]P does not compromise the stability of BRCA-1 mRNA, but represses transcriptional activity of a 1.69-kb BRCA-1 (pGL3-BRCA-1) promoter fragment that contains both exon-1A and exon-1B transcription start sites. The loss of BRCA-1 promoter activity was accompanied by accumulation of CYP1A1 and BAX-α mRNA and p53 and p21 protein, whereas levels of Bcl-2 mRNA were reduced. The aromatic hydrocarbon receptor ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is not metabolized, did not affect BRCA-1 promoter activity or the cellular levels of BRCA-1 and p53 protein, but it did induce a CYP1A1-like promoter. Conversely, treatment with the B[a]P metabolite 7r,8t-dihydroxy-9t,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) repressed BRCA-1 promoter activity and protein, while increasing p53 and p21 protein levels. Transient expression of dominant-negative p53 (175 Arg→His) counteracted the detrimental effects of BPDE on BRCA-1 promoter activity and protein levels. Similarly, treatment with B[a]P, TCDD, or BPDE failed to repress transcription from the pGL3-BRCA-1 construct transfected into ZR75.1 breast cancer cells containing mutated p53 (175 Pro→Leu). We conclude that activation of the aromatic hydrocarbon receptor is not sufficient for down-regulation of BRCA-1 transcription, which is, however, inhibited by the B[a]P metabolite BPDE through a p53-dependent pathway.

INTRODUCTION

The characterization of many germ-line mutations in familial breast and/or ovarian cancers has confirmed the role of BRCA-1 as a tumor suppressor gene (1–3). Nevertheless, only a small fraction of sporadic ovarian tumors (4) and no sporadic breast cancers (5) have been shown to harbor mutations in the BRCA-1 gene. These observations are indicative that alternative mechanisms other than coding mutations need to be considered for BRCA-1-mediated oncogenesis (6).

Loss of BRCA-1 expression may result from exposure to DNA-damaging agents (7) and methylation at 5’CpG islands in the BRCA-1 gene (6, 8, 9). We have directed our attention toward investigating the role of environmental xenobiotics such as PAHs3 as epigenetic disruptors of BRCA-1 expression. PAHs are classic DNA-damaging and tumor-promoting agents found in industrial pollution, auto exhaust, tobacco smoke, and coal tar (10). Exposure to PAHs elicits a number of genotoxic responses, including mammary tumors in rodents (11), oxidative damage (12), DNA adduct formation, (13–15), and base substitutions (16–18). We (19, 20) and others (21) have documented that estrogen increased cellular levels of BRCA-1 mRNA and protein in breast and ovarian cancer cells. In contrast, our group has recently reported that acute and chronic exposure to B[a]P, a prototype PAH, lowered both constitutive and estrogen-dependent expression of BRCA-1 in breast and ovarian cancer cells (22). The reduced potential for BRCA-1 expression correlated with S-phase and G2-M arrest and accumulation of p53, mdm2, and p21. The fact that cotreatment with the AhR antagonist α-naphthoflavone restored normal cell cycle distribution and BRCA-1 expression (23) suggests that the AhR pathway contributes to down-regulation of BRCA-1. In this study, we investigated the mechanisms through which B[a]P lowered BRCA-1 mRNA levels in MCF-7 breast cancer cells. We report that B[a]P repressed transcription of the BRCA-1 promoter in MCF-7 breast cancer, but not in ZR75.1 cells containing mutated p53. However, in MCF-7 cells, activation of the AhR pathway by B[a]P was not sufficient for down-regulation of BRCA-1 expression. Rather, BRCA-1 promoter activity was inhibited by the metabolite BPDE through a p53-dependent pathway.

MATERIALS AND METHODS

Cell Culture and Chemicals. MCF-7 and ZR75.1 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM/F12 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FCS (Hyclone Laboratories, Inc, Logan, UT) as described previously (19). B[a]P and actinomycin D were obtained from Sigma Chemical Co.), BPDE and TCDD were obtained from Midwest Research Institute (Kansas City, MO).

Semiquantitative RT-PCR and RNase Protection Assay. For mRNA studies, cells were plated at a density of 2 × 105 cells/100-mm tissue culture dish and maintained in DMEM/F12 plus 10% FCS. Three dishes were assigned to each experimental treatment. Details concerning the experimental conditions for semiquantitative RT-PCR analysis of BRCA-1 and CYP1A1 mRNA are described elsewhere (22). Briefly, total RNA (400 ng) was incubated with random hexamer primers, Moloney murine leukemia virus reverse transcriptase, RNase inhibitor (Life Technologies, Inc., Gaithersburg, MD), and reverse transcription buffer (Ambion Inc., Austin, TX) at 42°C for 1 h. cDNAs were amplified using the oligonucleotides summarized in Table 1. The amplification products were of the expected sizes, and their authenticity to the GenBank was confirmed by direct sequencing. Preliminary control experiments (data not shown) were carried out to assure that RT-PCR conditions allowed for linear amplification of PCR products.

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3 The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; B[a]P, benzo[a]pyrene; AhR, aromatic hydrocarbon receptor; BPDE, 7r,8t-dihydroxy-9t,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; RT-PCR, reverse transcription-PCR; RLU, relative luciferase unit(s); XRE, xenobiotic responsive element.
For amplification of the internal standard, 18S rRNA (488-bp), we used the Competitor oligonucleotide module from Ambion. The expression levels of BRCA-1 were quantified by Alpha Imagerr (Alpha Innotech Inc, San Diego, CA) analysis and corrected for the expression of the control mRNA (BRCA-1/18S). Details of the RNase protection assay for BRCA-1 mRNA are described elsewhere (22). Briefly, a 162-bp BRCA-1 riboprobe encoding a portion of exon 15 was transcribed in the antisense orientation from the transcription vector Triplescript (Ambion). As internal control for RNase protection assay, we transcribed a riboprobe for human cyclophilin from the pTRicyclphilin vector (Ambion). Relative phosphorimager units for BRCA-1 mRNA were corrected for the expression of the control, cyclophilin mRNA (BRCA-1/cyclophilin).

Western Blotting. Western blotting was performed as described previously (23). Cell extracts were normalized to protein content and separated by 4–12% gradient SDS-PAGE. Immunoblotting was carried out with antibodies against BRCA-1, p53, and p21 (from Oncogene Research Products, Cambridge, MA). Normalization of Western blots was confirmed by incubating immunoblots with β-actin antibody-1 (Oncogene Research Products). The immunocomplexes were detected by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

BRCA-1 Promoter Studies. Genomic DNA extracted from ovarian BG-1 cells (American Type Culture Collection) was used for PCR amplification of a 1.69-kb BRCA-1 promoter fragment, using the forward DRPR-F-5′-ATCGGTACCGGATTCCTGAACCAACAGACTT-3′ and reverse LHR-BglII-5′-ACTAGATCTACCTCATGACCAGCCGACGTT-3′ oligonucleotides. The BRCA-1 primers were designed with KpnI and BglII linkers, which after restriction digestion generated KpnI- and BglII-compatible cloning sites.

The authenticity to the BRCA-1 sequence deposited in the GenBank (accession no. HSU37574) was confirmed by direct sequencing of the PCR product, which spanned both exon-1A and exon-1B transcription start sites. The BRCA-1 promoter fragment was subcloned into the expression vector pGL3Basic (Promega Corporation, Madison, WI), which was previously digested to create compatible KpnI and BglII termini, thus generating the pGL3-BRCA-1 luciferase expression construct. For expression studies, the pGL3-BRCA-1 vector was transfected into MCF-7 and ZR75.1 cells by the Lipofectamine-Plus procedure, as described by the manufacturer (Life Technologies). Variations in transfection efficiency were accounted for by cotransfection with plasmids encoding for the β-galactosidase (RLU/β-gal) or renilla (RLU/renilla).

RESULTS

Effects of B[a]P, BPDE, and TCDD on Expression Profiles. RT-PCR analysis of total RNA from MCF-7 cells revealed that treatment with B[a]P and BPDE lowered BRCA-1 mRNA levels (Fig. 1A). These changes were accompanied by up-regulation of the CYPIA1 gene, which encodes for a member of the P450 family of metabolizing enzymes (24). The accumulation of CYPIA1 mRNA confirmed the functionality of the AhR pathway in MCF-7 cells, although B[a]P was more effective than BPDE in elevating the content of CYPIA1 transcripts. Treatment with B[a]P increased the levels of BAX-α mRNA, whereas transcripts for Bcl-2 were reduced, suggesting that B[a]P inversely regulated the expression of Bax-α and Bcl-2. However, neither the Bax-α nor the Bcl-2 mRNA level was affected by BPDE. These distinct expression patterns emphasized the fact that loss of BRCA-1 mRNA in cells treated with B[a]P or BPDE did not stem from a general effect on the transcriptional machinery.

Western blot analysis of cell extracts obtained from MCF-7 cells revealed that B[a]P lowered BRCA-1 protein levels, while increasing p53 (Fig. 1B). Expectedly, treatment with BPDE significantly reduced BRCA-1 protein, whereas the cellular p53 and p21 levels were elevated (Fig. 1C). In contrast, treatment with 10 nM TCDD did not alter BRCA-1, p53, or p21 protein levels, but elicited the accumulation of CYPIA1 mRNA levels (data not shown; Ref. 22).

B[a]P Does Not Compromise BRCA-1 mRNA Stability but Reduces Promoter Activity. The expression data of Fig. 1 indicated that B[a]P and BPDE activated multiple, perhaps overlapping, signal transduction pathways, which must be regarded as an integral part of a cellular network. In this context, we were interested in determining whether inhibition of BRCA-1 expression by B[a]P resulted from reduced stability of BRCA-1 mRNA. Data from RT-PCR (Fig. 1A) and RNase protection assay (Fig. 2A) experiments revealed that, compared with DEMEM, the levels of BRCA-1 mRNA corrected for the cyclophilin mRNA were reduced 3.0-fold by treatment of MCF-7 cells with B[a]P. To examine the effects of B[a]P on BRCA-1 mRNA stability, we compared the rate of decay of BRCA-1 transcripts in

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Table 1 Primers for RT-PCR

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Fig. 1. Effects of B[a]P, BPDE, and TCDD on Expression Profiles. A. MCF-7 cells were cultured for 24 h in basal DEMEM/F12 plus 10% FCS (DEMEM), or DEMEM plus 5 μM B[a]P or 500 nM BPDE. Semi-quantitative RT-PCR analysis was performed as described in “Materials and Methods.” Bands represent RT-PCR products for CYPIA1 (397 bp), BRCA-1 (712 bp), Bax-α (289 bp), Bcl-2 (293 bp), and control ribosomal 18S RNA (185; 488 bp) from input cDNA corresponding to 400 ng of total RNA. M/W, DNA molecular weight markers. B. Western blot analysis of BRCA-1 and p53 in cells cultured in DEMEM or DEMEM plus 5 μM B[a]P. C. bands are immunocomplexes for BRCA-1, p53, and p21 in cells cultured in DEMEM or DEMEM plus 500 nM BPDE or 10 nM TCDD. Bands for β-actin are control immunocomplexes.
control and B[a]P-treated cells. After MCF-7 were precultured for 24 h in DMEM/F12 containing 10% FCS (DMEM) or DMEM plus 5 μM B[a]P. At the end of the incubation period, cells were cultured for various periods of time (6, 9, 12, and 18 h) in the presence or absence of 5 μg/ml actinomycin-D (ActD). Cells were harvested, and BRCA-1 mRNA levels were measured by RNase protection assay in 10 μg of total RNA as described in “Materials and Methods.” A, bands are RNase-protected fragments for BRCA-1 or the internal standard cyclophilin. The doublet is the result of extended digestion of the BRCA-1 mRNA duplex. MW, RNA molecular weight standards (bp). B, decay of BRCA-1 mRNA in MCF-7 cells cultured in DMEM (○) or DMEM plus B[a]P (●). Data are expressed as the semilogarithmic value of BRCA-1/cyclophilin mRNA at each time point.

These results suggested that loss of BRCA-1 expression in cells treated with B[a]P was likely not due to increased degradation of BRCA-1 mRNA and prompted further investigations to assess whether B[a]P interfered with regulation of transcription at the BRCA-1 promoter. Fig. 3A diagrams the luciferase activity detected in MCF-7 cells transfected with pGL3-BRCA-1 in the presence or absence of B[a]P. Compared with the RLU measured in cells transfected with the empty pGL3Basic vector, luciferase units corrected for β-galactosidase increased, although not proportionally, 16.0- and 22.0-fold in cells transfected with 5 or 10 μg of pGL3-BRCA-1.

Fig. 3. B[a]P inhibits transcription activity of the BRCA-1 promoter. A, MCF-7 cells were transiently transfected with the empty pGL3Basic vector or vectors containing a luciferase reporter cassette under the control of the Simian SV40 (pGL3Control) or BRCA-1 (pGL3-BRCA-1) promoter. RLU were measured after cells were cultured for 24 h in DMEM/F12 plus 10% FCS (DMEM) or DMEM plus 5 μM B[a]P. B, effects of treatment for 24 h with various concentrations of B[a]P on RLU in MCF-7 cells transfected with 10 μg of pGL3-BRCA-1. C, induction by B[a]P (5 μM) of the promoter construct p1A1-4X-LEC (1 μg) containing four XREs. p1A1-LUC is the empty vector lacking the XREs. Columns represent mean RLU corrected for β-galactosidase ± SD (bars) from two independent experiments performed in triplicate.
However, after treatment with \( B[a]P \), the reporter activity was reduced by 2.2- and 2.0-fold in MCF-7 cells transfected with 5 or 10 \( \mu \)g of the pGL3-BRCA-1 vector, respectively. The RLU detected after transfection with 1 \( \mu \)g of the internal pGL3Control vector were not influenced by treatment with \( B[a]P \) and were 10.0-fold higher than those measured in cells transfected with the pGL3Basic lacking a promoter element.

In parallel experiments (Fig. 3B), we assessed the dose-dependent effects of \( B[a]P \) in MCF-7 cells transfected with 10 \( \mu \)g of the pGL3-BRCA-1 vector. A \( B[a]P \) concentration of 0.5 \( \mu \)mM did not influence RLU, whereas doses of 1 and 5 \( \mu \)M \( B[a]P \) significantly reduced luciferase activity by 1.5- and 1.6-fold, respectively. The reporter activity in control cells transfected with the positive control p1A1-4X-LUC was 4.0-fold higher than that produced by the p1A1-LUC vector lacking the four XREs and was increased an additional 10-fold in the presence of \( B[a]P \) (Fig. 3C).

**BPDE but not TCDD Represses BRCA1 Promoter Activity.** The data shown in Fig. 1C illustrated that, at least at the concentration (10 nM) used in this study, TCDD did not lower BRCA-1 protein levels. It should be pointed out that in previous studies (22), increasing the concentration of TCDD from 10 nM to 1000 nM affected neither BRCA-1 mRNA nor protein content in MCF-7 cells, although cell viability was reduced by 50 and 80% with 10 and 1000 nM TCDD, respectively. Because the affinity of TCDD for the AhR is \( \sim 100 \)-fold higher than that of \( B[a]P \) but TCDD is not metabolized (25), we envisioned that activation of the AhR pathway was not sufficient for \( B[a]P \)-mediated repression of BRCA-1 transcription. Rather, we formulated the hypothesis that products of \( B[a]P \) bioactivation, possibly BPDE, contributed to down-regulation of BRCA-1. To test this contention, we compared the effects of BPDE (100 and 500 nM) and TCDD (10, 100, and 500 nM) on BRCA-1 promoter activity in cells transiently transfected with the pGL3-BRCA-1 construct. In previous studies, concentrations up to 1.2 \( \mu \)M BPDE were used to investigate repair of DNA damage (26). However, we used lower concentrations, ranging from 100 to 500 nM BPDE, which in our hands have been effective in promoting S-phase arrest and loss of BRCA-1 expression in MCF-7 cells (23). The results shown in Fig. 4A indicate that treatment for 24 h with 100 or 500 nM BPDE inhibited by 1.5- and 2.2-fold, respectively, transcription from the BRCA-1 promoter. In contrast, the activity of the BRCA-1 reporter construct was not affected by treatment with TCDD at any of the concentrations tested in this study (Fig. 4B). The efficacy of the TCDD treatment was confirmed by evidence that it stimulated a dose-dependent increase in the reporter activity of the positive control, p1A1-4X-LUC (Fig. 4C). TCDD concentrations of 10 nM stimulated by 2.0-fold the activity of the 1A1-4X promoter compared with that measured in cells treated with equimolar concentrations of \( B[a]P \). This effect was attributed to the higher binding affinity of TCDD for the AhR. A TCDD concentration of 500 nM gave a response comparable to that obtained with 5 \( \mu \)M \( B[a]P \). These cumulative data suggested that the AhR is not involved directly in BRCA-1 repression but that the metabolite BPDE, or factors regulated by BPDE, contribute, at least in part, to \( B[a]P \)-dependent inhibition of the BRCA-1 transcription.

**Repression of BRCA-1 Promoter Activity by \( B[a]P \) and BPDE Requires Functional p53.** On the basis of our published observation that the AhR antagonist \( \alpha \)-naphthoflavone counteracted the S-phase arrest and loss of BRCA-1 expression induced by \( B[a]P \) while preventing the accumulation of p53 (23), we questioned whether the acquisition of p53 functions in MCF-7 cells treated with \( B[a]P \) contributed to repression of BRCA-1 promoter activity. To test this hypothesis, we cotransfected MCF-7 cells with a plasmid containing a cassette encoding for p53 mutated at position 175 (Arg to His) under the control of the cytomegalovirus promoter (pCVM53mut) cloned into pCMV (plasmids were gifts from Dr. Bert Vogelstein, The Johns Hopkins University School of Medicine, Baltimore, MD and made available by Dr. J. Martinez, The University of Arizona, Tucson, AZ). The cotransfection of the empty pCMV (data not shown) or
pCMV53mut vectors with pGL3-BRCA-1 did not influence BRCA-1 luciferase reporter activity in cells cultured in control medium (DMEM; Fig. 5A). In contrast, the concomitant transfection of pGL3-BRCA-1 with pCMV53mut, which encodes mutant p53, prevented the loss of BRCA-1 promoter activity (2.0-fold) induced by B[a]P. Positive evidence that the pCMV53mut construct expressed p53 was obtained by Western blot analysis (Fig. 5B). In control medium (DMEM), p53 levels were low in nontransfected cells or cells transfected with the empty pCMV vector, whereas p53 increased significantly in the presence of B[a]P. Conversely, accumulation of p53 was observed in cells cultured in DMEM after transfection with the pCMV53mut vector. The intensity of the p53 immunocomplex increased further after treatment with B[a]P, presumably because of coincident immunodetection of endogenous and recombinantly expressed p53.

We next examined the effects of BPDE on activity of the BRCA-1 reporter construct (Fig. 6A). The RLU detected in cells transfected with pGL3-BRCA-1 were reduced 1.8-fold by BPDE. In contrast, cotransfection with pCMV53mut restored luciferase activity to control levels. Similar results were obtained after cotransfection of a vector encoding for the human papilloma virus E6 protein, which prevented the loss of reporter activity elicited by BPDE (data not shown). Treatment with BPDE reduced BRCA-1 protein, whereas p53 and p21 levels were increased in cells transfected with the empty pCMV vector (Fig. 6B). However, in cells transfected with pCMV53mut, we detected constitutive expression of p53, whose levels were increased further by BPDE. More importantly, BRCA-1 protein was restored almost to control levels in cells expressing p53mut and treated with BPDE. The cellular content of p21 was elevated by BPDE in cells transfected with pCMV or pCMV53mut, but it was not altered by expression of exogenous mutant p53 in cells cultured in DMEM.

To further test the hypothesis that the metabolite BPDE inhibits BRCA-1 transcription via an effect that is mediated by p53, we examined the regulation on BRCA-1 transcriptional activity in ZR75.1 breast cancer cells, which contain mutated p53 (152 Pro→Leu)4 (27). The functionality of the AhR pathway in ZR75.1 cells was confirmed by evidence of transcriptional activation of the positive control, p1A1-4X-LUC construct, in the presence of 5 μM B[a]P (7.0-fold) and 500 nM TCDD (10.0-fold; Fig. 7A). Therefore, it appeared that ZR75.1 cells were more responsive than MCF-7 cells to stimulation with equimolar concentrations of TCDD or B[a]P.

4 Mohammed A. Khan, Laboratory of Human Carcinogenesis, National Cancer Institute (Bethesda, MD), personal communication.
slight increase (1.5-fold) in RLU was also observed in ZR75.1 cells transfected with p1A1-4X-LUC and treated with 500 nM BPDE. In contrast, transfection experiments with pGL3-BRCA-1 indicated that TCDD had no effects on BRCA-1 promoter activity (Fig. 7C), whereas RLU were increased 1.5-fold in ZR75.1 cells treated with 500 nM B[a]P (Fig. 7B) or 50 and 100 nM BPDE (Fig. 7D). This increase in transcriptional activity of the BRCA-1 promoter was similar to the increase observed in MCF-7 cells cotransfected with dominant-negative p53 (Figs. 5 and 6). Overall, these findings indicated that transcription of BRCA-1 was not repressed by B[a]P, BPDE, or TCDD in ZR75.1 breast cancer cells containing mutated p53.

**DISCUSSION**

The primary objective of this study was to shed some light on the mechanisms responsible for the reduction in BRCA-1 mRNA levels in MCF-7 breast cancer cells exposed to acute levels of B[a]P (22, 23). The environmental carcinogen B[a]P, a prototype PAH, has been implicated in the development of lung (15) and skin (26) tumors. A generally accepted concept is that the tumor-initiating and -promoting properties of B[a]P stem from its metabolic activation by detoxifying enzymes to a pool of end-products, including the highly mutagenic BPDE, which can form DNA adducts (28) and induce transversions at mutational hot spots (18, 29–31). Our working hypothesis is that the reactive metabolite BPDE, selected among many end-products of B[a]P metabolism, may alert regulatory cascades that repress BRCA-1 expression. Support for this hypothesis comes from the following: (a) No sporadic breast tumors have been shown to harbor mutations in the BRCA-1 gene (32) but express lower levels of BRCA-1 (33). This implies the existence of epigenetic mechanisms that reduce BRCA-1 expression in the absence of mutational alterations. (b) B[a]P and BPDE repress constitutive and estrogen-induced expression of BRCA-1 in breast and ovarian cancer cells (22, 23). This effect is not unique to B[a]P; other PAHs hamper, in a dose-dependent fashion, BRCA-1 protein levels in MCF-7 cells in the following order: 3-methylcholanthrene > B[a]P > benzo[e]pyrene.5 (c) Despite their high reactivity, PAH-DNA adducts correlate with low mutation frequencies (2%; Ref. 34), suggesting that physiological rather than genotoxic stresses may be implicated in PAH-dependent tumorigenesis (35). For example, exposure to PAHs contributes to disruption of cell cycle kinetics (19, 36), and PAHs have the ability to circumvent cellular defense mechanisms (18).

In mammalian models, activation of the AhR pathway elicits cell cycle arrest, apoptosis, and expression of genes encoding for enzymes in the cytochrome P450 family, which contribute to bioactivation of AhR ligands (37–39). The AhR is a ligand-activated factor that modulates transcription through interactions with XREs. The XRE-core recognition sequence (5′-GCGTG-3′) is harbored in the 5′ flanking region of several genes, including CYP1A1, CYP1A2, UDP-glucuronosyl-transferase, and the estrogen-inducible cathepsin-D (40, 41). The fact that the reporter activity of a PAH-inducible promoter (p1A1-4X-LUC) harboring a tandem of four XREs was greatly induced by B[a]P and TCDD provided confirmatory evidence that the AhR pathway was functional in MCF-7 and ZR75.1 cells under present experimental conditions. This notion is also supported by earlier reports documenting regulation by AhR ligands of XRE-containing promoter segments (42). Moreover, we observed that BPDE induces the production of CYP1A1 mRNA in MCF-7 cells and transcription activity of the p1A1-4X-LUC construct in ZR75.1 cells. The latter results deviated from the general model advocating binding of the ligand B[a]P to the AhR and the subsequent trans-activation of

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5. Our unpublished data.
CYP1A1. We excluded the possibility that the up-regulation of CYP1A1 was attributable to contamination of the BPDE preparation (purity >99%). One possible explanation is that BPDE may induce CYP1A1 via weak binding to the AhR or may be metabolized into a form that is, in turn, capable of binding to or activating the AhR (43). Alternatively, CYP1A1 expression could be induced through AhR-independent pathways (44). Regardless of the mechanism, the fact that accumulation of CYP1A1 mRNA in MCF-7 cells treated with B[a]P was accompanied by elevation of Bax-α and loss of Bcl-2 mRNA, whereas in the presence of BPDE the levels of these transcripts were unaltered, lent support to the conclusion that BPDE exerts specific effects on CYP1A1 expression.

With respect to XREs, which are known to confer responsiveness to PAHs, using computer-assisted analysis we have identified an array of candidate XRE consensus sequences in the 1.69-kb BRCA-1 promoter fragment. Although we cannot discard the possibility that binding of the activated AhR to XREs in the BRCA-1 gene contributed to its negative regulation, the data presented in this report were consistent with a model in which the metabolite BPDE mediated the negative effects of B[a]P on BRCA-1 transcription. Because TCDD failed to reduce BRCA-1 promoter activity but did induce transcription from a CYP1A1-like promoter in both MCF-7 and ZR75.1 cells, we concluded that effectors downstream of the AhR, such as BPDE, a product of B[a]P bioactivation (45), alerted cellular signals that repressed BRCA-1 promoter activity.

The tumor suppressor gene p53 encodes for one such effector, whose stability was increased significantly in MCF-7 cells treated with B[a]P or BPDE. An increase in p53 in MCF-7 cells exposed to PAH metabolites has been documented in previous reports (36) and was related to DNA adduct formation and delay in S-phase (46). However, more than one pathway has been implicated in the stabilization of p53 in response to DNA damage induced by BPDE, including DNA strand breaks (47) and phosphorylation and poly(ADP) ribosylation of the p53 protein (48). The p53 gene product has been shown to elicit transcription of several genes, including Bax, p21, and mdm2 (49), which are involved in cell cycle control and apoptosis (50). On the basis of published observations that expression of p53 and BRCA-1 may be regulated through a feedback loop (51–53), we tested whether inhibition of BRCA-1 transcription by B[a]P and BPDE resulted from a gain of p53 functions. In keeping with this concept, transfection of MCF-7 cells containing wild-type p53 (27) with a vector encoding for p53 mutated at amino acid position 175 (Arg to His) abrogated the negative effects of B[a]P and BPDE on BRCA-1 promoter activity. Our interpretation of these findings is that transient expression of mutant p53 interfered with normal p53 functions in a trans-dominant-negative fashion. Similarly, transient transfection of MCF-7 cells with an expression vector encoding for the E6 human papilloma virus counteracted the negative effects of BPDE on BRCA-1 promoter activity (data not shown). The E6 gene product binds to p53 and leads to suppression of its biological functions (54). From these cumulative data, we concluded that activation of the AhR pathway was required, but not sufficient for B[a]P-mediated inhibition of BRCA-1 transcription. Rather, the metabolite BPDE elevated p53, which in turn inhibited BRCA-1 promoter activity. Experiments with ZR75.1 cells corroborated the notion that repression of BRCA-1 transcriptional activity by B[a]P or BPDE could not be seen in breast cancer cells containing mutated p53 (152Pro→Leu; Ref. 27). Mutations such as those that occur in the core domain, which consists of amino acids from ~100 to 300, have been shown to result in loss of DNA binding activity of the p53 protein (55). Interestingly, the fact that in ZR75.1 cells exposure to low doses of BPDE (50–100 nM) tended to increase BRCA-1 promoter activity may imply the existence of dose-dependent mechanisms that activate BRCA-1 expression independent of p53.

Because it is known that levels of BRCA-1 vary during the cell cycle, with minimal expression in G0-G1 (56), one could argue that the decrease in BRCA-1 mRNA in response to B[a]P/BPDE was an indirect consequence of cell cycle arrest. However, we consider this possibility unlikely because we observed in a previous study (23) and in the present (data not shown) study that both B[a]P and BPDE induced accumulation of MCF-7 cells in S-phase, at which interval expression of BRCA-1 was expected to peak (56). This interpretation is in accord with data from other groups, documenting the arrest of MCF-7 cells in S-phase after exposure to benzo[a]pyrene dihydrodiol epoxides (46). In addition, we considered the possibility that by forming DNA adducts BPDE might impede progression of RNA polymerase II on the transcribed strand. However, evidence that transient expression of MCF-7 cells with mutated p53 restored BRCA-1 transcription and protein levels suggested that p53 mediated the negative effects of BPDE on BRCA-1 transcription. Furthermore, the distinct mRNA expression profiles presented in Fig. 1 and those obtained by cDNA microarray analysis of >1000 CancerArray genes (data not shown) confirmed that the loss of BRCA-1 mRNA in cells treated with B[a]P or BPDE was not associated with general disruption of the transcriptional machinery.

Loss of BRCA-1 in cells harboring DNA damage may destine cells to lethality (57–60). Previous reports from our laboratory (22) indicated that in HBL-100 cells expressing the SV40 large T antigen, which is known to inhibit the transcriptional transactivation functions of p53 (54), neither BRCA-1 expression nor proliferation was affected by B[a]P. Conversely, in addition to reducing BRCA-1 expression, acute exposure to B[a]P induced cell death of 70–80% of breast MCF-7 cells, which express wild-type p53 (21). The accumulation of Bax-α mRNA as well as p53 and p21 protein was paralleled in this study by loss of Bcl-2 mRNA, suggesting that proapoptotic pathways were alerted in response to exposure to B[a]P. However, because of deficient expression of caspase-3, a key player in the signaling of programmed cell death, MCF-7 cells may not succumb through classical apoptosis (57). A significant scenario emerging from these observations is one in which cells resistant to the cellular stresses induced by PAHs (61), but with a reduced potential for BRCA-1 expression, may be more likely to undergo neoplastic transformation (62).

In summary, the present study provides novel insights into the mechanisms through which PAHs may adversely affect transcriptional activity of the BRCA-1 gene. Activation of the AhR appears to be insufficient for repression of basal BRCA-1 transcription, which however, may be hampered after the bioactivation of AhR ligands to reactive metabolites, such as BPDE, and the gaining of p53 functions. The significance of these findings is that they offer a molecular basis for investigating the contribution of PAHs and structurally related compounds to dysregulation of the BRCA-1 gene as well as their role as a risk factor in the etiology of sporadic breast cancer.

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REFERENCES


Activation of the Aromatic Hydrocarbon Receptor Pathway Is Not Sufficient for Transcriptional Repression of BRCA-1: Requirements for Metabolism of Benzo[a]pyrene to 7r,8t-Dihydroxy-9t,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene


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