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13. Abstract ***(Maximum 200 Words)***
The purpose of this project is to investigate whether or not loss of expression of the BRCA-1 gene in breast epithelial cells exposed to polycyclic aromatic hydrocarbons (PAHs) is mediated by the aryl hydrocarbon receptor (AhR). *The scope* of the project is to examine whether or not the AhR complexed with the AhR-nuclear transporter (ARNT) protein, binds to several xenobiotic responsive elements (XRE) strategically located at –539 bp (CCGTGGAA=Cyp1A1-like) and +20base pairs (bp) (GCGTG=XRE-1) from the transcription start site on exon-1A. Two additional XREs (GCGTG) have been localized at –107 bp in the intervening sequence upstream (XRE-2) and +218 bp (XRE-3) into exon-1B. Findings of these experiments were: 1) Confirmed in transfection experiments with B[a]P that benzo[a]pyrene represses transcription at the BRCA-1 promoter; 2) Developed mutation constructs for CYP1A1, XRE-1, XRE-2 and double and triple mutants. 3) Developed deletion constructs for CYP1A1, XRE-1 and XRE-2. 3) Observed that the CYP1A1 element may be required (positive element) for constitutive activity of the BRCA-1 promoter and that the XRE-1 and XRE-2 may be negative cis-acting elements on basal BRCA-1 transcription. Finally, we report that the XRE-1 may act as a repressor of BRCA-1 transcription under conditions of estrogen stimulation.

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Introduction

The subject of the research is to investigate whether or not exposure to polycyclic aromatic hydrocarbons (PAHs) may be a risk factor in the onset of mammary neoplasia by repressing transcription of the tumor suppressor gene, BRCA-1. The *purpose* of this project is to investigate whether or not loss of expression of the BRCA-1 gene in breast epithelial induced by PAHs is mediated by the aryl hydrocarbon receptor (AhR). *The scope* of the project is to examine whether or not the AhR complexed with the AhR-nuclear transporter (ARNT) protein, binds to several xenobiotic responsive elements (XRE) strategically at –539 bp (CCGTGGAA=Cyp1A1-like) and +20base pairs (bp) (GCGTG=XRE-1) from the transcription start site on exon-1A. Two additional XREs (GCGTG) have been localized at –107 bp in the intervening sequence upstream (XRE-2) and +218 bp (XRE-3) into exon-1B.

Body

Synopsis

The tasks of Specific Aim#1 focused on developing internal deletion and mutation constructs in the context of the intact BRCA-1 promoter. The objective of these two tasks was that of investigating the contribution of the proposed binding sites for AhR-ligands to regulation of the BRCA-1 promoter. The sites of interest were a CYP1A1-like element (TCCGTGAGAA) homologous to an AhR-responsive domain found in the CYP1A1 gene and three consensus xenobiotic responsive elements (XRE) (GCGTG=XRE-1, XRE-2, XRE-3) spatially arranged upstream of the transcription start sites of exon-1A and exon-1B. As originally proposed in the Statement of Work, given the overlapping and complementary nature of these two tasks, experiments to develop mutation and deletion constructs have been conducted in parallel. As proposed, we have focused our initial efforts on the candidate elements surrounding exon-1A, whose expression is predominant in mammary tissue compared with transcripts originating from exon-1B. However, insertion of mutations into the DNA segment representing the BRCA-1 promoter has proceeded faster than the deletion of the segments of interest and we have already completed the construction of expression vectors containing single and combinatorial mutations for CYP1A1, XRE-1, XRE-2, CYP1A1 plus XRE-1, and CYP1A1 plus XRE-1 plus XRE-2. Also, we have successfully deleted the DNA region comprising CYP1A1, XRE-1, and XRE-2, whereas experiments are in progress to use these deletion constructs as templates to introduce combinatorial deletions. Consequently, a significant portion of transfection experiments testing the effects of single or combinatorial mutations in breast cancer cells have been completed meanwhile testing of deletion constructs is in progress. Nevertheless, we expect that all of the mutations and deletion constructs proposed in the Statement of Work be developed and tested before the end of year-2 of the award.

Role of CYP1A1, XRE-1 and XRE-2 responsive elements in regulation of basal BRCA-1 promoter activity and in response to benzo[a]pyrene

In previous studies, we reported on the regulation of BRCA-1 expression by estrogen (1) and inhibition of its basal and estrogen-induced expression in breast and ovarian cancer cells by the AhR-ligand benzo[a]pyrene (2,3), a prototype polycyclic aromatic hydrocarbon (PAH) known to induce mammary tumors (4,5). Specific Aim#1 of this award focused on investigating the role of candidate CYP1A1 and XRE as cis-acting elements mediating the effects of AhR-ligands. The data summarized in Fig. 1 indicated that activity of a positive control plasmid (p1A1-4X-LUC) containing an array of four XREs directing the expression of the luciferase reporter gene was induced significantly (10-fold) by treatment with 5 μM B[a]P for 24h. In contrast, activity of the wild-type BRCA-1 promoter was reduced by B[a]P (Fig. 2). These data confirmed the validity of the experimental conditions under which we tested the effects of B[a]P on activity of the wild-type and mutant BRCA-1 constructs. The results of the transfection experiments depicted in Fig. 3 are discussed with respect to individual elements:

CYP1A1: This element was mutated from the sequence TC**CGTG**AGAA to TC**C**ca**G**AGAA. That this sequence was mutated as planned was confirmed by direct sequencing. The corresponding plasmid was identified as pGL3-BRCA-1-cypMut. The bold nucleotides represent the core sequence of XRE in the CYP1A1 gene. Mutation of this sequence to CcaGA was successful in abrogating the responsiveness of the CYP1A1 element to AhR-ligands (6). In this study, mutation of the CYP1A1-like element reduced by 2.0-fold the basal reporter activity of the BRCA-1 promoter in MCF-7 cells cultured in control DMEM medium (Fig. 3B). Upon treatment with B[a]P luciferase units were reduced an additional 2.0-fold. Contrary to our expectations, it appeared that the CYP1A1 element was required for basal activity of the BRCA-1 promoter and that its mutation to a non-consensus sequence did not counteract the loss of BRCA-1 expression elicited by B[a]P.

XRE-1 and XRE-2: Mutation of the XRE-1 from GCGTG to GccaG resulted in a 1.5-fold increase in reporter activity in MCF-7 cells transfected with pGL3-BRCA-1-XRE-1Mut. In contrast, the reporter activity in cells treated with B[a]P did not differ from that measured in cells transfected with the wild-type BRCA-1 construct. Similarly, basal activity of the construct containing the mutated XRE-2 element was induced 1.8-fold compared with the wild-type BRCA-1 promoter. However, mutation of XRE-2 did not restore expression of BRCA-1 in the presence of B[a]P.

CYP1A1 plus XRE-1: Luciferase activity in cells transfected with the double-mutant CYP1A1 plus XRE-1 reduced basal activity to levels measured in cells transfected with the XRE-1 mutant construct. The same trend was evident in cells transfected with the triple-mutant pCYP1A1-XRE-1-XRE-2. The reporter activity was not restored in cells transfected with the double or triple-mutant in the presence of B[a]P.

Our interpretation of these results is that the CYP1A1 element may be required for constitutive activity of the BRCA-1 promoter. This interpretation is contrary to what expected, but it is in accord with published observations advocating the idea that DNA-binding activity is important in regulating CYP1A1 transcription and other gene promoters (7, 8), and that binding by the AhR receptor is required continuously to maintain transcription of various genes (9). This raises the possibility that basal activity of BRCA-1 is maintained by binding of the AhR to the CYP1A1 element. This contention will be tested as part of Specific Aim#2 of the Statement of Work. That basal activity of the BRCA-1 promoter at the CYP1A1 is regulated by B[a]P may offer important insights into the mechanisms that regulate transcription of the BRCA-1 gene. In fact, this may represent a defense mechanism against low-dose exposure to PAHs. In previous studies (2) we reported that exposure to non-cytotoxic levels of B[a]P (0.1 to 0.5 μM) tended to increase the cellular content of BRCA-1 mRNA in breast epithelial cells, whereas exposure to acute doses of B[a]P (1 to 5 μM) repressed BRCA-1 mRNA and protein levels. In addition, the fact that the CYP1A1 element in the BRCA-1 promoter is homologous to the consensus sequence for binding the AhR and AhR-nuclear translocator (ARNT) (6) suggests this element may act as bona-fide sentinel in response to exposure to PAHs. Whether or not the CYP1A1 element located in the BRCA-1

promoter represents a putative site for binding by the AhR-ARNT complex will be the subject of binding and electromobility shift assay experiments proposed in Specific Aim#2.

The fact that mutation of the XRE-1 and XRE-2 core sequences (GCGTG) resulted in increased basal activity suggests that binding to this DNA region in the BRCA-1 promoter, possibly by the AhR-ARNT complex, could exert a negative effect analogous to that produced by basic helix-loop-helix transcriptional repressors. The presence of the XRE-1 and XRE-2 in the promoter region of BRCA-1 could disrupt (e.g. by steric hindrance) the formation of active transcription complexes (10). Overall, it could be envisioned that the CYP1A1 and XREs may exert distinct functions in the context of regulation of the BRCA-1 gene. Precedents for this interpretation are offered by the fact that other DNA-binding transcriptional regulatory proteins can function in either a negative or positive fashion, depending upon the regulatory context. By analogy, the fact that mutation of the CYP1A1 and XRE-1/XRE-2 sequences produced negative and positive effects on basal activity of the BRCA-1 promoter might reflect the action of the liganded-AhR as a positive (CYP1A1) or negative (XRE-1/XRE2) regulator of constitutive transcription.

That mutations of the core sequence in the CYP1A1 and XRE-1/XRE-2 sequences failed to prevent the loss of BRCA-1 transcription induced by B[a]P suggest that other nucleotides outside these elements may be involved in this negative regulation. For example, nucleotides adjacent to the essential four base pairs 5'-CGTG-3' may determine whether the receptor-DNA interaction is productive. This contention may be tested by mutating the nucleotides adjacent to the essential four-base pairs, which influence the functional response to AhR-ligands. For example, mutation of the A/T base pair at position 9 to a G/C pair abolished function suggesting that a functional sequence may require an A/T pair at position 9 (10). This is the case of the CYP1A1 (5'-TCCGTGAGAA-3') and XRE-2 (5'-GGCGTGGGAG-3') elements, which comprise an A at position 9. To better address this aspect we would like to request permission to add these experiments to those proposed in the Statement of Work. Specifically, we propose of co-mutating the base pair A/T to a G/C and transfect the resulting mutation constructs into MCF-7 cells. Comparison of the reporter activity in the presence or absence of B[a]P will elucidate the role of these candidate elements in the inhibition of BRCA-1 transcription by B[a]P.

Role of CYP1A1, XRE-1 and XRE-2 in estrogen-dependent regulation of BRCA-1 promoter activity in response to benzo[a]pyrene

Published studies from our (1) and other laboratories (11, 12) have suggested that the BRCA-1 gene is inducible by estrogen in breast epithelial cells. Recently, we have reported (2) that treatment of breast cancer cells (MCF-7) with B[a]P abrogated estrogen-dependent induction of BRCA-1. These findings raised the possibility that the mechanism through which AhR-ligands may alter BRCA-1 expression may depend on the regulatory context. It has been documented that binding of the AhR to XRE alters the expression of estrogen-responsive genes, such as cathepsin-D. For example, binding of the AhR might disrupt by steric hindrance the formation of active transcription complexes at neighboring

sites (13). To further elucidate the contribution of the CYP1A1 and XREs to regulation of BRCA-1 by AhR ligands, the single and combinatorial mutation constructs were transfected in MCF-7 cells cultured in basal medium containing charcoal-stripped fetal calf serum, as described previously (1). After transfection of the appropriate constructs, cells were induced for 48 h with 10 nM estrogen in the presence or absence of 5 μM B[a]P. In previous studies (2) we found these concentrations to be effective in inducing (estrogen) or inhibiting (estrogen plus B[a]P) BRCA-1 expression. The data depicted in Fig. 4 indicated that the reporter activity of a promoter construct (p3xERE-TATA-LUC) containing an array of three consensus estrogen receptor elements (ERE) directing the expression of the luciferase gene was significantly induced by estrogen (10 nM, 48 h), whereas basal activity was not influenced by B[a]P (Fig. 4A). In contrast, the treatment with B[a]P counteracted the stimulatory effects of estrogen on the activity directed by the 3x-ERE promoter. These data offered positive confirmation of the efficacy of the estrogen and B[a]P treatments. The treatment of MCF-7 cells with estrogen for 48 h produced a 1.3-fold increase in reporter activity from the wild-type BRCA-1 promoter (Fig. 4B). This effect was counteracted by the presence of B[a]P, which lowered BRCA-1 transcription activity to basal (DMEM) levels. With respect to individual mutation constructs, mutation of the CYP1A1 element did not prevent the loss (2.0-fold) of reporter activity in the presence of B[a]P and contributed to lowering the responsiveness to estrogen. Conversely, mutation of the XRE-1 produced a dramatic increase in reporter activity in cells treated with estrogen, but was reduced (2.0-fold) upon treatment with estrogen plus B[a]P, although at levels higher (1.6-fold) than those observed for the wild-type BRCA-1. Finally, we observed that the XRE-2 mutant-luciferase construct was refractory to B[a]P. The addition of estrogen produced a 1.6-fold increase in transcription activity, which was repressed by 2.3-fold in the presence of B[a]P. Treatment with B[a]P plus estrogen reduced the reporter activity measured upon transfection with the double (CYP1A1-XRE-1) or triple (CYP1A1+XRE-1+XRE-2) mutants (Fig. 4C).

Our interpretation of these results is that in conditions that mimic estrogen stimulation, mutation at the XRE-1 appears to increase transcriptional activity at the BRCA-1 and counteract the inhibitory effects of B[a]P on estrogen-dependent BRCA-1 transcription. With respect to the candidate CYP1A1 element, mutation of the core sequence CGTG resulted in lower reporter activity in the presence of B[a]P, supporting the role of this element as a positive regulator of BRCA-1 transcription.

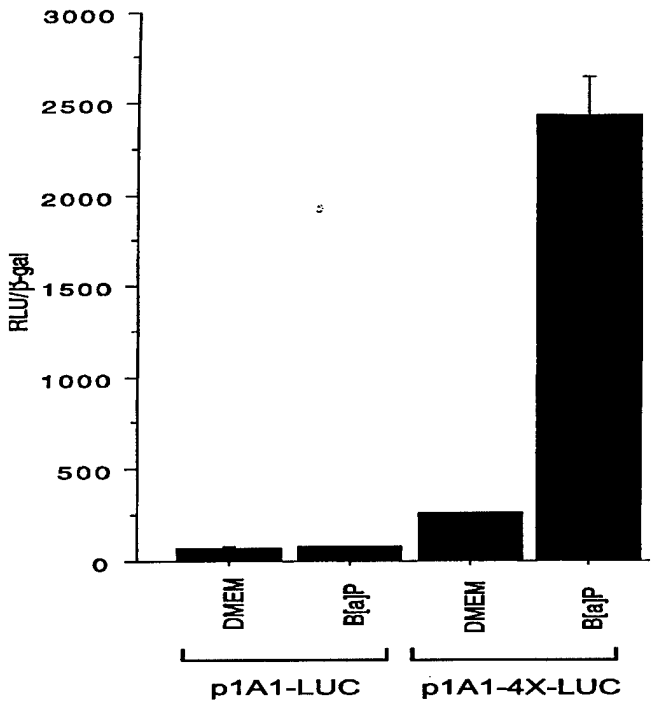


Figure 1. Induction by B[a]P (5 μM) of the promoter construct p1A1-4X-LUC (1 μg) containing four xenobiotic responsive elements (XRE). p1A1-LUC is the empty vector lacking the XREs. Bars represent mean RLU corrected for β-galactosidase ± standard deviations from two independent experiments performed in triplicate.

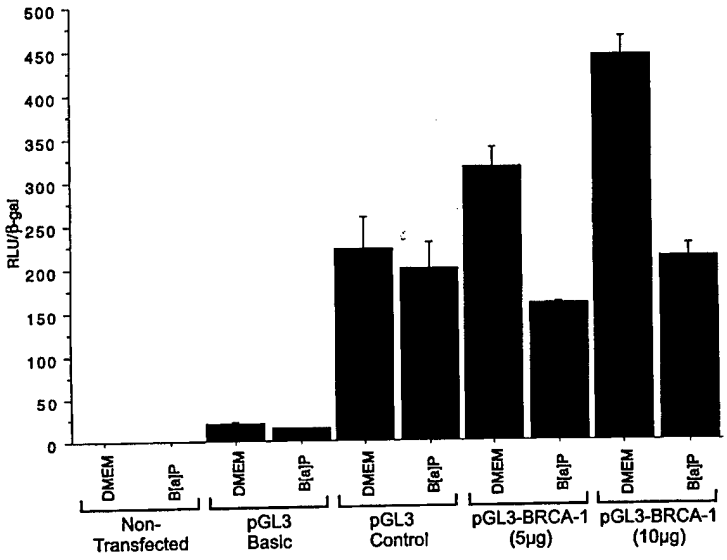
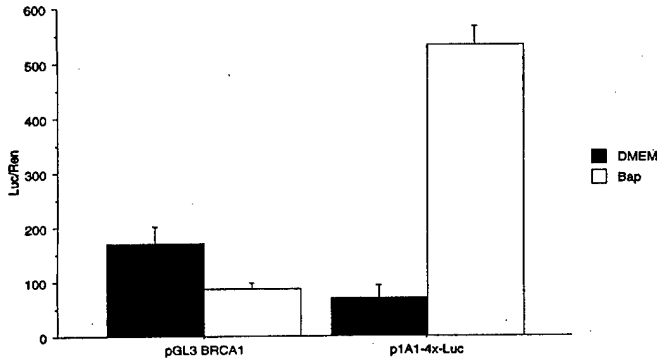


Figure 2. B[a]P inhibits transcription activity of the BRCA-1 promoter. MCF-7 cells were transiently transfected with the empty pGL3Basic vector, or vectors containing a luciferase reporter cassette under the control of the Simian SV40 (pGL3Control) or BRCA-1 (pGL3-BRCA-1) promoter. Relative luciferase units (RLU) were measured after cells were cultured for 24 h in DMEM/F12 plus 10% FCS or basal medium plus 5 μM B[a]P.

A



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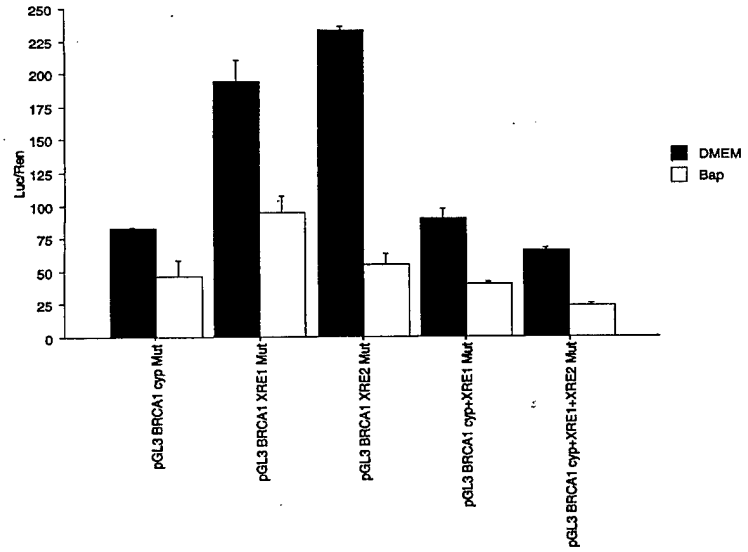
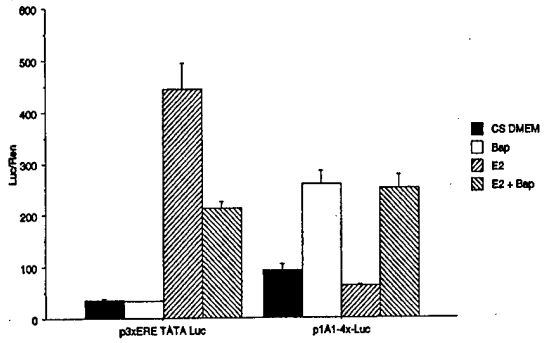


Figure 3. A) Induction of the positive control plasmid p4x-LUC by B[a]P (5 μM). B) Effects of mutations of Cyp1A1, XRE-1 and XRE-2 on constitutive and B[a]P-dependent transcription of BRCA-1 in MCF-7 cells. DMEM is control medium.

A



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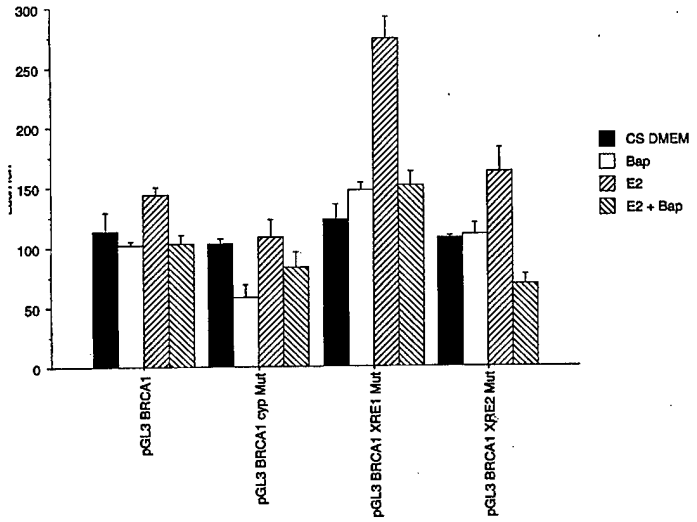

Figure 4. A) Estrogen stimulates the reporter activity of an estrogen-responsive-element (ERE). Treatment with B[a]P prevents activation by estrogen of the positive control plasmid p3x-ERE-TATA-LUC. B) Effects of mutations in the CYP1A1, XRE1, and XRE-2 elements on responsiveness of the BRCA-1 promoter to treatement with estrogen, B[a]P, and their combination. DMEM is control medium.

C

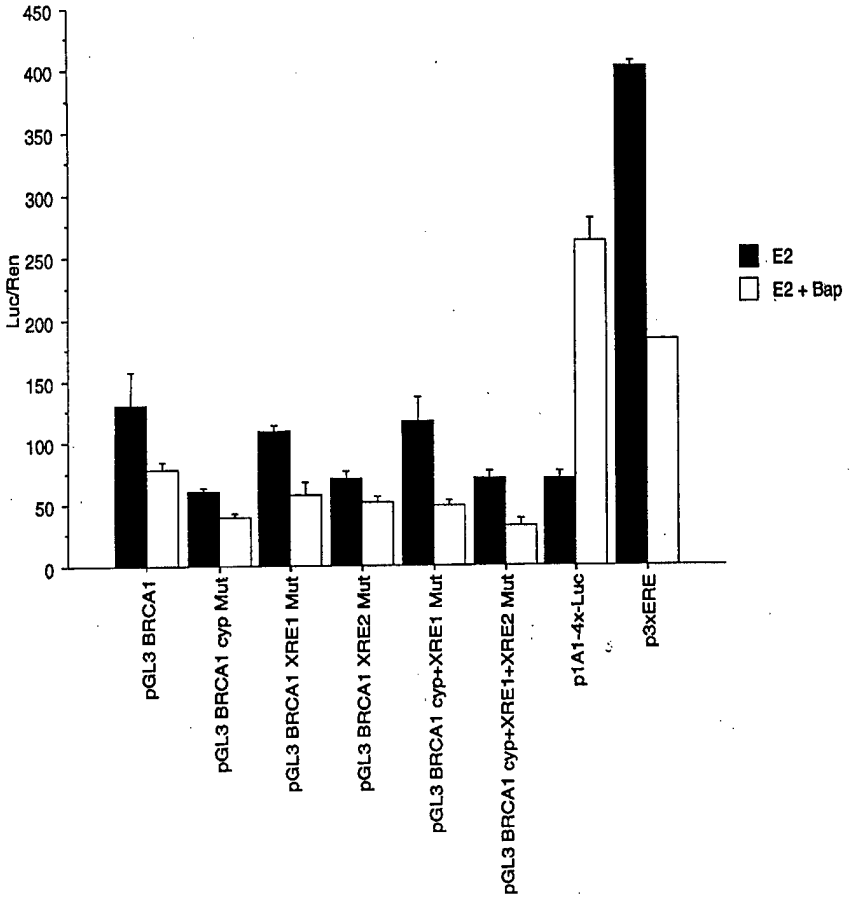

Figure 4. C) Effects of B[a]P on estrogen dependent regulation of the BRCA-1 promoter and BRCA-1-mutation constructs containing single or combinatorial mutations for CYP1A1, XRE-1, and XRE-2.

Key research Accomplishments

- Confirmed in dose-dependent experiments with B[a]P that this AhR-ligand represses transcription at the BRCA-1 promoter.
- Developed mutation constructs for CYP1A1, XRE-1, XRE-2 and double and triple mutants.
- Developed deletion constructs for CYP1A1, XRE-1 and XRE-2 (untested).
- Observed that the CYP1A1 element may be required (positive element) for constitutive activity of the BRCA-1 promoter.
- Observed that the XRE-1 and XRE-2 may be negative cis-acting elements on basal BRCA-1 transcription.
- Observed that the XRE-1 may act as a repressor of BRCA-1 transcription under conditions of estrogen stimulation.

Reportable Outcomes

1. Transcriptional repression of BRCA-1: requirements for metabolism of benzo[a]pyrene to BPDE. D. Jeffy, Ryan B. Chirnomas, Eddy J. Chen, Jean M. Gudas, and Donato F. Romagnolo. Manuscript submitted to Cancer Research and currently in revision for publication. A copy of this manuscript has been included in the Appendices section. The work presented includes data obtained in preliminary dose-response experiments of Specific Aim#1 aimed at characterizing the responsiveness of the BRCA-1 promoter to treatment with AhR-ligands. The support of the US Army Medical Research and Materiel Command has been acknowledged in the Acknowledgment section of the manuscript.
2. Abstract to be presented at the Meetings of the Breast Cancer and Environmental Mutagens Conference, Environmental Mutagen Society, Research Triangle Park, NC, September 22-25, 2001.
3. Poster to be presented at the Arizona Cancer Center Research Forum, September 11, 2001 The University of Arizona, Tucson, AZ
4. Abstract presented at the 2001 Meetings of the Southwest Environmental Health Sciences Center, The University of Arizona, Tucson, AZ.
5. The information being gathered through the execution of the experiments supported by this award will constitute the backbone of a Ph.D. project for Brandon Jeffy, who is currently working on this project in the laboratory of the P.I. Mr. Jeffy Ph.D. is a candidate in the Cancer Biology Interdisciplinary Program at the University of Arizona, Tucson, AZ.

Conclusions

Summary

Based on the data obtained through the completion of the experiments outlined in the Body section of this report, we can conclude that exposure to ligands of the aromatic hydrocarbon receptors represses transcription of the BRCA-1 gene. The mechanism being investigated is that through binding to AhR-binding domains the AhR alters the expression of BRCA-1. However, the candidate responsive elements (CYP1A1 and XREs) appear to have distinct functions. The CYP1A1 appears to be necessary for constitutive expression of BRCA-1 and may represent a mechanism for maintenance of BRCA-1 expression, possibly in response to low-dose exposure. This conclusion is supported by previous studies showing that regulation of constitutive gene expression required interactions of the AhR-ARNT complex with other proteins, such as the transcription factor SP-1. Both the AhR- and SP-1 binding domains were required for maximal activity in the absence of exogenous AhR-ligands (13). This conclusion is important because published studies have assigned the AhR the classic function of messenger of external exposure to AhR-ligands, whereas the data presented here and those published recently by other groups (13) suggest the AhR may function as keeper of constitutive expression. Interestingly, we have identified in the BRCA-1 promoter a putative SP-1 element (GGGCGG) 165-bp downstream of the CYP1A1 element, which may facilitate the binding of the AhR-ARNT heterocomplex to the CYP1A1 core sequence. Therefore, basal regulation of BRCA-1 may depend on the physical interaction between the AhR/ARNT and SP-1. This may explain why upon mutation of the CYP1A1 element we observed a significant reduction in transcription activity of the BRCA-1 promoter. Whether or not SP-1 facilitates the interaction of the AhR at the CYP1A1 site in the BRCA-1 promoter can be easily tested during the execution of binding and antibody experiments planned in Specific Aim#2.

In contrast, the XRE1- and XRE-2 may be negative regulators of basal BRCA-1 transcription. Finally, we found the XRE-2 may be a negative regulator of estrogen-dependent regulation of BRCA-1. The functionality of these sites as AhR-binding elements awaits confirmation by binding and electro-mobility shift assays proposed in Specific Aim#2.

Importance and Implications

The findings of this Annual Report confirm the original assumption that AhR-ligands may be a risk factor in the etiology of BRCA-1-mediated breast cancer by repressing basal and estrogen-stimulated BRCA-1 expression. We believe, however, that these conclusions need to be contrasted with the results of the transfections with deletion-mutation constructs and studies planned in Specific Aim#2. If confirmed, the knowledge gained through this studies will offer evidence that exposure to AhR-ligands may be a risk factor in environmental carcinogenesis of the breast. Because BRCA-1 is involved in DNA repair, loss of BRCA-1 protein may favor the accumulation of DNA damage and the onset of sporadic breast cancer. To date, no mutations in the BRCA-1 gene have been

identified in sporadic breast cancers, whereas the expression levels of BRCA-1 in breast tumors are lower than those observed in normal mammary tissue. The results outlined in this Annual Report suggest that exposure to polycyclic aromatic hydrocarbons, which are present in coal tar, tobacco smoke and industrial pollution, may contribute to repressing the expression of BRCA-1. This event may increase in women exposed to these environmental pollutants the susceptibility to developing mammary neoplasia. Finally, a significant implication of the findings reported here, is that basal expression of BRCA-1 may be positively regulated by the AhR. This may represent a mechanism of protection against low-dose exposure to AhR. If confirmed by experiments planned in Specific Aim#2 this inference may assign to the BRCA-1 gene a role of sentinel marker against low-dose/chronic exposure to polycyclic aromatic hydrocarbons.

References

1. Romagnolo, D., Annab, L.A., Lyon, T.T., Risinger, J.I., Terry, L.A., Barrett, J.C., and Afshari, C.A. Estrogen upregulation of expression of BRCA-1 with no effect on localization. Mol. Carcinogen., *22*, 102-109, 1998.
2. Jeffy, B.D., Schultz, E.U., Selmin, O., Gudas, J.M., Bowden, G.T., and Romagnolo, D. Inhibition of BRCA-1 expression by benzo[a]pyrene and its diol epoxide. Mol. Carcinogen., *26*, 100-118, 1999.
3. Jeffy, B.D., Chen, E.J., Gudas, J.M., and Romagnolo, D.F. Disruption of cell cycle kinetics by benzo[a]pyrene: Inverse expression patterns of BRCA-1 and p53 in MCF-7 cells arrested in S and G2. Neoplasia, *2*, 460-470, 2000.
4. Maher, V.M., Patton, J.D., Yang, J.L., Wang, Y.Y., Yang, L.L., Aust, A.E., Bhattacharyya, N., and McCormick, J.J. Mutations and homologous recombination induced in mammalian cells by metabolites of benzo[a]pyrene and 1-nitropyrene. Environ. Health Perspect., *76*, 33-39, 1987.
5. Ronai, Z., Gradia, S. el-Bayoumy, K., Amin, S., and Hecht, S.S. Contrasting incidence of ras mutations in rat mammary and mouse skin tumors induced by anti-benzo[c]phenanthrene-3,4-diol-1,2-epoxide. Carcinogenesis, *15*, 2113-2116, 1994.
6. Fukunaga, B.N., and Hankinson, O. Identification of a novel domain in the aryl hydrocarbon receptor required for DNA binding. J.Biol. Chem. 271:3743-3749, 1996.
7. Zhang, L. Savas, U., Alexander, D.L., and Jefcoate, C.R. Characterization of the mouse Cyp1B1 gene. Identification of an enhancer region that directs aryl hydrocarbon receptor-mediated constitutive and induced expression. J. Biol. Chem. 273:5174-5183. 1998.
8. Boesch, J.S., Miskimins, R., Miskimins, W.K., Lindahal, R. The same xenobiotic response element is required for constitutive and inducible expression of the mammalian aldehyde dehydrogenase-3-gene. Arch. Biochem. Biophys. 361:223-230. 1999.
9. Reick, M., Robertson, R.W., Pasco, D.S., Fagan, J.B. Down-regulation of nuclear aryl hydrocarbon receptor DNA-binding and transactivation functions: requirement for a labile or inducible factor. Mol. Cell. Biol. 14:5653-5660.
10. Lusska, A., Shen, E., Whitlock, J.P. Jr. Protein-DNA interactions at a dioxin-responsive enhancer. J. Biol. Chem. 268:6575-6580. 1993.
11. Gudas, J.M., Nguyen, H., Li, T., and Cowen, K.H., Hormone-dependent regulation of BRCA-1 in human breast cancer cells. Cancer Res., *55*, 4561-4565, 1995.
12. Spillman, M.A. and Bowock, A.M. BRCA1 and BRCA2 mRNA levels are coordinately elevated in human breast cancer cells in response to estrogen. Oncogene, *12*, 1639-1645, 1996.
13. Wang, F., Wang, W. and Safe, S. Regulation of constitutive gene expression through interactions of Sp1 protein with the nuclear aryl hydrocarbon receptor complex. Biochem. 38:11490-11500. 1999.

Appendices

1. Romagnolo, D., Annab, L.A., Lyon, T.T., Risinger, J.I., Terry, L.A., Barrett, J.C., and Afshari, C.A. Estrogen upregulation of expression of BRCA-1 with no effect on localization. Mol. Carcinogen., *22*, 102-109, 1998.
2. Jeffy, B.D., Schultz, E.U., Selmin, O., Gudas, J.M., Bowden, G.T., and Romagnolo, D. Inhibition of BRCA-1 expression by benzo[a]pyrene and its diol epoxide. Mol. Carcinogen., *26*, 100-118, 1999.
3. Jeffy, B.D., Chen, E.J., Gudas, J.M., and Romagnolo, D.F. Disruption of cell cycle kinetics by benzo[a]pyrene: Inverse expression patterns of BRCA-1 and p53 in MCF-7 cells arrested in S and G2. Neoplasia, *2*, 460-470, 2000.
4. Transcriptional repression of BRCA-1: requirements for metabolism of benzo[a]pyrene to BPDE. D. Jeffy, Ryan B. Chirnomas, Eddy J. Chen, Jean M. Gudas, and Donato F. Romagnolo (manuscript submitted to Cancer Research on June 12, 2001 and currently in revision for publication)

MOLECULAR CARCINOGENESIS 22:102–109 (1998)

Estrogen Upregulation of BRCA1 Expression with No Effect on Localization

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Alterations in the expression of the breast and ovarian cancer susceptibility gene *BRCA1* may contribute to the development of mammary and ovarian neoplasia. The sex-steroid estrogen modulates cell proliferation of normal and neoplastic breast and ovarian epithelial cells, but the role of estrogen regulation on the expression of BRCA1 remains to be defined. In this study, estrogen-regulated BRCA1 expression was examined in breast and ovarian cancer cells. Estrogen stimulated the proliferation of estrogen receptor (ER)–positive breast MCF-7, C7-MCF-7, and ovarian BG-1 cells as well as the expression of the estrogen-inducible *pS2* gene. This was concomitant with upregulation of *BRCA1* mRNA (2.5- to 5.0-fold) and a 3- to 10-fold induction of BRCA1 protein (230 kDa). Cell fractionation studies localized the BRCA1 protein to the nucleus in both unstimulated and estrogen-stimulated cells. The antiestrogen ICI-182780 inhibited estrogen-induced cell proliferation, *BRCA1* mRNA induction, and BRCA1 protein expression in ER-positive cells. Conversely, estrogen did not influence expression of BRCA1 in HBL-100 cells that lacked the estrogen receptor, although the constitutive levels of *BRCA1* mRNA (but not protein) in these cells were 5- to 30-fold higher than in other breast and ovarian cancer cells. Secretion of the BRCA1 protein into the cell medium did not account for the discrepancy between the mRNA and protein levels in HBL-100 cells. Proliferation of HBL-100 cells was not affected by either estrogen or ICI-182780. Taken together, these data support a role for the steroid estrogen and the involvement of the estrogen receptor pathway in the modulation of expression of BRCA1. We therefore propose that stimulation of cell proliferation may be a prerequisite for upregulation of BRCA1 in breast and ovarian cancer cells. *Mol. Carcinog. 22:102–109, 1998.* © 1998 Wiley-Liss, Inc.†

Key words: Hormone; breast cancer; ovarian cancer; BRCA1

INTRODUCTION

Growth and differentiation of mammary and ovarian tissue are coordinately regulated by sex steroids, peptide hormones, and growth factors [1]. Specifically, the ovarian steroids estrogen and progesterone, in concert with other endocrine and autocrine factors, modulate the growth and development of breast and ovarian epithelial cells [2,3]. In normal tissue, ovarian hormones initiate morphogenesis and differentiation through cycles of cell proliferation [4]. These effects may be counterbalanced by feedback mechanisms that suppress normal and neoplastic proliferation. However, the loss of expression of tumor suppressor genes, such as *BRCA1*, may favor proliferative pathways and the consequent development of breast and ovarian malignancies [5].

The breast and ovarian cancer susceptibility gene *BRCA1* encodes a protein of 1863 amino acids [6,7]. Loss of heterozygosity along chromosome 17q in familial and sporadic tumors provided indirect evidence that *BRCA1* is indeed a tumor suppressor [8,9]. Stronger evidence is provided by studies in which retroviral introduction of *BRCA1* into breast tumor cells resulted in growth suppression or cell death [10–12]. Mutations in this gene, whether inherited [13–16] or somatic [17–19], combined with alterations in the expression level of BRCA1 [10], may contribute to the onset of mammary and ovarian malignancies.

Previous investigations indicated that expression of *BRCA1* mRNA is stimulated by estrogen in estrogen receptor (ER)–positive breast MCF-7 and T47-D cells [20] and in ovariectomized mice in the presence of estrogen plus progesterone [21]. Other studies indicated that estrogen indirectly regulates BRCA1 expression in breast cancer cells [22,23]. However, no comparable studies of ovarian cells have been reported.

There has been a remarkable controversy over the pattern of BRCA1 protein expression. This controversy has encompassed debate on the molecular weight of the protein; there have been some reports

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Abbreviations: ER, estrogen receptor; DMEM, Dulbecco's modified Eagle's medium; SFCS, charcoal-stripped fetal calf serum; SDS, sodium dodecyl sulfate.

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of a 170–190 kDa protein [20,24,25] and others indicating that the size of the BRCA1 protein is 220–230 kDa [26–30]. In addition, the protein has been localized to different cell compartments, including the plasma membrane, the cytosol, and the nucleus [25–27,29]. It has been reported that a conserved granin motif is present within the *BRCA1* cDNA and that this motif directs secretion of the protein [25]. Furthermore, the amino terminus of BRCA1 has a characteristic ring structure homologous to DNA-binding or protein-protein interaction domains [31], and transcriptional transactivation activity has been assigned to the carboxy-terminal domain of the protein [32,33]. However, no study to date has attempted to determine whether BRCA1 location is affected by hormonal stimulation.

Understanding the relationships between regulation of BRCA1 expression and cell proliferation is central to defining the function of this gene in controlling the pathways that lead to the development of breast and ovarian cancer. Therefore, in this study we investigated correlations between estrogen-stimulated cell growth and BRCA1 expression, the involvement of the ER pathway in modulation of BRCA1 expression in breast and ovarian cancer cells, and the location of the BRCA1 protein after estrogen stimulation. We provide evidence that in ER-positive breast and ovarian cell lines, proliferation and expression of BRCA1 are coordinately regulated by estrogen and involve the participation of the ER pathway. We suggest that upregulation of BRCA1 may represent a feedback mechanism specific for rapidly proliferating cells.

MATERIALS AND METHODS

Cell Culture and Proliferation Assay

The regulation of expression of *BRCA1* was investigated in breast ER-positive (MCF-7) and ER-negative cells (HBL-100) and ovarian cancer ER-positive cells (BG-1) obtained from American Type Culture Collection (Rockville, MD). The ER-positive cell line C7-MCF-7 was a kind gift from A. Soto; this line, derived from MCF-7 cells, contains a higher number of ER molecules than the parental MCF-7 line [34]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal calf serum. Before the experimental treatments, the cells were preconditioned for 5 d in phenol red–free medium containing 5% charcoal-stripped fetal calf serum (SFCS) (Hyclone Laboratories, Inc., Logan, UT), as described previously [35]. The cells were plated at a density of 2×10^6 cells/100-mm tissue culture dish and maintained overnight in DMEM/F12 plus 5% SFCS. Then, the cells were either harvested (0 h) or subjected to treatment with estrogen (10 nM), ICI-182780 (1 μM), and estrogen plus ICI-182780 for 24, 48, 72, and 144 h. Three dishes were assigned to each experimental treatment, and at the end of the incubation periods, the cells were counted in triplicate (n = 9).

Ribonuclease Protection Assay

Total RNA was extracted by using a guanidine thiocyanate procedure [36], and BRCA1 transcript levels were measured by using ribonuclease protection assay and the HybSpeed hybridization procedure (Ambion Inc., Austin, TX). A *BRCA1* fragment of 170 bp encoding the 3′ region of exon 20, the entire coding sequence of exon 21, and a 5′ portion of exon 22 was amplified from normal human mammary gland cDNA and cloned in the antisense orientation into the transcription vector Triplescript (Ambion Inc.) to generate the transcription construct pBRCA1-20/22. The primers used for amplification were 5′-TGAAGTCAGAGGAGATGTGGT-3′ (forward) and 5′-ACAGAAGCACCACACAGCTGT-3′ (reverse). Sequencing of the amplified *BRCA1* fragment confirmed its identity with the *BRCA1* sequence deposited in the GeneBank (accession number U14680). The construct pBRCA1-20/22 transcribed a riboprobe of the expected size that, after gel purification, hybridized with *BRCA1* mRNA to protect a fragment of the expected length. Preliminary control experiments were performed to assure that an excess of radiolabeled probes was added to the hybridization cocktail. The presence of two bands was evident in some experiments with the *BRCA1* probe. This was due to incomplete digestion of the protected fragments. To optimize the assay, a balance between digestion time, amount of RNAse, and quantity of RNA was achieved. Increasing the RNAse concentration and the digestion time led to overdigestion of the RNA complex; therefore, we used conservative conditions that sometimes led to incomplete digestion and the appearance of a doublet. *BRCA1* mRNA was quantified by phosphorimage analysis with arbitrary units corrected for the expression of the control, cyclophilin mRNA (BRCA1/cyclophilin) [37].

Western Blot Analysis

Western blot analysis was performed as previously described [20]. Protein extracts were prepared by lysing cells in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis sample buffer, and protein extract, normalized to cell number, was electrophoresed on 6% SDS-polyacrylamide gels. After transfer to nitrocellulose membranes, the blots were blocked with Tris-buffered saline containing 10% dried milk and 0.05% Tween-20. BRCA1 immunoblotting was performed by using the C-20 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), which was raised against the carboxy terminus (residues 1843–1862), or BRCA1-Ab1 and BRCA1-Ab2 (Oncogene Research Products, Cambridge, MA), which were raised against an amino-terminal fusion protein, diluted 1:50 in 2% milk in Tris-buffered saline with Tween. The

blots were incubated with anti-BRCA1 antibodies for 2–3 h at room temperature and then with secondary antibody (Boehringer Mannheim Corp., Indianapolis, IN). Immunocomplexes were detected by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Cell fractionation was performed according to previously published protocols [27]. Briefly, 1×10^6 cells were collected and washed with phosphate-buffered saline. Then three-pellet-volumes of suspension buffer (10 mM HEPES, pH 7.9; 10 mM KCL; 0.1 mM EDTA; 0.1 mM EGTA; and 1 mM dithiothreitol plus protease inhibitors) was added to the pellet. The cells were allowed to swell on ice for 15 min, and then Nonidet P40 was added to a final concentration of 0.6%. The pellet was vortexed vigorously for 15 s, and then the nuclei were pelleted by microfuge centrifugation for 30 s. The supernatant was removed and retained as the cytoplasmic/membrane fraction, and the pellet contained nuclear protein. The nuclear pellet was resuspended in 50 µL of 20 mM HEPES pH 7.9, 0.4 M NaCL; 1 mM EDTA; 1 mM dithiothreitol, and protease inhibitors; vigorously rocked for 15 min at 4°C; and then microcentrifuged for 5 min. The supernatant was retained as the nuclear fraction. Fractions were mixed 1:1 with 2× Laemmli buffer and loaded on 6% SDS-polyacrylamide gels as described above.

Conditioned medium was collected from cells for 72 h. The medium was then concentrated 15- to 44–fold by using Centricon filters as described by the manufacturer (Amicon, Inc., Berely, MA). Concentrated medium was mixed 1:1 with 2× Laemmli sample buffer and run on 6% SDS-polyacrylamide gels as described above. The blots were stained with Ponceau S immediately after transfer to verify the presence of protein on the membranes.

Expression of pS2

Expression of the estrogen-inducible *pS2* gene was investigated by northern hybridization of a ^{32}P-labeled *pS2* cDNA probe (American Tissue Type Collection) to 10 µg of total RNA linked to nitrocellulose membranes.

RESULTS

Cell Proliferation and Expression of BRCA1

We examined the effects of estrogen on cell proliferation and regulation of BRCA1 expression in the ER-positive breast cancer cell lines MCF-7 and C7-MCF-7, ovarian BG-1 cancer cells, and ER-negative HBL-100 immortal breast cells. Estrogen stimulated the proliferation of MCF-7, C7-MCF-7, and BG-1 cells but had no effect on the growth of HBL-100 cells (Figure 1). In MCF-7 and BG-1 cells, significant growth stimulation by estrogen was detectable after 48 h. However, by 144 h, BG-1 cells were induced tenfold by estrogen compared with fourfold for MCF-7 cells. Interestingly, the ER-positive C7-MCF-7 cells proliferated faster than the parental cell line, MCF-7. We attributed this effect to the higher number of ERs in C7-MCF-7 [34].

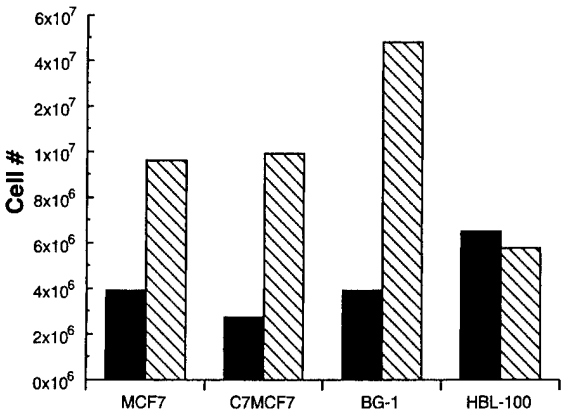

Figure 1. Effect of estrogen on cell proliferation of breast and ovarian cells. Cells were cultured in DMEM/F12 medium containing 5% SFCS as described in Materials and Methods. The cells were plated at a density of 2×10^6 cells/100-mm tissue culture dish and maintained overnight in DMEM/F12 plus 5% SFCS. Then, the cells were either harvested (solid bars) or treated with estrogen (10 nM) for 144 h (striped bars).

We compared the expression of *BRCA1* mRNA in cells cultured for 72 h in DMEM and 5% SFCS with or without estrogen by using a ribonuclease protection assay (Figure 2A). In all ER-positive cell lines, expression of *BRCA1* mRNA was induced from 2.5- to 5.0-fold as measured by the ribonuclease protection assay directed to *BRCA1* exons 20, 21, and 22 (Figure 2B). The cell line C7-MCF-7 expressed the highest levels of *BRCA1* mRNA in response to estrogen. Conversely, in HBL-100 cells, *BRCA1* transcript levels were not affected by estrogen, although the constitutive levels of *BRCA1* mRNA in this cell line were 5- to 30-fold higher (control DMEM vs estrogen) than in breast and ovarian cancer cells (Figure 2B).

Western blotting confirmed the stimulatory effects of estrogen on expression of BRCA1 protein (230 kDa). The BRCA1 protein induction ranged from threefold in MCF-7 cells up to greater than 20.0-fold in C7-MCF-7 (Figure 2C). The same band was recognized by a variety of antibodies (Santa Cruz C-20 and Oncogene Research BRCA1-Ab1 and BRCA1-Ab2) raised against the carboxy- and amino-terminal regions, respectively, of BRCA1 (data not shown). Nevertheless, in HBL-100 cells, BRCA1 protein expression levels were not affected by estrogen, in accordance with the absence of ER expression in these cells.

The induction of *BRCA1* mRNA expression by estrogen reached a maximum after 24 h in ovarian BG-1 cells and then declined, as measured by ribonuclease protection assay (Figure 3A). Conversely, in breast MCF-7 cells (Figure 3B), the levels of *BRCA1* mRNA did not peak until 72 h after stimulation with

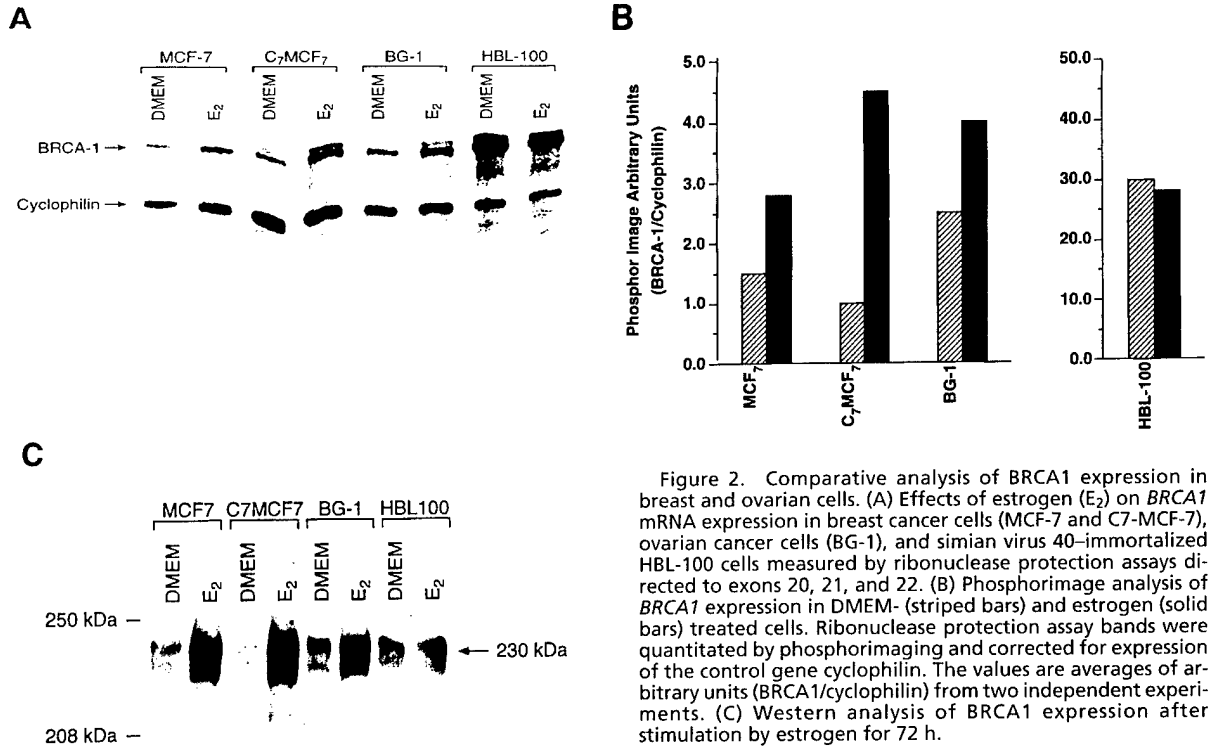

Figure 2. Comparative analysis of BRCA1 expression in breast and ovarian cells. (A) Effects of estrogen (E_2) on *BRCA1* mRNA expression in breast cancer cells (MCF-7 and C7-MCF-7), ovarian cancer cells (BG-1), and simian virus 40–immortalized HBL-100 cells measured by ribonuclease protection assays directed to exons 20, 21, and 22. (B) Phosphorimage analysis of *BRCA1* expression in DMEM- (striped bars) and estrogen (solid bars) treated cells. Ribonuclease protection assay bands were quantitated by phosphorimaging and corrected for expression of the control gene cyclophilin. The values are averages of arbitrary units (BRCA1/cyclophilin) from two independent experiments. (C) Western analysis of BRCA1 expression after stimulation by estrogen for 72 h.

estrogen. These differences suggest that estrogen-dependent pathways for regulation of expression of BRCA1 may follow different kinetics in ovarian and breast tissue and may be influenced by the rate of cell proliferation.

Expression of estrogen-inducible *pS2* mRNA was stimulated by estrogen in the ER-positive BG-1, C7-MCF-7, and MCF-7 cells (Figure 4). This indicated that our experimental conditions were favorable for investigating estrogen regulation of BRCA1. Maximum induction of pS2 by estrogen was achieved within 24 h in both cell types, with no further upregulation up to 144 h after stimulation (data not shown).

ICI-182780 Inhibition of *BRCA1* Expression

The antiestrogen ICI-182780 inhibited estrogen-dependent cell proliferation (Figure 5A and B), induction of *BRCA1* mRNA (Figure 5C), and *pS2* expression (Figure 4) in MCF-7 and BG-1 cells. However, neither cell growth nor expression of *BRCA1* in the HBL-100 cells were affected by estrogen or ICI-182780 (data not shown). The unresponsiveness of HBL-100 to estrogen and estrogen plus the ICI compound were expected because this line does not express ERs [39]. We confirmed the lack of expression of transcripts for the ER in HBL-100 by ribonuclease protection assay (data not shown). Treatment of MCF-7 and BG-1 cells with ICI-182780 attenuated the estrogen stimulation of BRCA1 protein (Figure 5E, lanes 3 and 6), in agreement with the inhibition of expression of *BRCA1* mRNA by ICI-182780 (Figure 5D).

BRCA1 Localization after Estrogen Stimulation

An earlier study indicated that BRCA1 may be located in both the cytoplasm and the nucleus [27].

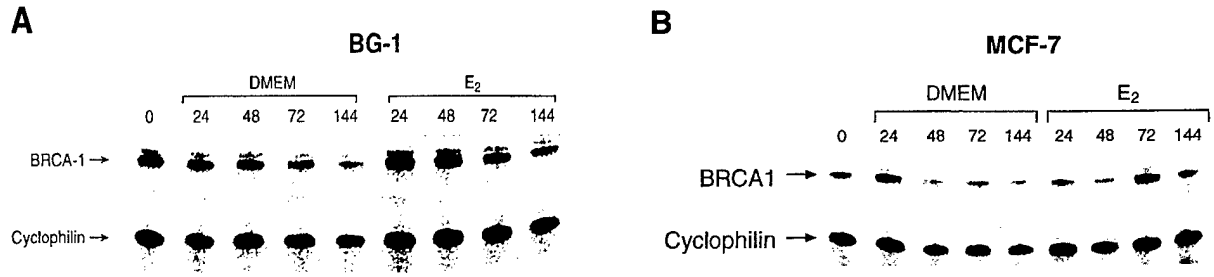

Figure 3. Time course of estrogen (E_2) stimulation of BRCA1 mRNA expression. Ribonuclease protection assays directed to exons 20, 21, and 22 of BG-1 ovarian (A) and MCF-7 breast cancer cells (B) after treatment with estrogen.

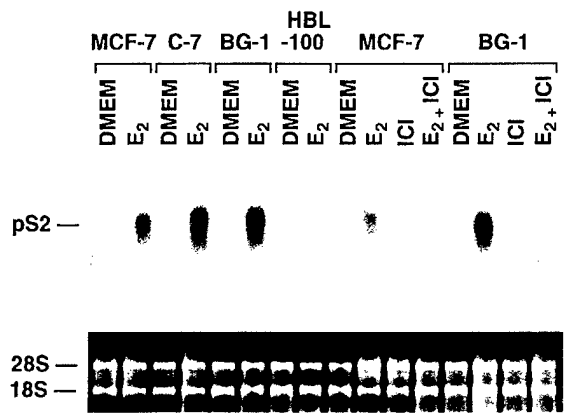

Figure 4. Induction of *pS2* mRNA by estrogen. Cells were treated as described in Figures 1–3 and analyzed by northern blotting with a *pS2*-specific probe. The image of the ethidium bromide stained gel is shown to indicate equivalent loading, as seen by staining of the 28S and 18S rRNAs.

However, those studies compared a variety of cell types without consideration of growth or hormonal influences. Therefore, we examined whether hormonal stimulation of the ovarian carcinoma cell line BG-1 affected the location of the BRCA1 protein. We found that while estrogen drastically increased BRCA1 expression (Figure 6, lanes 5 and 6), most of the full-length BRCA1 protein remained nuclear (Figure 6, lane 3). This was similar to what was observed in the immortal breast cell line HBL-100 (Figure 6, lane 1) and in normal mammary epithelial cells (data not shown). The efficacy of the fractionation protocol was determined by analysis of tubulin and retinoblastoma protein for purity of the cytoplasmic and nuclear fractions, respectively (data not shown).

A previous report indicated that BRCA1 may be secreted from normal breast epithelial cells [25]. We examined whether any BRCA1 was secreted into the medium of the estrogen-stimulated BG-1 cells, but no BRCA1 protein was apparent in the concentrated medium conditioned by these cells (Figure 6, lane 8). In the immortal nontumorigenic breast cell line HBL-100, we observed a very high level of *BRCA1* mRNA but not BRCA1 protein, as compared with the other tumor cell lines examined (Figure 2B). Therefore, the possibility that BRCA1 was being secreted from this cell line into the tissue-culture medium was also investigated. Conditioned medium was collected from HBL-100 cells, concentrated 40-fold, and run on western blots. No BRCA1 protein was detected in the medium conditioned from these cells (Figure 6, lane 7) or from 15-fold concentrated medium collected from normal breast epithelial cells (data not shown).

DISCUSSION

In defining the function of *BRCA1* as a tumor suppressor gene, it is imperative to assess the relationship between stimulation of cell proliferation and regulation of BRCA1 expression. Consequently, we focused our efforts on investigating whether increased expression of BRCA1 is a consequence of stimulated proliferation and represents a potential feedback mechanism to control cell growth. Our interest in estrogen regulation derived from the notion that this ovarian steroid stimulates the growth of normal and breast cancer cells [35]. We report here that estrogen stimulated the expression of *BRCA1* mRNA and protein in both ER-positive breast and ovarian cancer cells. Conversely, ER-negative, simian virus 40–immortalized HBL-100 breast cells expressed constitutively high levels of BRCA1 mRNA but a modest amount of protein and that both were not affected by estrogen. Although it still remains unclear whether expression of *BRCA1* is enhanced directly by estrogen or through indirect mechanisms due to stimulation of proliferative pathways [22–23], our data do support a role for estrogen and the involvement of the ER pathway in the regulation of this protein.

Our findings are in agreement with earlier observations documenting BRCA1 stimulation by estrogen in breast cancer cells [20,22,23]. We ascertained that upregulation of BRCA1 expression was mediated by the ER. This observation was tested by antagonizing estrogen induction using the pure antiestrogen ICI-182780. In these studies, we demonstrated that the ICI compound inhibited estrogen-dependent cell proliferation, *pS2* mRNA expression, and BRCA1 expression both at the mRNA and protein levels. From these results, we concluded that BRCA1 stimulation may be a response to stimulation of cell growth by estrogen. Consequently, alterations in the normal functions of BRCA1 may predispose to the development of mammary neoplasia in the presence of estrogen.

HBL-100 cells were refractory to estrogen treatment. This was not surprising, as these cells do not bind estrogen or express estrogen receptor protein [39] and did not express transcripts for the ER, as assessed by ribonuclease protection assay (data not shown). However, it is intriguing that HBL-100 cells contained higher constitutive levels of *BRCA1* mRNA (but not protein) than breast and ovarian cancer cells induced with estrogen. It is tempting to speculate that this may be in keeping with the fact that levels of *BRCA1* mRNA are higher in normal mammary cells than in breast cancer cells [19]. We explored the possibility that the levels of expression of mRNA versus protein were uncoupled in this line because BRCA1 was secreted. However, we found no evidence for secretion of BRCA1 by this cell line, normal mammary epithelial cells, or estrogen-stimulated ovarian cancer cells despite the presence of a conserved granin motif within the *BRCA1* cDNA [25]. Alternatively, it is possible that expression of *BRCA1* mRNA in HBL-100 may be al-

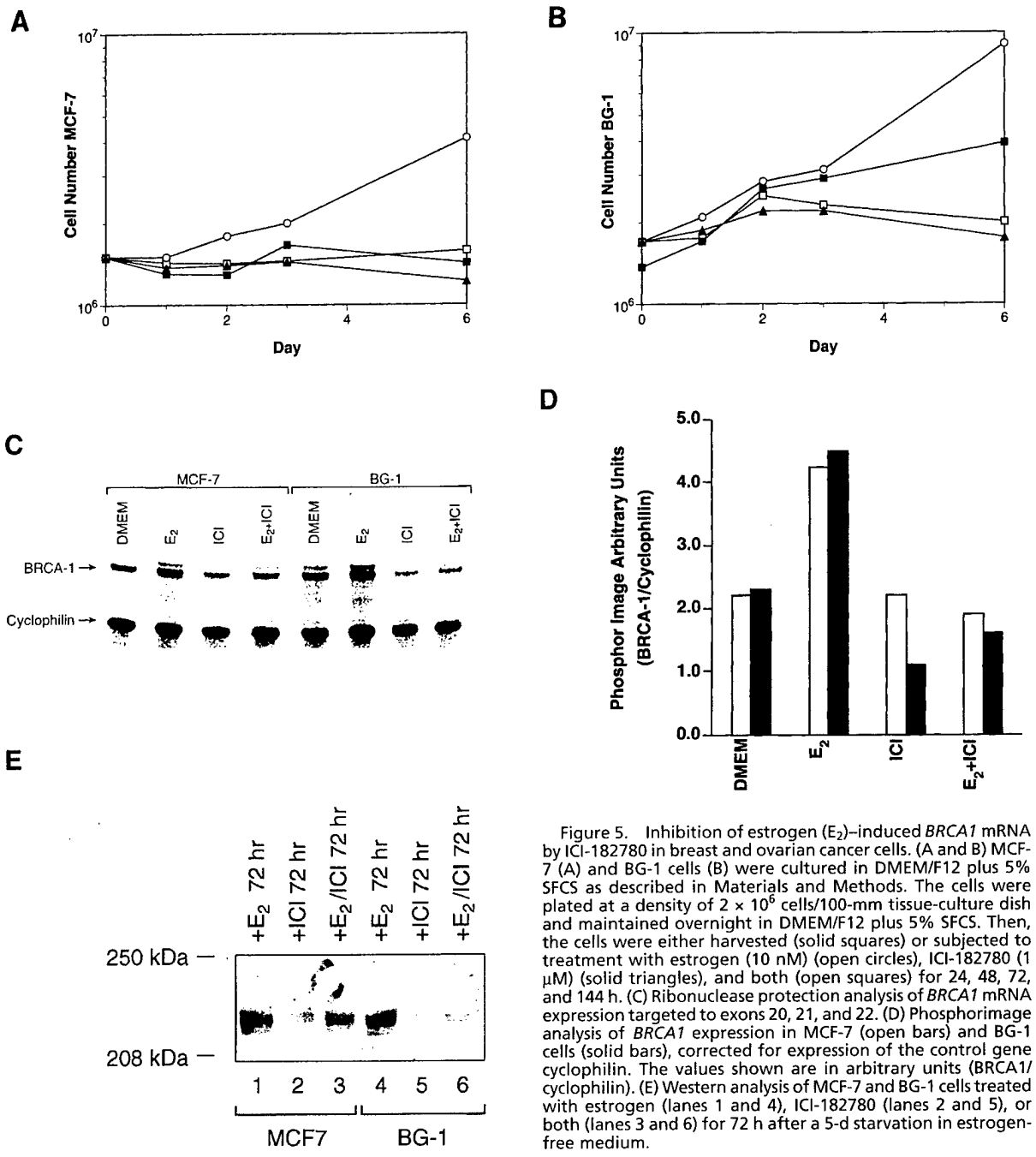

Figure 5. Inhibition of estrogen (E_2)–induced *BRCA1* mRNA by ICI-182780 in breast and ovarian cancer cells. (A and B) MCF-7 (A) and BG-1 cells (B) were cultured in DMEM/F12 plus 5% SFCS as described in Materials and Methods. The cells were plated at a density of 2×10^6 cells/100-mm tissue-culture dish and maintained overnight in DMEM/F12 plus 5% SFCS. Then, the cells were either harvested (solid squares) or subjected to treatment with estrogen (10 nM) (open circles), ICI-182780 (1 μM) (solid triangles), and both (open squares) for 24, 48, 72, and 144 h. (C) Ribonuclease protection analysis of *BRCA1* mRNA expression targeted to exons 20, 21, and 22. (D) Phosphorimage analysis of *BRCA1* expression in MCF-7 (open bars) and BG-1 cells (solid bars), corrected for expression of the control gene cyclophilin. The values shown are in arbitrary units (BRCA1/cyclophilin). (E) Western analysis of MCF-7 and BG-1 cells treated with estrogen (lanes 1 and 4), ICI-182780 (lanes 2 and 5), or both (lanes 3 and 6) for 72 h after a 5-d starvation in estrogen-free medium.

tered at the transcriptional level by mechanisms related to the simian virus 40 immortalization of these cells. Investigation of other virally transformed cells may indicate whether the presence of viral proteins stabilizes the BRCA1 transcript.

In addition to examining whether BRCA1 was secreted into the medium in estrogen-stimulated cells, we determined that the nuclear location of the BRCA1 protein did not change in cells stimulated with estrogen. Initially, there were conflicting reports that indicated that BRCA1 was either expressed exclusively in the nucleus [26] or expressed in both the cytoplasm and the nucleus [27]. While technical reasons related to antibodies, fractionation procedures, and immunofluorescence protocols [26–28] may explain this data, there may also be biological explanations. Since these initial reports, additional experiments have shown that BRCA1 is predominantly a nuclear protein [40,41]. One report suggests that BRCA1 is associated with cytoplasmic components that penetrate the nucleus [42]. Some investigators have examined the localization of

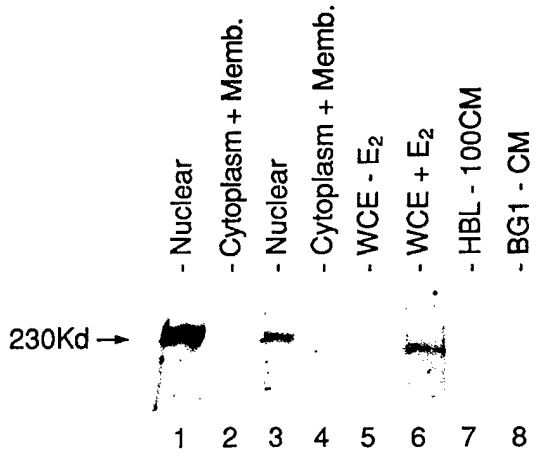

Figure 6. Localization of BRCA1 protein in estrogen stimulated BG-1 ovarian cancer cells. Cellular proteins from logarithmically growing HBL-100 cells (lanes 1 and 2) or 72 hr-estrogen stimulated BG-1 cells (lanes 3 and 4) were fractionated into nuclear (lanes 1 and 3) or cytoplasmic and membrane (lanes 2 and 4) components. Whole-cell extract from unstimulated (lane 5) and estrogen-stimulated (lane 6) BG-1 cells were run as loading controls. In addition, conditioned medium (CM) from HBL-100 and estrogen-stimulated BG-1 cells was concentrated and run in lanes 7 and 8, respectively.

BRCA1 after DNA damage and have found that while the location within the nucleus changes, BRCA1 remains nuclear after damage [40,43]. We were interested in investigating whether the proliferative status and expression level of BRCA1 resulted in a change in the location of the protein, specifically in the appearance of BRCA1 in the cytoplasmic/membrane compartment. We did not observe any change in the location of BRCA1 after estrogen stimulation. Little or no cytoplasmic BRCA1 was detected. More recent data suggest that some cells express an alternately spliced form of BRCA1 that produces a protein that has no nuclear localization signals. It has been shown in transfection experiments that this form of BRCA1 remains cytoplasmic [12]. We are currently examining the expression of this variant form after estrogen treatment of our cells.

The kinetics of accumulation of *BRCA1* mRNA differed in ovarian and breast cells. Early accumulation of *BRCA1* in ovarian BG-1 cells occurred within 24 h, whereas *BRCA1* mRNA levels in MCF-7 cells peaked at 72 h in response to estrogen. It is interesting that BG-1 cells grew faster than MCF-7 cells did. Consequently, we speculate that early expression of *BRCA1* in BG-1 cells may be related to faster cell growth. Similarly, when we compared the patterns of *BRCA1* expression and cell growth of C7-MCF-7 with those of parental MCF-7 cells, we observed that C7-MCF-7 cells proliferated faster (Figure 1) and expressed higher levels of *BRCA1* within 24 h. We interpreted this early expression of *BRCA1* in C7-MCF-7 cells as a consequence of the higher number of ERs, which may have accelerated estrogen-induced proliferation. These data are evidence of an association between the rate of cell growth and stimulation of *BRCA1* expression in breast and ovarian cancer ER-positive cells. This notion is central to the concept that *BRCA1* encodes peptides with growth-inhibitory properties [10], and accelerated proliferation may trigger early BRCA1 expression in an effort to hamper cell growth. Therefore, despite cell- and tissue-specific mechanisms that may be specific to ovarian and breast tissue, our findings suggest that upregulation of BRCA1 may be a feedback mechanism related to rate of cell proliferation and, therefore, cell density. On the other hand, early induction of BRCA1 in BG-1 cells may imply direct responsiveness of BRCA1 to estrogen in ovarian but not breast cells. In fact, in MCF-7 cells expression of *pS2* reached a maximum within 24 h, whereas *BRCA1* mRNA did not peak until 72 h. It is conceivable that late stimulation of BRCA1 in MCF-7 cells may be the result of cell growth induction by autocrine/paracrine pathways triggered by estrogen [44].

Overall, our observations support the contention that estrogen-stimulated cell proliferation may be a prerequisite for upregulation of expression of BRCA1. A previous study indicated that BRCA1 inhibits breast and ovarian cancer cell growth and, therefore, confirmed that BRCA1 is a growth inhibitor and tumor suppressor gene [10]. In addition, antisense experiments indicated that BRCA1 is a protective negative feedback regulator of cell growth [19,24]. We have preliminary data that show the expression of antisense *BRCA1* mRNA in BG-1 cells inhibits endogenous accumulation of *BRCA1* transcripts and permits accelerated growth of antisense BG-1 cells in response to estrogen [45]. Similarly, induction of *BRCA1* expression with antisense oligonucleotides accelerates the growth of normal and malignant mammary epithelial cells [19] and provided evidence that BRCA1 indeed may act as a negative regulator of mammary epithelial cell proliferation. Whether estrogen induction of BRCA1 is due to activation of regulatory elements within the *BRCA1* sequence [46] or to autocrine/paracrine loops induced by estrogen necessitates further investigation.

REFERENCES

1. Topper YJ, Freeman CS. Multiple hormone interactions in the developmental biology of the mammary gland. Physiol Rev 60:1049–1060, 1980.
2. Nandi S, Guzman RC, Yang J. Hormones and mammary carcinogenesis in mice, rats, and humans: A unifying hypothesis. Proc Natl Acad Sci USA 92:3650–3657, 1995.
3. Rao BR, Slotman BJ. Endocrine factors in common epithelial ovarian cancer. Endocr Rev 12:14–26, 1991.
4. Forsyth IA. Mammary development. Proc Nutr Soc 48:17–22, 1989.
5. King M-C, Rowell S, Love SM. Inherited breast and ovarian cancer. What are the risks? What are the choices? JAMA 269:1975–1980, 1993.
6. Miki Y, Swensen J, Shattuck-Eidens D, et al. A strong candidate

for the breast and ovarian cancer susceptibility gene BRCA1. Science 266:66–71, 1994.
7. Futreal PA, Liu Q, Shattuck-Eidens D, et al. BRCA1 mutations in primary breast and ovarian carcinomas. Science 266:120–122, 1994.
8. Lee JH, Kavanagh JJ, Wildrick DM, Wharton JT, Blick M. Frequent loss of heterozygosity on chromosomes 6q, 11, and 17 in human ovarian carcinomas. Cancer Res 50:2724–2728, 1990.
9. Eccles DM, Cranston G, Steel CM, Nakamura Y, Leonard RCF. Allele losses on chromosome 17 in human epithelial ovarian carcinoma. Oncogene 5:1599–1601, 1990.
10. Holt JT, Thompson ME, Szabo C, et al. Growth retardation and tumor inhibition by BRCA1. Nat Genet 12:298–302, 1996.
11. Shao N, Chai YL, Shyam E, Reddy P, Rao VN. Induction of apoptosis by the tumor suppressor protein BRCA1. Oncogene 13:1–7, 1996.
12. Wilson CA, Payton MN, Elliott GS, et al. Differential subcellular localization, expression and biological toxicity of BRCA1 and the splice variant BRCA1-Δ11b. Oncogene 14:1–16, 1997.
13. Easton DF, Bishop DT, Ford D, Crockford GP. Genetic linkage analysis in familial breast and ovarian cancer: Results from 214 families. Am J Hum Genet 52:678–701, 1993.
14. Castilla LH, Couch FJ, Edros MR, et al. Mutations in the BRCA1 gene in families with early-onset breast and ovarian cancer. Nat Genet 8:387–391, 1994.
15. Simard J, Tonin P, Durocher F, et al. Common origins of BRCA1 mutations in Canadian breast and ovarian cancer families. Nat Genet 8:392–398, 1994.
16. Struewing JP, Abeliovich D, Peretz T, et al. The carrier frequency of the BRCA1 185delAG mutation is approximately 1 percent in Ashkenazi Jewish individuals. Nat Genet 11:198–200, 1995.
17. Hosking L, Trowsdale JA. A somatic BRCA1 mutation in an ovarian tumour. Nat Genet 9:343–344, 1995.
18. Merajver SD, Pham TM, Caduff RF, et al. Somatic mutations in the BRCA1 gene in sporadic ovarian tumors. Nat Genet 9:439–450, 1995.
19. Thompson ME, Jensen RA, Obermiller PS, Page PS, Holt DL. Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nat Genet 9:444–450, 1995.
20. Gudas JM, Nguyen H, Li T, Cowan KH. Hormone-dependent regulation of BRCA1 in human breast cancer cells. Cancer Res 55:4561–4565, 1995.
21. Marquis ST, Jayant VR, Wynshaw-Boris A, et al. The developmental pattern of BRCA1 expression implies a role in differentiation of the breast and other tissues. Nat Genet 11:17–26, 1995.
22. Marks JR, Huper G, Vaughn JP, et al. BRCA1 expression is not directly responsive to estrogen. Oncogene 14:115–121, 1997.
23. Spillman MA and Bowock AM. BRCA1 and BRCA2 mRNA levels are coordinately elevated in human breast cancer cells in response to estrogen. Oncogene 13:1639–1645, 1996.
24. Rao VN, Shao N, Ahmad M, Shyam E, Reddy P. Antisense RNA to the putative tumor suppressor gene BRCA1 transforms mouse fibroblasts. Oncogene 12:523–528, 1996.
25. Jensen RA, Thompson ME, Jetton TL, et al. BRCA1 is secreted and exhibits properties of a granin. Nat Genet 12:303–308, 1996.
26. Scully R, Ganesan S, Brown M, et al. Location of BRCA1 in human breast and ovarian cell lines. Science 272:123–125, 1996.
27. Chen Y, Chen C-F, Riley DJ, et al. Aberrant subcellular localization of BRCA1 in breast cancer. Science 270:789–791, 1995.
28. Wilson CA, Payton MN, Pekar SK, et al. BRCA1 protein products: Antibody specificity. Nat Genet 13:264–265, 1996.
29. Thomas JE, Smith M, Rubinfeld B, Gutowski M, Beckmann RP, Polakis P. Subcellular localization and analysis of apparent 180-kDa and 220-kDa proteins of the breast cancer susceptibility gene, BRCA1. J Biol Chem 271:28630–28635, 1996.
30. Aprelikova O, Kuthiala A, Bessho M, Ethier S, Liu E. BRCA1 protein level is not affected by peptide growth factors in MCF10A cell line. Oncogene 13:2487–2491, 1996.
31. Bienstock RJ, Darden T, Wiseman R, Pedersen L, Barrett JC. Molecular modeling of the amino-terminal zinc ring domain of BRCA1. Cancer Res 56:2539–2545, 1996.
32. Chapman MS, Verma IM. Transcriptional activation by BRCA1. Nature 382:678–679, 1996.
33. Monteiro ANA, August A, Hanafusa H. Evidence for a transcriptional activation function of BRCA1 C-terminal region. Proc Natl Acad Sci USA 93:13595–13599, 1996.
34. Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. Environ Health Perspect 103:113–122, 1995.
35. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: Implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 83:2496–2500, 1986.
36. Puissant C, Houdebine LM. An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Biotechniques 8:148–149, 1990.
37. Haendler B, Hofer E. Characterization of the human cyclophilin gene and of related processed pseudogenes. Eur J Biochem 190:477–482, 1990.
38. Schreiber E, Matthias P, Muller MM, Schaffner. Eukaryotic expression vectors for the analysis of mutant proteins. Nucleic Acids Res 17:6418, 1989.
39. Zajchowski DA, Sager R. Induction of estrogen-regulated genes differs in immortal and tumorigenic human mammary epithelial cells expressing a recombinant estrogen receptor. Mol Endocrinol 5:1613–1623, 1991.
40. Thomas JE, Smith M, Tonkinson JL, Rubinfeld B, Polakis P. Induction of phosphorylation on BRCA1 during the cell cycle and after DNA damage. Cell Growth Differ 8:801–809, 1997.
41. Ruffner H, Verma IM. BRCA1 is a cell cycle-regulated nuclear phosphoprotein. Proc Natl Acad Sci USA 94:7138–7143, 1997.
42. Coene E, Van Oostveldt P, Willems K, van Emmelo J, De Potter CR. BRCA1 is localized in cytoplasmic tube-like invaginations in the nucleus. Nat Genet 16:122–124, 1997.
43. Scully R, Chen J, Ochs RL, et al. Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. Cell 90:1–20, 1997.
44. Reddy KB, Yee D, Hilsenbeck SG, Coffey RJ, Osborne CK. Inhibition of estrogen-induced breast cancer cell proliferation by reduction in autocrine transforming growth factor alpha expression. Cell Growth Differ 5:1275–1281, 1994.
45. Annab L, Lyon T, Solomon G, et al. Inhibition of endogenous BRCA1 by antisense in an ER positive ovarian cell line results in proliferation and protection from cell death during estrogen deprivation. Abstract. Proceedings of the American Association for Cancer Research 37:585, 1996.
46. Norris J, Fan D, Aleman C, et al. Identification of a new subclass of Alu DNA repeats which can function as estrogen receptor-dependent transcriptional enhancers. J Biol Chem 270:22777–22782, 1995.

MOLECULAR CARCINOGENESIS 26:100–118 (1999)

Inhibition of BRCA-1 Expression by Benzo[*a*]pyrene and its Diol Epoxide

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The objective of this study was to investigate whether polycyclic aromatic hydrocarbons (PAHs) contribute to the etiology of sporadic breast cancer by altering the expression of BRCA-1. Acute exposure to the PAH benzo[*a*]pyrene (B[a]P) inhibited in a time- and dose-dependent fashion cell proliferation and levels of *BRCA-1* mRNA and protein in estrogen receptor (ER)–positive breast MCF-7 and ovarian BG-1 cancer cells. Moreover, the acute exposure to B[a]P abrogated estrogen induction of BRCA-1 in MCF-7 cells. The loss of BRCA-1 expression was prevented by the aromatic hydrocarbon receptor (AhR) antagonist α-naphthoflavone, suggesting participation of the AhR pathway. *BRCA-1* exon 1a transcripts were downregulated by B[a]P faster than exon 1b mRNA was. Long-term exposure to B[a]P (40 nM for 15 mo) lowered *BRCA-1* mRNA levels in subclones of MCF-7 and BG-1 cells, whereas expression of *BRCA-1* in these clones was reverted to normal levels by washing out of B[a]P. The mechanisms of BRCA-1 repression by B[a]P were further investigated by examining the effects of the halogenated aryl hydrocarbon 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and the B[a]P metabolite 7r,8t-dihydroxy-9t,10t-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE). While TCDD did not influence basal *BRCA-1* mRNA and protein levels at any of the doses (from 10 nM to 1 μM) tested in this study, treatment with 50 nM BPDE drastically reduced *BRCA-1* mRNA levels, indicating that metabolism of B[a]P to BPDE may contribute to downregulation of *BRCA-1*. Conversely, ER-negative breast MDA-MB-231 and HBL-100 cancer cells were refractory to treatment with B[a]P or TCDD and expressed constant levels of *BRCA-1* mRNA and protein. We conclude that B[a]P may be a risk factor in the etiology of sporadic breast cancer. *Mol. Carcinog. 26:100–118, 1999.* © 1999 Wiley-Liss, Inc.

Key words: benzo[*a*]pyrene; BRCA-1; sporadic breast cancer; aromatic hydrocarbon receptor

INTRODUCTION

Significant efforts have been devoted to dissecting the biological function of the breast and ovarian cancer susceptibility gene, *BRCA-1*, since its discovery in 1994 [1,2]. The characterization of many germline mutations in familial breast and ovarian cancer has confirmed the role of *BRCA-1* as a tumor suppressor gene [3]. Although the presence of zinc-finger and nuclear-localization domains [4] imply that BRCA-1 has transcription-activation functions [5], alternative subcellular compartmentalization of BRCA-1 peptides may be linked to the onset of sporadic breast cancer [6] or related to unknown functions of BRCA-1 [7,8]. In mitotic and meiotic cells, both BRCA-1 and BRCA-2 interact with rad51, which is involved in repairing double-stranded breaks and recombination-linked repair [9,10], suggesting that both BRCA-1 and BRCA-2 function as caretakers in the maintenance of genome integrity [11]. Additionally, recent findings provided important evidence that BRCA-1 is involved in transcription-coupled repair of oxidative DNA damage [12].

Family history and dietary, reproductive, hormonal, and environmental factors may contribute to breast cancer risk. Specific mutations in the zinc-finger domain localized to the amino-terminus of the BRCA-1 peptide have been identified in inherited breast and sporadic ovarian cancer [13]. However, to date there is no evidence of a causal relationship between mutations in the coding region of *BRCA-1* and the incidence of sporadic breast cancer. Therefore, non-mutational events

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Abbreviations: PAH, polycyclic aromatic hydrocarbon; ER, estrogen receptor; B[a]P, benzo[*a*]pyrene; AhR, aromatic hydrocarbon receptor; ARNT, AhR nuclear translocator; CYP, cytochrome P450; BPDE, 7r,8t-dihydroxy-9t, 10t-epoxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; B[e]P, benzo[*e*]pyrene; ANF, α-naphthoflavone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CS-FCS, charcoal-stripped fetal calf serum; PCR, polymerase chain reaction; RT, reverse transcription; XRE, xenobiotic-responsive element.

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that alter the expression of BRCA-1, including environmental insults, may be responsible for the onset of sporadic mammary neoplasia. In keeping with this notion, the levels of *BRCA-1* mRNA are decreased in sporadic breast tumors [14], whereas in familial breast carcinomas, loss of *BRCA-1* is linked to regulatory mutations [15].

Previous investigations have documented down-regulation of *BRCA-1* mRNA in human breast [16] and ovarian [17] cancer cells by DNA-damaging and cytotoxic agents, including doxorubicin and ultraviolet radiation. Our goal was to investigate the effects of acute and chronic exposure to polycyclic aromatic hydrocarbons (PAHs) on expression of BRCA-1. PAHs, which have been shown to induce mammary tumors in rodents [18], are ubiquitous contaminants found in tobacco smoke, industrial pollution, coal tar, and auto exhaust [19]. The exposure to PAHs may disrupt endocrine functions by altering the expression of estrogen-inducible genes [20], the oxidative metabolism of estrogen [21], and the expression of progesterone, epidermal growth factor, and estrogen receptor (ER) [22]. The PAH benzo[*a*]pyrene (B[a]P) is a classic DNA-damaging carcinogen [23] found in amounts of 20–40 ng per cigarette [24]. B[a]P displays high affinity for the aromatic hydrocarbon receptor (AhR). The AhR is a ligand-activated transcription factor that regulates transcription of several PAH-responsive genes and the induction of apoptosis by dioxin-like compounds in immature thymocytes [25,26]. Activation of the AhR signal transduction pathway requires the assembly of a heterocomplex comprised of an AhR ligand, the AhR, and a nuclear translocator protein (AhR nuclear translocator (ARNT)) [27]. The AhR heterocomplex transactivates promoter elements of a multitude of genes, such as those encoding enzymes of the cytochrome P450 (CYP) family, which, along with other modifying enzymes, metabolize B[a]P to a number of end products, including the mutagenic diol-epoxide 7r,8t-dihydroxy-9t,10t-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) [28]. The metabolite BPDE has been shown to form stable DNA adducts at mutational hot-spots in the *p53* [24] and Ha-*ras* [29] genes and to disrupt Sp1-dependent transcription and the binding of E2F heterodimers to DNA [28]. In addition, BPDE has been shown to induce growth arrest and DNA damage in murine 3T3 fibroblasts [30].

In previous studies, we have documented that estrogen induces the expression of BRCA-1 in ER-positive breast and ovarian cancer cells through mechanisms that involve the ER [31,32]. Here, we present evidence that both acute and chronic exposure to B[a]P abrogate the expression of BRCA-1 in ER-positive breast MCF-7 and ovarian BG-1 cancer cells but not in ER-negative breast MDA-MB-231 and HBL-100 cancer cells. Also, we document that the reactive metabolite BPDE repressed *BRCA-1* transcripts in MCF-7 cells. The results presented in this report support the notion that in ER-positive cells, activation of the AhR by B[a]P may be a risk factor in the etiology of sporadic breast cancer.

MATERIALS AND METHODS

Cell Culture and Proliferation Assay

Regulation of expression of BRCA-1 by AhR ligands was investigated in ER-positive breast MCF-7 and ovarian BG-1 cancer cells and ER-negative breast MDA-MB-231 and HBL-100 cancer cells obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories, Inc., Logan, UT). B[a]P, benzo[*e*]pyrene (B[e]P), α-naphthoflavone (ANF), and 17β-estradiol were obtained from Sigma Chemical Co., BPDE and TCDD were obtained from Midwest Research Institute (Kansas City, MO). For proliferation studies, cells were plated at a density of 2×10^6 cells/100-mm tissue-culture dish and maintained overnight in DMEM/F12 plus 10% FCS. Three dishes were assigned to each experimental treatment. At the end of the incubation periods, the cells were counted in triplicate ($n = 9$). Before treatment with estrogen, the cells were preconditioned for 5 d in phenol red–free medium containing 5% charcoal-stripped FCS (CS-FCS) (Hyclone Laboratories, Inc.) as previously described [32]. Then, the cells were cultured in DMEM for the designated time in 5% CS-FCS free of phenol red in the presence or absence of 10 nM estrogen.

Ribonuclease Protection Assay

Total cellular RNA was extracted by using a guanidinium thiocyanate procedure [33]. The integrity of the total RNA was confirmed by electrophoretic analysis of ribosomal 28S and 18S subunits (data not shown). Changes in *BRCA-1* mRNA were measured by ribonuclease protection assay as described previously [32] by using the Hybspeed kit (Ambion Inc., Austin, TX). Briefly, a DNA fragment of 162 bp encoding a portion of exon 15 was amplified by using the forward DF15 and reverse DR14 oligonucleotides (Table 1). That the fragment was identical to the *BRCA-1* sequence deposited in GenBank was ascertained by direct sequencing of the polymerase chain reaction (PCR) product, which was cloned in the antisense orientation into the transcription vector Triplescript (Ambion Inc.). In vitro transcription from this construct generated a riboprobe that hybridized with *BRCA-1* mRNA to protect a fragment of the expected length (data not shown). As an internal control, we transcribed a riboprobe for human cyclophilin by using the pTRI-

Table 1. Primers for *BRCA-1* and *CYP1A1*

Gene	Primers	Primer sequence
BRCA-1 exon 1a	DF1	5′-AGCTCGCTGAGACTTCCTGGA-3′
BRCA-1 exon 1b	LH9-F	5′-ACGTCGGCTGGTCATGAG-3′
BRCA-1 exon 2	MDF1	5′-TAATACGACTCACTATAGGGAGGAGCTCGCTGAGACTTCCT-GGATATCTGCTCTTCGCGTTGAAG-3′*
BRCA-1 exon 8	DR6	5′-CAATTCAATGTAGACAGACGT-3′
	MDR6	5′-T_{20}CAATTCAATGTAGACAGACGT-3′
BRCA-1 exon 15	DF15	5′-ATGATAGGTGGTACATGCACA-3′
	DR14	5′-CTAGATCTTGCCTTGGCAAGT-3′
CYP1A1	A1AF	5′-TAACATCGTCTTGGACCTCTTTG-3′
	A1AR	5′-GTCGATAGCACCATCAGGGGT-3′

*The underlined sequence represents the T7 RNA polymerase promoter.

cyclophilin template (Ambion Inc.), which, upon digestion with RNAse, protects a fragment of 103 bp. *BRCA-1* mRNA was quantitated by phosphorimage analysis in arbitrary units corrected for the expression of the control, cyclophilin mRNA (*BRCA-1*/cyclophilin).

Competitive Reverse Transcription–PCR

For competitive reverse transcription (RT)–polymerase chain reaction, we used the strategy described in the QuantumRNA Module (Ambion Inc.). We constructed a competitive DNA template from which we generated by PCR a 582-bp product, 130 bp smaller than the endogenous 712-bp *BRCA-1* fragment amplified by using the forward (DF1) and reverse (DR6) oligonucleotides (Table 1). The competitive forward primer (MDF1) was designed to bind 152 bp downstream from DF1 and included a T7 polymerase promoter at the 5′ end. The reverse competitive primer (MDR6) contained the oligonucleotide DR6 and a 20-bp poly(T) tail. The competitive DNA fragment generated by PCR was used as a template in a transcription reaction using the MAXIscript kit (Ambion Inc.). After DNAse treatment, the synthetic RNA was purified by phenol-choloroform extraction and ethanol precipitated. The synthetic RNA was quantitated and added to RT reaction mixtures containing total RNA from MCF-7 cells cultured in DMEM/F12 in the presence or absence of 5 μM B[a]P for 48 h. Total cellular and synthetic RNAs were incubated with random hexamer primers, Moloney murine leukemia virus reverse transcriptase, RNAse inhibitor (Life Technologies/Gibco BRL, Gaithersburg, MD), and RT buffer (Ambion Inc.) at 42°C for 1 h. After RT-PCR with DF1 and DR6 as the forward and reverse oligonucleotides, respectively, the *BRCA-1* transcripts were quantitated by logarithm transformation of the ratio between endogenous and competimer *BRCA-1* mRNA and plotted against the logarithm of input competimer *BRCA-1* mRNA.

Semiquantitative RT-PCR

For semiquantitative RT-PCR, DNA was amplified by PCR with the primers described in Table 1. The PCR products were of the expected sizes, and that they were identical to the *BRCA-1* (accession no. U14680) and *CYP1A1* (accession no. K03191) sequences deposited in GenBank was confirmed by direct sequencing. Oligonucleotides for *BRCA-1* were designed to amplify a 712-bp PCR product spanning exon 1a (DF1, forward) to exon 8 (DR6, reverse). The primers for the internal standards S15 and 18S ribosomal RNA were from Ambion Inc. The levels of 18S ribosomal RNA (488 bp) and S15 (488 bp) PCR products were used as controls for the PCR conditions and equal loading. PCRs were performed by using Vent DNA polymerase (New England Biolabs, Beverly, MA). Preliminary relative semiquantitative pilot experiments were performed to assure that the PCR products were generated in a linear range. The expression levels of *BRCA-1* mRNA were quantified by Alpha Imager (Alpha Innotech Inc., San Diego, CA) analysis in arbitrary units corrected for expression of the control mRNA (*BRCA-1*/18S).

Western Blot Analysis

Western blot analysis was performed as previously described [31]. Protein extracts were prepared by lysing cells in sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer. Cell extracts were normalized to protein content and separated by 4–12% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After transfer to nitrocellulose membranes, the blots were blocked with Tris-buffered saline containing 10% dried milk and 0.1% Tween-20. BRCA-1 immunoblotting was performed with the BRCA-1-Ab2 antibody (Oncogene Research Products, Cambridge, MA), which was raised against an amino-terminal fusion protein, diluted 1:50 in 2% milk in Tris-buffered saline

with Tween-20. The immunoblots were incubated with anti–BRCA-1 antibodies for 2 h at room temperature and then with a secondary antibody (Boehringer Mannheim Corp., Indianapolis, IN). Normalization of western blots was performed by incubating them with actin antibody-1 (Oncogene Research Products). The immunocomplexes were detected by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

RESULTS

Lethal Effects of B[a]P on ER-Positive cells

Because expression of BRCA-1 is upregulated in proliferating breast cancer cells [32], we first studied the relationships between acute exposure to B[a]P and cell proliferation of ER-positive breast (MCF-7) and ovarian (BG-1) cancer cells and ER-negative breast (MDA-MB-231 and HBL-100) cancer cells. We exposed the cells to various concentrations of B[a]P ranging from 50 nM to 10 μM, a range similar to those used to induce cell transformation of primary mammary epithelial cells [34] and modulate the expression of c-Ha-*ras* mRNA [35]. In preliminary studies, we found that the exposure to B[a]P compromised cell viability (as determined by trypan blue exclusion) of ER-positive MCF-7 breast cells in a dose-dependent fashion. Compared with the viability of cells cultured in control DMEM plus 10% FCS, the viabilities of MCF-7 cells treated for 72 h with 1 and 10 μM B[a]P were reduced 3.6- and 10-fold, respectively (data not shown). In time-course experiments, 5 μM B[a]P inhibited growth of MCF-7 and BG-1 cells by 24 h (Figure 1). Growth arrest was followed by cell death at 48–72 h. Conversely, ER-negative MDA-MB-231 and HBL-100 cells were refractory to all of the B[a]P doses tested in this study. In fact, in the presence of 5 μM B[a]P for 72 h, there was a 2.5-fold increase in cell number for both ER-negative cell lines, whose growth did not differ from that of cells grown in control DMEM/F12 plus 10% FCS medium (Figure 1C and D). These data indicated that sensitivity to B[a]P was cell specific, as only the viabilities of ER-positive MCF-7 and BG-1 cells were compromised by treatment with B[a]P.

Upregulation of *CYP1A1* by B[a]P Associated with Loss of *BRCA-1* mRNA and Protein Expression in ER-Positive Cells

The levels of *CYP1A1* mRNA were elevated by treatment with 5 μM B[a]P in MCF-7, MDA-MB-231, and HBL-100 cells (Figure 2A) and in BG-1 cells (data not shown) irrespective of ER status, providing evidence that treatment with B[a]P activated the expression of the AhR-dependent gene *CYP1A1*. Next, we investigated whether the acute exposure to B[a]P influenced the expression of *BRCA-1*. For this purpose, we extracted total RNA from asynchronous ovarian (BG-1) and breast (MCF-7) cancer cells exposed to increasing concentrations of B[a]P for various periods of time. Ribonuclease protection analysis of total RNA documented downregulation of *BRCA-1* mRNA by B[a]P in MCF-7 cells (Figure 2B). Phosphorimage analysis of *BRCA-1* transcripts corrected for the internal control cyclophilin mRNA indicated that the *BRCA-1* levels were reduced 2.2-fold in the presence of B[a]P.

The negative effects of B[a]P on expression of BRCA-1 were confirmed by competitive RT-PCR. Figure 2C shows that the addition of increasing amounts of exogenous *BRCA-1* mRNA to the RT reactions resulted, as expected, in lower yields of endogenous *BRCA-1* products. However, by comparing the log values of the ratios of the 712-bp RT-PCR product spanning exon 1a (type α) to exon 8 and the 582-bp exogenous *BRCA-1* competimer, we ascertained that *BRCA-1* mRNA was less abundant in cells exposed to B[a]P. The slopes of the two straight lines were similar for cells grown in DMEM alone (−0.55, $R^2 = 0.996$) and in B[a]P (−0.435, $R^2 = 0.984$), indicating accurate quantification of exogenous RNA and equal amplification efficiency. However, the points of intersection of the two straight lines at log = 0, at which the ratios between endogenous and competimer products are equal to 1, indicated that the relative amounts of *BRCA-1* mRNA in cells cultured in the presence of B[a]P were lower than those found in control (DMEM-treated) cells.

For semiquantitative RT-PCR, we ascertained in pilot experiments that a 30-cycle PCR amplification of *BRCA-1* required a minimum input of cDNA synthesized from 200 ng of total RNA (Figure 2D). Increasing the input cDNA to that from 400 and 600 ng of total RNA obtained from MCF-7 cells cultured in DMEM resulted in a linear increase in *BRCA-1* amplification. In contrast, the levels of *BRCA-1* mRNA in MCF-7 cells treated with B[a]P were reduced significantly, irrespective of the amount of input cDNA. Therefore, we concluded that under the experimental conditions of this study, the lower PCR yields from cells exposed to B[a]P were a consequence of reduced concentration of *BRCA-1* transcripts in total RNA. For subsequent experiments, RT-PCR was performed with input cDNA from 400 ng of total RNA.

Our results indicated that B[a]P reduced *BRCA-1* type α transcripts in BG-1 (Figure 2E) and MCF-7 cells in a dose-dependent (Figure 2F) and time-dependent (Figure 2G) fashion. The time-dependent effects were evident within 12–24 h after treatment with 5 μM B[a]P. Conversely, in untreated MCF-7 cells, the expression of *BRCA-1* mRNA peaked at 12 h and remained detectable later. Loss of *BRCA-1* mRNA was paralleled by downregulation of BRCA-1 protein (Figure 2H), which, in agreement with the expected size of the full-length peptide, was immunoblotted as a band of approximately 220 kDa.

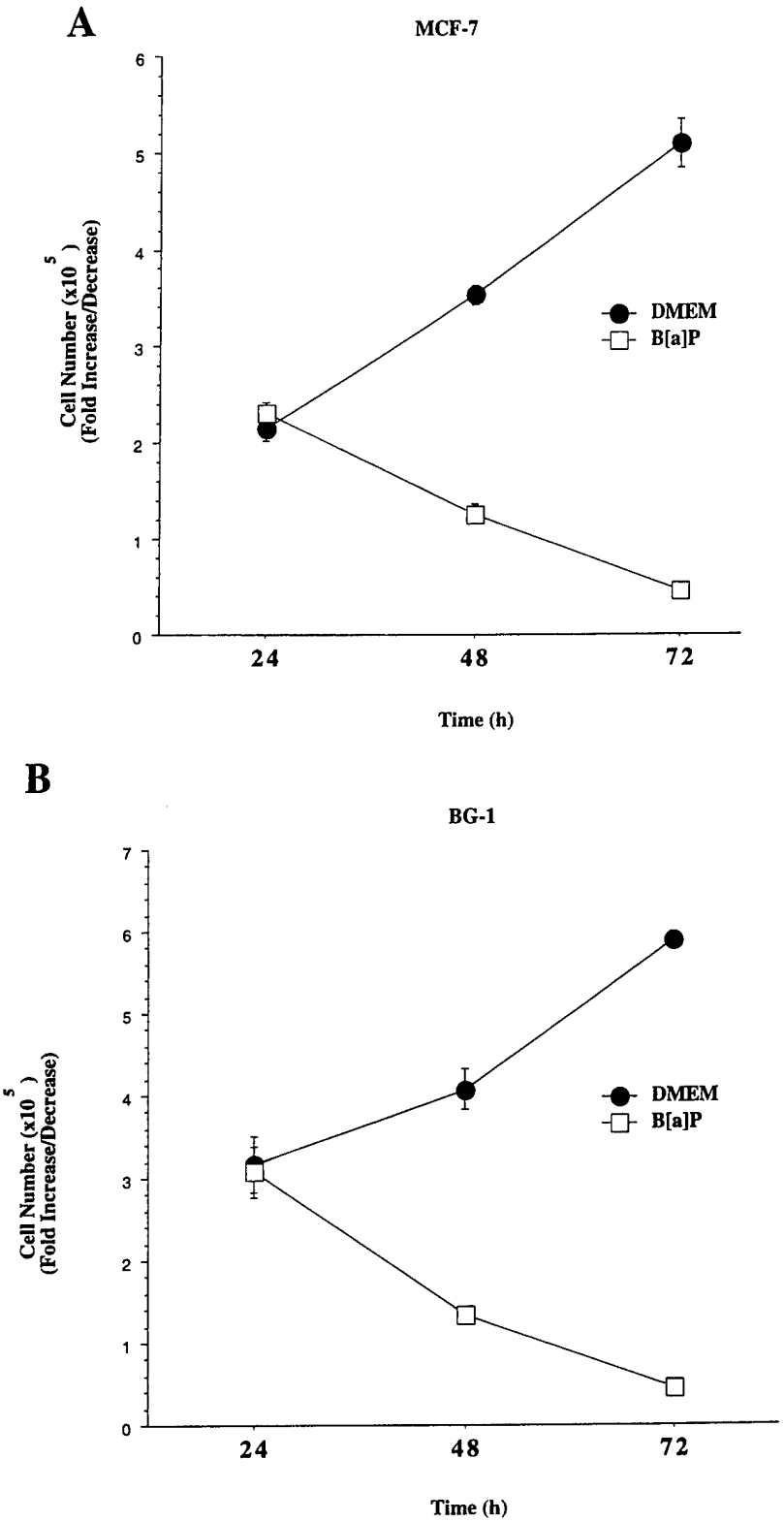
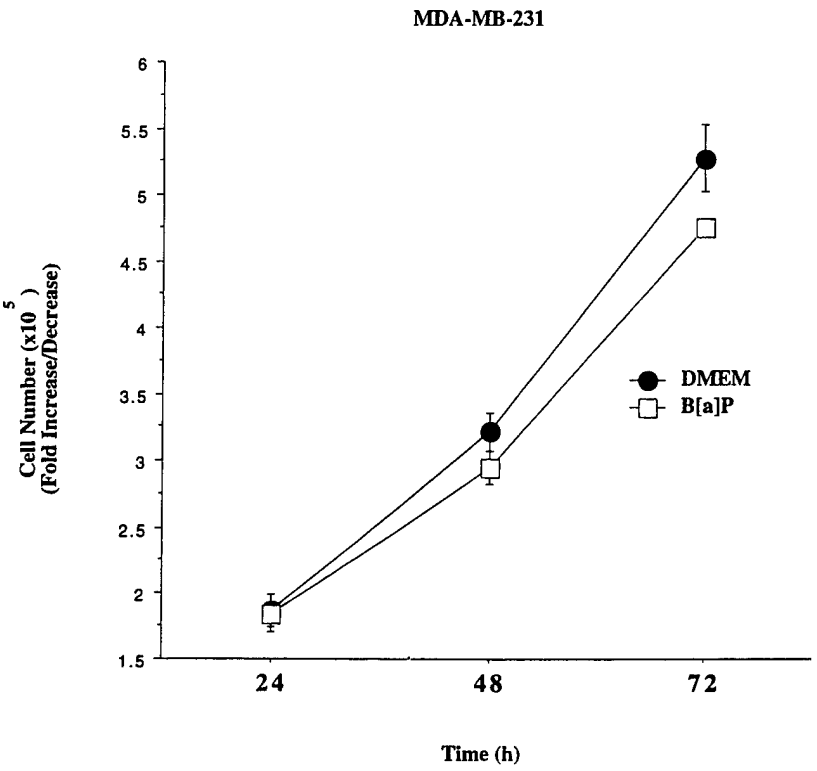


Figure 1. Effect of B[a]P on cell proliferation of breast and ovarian cancer cells. MCF-7 (A), BG-1 (B), MDA-MB-231 (C), and HBL-100 (D) cells were cultured in DMEM/F12 plus 10% FCS in the absence (filled circles) or presence (open squares) of 5 μM B[a]P for 72 h as described in Materials and Methods. The data represent mean cell numbers ± standard deviations of triplicate samples from two independent experiments.

C



D

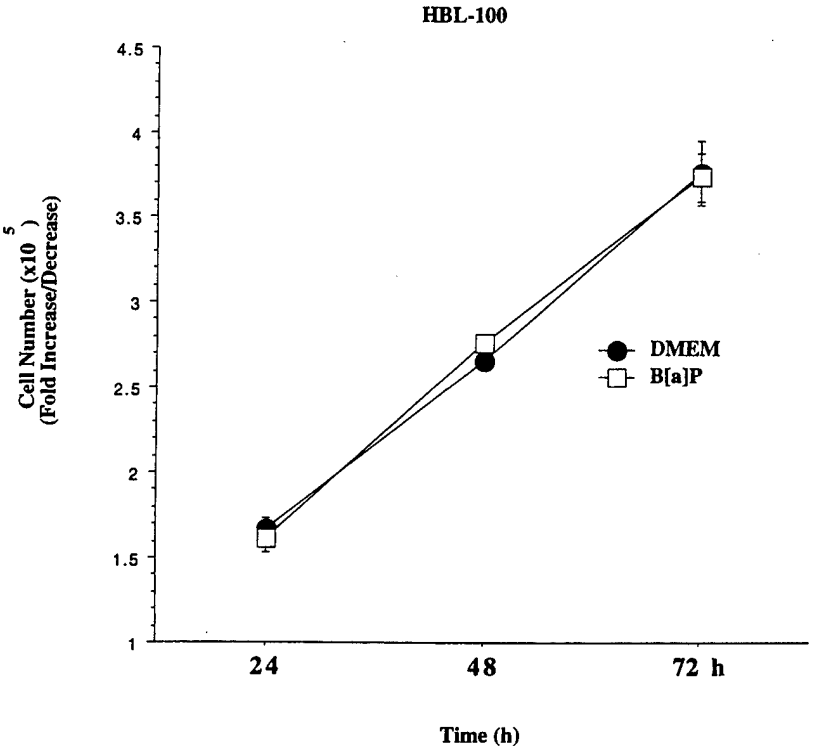


Figure 1. (*continued*)

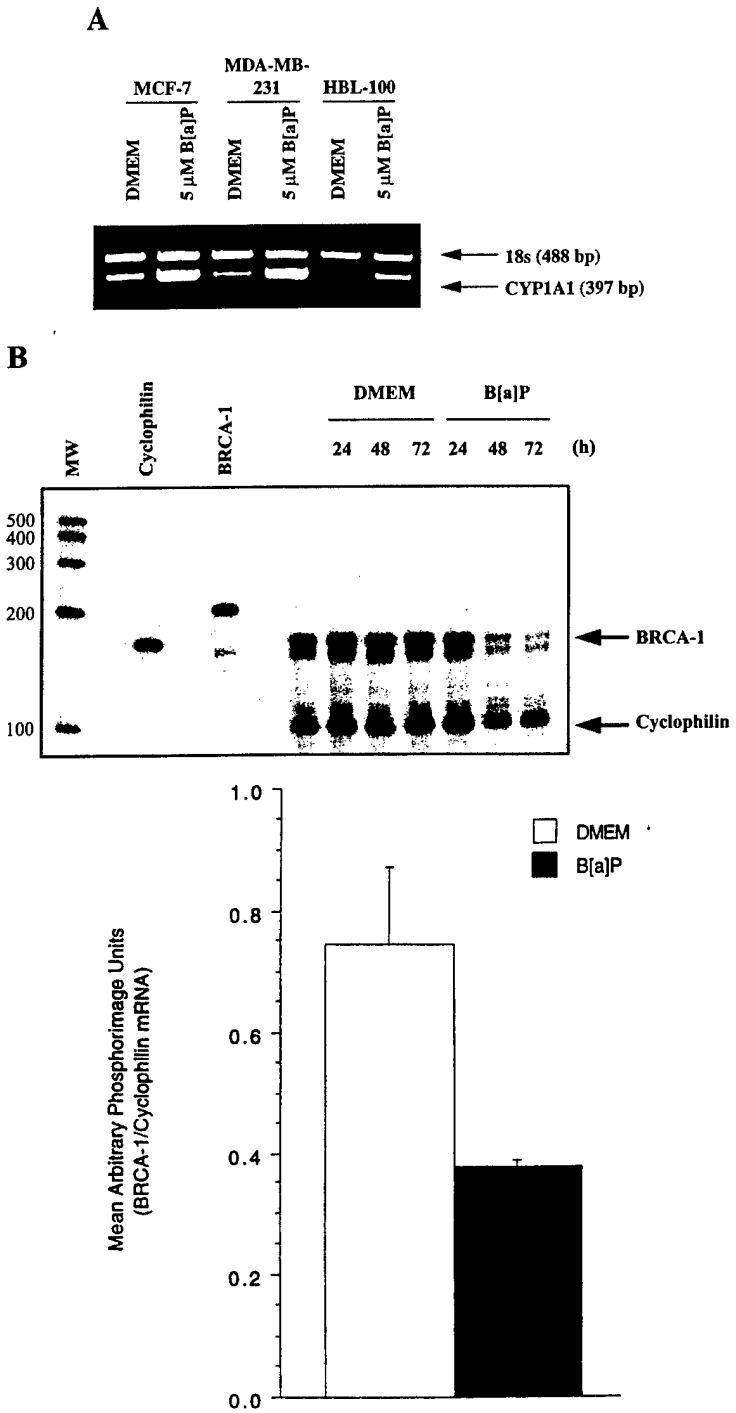
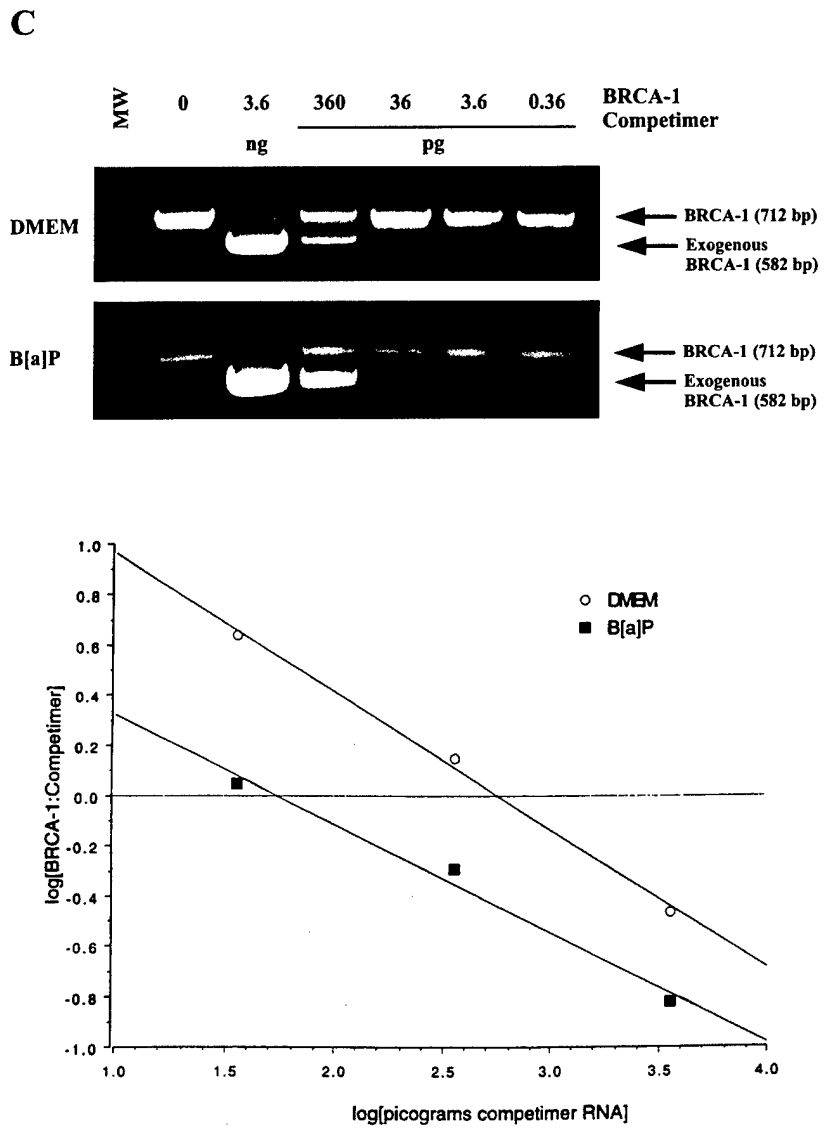


Figure 2. Induction of *CYP1A1* by B[a]P and inhibition of *BRCA-1* type α mRNA in ovarian and breast cancer cells. (A) Treatment with B[a]P activated the expression of *CYP1A1* mRNA in MCF-7, MDA-MB-231, and HBL-100 breast cancer cells. The bands represent *CYP1A1* (397 bp) and ribosomal 18S RNA (488 bp) RT-PCR products from total RNA extracted from cells cultured in control DMEM/F12 plus 10% FCS in the absence or presence of 5 μM B[a]P for 72 h. (B) Ribonuclease protection analysis of *BRCA-1* mRNA in MCF-7 cells. The bars represent the means ± standard deviations of *BRCA-1*/cyclophilin arbitrary phosphorimage units from two independent experiments. (C) Competitive RT-PCR yields from various amounts of synthetic *BRCA-1* mRNA. The diagram shows quantitation of *BRCA-1* mRNA in MCF-7 cells in DMEM only or in the presence of 5 μM B[a]P. (D) Semiquantitative RT-PCR analysis of *BRCA-1* expression from increasing amounts of input total RNA from control and B[a]P-treated MCF-7 cells. (E) *BRCA-1* (712 bp) and control S15 (361 bp) RT-PCR products from total RNA extracted from BG-1 cells cultured in control DMEM/F12 plus 10% FCS in the absence or presence of 5 μM B[a]P for 24, 28, and 72 h. (F) Expression of *BRCA-1* mRNA in MCF-7 cells treated with increasing concentrations of B[a]P for 72 h, measured by RT-PCR as described in Materials and Methods. (G) RT-PCR analysis of the time-dependent effects of 5 μM B[a]P on *BRCA-1* mRNA in MCF-7 cells. (H) Western analysis of BRCA-1 expression after treatment with 5 μM B[a]P for various periods of time. The control bands are β-actin immunocomplexes visualized by incubating the blots with antibody-1.



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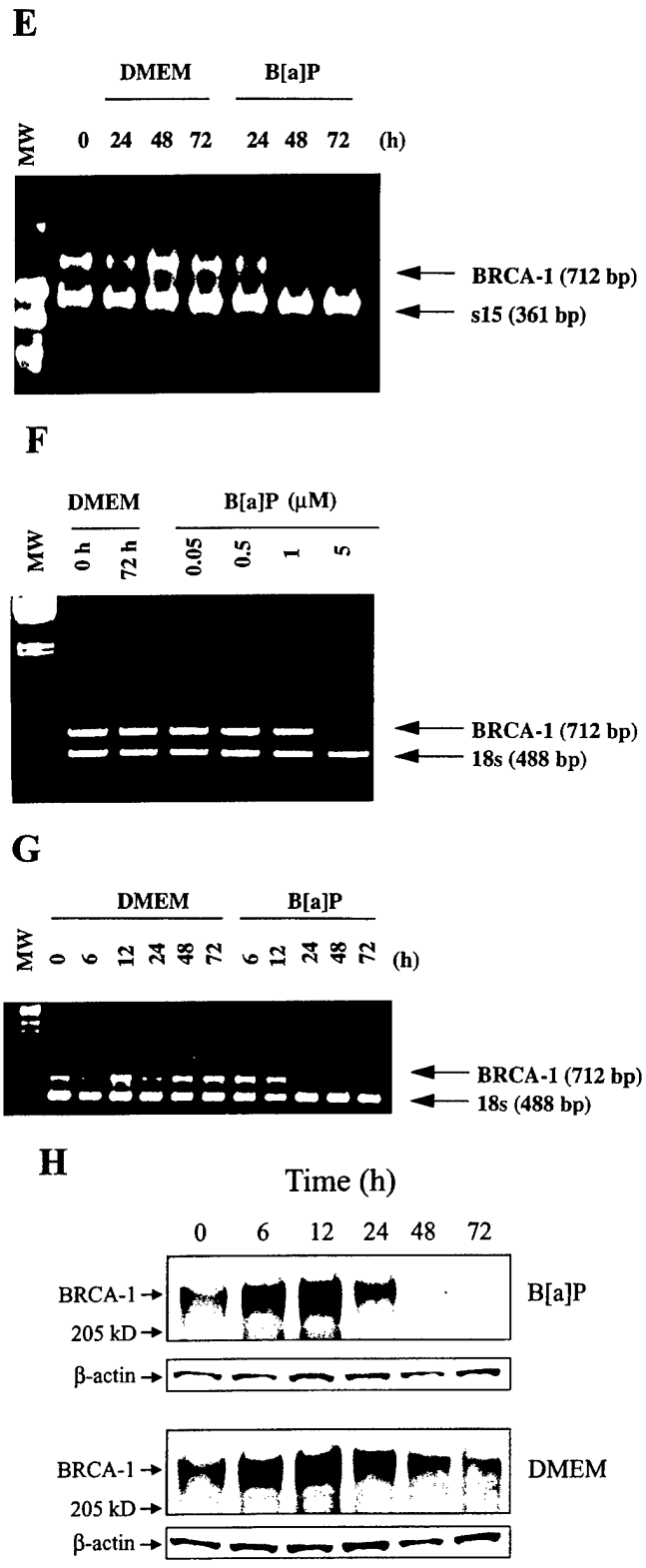

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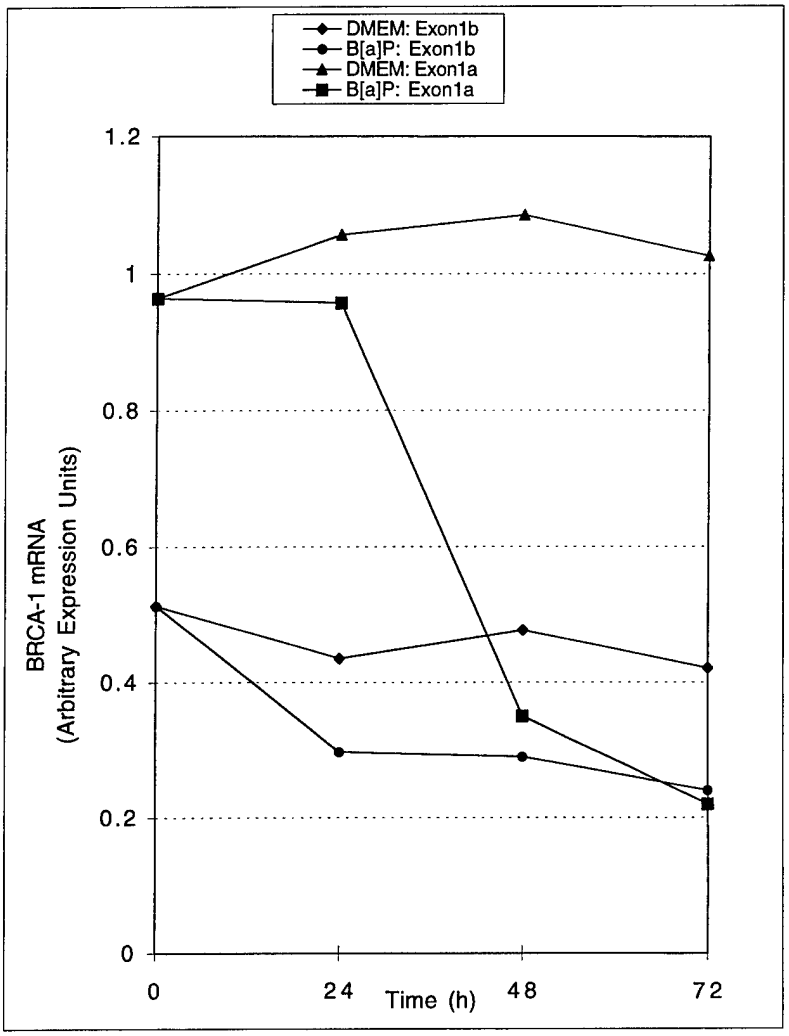


Figure 3. Effect of B[a]P on levels of type α and β *BRCA-1* mRNA. Total RNA was collected after treating MCF-7 cells with 5 μM B[a]P for 24, 48, and 72 h. RT-PCR was performed as described in Materials and Methods. The expression levels of *BRCA-1* mRNAs were quantified by phosphorimager analysis in arbitrary units corrected for expression of the control 18S ribosomal RNA.

Overall, the temporal profiles of expression of *BRCA-1* suggested that downregulation of *BRCA-1* mRNA in MCF-7 cells preceded loss of cell viability by approximately 12–24 h. With respect to the variation in BRCA-1 protein at different time points, we detected increased expression of BRCA-1 at 12 h in both control and B[a]P-treated cells. This was somewhat expected, as upregulation of BRCA-1 is typically seen in rapidly proliferating cells [31]. Nevertheless, in the presence of B[a]P, the levels of BRCA-1 protein declined more rapidly and were not detectable at 48 and 72 h. Therefore, while expression of *CYP1A1* mRNA was upregulated by B[a]P in all of the breast cancer cell lines tested in this study, loss of *BRCA-1* mRNA and protein suggested a negative effect of B[a]P on BRCA-1 expression in ER-positive breast and ovarian cancer cells.

Because exons 1a and 1b are constitutively transcribed from promoters α and β, respectively, we tested whether B[a]P differently altered transcription from the two *BRCA-1* promoters. After RT, exon 1b (type-β) cDNAs were amplified by using the forward oligonucleotide LH9F [36] and the reverse oligonucleotide DR6 (exon 8) (Table 1). As depicted in Figure 3, in control cells, basal expression of exon 1b transcripts was lower than that of exon 1a transcripts, confirming higher levels of type α mRNA in mammary epithelial cells. However, in cells treated with B[a]P, the levels of both transcripts rapidly decreased, although type α mRNA was reduced at a faster rate than was type β mRNA (5.0- vs 2.0-fold).

No Downregulation of *BRCA-1* by B[a]P in ER-Negative Breast Cancer Cells

Consistent with our proliferation data, B[a]P had no effect on expression of *BRCA-1* in MDA-MB-231 (Figure 4) and HBL-100 (data not shown) cells, which contained constant levels of *BRCA-1* mRNA. Therefore, these results suggested that the loss of

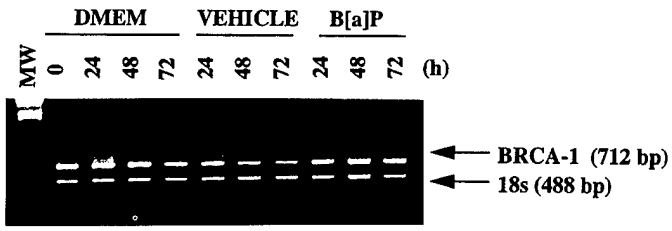
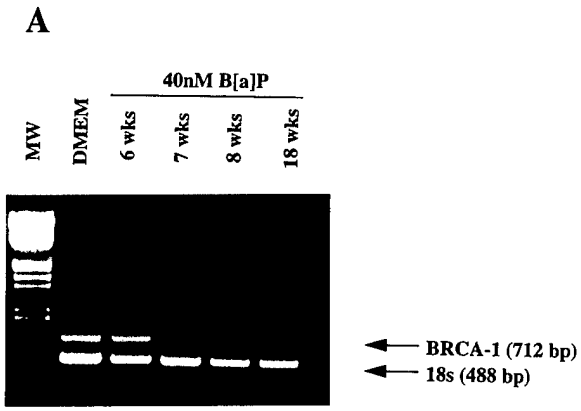


Figure 4. Lack of effect of B[a]P on expression of BRCA-1 in ER-negative MDA-MB-231 cells. Total RNA was extracted from MDA-MB-231 breast cancer cells treated with 5 μM B[a]P for 24, 48, and 72 h. The levels of *BRCA-1* type α and 18S ribosomal RNA were examined by RT-PCR as described in Materials and Methods.



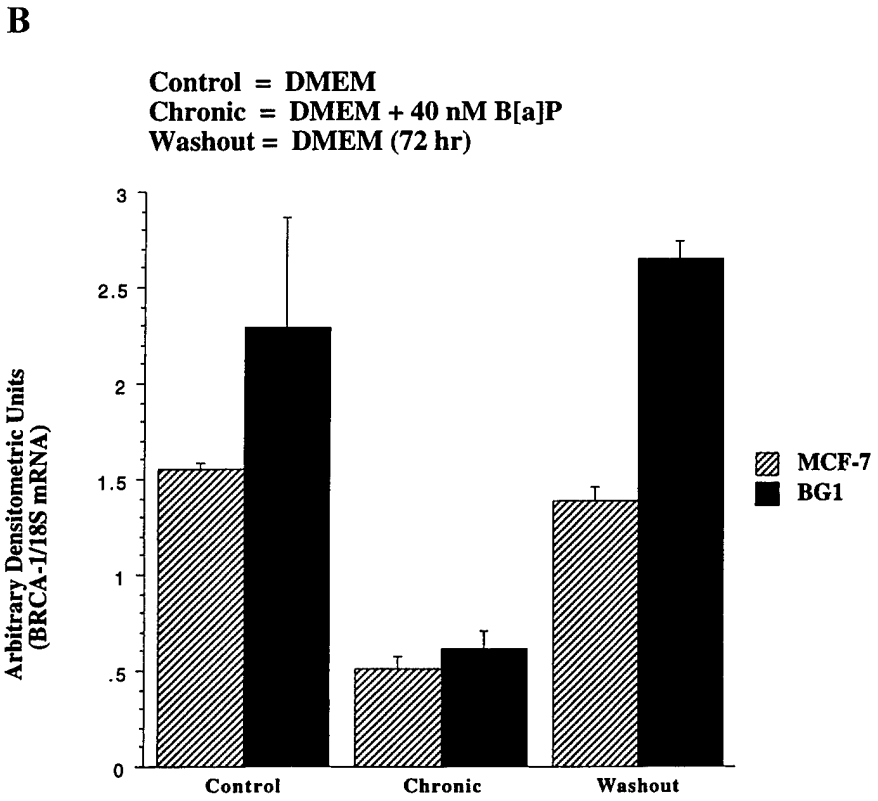


Figure 5. Downregulation of BRCA-1 in breast and ovarian cancer cells by chronic exposure to B[a]P. (A) MCF-7 and BG-1 cells were exposed to 40 nM B[a]P for 6, 7, 8, and 18 wk. At the end of each incubation period, total RNA was extracted and RT-PCR performed as described in Materials and Methods. The bands represent the levels of type α *BRCA-1* and 18S ribosomal RNA. (B) Wash-out experiment. Five each subclones of MCF-7 and BG-1 cells maintained in the presence of 40 nM B[a]P for 15 mo were plated in DMEM/F12 plus 10% FCS and cultured for 72 h in the absence of B[a]P. The bars represent mean levels of *BRCA-1* type α mRNA ± standard deviations measured by RT-PCR of total RNA extracted from five independent subclones and corrected for expression of the control 18S ribosomal RNA.

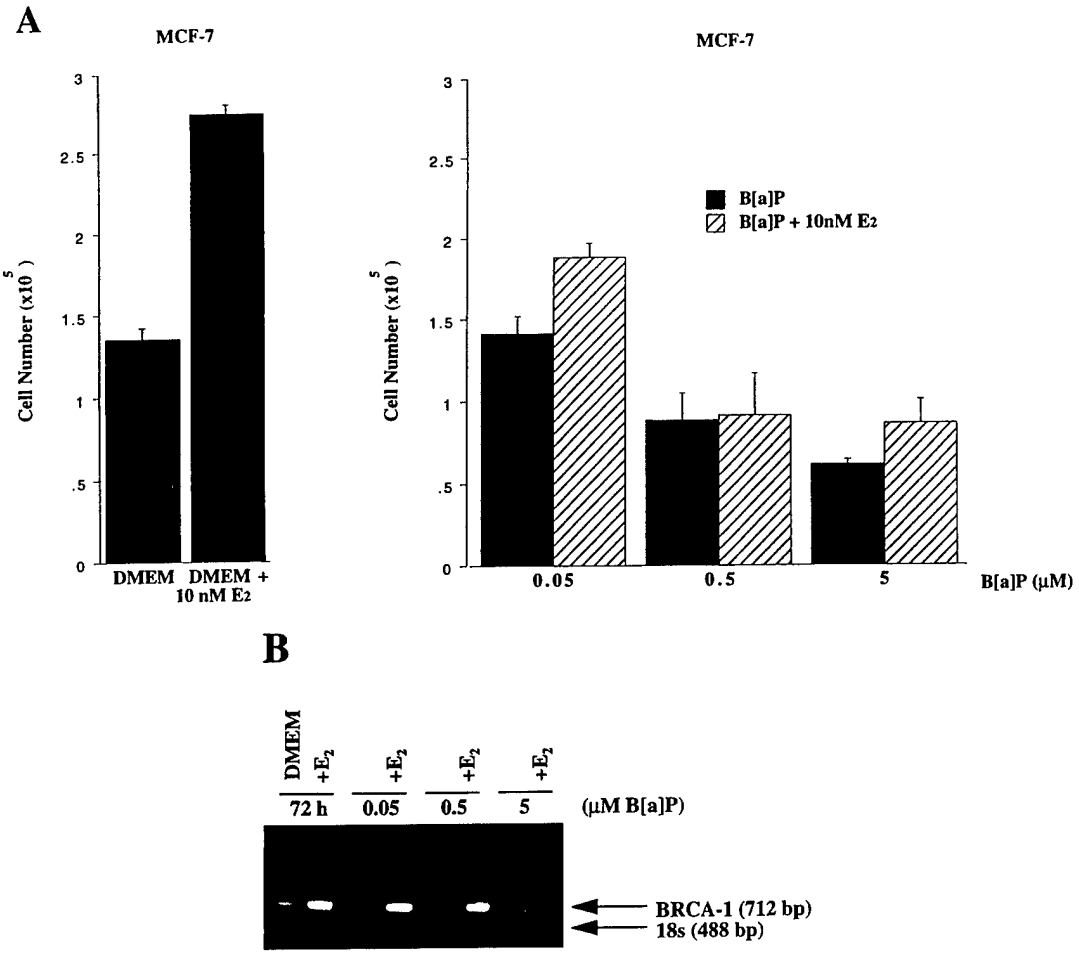


Figure 6. B[a]P disruption of estrogen-dependent proliferation and expression of *BRCA-1*. (A) MCF-7 cells preconditioned in 5% CS-FCS phenol red–free medium were induced with 10 nM estrogen (E_2) in the absence or presence of increasing amounts of B[a]P for 72 h. At the end of the incubation period, the cells were counted in triplicate (n = 9). The bars represent mean cell numbers ± standard deviations from two independent experiments. (B) RT-PCR analysis of estrogen-dependent expression of *BRCA-1* type α mRNA in MCF-7 cells cultured for 72 h in the absence or presence of 10 nM estrogen (E_2) plus increasing amounts of B[a]P.

BRCA-1 was probably not a consequence of the general cytotoxicity of B[a]P. B[a]P may trigger downregulation of BRCA-1 through mechanisms that are unique to ER-positive cells, as neither cell viability nor expression of BRCA-1 was affected by B[a]P in ER-negative breast cancer cells.

Long-Term Exposure to B[a]P

To examine whether perturbations in expression of *BRCA-1* could also be induced by long-term exposure to low levels of B[a]P, we exposed MCF-7 and BG-1 cells to 40 nM B[a]P for various periods of time. After continuous passage for 6, 7, 8, and 18 wk in B[a]P-containing medium, we measured the expression of *BRCA-1* mRNA in subclones of MCF-7 and BG-1 cells. As depicted in Figure 5, the chronic exposure to B[a]P significantly reduced the levels of *BRCA-1* mRNA, in the absence of any effects on cell viability (data not shown). Next, we examined whether expression of *BRCA-1* could be restored by washing out B[a]P. Five subclones each of MCF-7 and BG-1 cells maintained in the presence of 40 nM B[a]P for 15 mo were plated in DMEM/F12 plus 10% FCS and cultured for 72 h without B[a]P. Removal of B[a]P restored *BRCA-1* mRNA expression. These findings supported the notion that chronic exposure to B[a]P may repress BRCA-1 expression, perhaps through regulatory mechanisms (Figure 5B).

Disruption of Estrogen Induction of *BRCA-1* in MCF-7 Cells by B[a]P

Published observations [31] and our work [32] have documented that BRCA-1 expression is induced by estrogen in breast and ovarian cancer cells. Because PAHs may disrupt estrogen-dependent expression, we tested whether B[a]P influenced estrogen regulation of *BRCA-1*. Breast MCF-7 cells were preconditioned for 5 d in phenol red–free DMEM/F12 plus 5% CS-FCS. The cells were then treated for 72 h with 10 nM estrogen in the absence

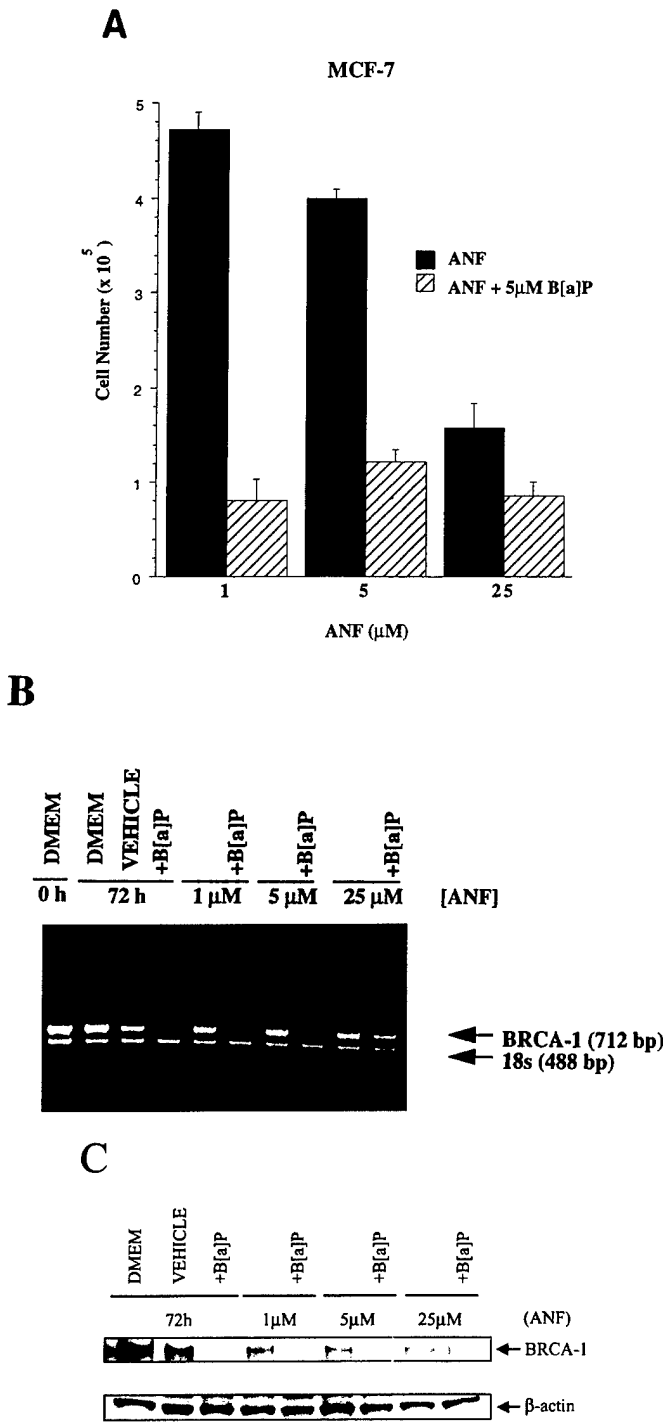


Figure 7. Treatment with ANF counteracted the inhibitory effects of B[a]P on cell proliferation and expression of *BRCA-1*. (A) MCF-7 cells were cultured for 72 h in DMEM/F12 plus 10% FCS in the absence or presence of 5 μM B[a]P and increasing amounts of ANF. At the end of the incubation period, the cells were counted in triplicate (n = 9). The bars represent mean cell numbers ± standard deviations from two independent experiments. (B and C) RT-PCR (B) and western blotting (C) analyses of BRCA-1 expression after stimulation with B[a]P in the absence or presence of increasing amounts of ANF. The control bands in panel C are β-actin immunocomplexes visualized by incubating blots with antibody-1.

or presence of 5 μM B[a]P. As expected, estrogen stimulated cell proliferation (2.1-fold) of MCF-7 cells (Figure 6A). Nevertheless, the addition of B[a]P to the culture medium reduced in a dose-dependent fashion the number of viable cells. Cell loss was counteracted in part by treatment with estrogen. With respect to *BRCA-1* expression, exposure to 0.05 or 0.5 μM B[a]P plus estrogen did not alter *BRCA-1* expression, whereas 5 μM B[a]P abrogated induction of *BRCA-1* mRNA by estrogen (Figure 6B).

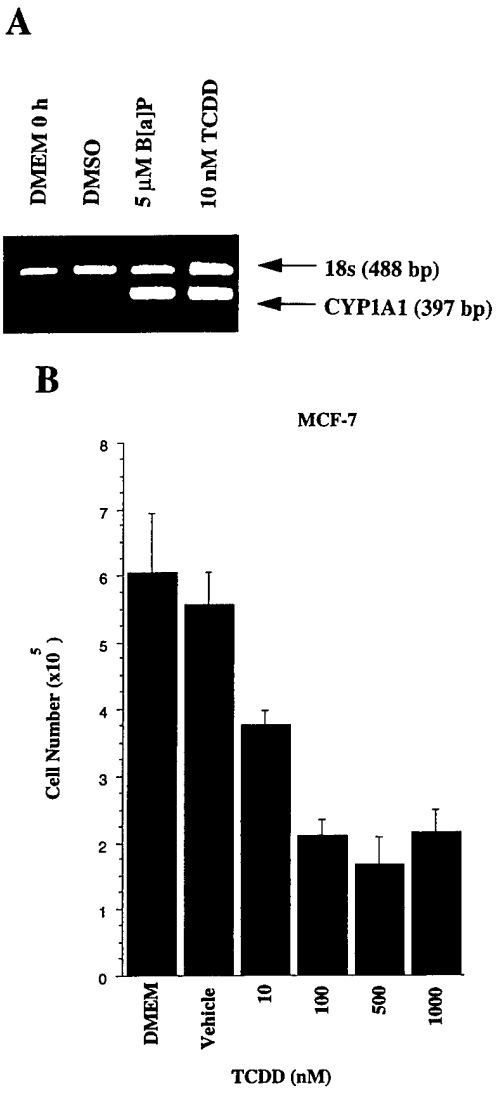


Figure 8. Effects of TCDD and BPDE on cell proliferation and BRCA-1 expression. (A) Expression of *CYP1A1* in MCF-7 cells cultured in DMEM/F12 plus 10% FCS in the absence or presence of 10 nM TCDD for 72 h. (B) MCF-7 cells were cultured in the presence of increasing concentrations of TCDD for 72 h. At the end of the incubation period, the cells were counted in triplicate (n = 9). The bars represent mean cell numbers ± standard deviations from two independent experiments. (C and D) RT-PCR (C) and western (D) analyses of BRCA-1 expression in MCF-7 cells after treatment with TCDD for 72 h. The control bands are β-actin immunocomplexes visualized by incubating blots with antibody-1. (E and F) Growth arrest (E) and RT-PCR (F) analyses of BRCA-1 expression in MCF-7 cells cultured in the presence of increasing amounts of BPDE for 24 h.

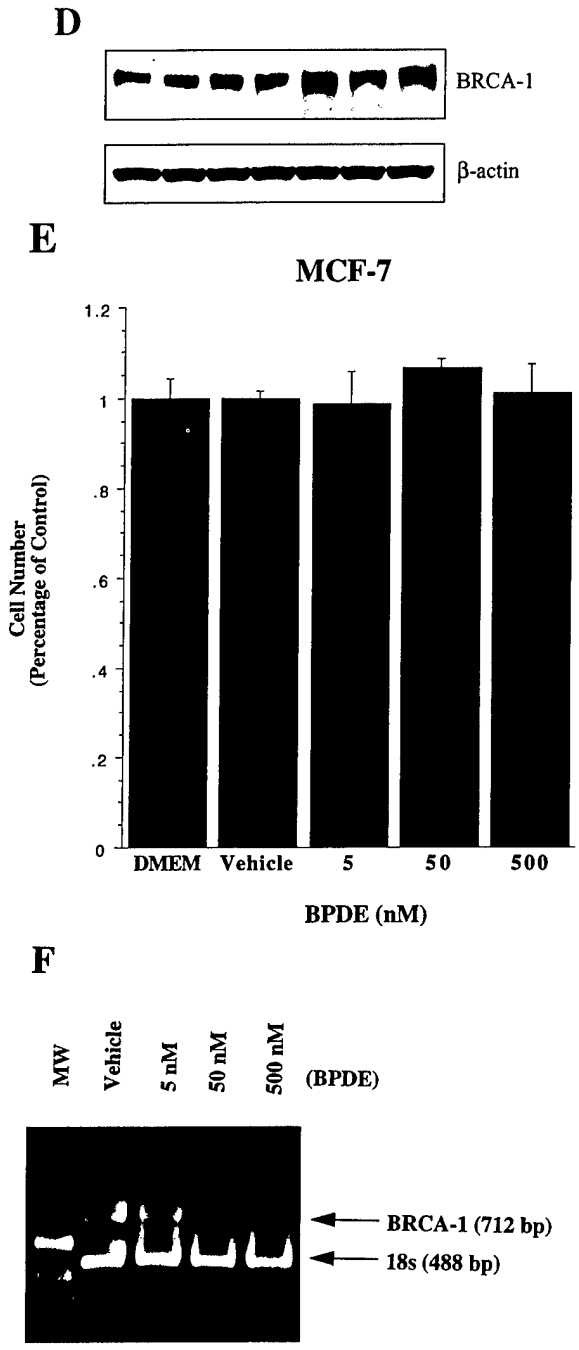


Figure 8. (*Continued*)

Involvement of the AhR Pathway in Downregulation of *BRCA-1* by B[a]P

To investigate whether the deleterious effects of B[a]P on *BRCA-1* expression involved the AhR pathway, we exposed cells to B[e]P. B[e]P is a PAH that differs from B[a]P in that it binds with very low affinity to the AhR [37] and does not inhibit DNA synthesis in rat hepatocytes in primary culture [38]. Treatment with 5 μM B[e]P did not influence cell viability or the expression of BRCA-1 in MCF-7 cells (data not shown). Positive direct evidence of the involvement of the AhR pathway was obtained by competition experiments in which MCF-7 cells were exposed to 5 μM B[a]P, alone or in combination with increasing amounts of the AhR antagonist ANF. Concentrations of ANF ranging from 1 to 5 μM reduced the negative effects of B[a]P on cell viability (Figure 7A) without affecting *BRCA-1* expression. However, at doses of 25 μM, ANF restored *BRCA-1* mRNA (Figure 7B) and protein (Figure 7C) expression, counteracting the negative effects of B[a]P. These findings supported the hypothesis that downregulation of *BRCA-1* by B[a]P may involve the participation of the AhR pathway.

Loss of *BRCA-1* mRNA Expression Induced by BPDE but not TCDD

To further characterize the mechanisms of regulation of *BRCA-1* by AhR ligands, we exposed MCF-7 cells to the halogenated aryl hydrocarbon TCDD, which is not metabolized but has a higher affinity for the AhR than B[a]P does [39]. The treatment with TCDD induced *CYP1A1* mRNA (Figure 8A) while reducing the number of viable MCF-7 cells in a time-dependent and dose-dependent (Figure 8B) fashion starting at concentrations of 10 nM. However, loss of cell viability was not coupled with loss of *BRCA-1* mRNA (Figure 8C) and protein (Figure 8D) at any of the TCDD concentrations tested in this study. Therefore, activation of the AhR pathway by TCDD, as assessed by induction of *CYP1A1* mRNA, did not result in loss of *BRCA-1* expression. These data contradicted those obtained with B[a]P, which compromised both cell viability and expression of *BRCA-1*, and raised the question of whether downregulation of *BRCA-1* requires metabolism of B[a]P. Treatment with the metabolite BPDE at concentrations ranging from 5 to 500 nM caused growth arrest of MCF-7 (Figure 8E) and BG-1 cells (data not shown), whereas 50 nM BPDE was sufficient to inhibit the expression of *BRCA-1* mRNA (Figure 8F). These observations suggested that metabolism of B[a]P to the reactive metabolite BPDE may contribute to loss of *BRCA-1* expression.

DISCUSSION

Cigarette smoke and environmental pollution are vehicles of a complex mixture of compounds, including carcinogenic PAHs, aromatic amines, and nitrosoamines, all of which after metabolic activation may induce DNA damage. PAHs are well-characterized DNA-damaging and tumor-promoting agents [40]. Of the many PAHs present in tobacco smoke, B[a]P is considered a prototype carcinogen [41]. Population studies documented that smokers have more PAH-DNA adducts than nonsmokers do and suggested a potential correlation between tobacco smoking and the develop-

ment of breast cancer [42]. In the absence of efficient DNA repair, chromosomal aberrations may initiate carcinogenesis [23]. For example, transversion mutations in the *p53* tumor suppressor gene are associated with various types of tumors related to defective repair of DNA adducts [24]. Removal of many types of DNA damage is highly efficient in transcriptionally active DNA, where lesions in the transcribed strand are rapidly repaired. Consequently, downregulation of BRCA-1 may compromise its participation in transcription-coupled DNA repair and so lead to accumulation of mutations and neoplastic transformation [11].

Breast cancer ER-positive MCF-7 cells express the AhR and have been used extensively to study the effects of B[a]P and other PAHs on regulation of expression of *CYP1A1* and estrogen-inducible [20] and tumor suppressor genes [24]. In this study, we observed constitutive expression of the AhR in all of the epithelial cell lines tested, including the ER-negative MDA-MB-231 and HBL-100 lines (unpublished observation). Our findings were in agreement with published data documenting induction of CYP1A1 by TCDD in MDA-MB-231 cells [43], which contain approximately 40-fold more *AhR* mRNA than MCF-7 cells do [44]. Thus, the induction of CYP1A1 by B[a]P and TCDD observed in our studies confirmed that the AhR pathway was operative in these cell systems. However, upregulation of CYP1A1 correlated with loss of BRCA-1 only in ER-positive breast MCF-7 and ovarian BG-1 cells. Interestingly, a comparison of levels of expression of type α and β mRNAs ascertained that both *BRCA-1* transcripts were downregulated by B[a]P in MCF-7 cells. Specifically, the levels of type α mRNA decreased faster in the presence of B[a]P, perhaps suggesting differential regulation of the two *BRCA-1* promoters.

In examining the time-dependent effects of B[a]P, we found that loss of BRCA-1 occurred 12–24 h after treatment with B[a]P and preceded by approximately 24 h the death of MCF-7 and BG-1 cells. Therefore, downregulation of BRCA-1 was probably not due to mutational events, for which at least two rounds of replication are required. These events are also accompanied by a drastic reduction in the expression of *bcl-2* mRNA and increased 7-amino actinomycin D staining [unpublished observations], both of which are indicative of apoptosis [45,46]. In previous investigations, growth of early embryos was suppressed in the absence of BRCA-1, possibly because of failure to repair DNA damage [47,48]. Therefore, it is conceivable that in the absence of BRCA-1, cells may not respond to DNA damage caused by B[a]P and so enter programmed cell death. This hypothesis is being investigated in our laboratory and is consistent with previous findings documenting induction of apoptosis in MCF-7 cells in response to antiestrogens [49] and AhR ligands [26]. Here, cotreatment of ER-positive cells with ANF counteracted in a dose-dependent fashion the deleterious effects of B[a]P on cell viability and restored BRCA-1 mRNA and protein expression. The antagonist ANF competes for the cytosolic AhR binding sites, thus eliciting a conformational change that lowers the affinity for DNA [50]. Therefore, our data provide additional evidence that the AhR may act as a negative transcription factor [20].

Under normal conditions, individuals are probably exposed to PAHs at concentrations much lower than those used for acute experimental treatment. Here, the chronic exposure to B[a]P elicited a significant reduction in *BRCA-1* mRNA in MCF-7 and BG-1 cells. In contrast, the withdrawal of B[a]P restored BRCA-1 to control levels, indicating that continuous passage in B[a]P-containing medium may inhibit BRCA-1, perhaps through regulatory mechanisms. These observations may be important in examining the role of intensity and duration of exposure to environmental PAHs and possibly the role of B[a]P present in tobacco smoke in the etiology of sporadic breast cancer. Epidemiological evidence points to tobacco smoking as a possible risk factor in breast carcinogenesis as an early-stage promoter in the process that may lead later in life to the development of mammary neoplasia [42,51,52]. In mammalian cells, maintenance of genome integrity is the result of production and repair of DNA damage. Consequently, repression of BRCA-1 due to prolonged exposure to PAHs may predispose to fixation of mutations and tumor development.

Our results illustrate that B[a]P abrogates the stimulatory effects of estrogen on BRCA-1 expression. B[a]P induces the expression of the CYP1A1 enzyme, which, in turn, stimulates the hydroxylation of endogenous estrogen at the C2, -6α, and -15α positions [21]. The enzyme CYP1B1, another member of the Ah gene family, catalyzes the hydroxylation of estrogen to 4-hydroxyestradiol [53]. Therefore, the oxidation of estrogen catalyzed by enzymes of the CYP family may be responsible for the disruption of estrogen-induced BRCA-1. Cooperativity between the ER and AhR pathways was elegantly investigated in previous studies in which transfection of expression vectors encoding the ER into ER-negative breast cancer MDA-MB-231 cells compensated for the expression in this cell line of a variant ARNT [7,54,55]. Nevertheless, recent observations suggested that the ER may play an ancillary role in the regulation of transcription of estrogen-responsive genes and attributed a pivotal function to transcription factors or receptors that bind to the ER [56]. For example, the transcription factor Sp1 is ubiquitous and plays both a stimulatory role and an inhibitory role in a cell- and promoter-specific manner [57]. This selectivity is influenced by the availability of nuclear ligands. The C-

terminal region of the Sp1 protein interacts with the ER, forming an ER-Sp1 complex that transactivates responsive elements. However, in the presence of AhR ligands, the AhR and ARNT interact with the zinc-finger domain of Sp1 via their basic helix-loop-helix domains, enhancing binding of the AhR heterocomplex to xenobiotic-responsive elements (XREs) [58] that may silence estrogen-dependent expression [20]. Promoter studies have mapped to the 5′ flanking region of the *BRCA-1* gene potential ER and Sp1 domains [59] that may cooperate with the AhR heterocomplex. With respect to XREs that are known to be responsive to PAHs [60], we have identified an array of consensus sequences for XRE-binding proteins by computer-assisted analysis of the *BRCA-1* promoter region (unpublished data). The role of these candidate XREs in transcriptional regulation of the *BRCA-1* promoter and cross talk with ER/Sp1 sites is currently being examined.

To further dissect the mechanisms of downregulation of BRCA-1 by B[a]P, we investigated the effects of treatment with TCDD. TCDD is a dioxin-like halogenated hydrocarbon that is not metabolized but displays higher affinity for the AhR than B[a]P does [39]. Previous studies have documented that transcriptional activation of CYP1A1 by TCDD is an AhR-dependent event [61]. Our results illustrated that treatment of MCF-7 cells with TCDD upregulated CYP1A1 expression and compromised cell viability in a dose-dependent fashion without, however, affecting the levels of *BRCA-1* mRNA. This apparent paradox may be explained by the fact that TCDD significantly decreases nuclear ER levels [62], which may contribute to estrogen-dependent upregulation of BRCA-1 [32]. The ER may be necessary for cross talk with components of the AhR pathway. Specifically, TCDD may not simply influence basal transcription of estrogen-responsive genes. For instance, only upon treatment with TCDD plus estrogen does the formation of an ER-Sp1 complex facilitate accessibility of the TCDD-AhR heterocomplex to an XRE site in the cathepsin-D promoter region [20].

The ineffectiveness of TCDD suggested that activation of the AhR pathway may be necessary but not sufficient to modulate the expression of BRCA-1 and prompted us to consider the possible role of BPDE. Treatment of MCF-7 cells with 50 nM BPDE inhibited *BRCA-1* mRNA, confirming that this metabolite is more reactive than its parental precursor, B[a]P. More importantly, these data provided evidence that the suppressive effects of B[a]P could be mediated by BPDE, which is known to inhibit progression of RNA polymerase [28]. Alternatively, reduced expression of BRCA-1 may be related to molecular hijacking of transcription factors to non-target sites in DNA modified by BPDE [63].

At least three lines of evidence suggested that downregulation of BRCA-1 is not a consequence of general cytotoxicity. First, loss of BRCA-1 was coupled to upregulation of p53 protein (data not shown) and *CYP1A1* mRNA. Upregulation of p53 agrees with the known function of this tumor suppressor in cell-cycle arrest in response to DNA damage. The CYP1A1 gene product is a CYP enzyme involved in metabolism of B[a]P to BPDE. Therefore, downregulation of *BRCA-1* mRNA may not be simply related to general repression of the transcription machinery. In contrast, the loss of BRCA-1 in ER-positive cells was reversed by addition of ANF despite its negative effects on cell proliferation. Second, breast ER-negative cells were refractory to treatment with B[a]P and contained unaltered levels of *BRCA-1* mRNA, suggesting that simple exposure to B[a]P is not sufficient to abrogate cell proliferation and adversely affect levels of *BRCA-1* transcripts. Third, exposure to TCDD caused cell death in a dose-dependent fashion without affecting BRCA-1 mRNA and protein. Thus, binding to the AhR can induce loss of cell viability but may not be sufficient to repress BRCA-1. Taken together, these results suggest that B[a]P may suppress the expression of BRCA-1, preventing its participation in repair of DNA damage. A possible outcome may be cell death by apoptosis. Downregulation of BRCA-1 by B[a]P could be elicited through several not mutually exclusive mechanisms, including interaction of the B[a]P-AhR heterocomplex with XREs in the promoter region of BRCA-1 and the formation of BPDE-DNA adducts, which may sequester transcription factors required for BRCA-1 expression. Critical questions that need to be addressed to are whether susceptibility to B[a]P is the result of cells expressing the ER and what the role of cross talk between the ER and AhR pathways is. While further investigation is necessary to distinguish between regulation of BRCA-1 by the AhR and regulation of BRCA-1 in response to DNA damage, here we have provided novel evidence that exposure to B[a]P and its metabolite BPDE may be a risk factor in the etiology of sporadic breast cancer by disrupting the expression of BRCA-1.

ACKNOWLEDGMENTS

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REFERENCES

1. Miki Y, Swensen J, Shattuck-Eidens D, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 1994;266:66–71.
2. Futreal PA, Liu Q, Shattuck-Eidens D, et al. BRCA1 mutations in primary breast and ovarian carcinomas. Science 1994;266:120–122.

3. Castilla LH, Couch FJ, Edros MR, et al. Mutations in the BRCA1 gene in families with early-onset breast and ovarian cancer. Nat Genet 1994;8:387–394.
4. Rao VN, Shao N, Ahmad M, Shyam C, Reddy P. Antisense RNA to the putative tumor suppressor gene BRCA1 transforms mouse fibroblasts. Oncogene 1996;12:523–528.
5. Monteiro ANA, August A, Hanafusa H. Evidence for a trans-criptional activation function of BRCA-1 C-terminal region. Proc Natl Acad Sci USA USA 1996;93: 13595–13599.
6. Chen Y, Farmer AA, Chen CF, Jones DC, Chen PL, Lee WF. BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner. Cancer Res 1996;56:3168–3172.
7. Wilson CL, Thomsen J, Hoivik DJ, et al. Aryl hydrocarbon (Ah) nonresponsiveness in estrogen receptor-negative MDA-MB-231 cells is associated with expression of a variant ARNT protein. Arch Biochem Biophys 1997;346:65–73.
8. Coene E, Van Oostveldt P, Willems K, van Emmelo J, De Potter CR. BRCA-1 is localized in cytoplasmic tube-like invaginations in the nucleus. Nat Genet 1997;16:122–124.
9. Kinzler KW, Vogelstein B. Cancer-susceptibility genes: Gatekeepers and caretakers. Nature 1997;386:761.
10. Scully R, Chen J, Ochs RL, et al. Dynamic changes of BRCA1 subnuclear localization and phosphorylation state are initiated by DNA damage. Cell 1997;90:1–20.
11. Sharan SK, Morimatsu M, Albrecht U, et al. Embyonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking BRCA2. Nature 1997;386:804–810.
12. Gowen LC, Avrutskaya AV, Latour AM, Koller BH, Leadon SA. BRCA1 required for transcription-coupled repair of oxidative DNA damage. Science 1998;281:1009–1012.
13. Merajver SD, Pham TM, Caduff RF, et al. Somatic mutations in the BRCA1 gene in sporadic ovarian tumors. Genetics. 1995;9:439–443.
14. Thompson ME, Jensen RA, Obermiller PS, Paige PS, Holt DL. Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nat Genet 1995;9:444–450.
15. Xu CF, Solomon E. Mufations of the BRCA1 gene in human cancer. Semin Cancer Biol 1996;7:33–40.
16. Andres JL, Fan S, Turkel GJ, et al. Regulation of BRCA1 and BRCA2 expression in human breast cancer cells by DNA-damaging agents. Oncogene 1998;16;2229–2241.
17. Fan S, Twu N-F, Wang J-A, et al. Down-regulation of BRCA1 and BRCA2 in human ovarian cancer cells exposed to adriamycin and ultraviolet radiation. Int J Cancer 1998;77: 600–609.
18. Ronai Z, Gradia S, el-Bayoumy K, Amin S, Hecht SS. Contrasting incidence of ras mutations in rat mammary and mouse skin tumors induced by anti-benzo[c]phenanthrene-3,4-diol-1,2-epoxide. Carcinogenesis 1994;15(10): 2113–2116.
19. Batsch H, Hietanen E. The role of individual susceptibility in cancer burden related to environmental exposure. Environ Health Perspect 1996;104:569–577.
20. Krishnan V, Porter W, Santostefano M, Wang X, Safe S. Molecular mechanism of inhibition of estrogen-induced cathepsin D gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in MCF-7 cells. Mol Cell Biol 1995;15(12): 6710–6719.
21. Spink DC, Eugster HP, Lincoln DW, et al. 17-Beta-estradiol hydroxylation catalyzed by human cytochrome P4501A1: A comparison of the activities induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in MCF-7 cells with those from heterologous expression of the cDNA. Arch Biochem Biophys 1992;293:342–348.
22. Hoivik D, Willett K, Wilson C, Safe S. Estrogen does not inhibit 2,3,7,8-tetrachlorodibenzo-p-dioxin-mediated effects in MCF-7 and Hepa 1c1c7 cells. J Biol Chem 1997;272:30270–30274.
23. Wei Q, Gu J, Cheng L, et al. Benzo[a]pyrene diol epoxide-induced chromosomal aberrations and risk of lung cancer. Cancer Res 1996;56:3975–3979.
24. Denissenko MF, Pao A, Tang M, Pfeifer GP. Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in p53. Science 1996;274:430–432.
25. Harper N, Wang X, Liu H, Safe S. Inhibition of estrogen-induced progesterone receptor in MCF-7 human breast cancer cells by aryl hydrocarbon (Ah) receptor agonists. Mol Cell Endocrinol 1994;104:47–55.
26. McConkey DJ, Harzell P, Duddy SK, Hakansson H, Orrenius S. 2,3,7,8-Tetrachlorodibenzo-p-dioxin kills immature thymocytes by Ca^{2+}-mediated endonuclease activation. Science 1988;242:256–259.
27. Hord NG, Perdew GH. Physicochemical and immunocytochemical analysis of the aryl hydrocarbon receptor nuclear translocator: Characterization of two monoclonal antibodies to the aryl hydrocarbon receptor nuclear translocator. Mol Pharmacol 1994;46:618–626.
28. Butler AP, Johnson DG, Kumar AP, Narayan S, Wilson SH, MacLeod MC. Disruption of transcription in vitro and gene expression in vivo by DNA adducts derived from a benzo[a]pyrene diol epoxide located in heterologous sequences. Carcinogenesis 1997;18:239–244.
29. Chakravarti D, Pelling JC, Cavalieri EL, Rogan EG. Relating aromatic hydrocarbon-induced DNA adducts and c-H-ras mutations in mouse skin papillomas: The role of apurinic sites. Proc Natl Acad Sci USA 1995;92:10422–10426.
30. Vaziri C, Faller DV. A benzo[a]pyrene-induced cell cycle checkpoint resulting in p53-independent G1 arrest in 3T3 fibroblasts. J Biol Chem 1997;272:2762–2769.
31. Gudas JM, Nguyen H, Li T, Cowen KH. Hormone-dependent regulation of BRCA1 in human breast cancer cells. Cancer Res 1995;55:4561–4565.
32. Romagnolo D, Annab LA, Lyon TT, et al. Estrogen upregulation of expression of *BRCA1* with no effect on localization. Mol Carcinog 1998;22:102–109.
33. Puissant C, Houdebine LM. An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Biotechniques 1990;8:148–149.
34. Stampfer MR, Bartley JC. Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. Proc Natl Acad Sci USA 1985;82:2394–2398.
35. Bral CM, Ramos KS. Identification of benzo[a]pyrene-inducible cis-acting elements within c-Ha-ras transcriptional regulatory sequences. Mol Pharmacol 1997;52:974–982.
36. Xu C, Brown MA, Chambers JA, Griffiths B, Nicolai H, Solomon E. Distinct Transcription start sites generate two forms of BRCA1 mRNA. Hum Mol Genet 1995;4: 2259–2264.
37. Kamps C, Safe S. Binding of polynuclear aromatic hydrocarbons to the rat 4S cytosolic binding protein: Structure-activity relationships. Cancer Lett 1987;4:129–137.
38. Zhao W, Ramos KS. Inhibition of DNA synthesis in primary cultures of adult rat hepatocytes by benzo[a]pyrene and related aromatic hydrocarbons: Role of Ah receptor-dependent events. Toxicology 1995;99:179–189.
39. Piskorska-Pliszczynska J, Keys B, Safe S, Newman MS. The cytosolic receptor binding affinity and AhR induction potencies of 29 polynuclear aromatic hydrocarbons. Toxicol Lett 1986;34:67–74.
40. Maher VM, Patton JD, Yang JL et al. Mutations and homologous recombination induced in mammalian cells by metabolites of benzo[a]pyrene and 1-nitropyrene. Environ Health Perspect 1987;76:33–39.
41. Leadon SA, Stampfer MR, Bartley J. Production of oxidative DNA damage during the metabolic activation of benzo[a]-pyrene in human mammary epithelial cells correlates with cell killing. Proc Natl Acad Sci USA 1988;85:4365–4368.

42. Calle EE, Miracle-McMahill HL, Thun MJ, Heath CW Jr. Cigarette smoking and risk of fatal breast cancer. Am J Epidemiol 1994;139:1001–1007.
43. Spink DC, Spink BC, Cao JQ, et al. Differential expression of CYP1A1 and CYP1B1 in human breast epithelial and breast tumor cells. Carcinogenesis 1998;19:291–298.
44. Dohr O, Vogel C, Abel J. Different response of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-sensitive genes in human breast cancer MCF-7 and MDA-MD-231 cells. Arch Biochem Biophys 1995;321:405–412.
45. Philpott NJ, Turner AJC, Scopes J et al. The use of 7-amino actinomycin D in identifying apoptosis: Simplicity of use and broad spectrum of application compared with other techniques. Blood 1996;87:2244–2251.
46. Yang J, Liu X, Bhalla K, et al. Prevention of apoptosis by Bcl-2: Release of cytochrome C from mithocondria blocked. Science 1997;275:1129–1132.
47. Ludwig T, Chapman DL, Papaioannou VE, Efstratiadis A. Targeted mutations of breast cancer susceptibility gene homologs in mice: Lethal phenotypes of BRCA1, BRCA2, BRCA1/BRCA2, BRCA1/p53, and BRCA2/p53 nullizygous embryos. Genes Dev 1997;11:1226–1241.
48. Hakem R, de la Pompa JL. Elia A, Potter J, Mak TW. Partial rescue of BRCA1 (5-6) early embryonic lethality by p53 or p21 null mutation. Nat Genet 1997;16:298–302.
49. Welsh J. Induction of apoptosis in breast cancer cells in response to vitamin D and antiestrogens. Biochem Cell Biol 1994;72:537–545.
50. Gasiewicz TA, Rucci G. α-Naphthoflavone acts as an antagonist 2,3,7,8-tetrochlorodibenzo-p-dioxin by forming an inactive complex with the Ah receptor. Mol Pharmacol 1991;40:607–612.
51. Horton AW. Epidemiologic evidence for the role of indoor tobacco smoke as an initiator of human breast carcinogenesis. Cancer Detect Prev 1992;16:119–127.
52. Lemon HM. Breast cancer and cigarette smoking: A hypothesis. Am J Epidemiol 1992;135:1184–1185.
53. Liehr JG, Ricci MJ. 4-Hydroxylation of estrogens as marker of human mammary tumors. Proc Natl Acad Sci USA 1996;93:3294–3296.
54. Safe S, Krishnan V. Chlorinated hydrocarbons: Estrogens and antiestrogens. Toxicol Lett 1995;731–736.
55. Thomsen JS, Wang X, Hines RN, Safe S. Restoration of aryl hydrocarbon (Ah) responsiveness in MDA-MB-231 human breast cancer cells by transient expression of the estrogen receptor. Carcinogenesis 1994;15:933–937.
56. Spink DC, Spink BC, Cao JQ, et al. Differential expression of CYP1A1 and CYP1B1 in human breast epithelial cells and breast tumor cells. Carcinogenesis 1998;19: 291–298.
57. Porter W, Saville B, Hoivik D, Safe S. Functional synergy between the transcription factor Sp1 and the estrogen receptor. Mol Endocrinol 1997;11:1569–1580.
58. Kobayashi A, Sogawa K, Fujii-Kuriyama Y. Cooperative interaction between AhR-Arnt and Sp1 for the drug-inducible expression of CYP1A1 gene. J Biol Chem 1996;271:12310–12316.
59. Xu C, Chambers JA, Solomon E. Complex regulation of the BRCA1 gene. J Biol Chem 1997;272:20994–20997.
60. Fukunaga BN, Probst MR, Reisz-Porszasz S, Hankinson O. Identification of functional domains of the aryl hydrocarbon receptor. J Biol Chem 1995;270:29270–29278.
61. Nebert DW, Petersen DD, Fornace JrAJ. Cellular responses to oxidative stress: The Ah gene battery as a paradigm. Environ Health Perspect 1990;88:13–25.
62. Wang X, Porter W, Krishnan V, Narasimhan, TR, Safe S. Mechanism of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-mediated decrease of the nuclear estrogen receptor in MCF-7 human breast cancer cells. Mol Cell Endocrinol 1993; 96:159–166.
63. MacLeod MC, Powell KL, Tran N. Binding of the transcription factor Sp1 to non-target sites in DNA modified by benzo[a]pyrene diol epoxide. Carcinogenesis 1995;16: 975–983.

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Disruption of Cell Cycle Kinetics by Benzo[*a*]pyrene: Inverse Expression Patterns of BRCA-1 and p53 in MCF-7 Cells Arrested in S and G_2

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Abstract

The effects of a ligand of the aromatic hydrocarbon receptor (AhR), benzo[*a*]pyrene (B[*a*]P), and its metabolite, BPDE (7*r*,8*t*-dihydroxy-9*t*,10*t*-epoxy-7,8,9,10-tetrahydro-benzo[*a*]pyrene), on BRCA-1 levels and cell cycle kinetics were determined in MCF-7 breast cancer cells. Exposure of asynchronous MCF-7 cells for 72 hours to a non-cytotoxic dose of 0.5 μM B[*a*]P triggered a three-fold reduction in BRCA-1 protein. In MCF-7 cells resistant (20% to 30%) to genotoxic concentrations of B[*a*]P (1 to 5 μM), the loss of BRCA-1 protein was coupled with pausing in S-phase and G_2/M, and accumulation of p53, mdm2 and p21. Treatment of MCF-7 cells synchronized in S-phase (72%) with B[*a*]P prolonged the arrest in S-phase, although this checkpoint was transient since cells resumed to G_2/M after 12 hours with reduced levels of BRCA-1. In these cells, levels of p53 were increased, whereas the cellular content of p21 remained unaltered. In contrast, the co-treatment with the AhR antagonist, α-naphthoflavone (ANF), abrogated the deleterious effects of B[*a*]P on BRCA-1 expression, while preventing the accumulation of p53 and disruption of cell cycle profile. These findings suggest that the AhR mediated the inverse expression patterns of BRCA-1 and p53 upon exposure to B[*a*]P. The treatment with BPDE induced S-phase arrest and reduced BRCA-1 mRNA levels. The negative effects of BPDE on BRCA-1 expression were not transient since removal of BPDE did not allow complete reversal of the repression. These cumulative data suggest that the B[*a*]P metabolite, BPDE, may play a key role in disruption of BRCA-1 expression and cell cycle kinetics in breast epithelial cells. *Neoplasia* (2000) **2,** 460–470.

Keywords: benzo[*a*]pyrene, BPDE, BRCA-1, p53, cell cycle kinetics, sporadic breast cancer.

Introduction

The BRCA-1 protein participates in transcription-coupled repair of oxidative damage [1], co-localizes with Rad51 [2], BRCA-2 [3], and the hRad50–hMre11–p95 complex [4]. In addition, an important role in embryogenesis has been attributed to BRCA-1 since nullizygous embryos die early in development, possibly because of accumulation of DNA damage [5,6]. The latter affects subnuclear location and ATM-dependent phosphorylation of BRCA-1 [7–9].

It has been proposed that BRCA-1 may be involved in cell cycle control since the cellular levels of BRCA-1 protein peak in S- and M- phases [10]. This inference is supported by the fact that BRCA-1 contributes to p53-dependent gene expression [11] and the *trans*-activation of p21 [12,13]. Moreover, the overexpression of mutant BRCA-1 encoding for COOH-terminal residues decreases the doubling time of 184A1 human breast epithelial cells, while inducing the loss of G_2/M block by colchicine [14]. These cumulative data are consistent with a role for BRCA-1 in S- and G_2/M-phases control [15].

One of the cellular responses to DNA damage is the activation of G_1/S and G_2/M checkpoints. Stabilization of p53 at the G_1/S boundary elicits the *trans*-activation of a number of genes, including mdm2 and the cyclin-dependent kinase inhibitor p21, which, in concert, controls entry into the S-phase [16]. While upregulation of p21 can occur through a p53-independent pathway, both p53 and p21 are essential for maintaining the G_2 checkpoint after DNA damage [17]. Nevertheless, the disruption of these cell cycle regulators by drugs and chemical agents may compromise the fidelity of DNA replication and allow progression of cells harboring genomic aberrations. Alternatively, severe DNA damage may destine cells to lethality [18].

One of the puzzles in breast carcinogenesis is that BRCA-1 is not mutated in sporadic breast tumors [3]. We hypothesized that epigenetic effectors capable of inducing genotoxic damage may cause a concomitant loss of BRCA-1, thus preventing its participation in DNA repair functions. An

Abbreviations: PAH, polycyclic aromatic hydrocarbon; B[*a*]P, benzo[*a*]pyrene; AhR, aromatic hydrocarbon receptor; BPDE, 7*r*,8*t*-dihydroxy-9*t*,10*t*-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ANF, α-naphthoflavone; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium.

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outcome may be the propagation of mutations or chromosomal aberrations that contribute to drug resistance and cancer development.

Although recent studies have investigated the regulation of BRCA-1 expression by DNA-damaging agents [19], knowledge concerning the contribution of polycyclic aromatic hydrocarbons (PAHs) to the regulation of BRCA-1 is limited. PAHs are known to induce a number of biological responses including G_1 arrest [20] and genotoxic stress [21]. Recently [22], we reported that benzo[*a*]pyrene (B[*a*]P), a prototype PAH and ligand of the aromatic hydrocarbon receptor (AhR), inhibited the expression of BRCA-1 in estrogen receptor-positive breast (MCF-7) and ovarian (BG-1) cancer cells. Here, we present evidence that in MCF-7 cells, the expression of BRCA-1 and p53 is inversely regulated by B[*a*]P, which contributes to transition from S- to G_2/M-phases in the presence of reduced levels of BRCA-1. We document that disruption of BRCA-1 expression and cell cycle kinetics by B[*a*]P are mediated, at least in part, by the reactive metabolite, 7*r*,8*t*-dihydroxy-9*t*,10*t*-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE).

Methods

Cell Culture

Effects of B[*a*]P on cell proliferation were investigated as described previously [23] in MCF-7 cells obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories Inc., Logan, UT). B[*a*]P, α-naphthoflavone (ANF), and colchicine were obtained from Sigma. Aphidicolin was purchased from Calbiochem Co. (La Jolla, CA). BPDE was obtained from Midwest Research Institute (Kansas City, MO). For cell viability studies, cells were plated at a density of 2×10^6 cells/100 mm tissue culture dish and maintained overnight in DMEM/F12 plus 10% FCS. Three dishes were assigned to each experimental treatment. At the end of the incubation periods, cell viability was assessed in triplicate (n = 9) by trypan blue exclusion.

Flow Cytometry

Flow cytometry was performed as previously described [19]. Briefly, cells were harvested with trypsin and washed in PBS. Then cells were treated with RNAse and stained with propidium iodide (50 μg/ml in PBS). Cell cycle distribution profiles were recorded with a FACscan (Becton-Dickinson, Franklin Lakes, NJ), using a CELLQuest program. Data were analyzed with the MODFIT.2 software at the Flow Cytometry Laboratory of the Arizona Cancer Center.

Western Blotting

Western blotting was performed as previously described [24]. Cell extracts were normalized to protein content and separated by 4% to 12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunoblotting was carried out with antibodies raised against BRCA-1 (Ab-2), p53 (Ab-2), p21 (Ab-1), and mdm2 (Ab-1) obtained from Oncogene Research Products (Cambridge, MA) and p27 (C-19) obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). 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).

Semi-Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Details concerning the experimental conditions for semi-quantitative RT-PCR analysis of BRCA-1 mRNA are

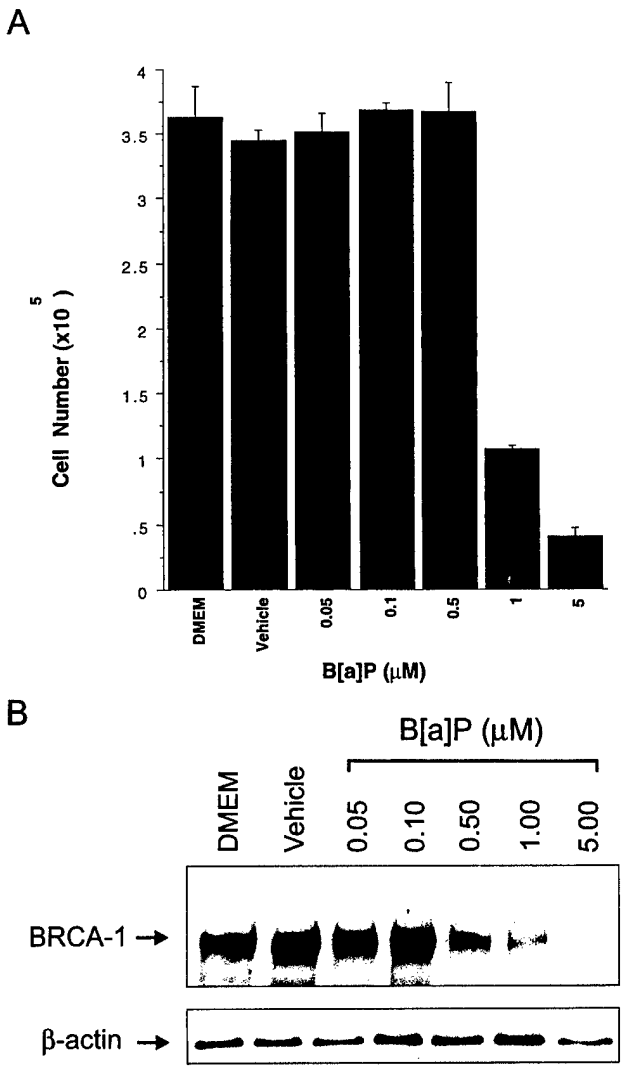


Figure 1. *Dose-response effects of B[a]P on cell viability and BRCA-1. (A) MCF-7 cells were cultured in the presence of increasing concentrations of B[a]P for 72 hours. Bars represent average cell number±SD from three independent wells counted in triplicate (n = 9). (B) Western blotting of BRCA-1 protein. Cell extracts were obtained from MCF-7 cells cultured for 72 hours in control DMEM/F12 containing 10% FCS, basal medium plus vehicle (DMSO), or increasing concentrations of B[a]P. The bands in panel B are immunocomplexes visualized by incubating Western blots with BRCA-1 (Ab-2) or β-actin (Ab-1) antibodies.*

described elsewhere [22]. Briefly, total RNA (400 ng) was incubated with random hexamer primers, Moloney murine leukemia virus RT, RNase inhibitor (Life Technologies/Gibco BRL, Gaithersburg, MD), and RT buffer (Ambion Inc., Austin, TX) at 42°C for 1 hours. cDNA was amplified using the forward 5′-AGCTCGCTGAGACTTCCTGGA-3′ and reverse 5′-CAATTCAATGTAGACAGACGT-3′ primers, which produced fragments spanning exon-1A to exon-8. The amplification products were of the expected size (712 bp), and their authenticity to the BRCA-1 sequence (GenBank accession no. U1460) was confirmed by direct sequencing. The primers for the internal standard 18*S* ribosomal RNA (488 bp) were from Ambion Inc. The expression levels of BRCA-1 were quantified by Alpha Imager (Alpha Innotech Inc., San Diego, CA) analysis and corrected for the expression of the control mRNA (BRCA-1/18*S*).

Results

Dose-Dependent Effects of B[a]P on Cell Viability and BRCA-1

The data depicted in Figure 1 demonstrate a strong correlation between cell number (Figure 1*A*) and levels of BRCA-1 protein (Figure 1*B*) in cells treated with varying concentrations of B[*a*]P. MCF-7 cells treated with 0.05 to 0.5 μM B[*a*]P proliferated at the same rate as control cells, even though BRCA-1 protein levels were reduced by three-fold at concentrations of 0.5 μM B[*a*]P (Figure 1*B*). This observation indicates that non-cytotoxic concentrations of B[*a*]P might abrogate the expression of BRCA-1 in circumstances of chronic exposure. However, acute doses of 1 and 5 μM B[*a*]P reduced cell viability 3.5- and 10-fold, respectively, after 72 hours. BRCA-1 protein was reduced six-fold in the presence of 1 μM

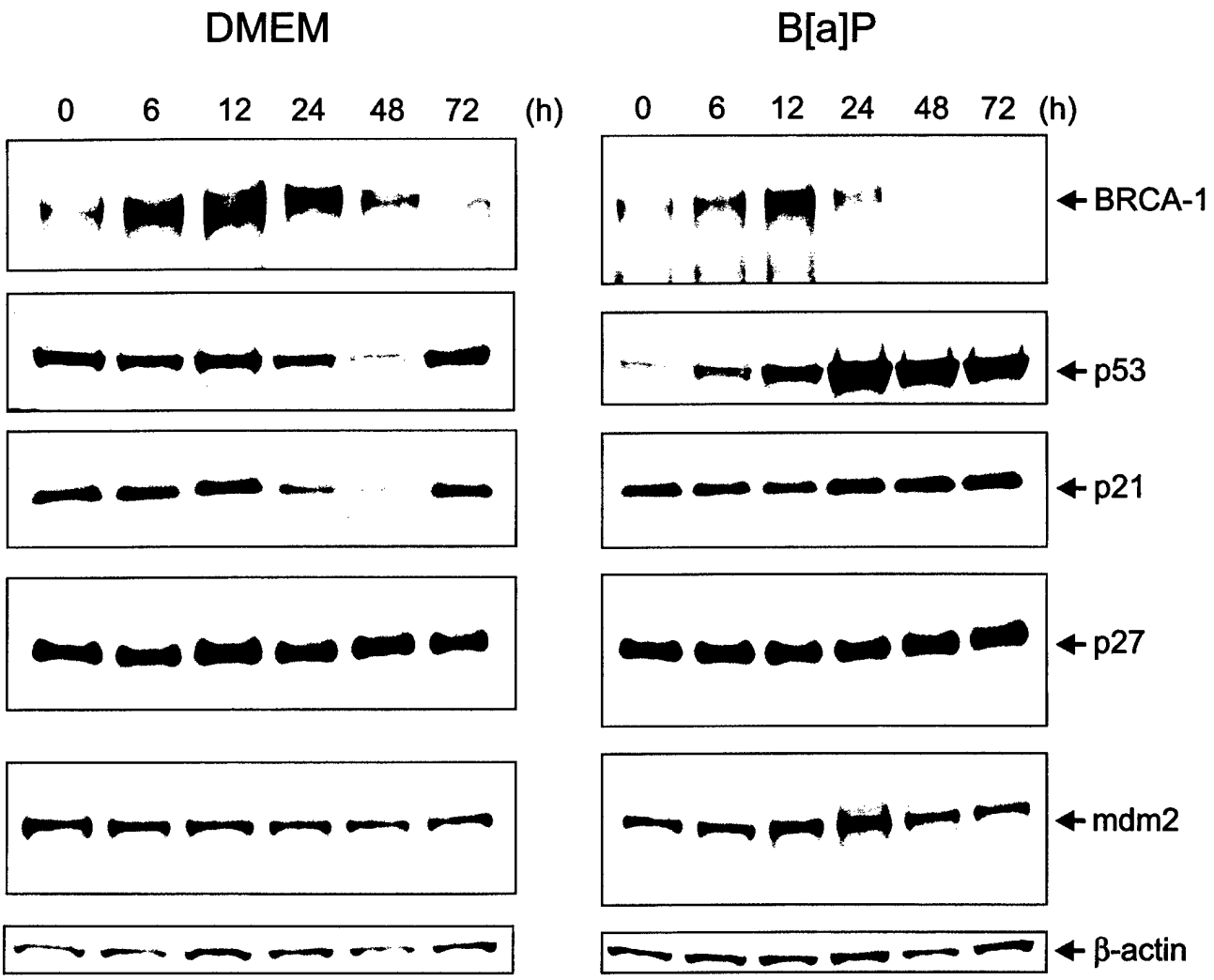


Figure 2. *Time-dependent effects of B[a]P on BRCA-1 and cell cycle checkpoints. Asynchronous MCF-7 cells were cultured for various periods of time in basal DMEM/F12 plus 10% FCS or basal medium containing 5 μM B[a]P. At the end of the incubation periods, cell extracts were analyzed for their content in BRCA-1, p53, p21, and mdm2 protein. The control bands are β-actin immunocomplexes.*

B[a]P, whereas at 5 μM B[a]P, BRCA-1 immunocomplexes were nearly undetectable. These data lend support to our previous findings [22] that non-lethal doses of B[a]P may reduce BRCA-1 protein, whereas at concentrations higher than 0.5 μM, the loss of BRCA-1 is paralleled by decreased cell viability. The finding that 20% to 30% of cells did not succumb to treatment with cytotoxic levels of B[a]P, but contained little or no BRCA-1, triggered our interest in examining the dynamic changes in BRCA-1 expression and cell cycle kinetics in this sub-cell population.

We performed Western blot analysis of cell extracts obtained from asynchronous MCF-7 cells cultured in control medium or medium supplemented with 5 μM B[a]P for various periods of time (Figure 2). In both control and B[a]P-treated cells, BRCA-1 protein peaked at 12 hours. This induction was attributed to stimulated expression of BRCA-1, which is characteristic of rapidly proliferating cells [24]. Whereas BRCA-1 resumed to basal levels in control cells at 24 hours, the presence of B[a]P drastically reduced BRCA-1 levels at both 48 and 72 hours. Between 6 and 12 hours, we detected fluctuations in the levels of p53 in cells cultured in basal medium. Treatment with B[a]P for 12 hours induced a significant increase in the cellular content of p53, which was further augmented at 24 hours, and remained significantly higher than the amount detected in control cells. The accumulation of p53 in B[a]P-treated cells was accompanied by an increase in the level of cyclin-dependent kinase inhibitor p21. In contrast, there were no detectable changes in the cellular content of p27, in the presence or absence of B[a]P. Cellular levels of the p53 regulator, mdm2, remained unchanged throughout the experiment in MCF-7 cells cultured in DMEM/F12, whereas the mdm2 protein accumulated between 12 and 24 hours after treatment with B[a]P. Thus, when comparing the temporal profiles of expression of BRCA-1 and p53, the accumulation of p53 at 12 hours in B[a]P-treated cells preceded by approximately 12 hours the loss of BRCA-1. At 48 and 72 hours after treatment with B[a]P, BRCA-1 was not detectable, whereas the cellular levels of p53 and p21 remained elevated.

B[a]P Alters Cell Cycle Kinetics

The concomitant loss of BRCA-1, along with the changes in p53 and p21 proteins, prompted us to examine the effects of B[a]P on cell cycle progression. Cell cycle distribution was determined by flow cytometric analysis of propidium iodide stained cells. Treatment of asynchronous MCF-7 cells with B[a]P, when 55% of cells were in G_0/G_1, induced within a period of 24 hours a significant enrichment in S-phase compared with control cells (53.1% vs. 32.0%). The accumulation in S-phase was paralleled by a reduction in the fraction of cells in G_0/G_1 (36.3% vs. 54.0%) (Figure 3) and a 2.5- and 4.0-fold increase in the percentage of cells positioned in G_2/M at 48 and 72 hours, respectively. Thus, loss of BRCA-1 and stabilization of p53 and p21 correlated with pausing of cells in S-phase and subsequent arrest in G_2/M.

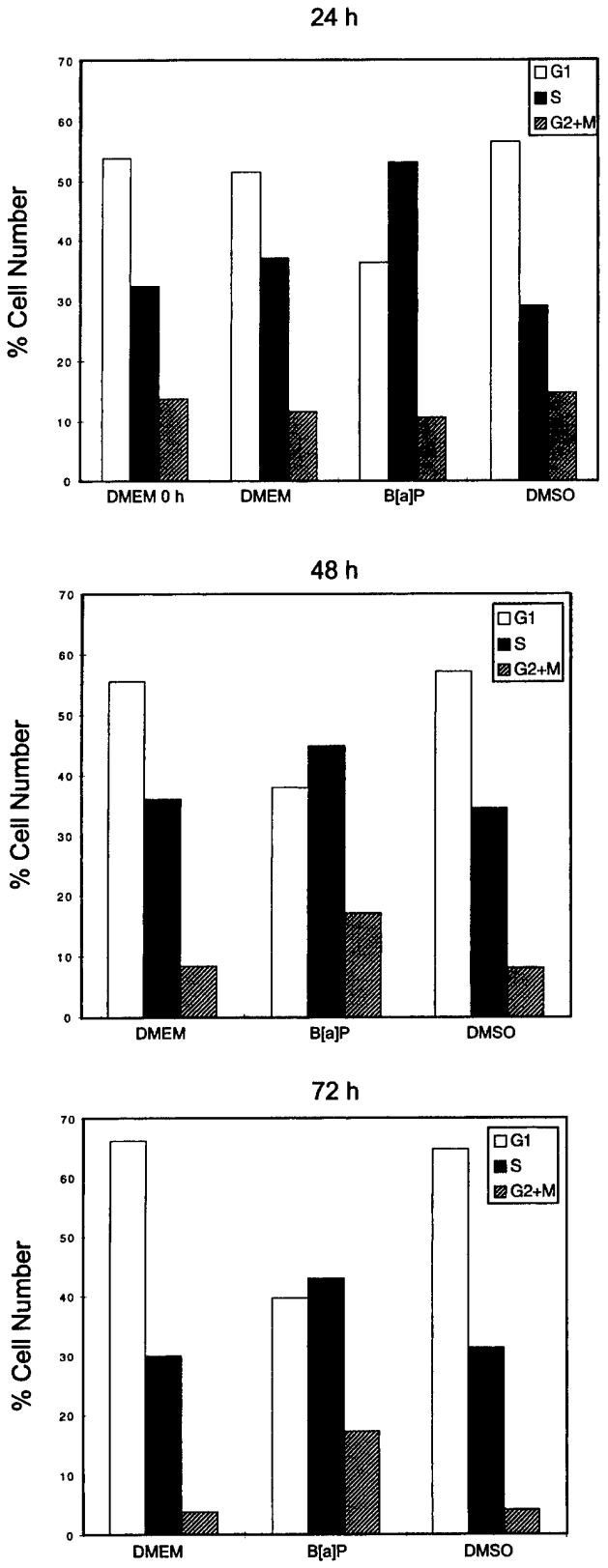


Figure 3. *B[a]P induces S-phase and G_2/M accumulation. Asynchronous MCF-7 cells were cultured for 24, 48, and 72 hours in basal DMEM/F12 plus 10% FCS, basal medium containing the vehicle (DMSO) or vehicle plus 5 μM B[a]P. Cell cycle profiles were analyzed by flow cytometry as described in Materials and Methods section. Bars represent percentages of cells in G_0/G_1, S, and G_2/M representative of three separate experiments with standard deviations lower than 3%.*

Because levels of BRCA-1 normally peak in S-phase [10], we wished to characterize the effects of B[a]P on levels of BRCA-1 protein and cell cycle progression in MCF-7 cells synchronized in S-phase. After synchronization with 1 μg/ml aphidicolin for 24 hours, cells were released from S-phase arrest by replacing the culture medium with fresh DMEM/F12 plus 10% FCS or medium supplemented with 5 μM B[a]P. The data depicted in Figure 4 showed that, at 12 hours after release, the addition of B[a]P induced arrest in S-phase (36.1% vs. 21.2%) (Figure 4*E*) and reduced the fraction (27.4%) that progressed to G_2/M compared with cells (42%) cultured in basal DMEM/F12 medium. However, while a significant percentage of cells released into control medium transitioned to G_0/G_1 (72.4%) by 24 hours and assumed the characteristic asynchronous distribution (Figure 4*A*), the treatment with B[a]P sustained significant accumulation in G_2/M (29.4% vs. 5.6%) (Figure 4*F*). Overall, B[a]P appeared to alter normal cell cycling by

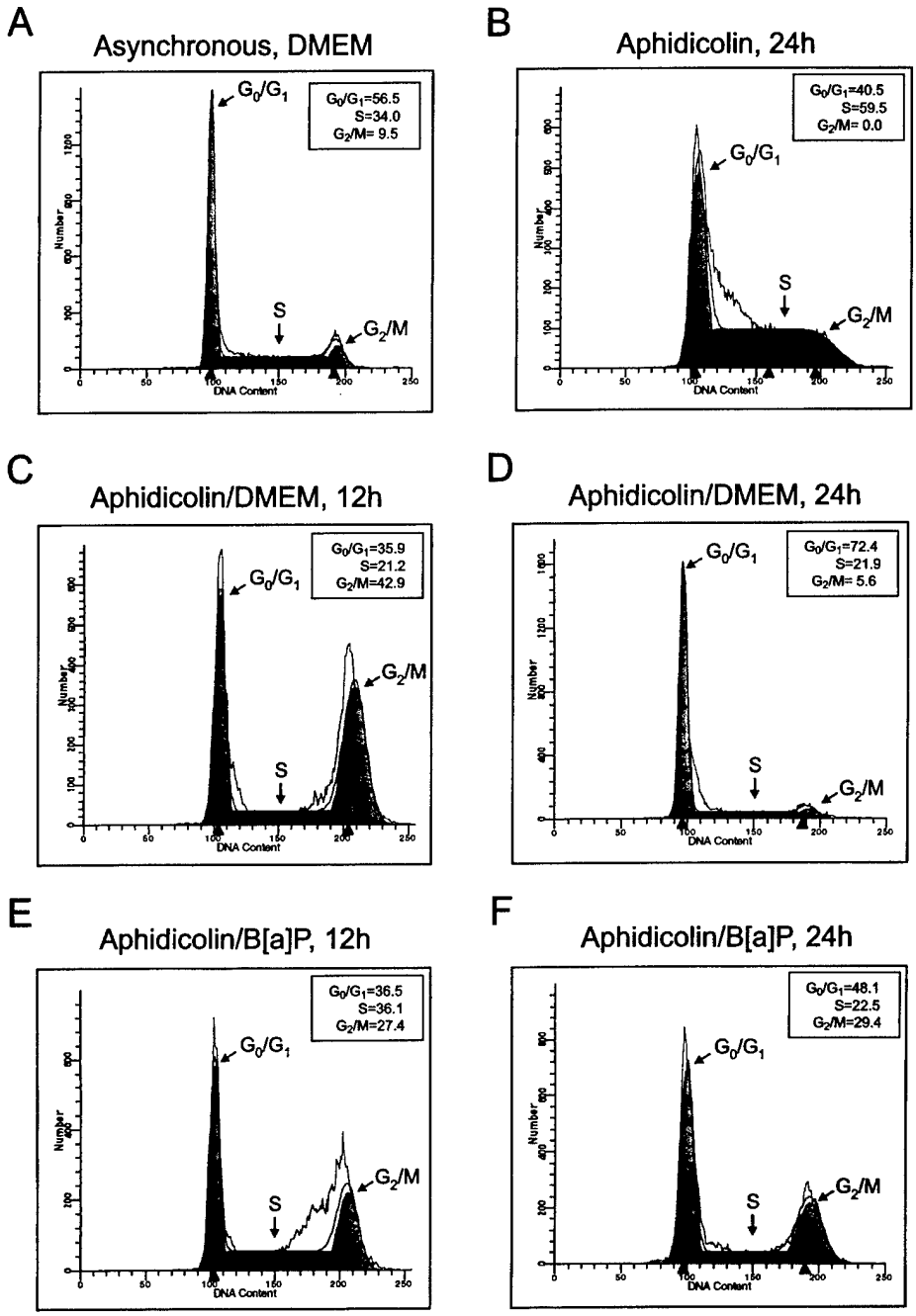
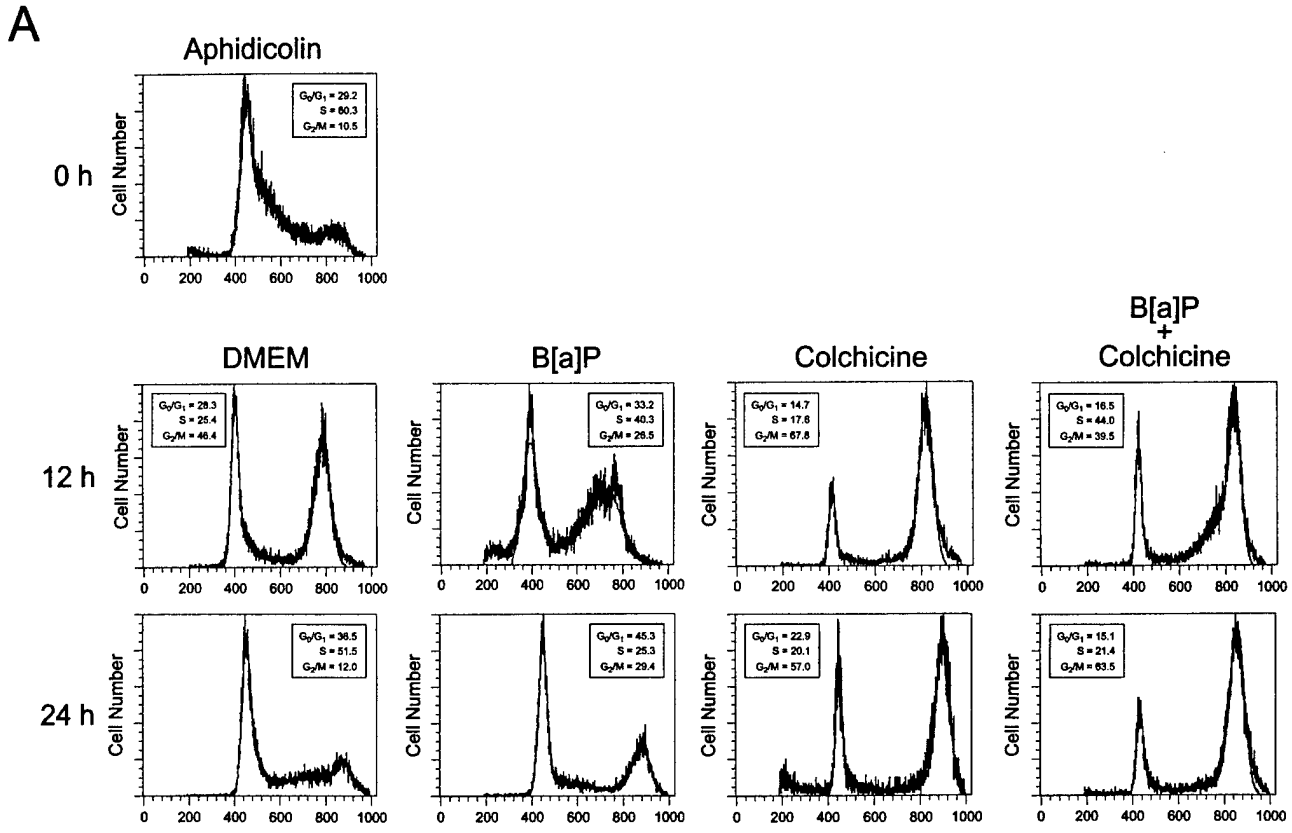


Figure 4. *B[a]P delays escape from S-phase and extends transit through G_2/M. MCF-7 cells were synchronized in S-phase with aphidicolin (1 μg/ml) for 24 hours, after which cells were released into (C and D) basal DMEM/F12 plus 10% FCS (aphidicolin/DMEM) or (E and F) basal medium plus 5 μM B[a]P (aphidicolin/B[a]P). Cells were harvested at 12 and 24 hours after release. Cell cycle distribution was examined by flow cytometry after propidium-iodide staining. Arrowheads below the DNA histograms represent channels with most events for specific phases of the cell cycle. The profiles are representative of three independent experiments.*



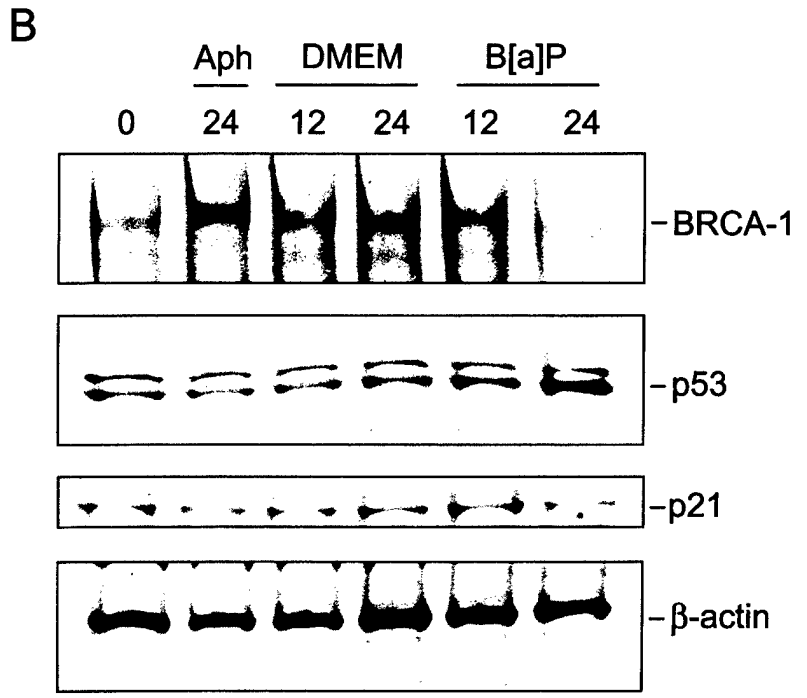


Figure 5. *B[a]P-dependent accumulation in G_2/M coincides with loss of BRCA-1. (A) MCF-7 cells were synchronized in S-phase with aphidicolin (1 μg/ml) for 24 hours, after which cells were released into basal DMEM/F12 plus 10% FCS, or basal medium plus 5 μM B[a]P, colchicine (0.25 μM), or colchicine plus B[a]P. Cells were harvested at 12 and 24 hours after release. Cell cycle distribution was examined by flow cytometry after propidium-iodide staining. The profiles are representative of three independent experiments. (B) Western blotting of BRCA-1, p53 and p21. After synchronization in S-phase with aphidicolin (1 μg/ml) for 24 hours, cells were released into DMEM/F12 plus 10% FCS or basal medium containing 5 μM B[a]P. Cell extracts were collected at 12 and 24 hours after release. The control bands are β-actin immunocomplexes.*

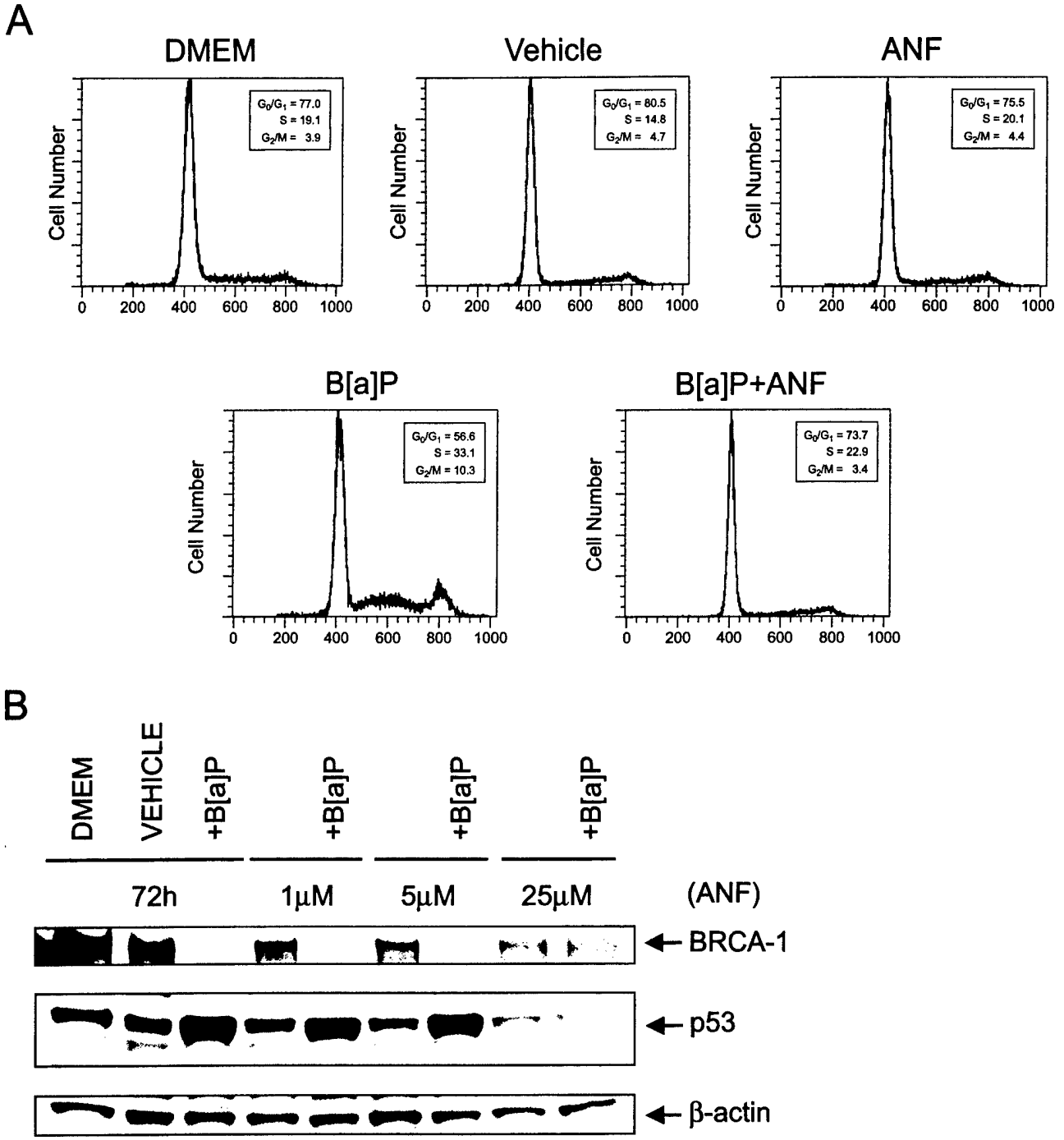


Figure 6. *ANF restores normal cell cycle distribution, BRCA-1, and inhibits the accumulation p53. Asynchronous MCF-7 cells were cultured for 72 hours in basal DMEM/F12, basal medium containing vehicle (DMSO), or 5 μM B[a]P plus increasing concentrations of ANF. Bands represent immunocomplexes of BRCA-1 and p53. The control bands are β-actin immunocomplexes.*

lengthening the transition through S and inducing subsequent arrest in G_2/M. This inference was confirmed in a G_2/M trapping experiment in which cells previously synchronized in S-phase with aphidicolin were released into culture media containing colchicine (0.25 μM) to prevent cycling beyond G_2/M. These experiments illustrated that, upon treatment with B[*a*]P, cells resumed to G_2/M with an approximate 12 hours delay compared with cells released in control DMEM/F12 (Figure 5*A*). In fact, in the presence of B[*a*]P plus colchicine, 44.0% of cells were positioned in S-phase at 12 hours compared with only 17.6% when cells were treated with colchicine alone. Nevertheless, flow cytometry profiles confirmed that by 24 hours after release, a significant percentage of cells treated with B[*a*]P plus colchicine had escaped S-phase arrest and occupied the G_2/M window at levels (63.5%) similar to those elicited by colchicine (57.0%). Therefore, although B[*a*]P induced S-phase arrest, this checkpoint was

relaxed since cells resumed cycling to G_2/M within a 24-hour interval.

The synchronization in S-phase with aphidicolin elicited the accumulation of BRCA-1 protein, as indicated by Western blotting of protein extracts (Figure 5*B*). The release from S-phase into basal DMEM was accompanied at 12 hours by a slight reduction in BRCA-1, whose levels increased at 24 hours (lane 4) when 51.5% of cells occupied the S-phase. These dynamic changes in BRCA-1 are in keeping with the notion that levels of BRCA-1 fluctuate during cell progression, but that the BRCA-1 protein is most abundant during transit through S-phase [10]. In contrast, BRCA-1 was significantly reduced after treatment with B[*a*]P for 24 hours, whereas levels of p53 were increased. Interestingly, the accumulation of p53 did not yield a corresponding increase in p21, whose levels remained unchanged at 12 and 24 hours after release from S-phase. These data indicated that cells containing increased levels of p53 paused transiently in S-phase, but subsequent transition to G_2/M occurred with reduced cellular levels of BRCA-1.

ANF Counteracts the Loss of BRCA-1 Expression and Disruption of Cell Cycle Kinetics

We wished to obtain direct evidence of the involvement of the AhR pathway in the disruption of cell cycle kinetics by B[*a*]P. Therefore, we tested whether co-treatment with the AhR antagonist, ANF, prevented the perturbations in cell cycle kinetics triggered by B[*a*]P, and restored BRCA-1 expression. The co-treatment with ANF abrogated the transient arrest of MCF-7 cells in S-phase and the subsequent accumulation in G_2/M, suggesting that the AhR pathway mediated the disruptive effects of B[*a*]P on cell cycle progression. In addition to restoring normal cell cycle distribution (Figure 6*A*), the co-treatment with ANF counteracted the loss of BRCA-1 and accumulation of p53 (Figure 6*B*). Taken together, these data confirmed that the changes in cell cycle kinetics as well as the fluctuations in BRCA-1 and p53 involved the participation of the AhR pathway, and attributed to ANF a protective effect against B[*a*]P. Furthermore, these findings suggest the existence of an inverse relationship between BRCA-1 and p53 status in MCF-7 cells with regard to exposure to B[*a*]P.

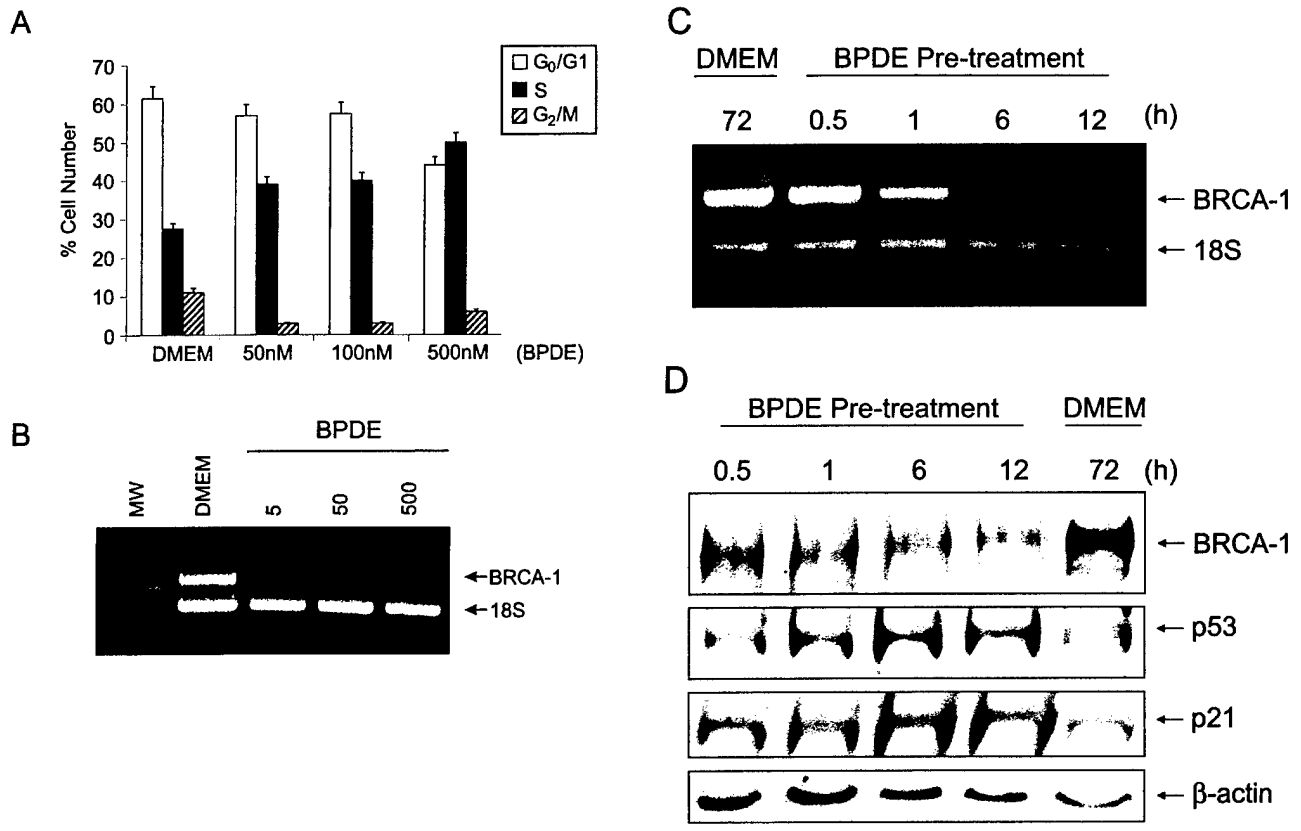


Figure 7. *BPDE induces S-phase arrest and reduces the potential for BRCA-1 expression. (A) Flow cytometry profile of MCF-7 cells cultured for 24 hours in basal DMEM/F12 plus 10% FCS, or basal medium plus increasing concentrations of BPDE. Bars represent average percentage of cells in G_0/G_1, S, and G_2/M from two separate experiments. (B) RT-PCR analysis of BRCA-1 mRNA from MCF-7 cells treated with nanomolar concentrations of BPDE for 24 hours. (C) MCF-7 cells were treated for 72 hours in basal medium, or pre-treated in BPDE (500 nM)-containing medium for 0.5 1, 6 and 12 hours. At the end of the pre-incubation periods, cells were washed and cultured up to 72 hours in fresh DMEM/F12 plus 10% FCS free of BPDE. In (B) and (C), changes in BRCA-1 mRNA were assessed by RT-PCR analysis of total RNA. The PCR products represent BRCA-1 (712 bp) and control ribosomal 18S RNA (488 bp) from input cDNA corresponding to 400 ng of total RNA. Experimental conditions for semi-quantitative RT-PCR analysis of BRCA-1 were those described in Material and Methods section [22]. (D) Western blot analysis of BRCA-1 and p53. MCF-7 cells were pre-treated with BPDE (500 nM) for various periods of time and then cultured up to 72 hours in basal DMEM/F12 plus 10% FCS medium. Bands represent immunocomplexes of BRCA-1, p53 and p21. The control bands are β-actin immunocomplexes.*

BPDE Induces S-Phase Arrest and Reduces the Potential for BRCA-1 Expression

In previous studies [22], we reported that B[*a*]P, but not 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), reduced the expression of BRCA-1. The fact that TCDD binds with high affinity to the AhR, but is not metabolized, led us to consider the possibility that downregulation of BRCA-1 may be mediated, at least in part, by metabolites of B[*a*]P. Therefore, we investigated further the effects of the metabolite BPDE on cell cycle progression and expression of BRCA-1.

Flow cytometry analysis of MCF-7 cells documented that, similar to B[*a*]P, BPDE induces a dose-dependent arrest of cells in S-phase. The fraction of cells positioned in S-phase at 24 hours increased from 28% for the DMEM control to 50% for MCF-7 cells treated with 500 nM BPDE (Figure 7*A*). The BPDE-dependent accumulation in S-phase was paralleled by a reduction in the percentage of cells positioned in G_0/G_1 (from 60% to 44%) and G_2/M (from 10% to 5%). RT-PCR analysis of total RNA revealed that treatment with increasing amounts of BPDE elicited a dose-dependent loss of BRCA-1 transcripts (Figure 7*B*), which were nearly undetectable at concentrations equal to 500 nM BPDE.

The effects of BPDE on BRCA-1 expression were further examined in a washout experiment in which MCF-7 cells were pre-treated with 500 nM BPDE for 0.5, 1, 6 and 12 hours. Then, after washing out of media containing the BPDE, cells were cultured in fresh DMEM/F12 plus 10% FCS medium up to 72 hours. At the end of the incubation period, we analyzed the levels of BRCA-1 mRNA in total RNA, and protein in cell extracts. While at 72 hours the levels of BRCA-1 mRNA in control cells were similar to those observed in cells harvested at the time of induction (data not shown), BRCA-1 transcripts were reduced five-fold in cells pre-treated with BPDE for 6 to 12 hours (Figure 7*C*). Similarly, BRCA-1 protein levels were reduced significantly by the pre-treatment for 6 to 12 hours with BPDE (Figure 7*D*). The loss of BRCA-1 at these time points was paralleled by accumulation of p53 and p21. Because the half-life of BDPE is approximately 5 to 20 minutes [25], we discounted the likelihood that residual BPDE may have been responsible for the reduction in BRCA-1 mRNA and protein. In fact, cells were washed twice and then cultured in fresh medium for at least 60 hours after removal of media containing the BPDE. Based on these considerations, we concluded that the short-term exposure to BPDE exerted a signature effect by reducing the potential for BRCA-1 expression and increasing the cellular levels of p53 and p21.

Discussion

In the absence of a causal relationship between the occurrence of sporadic breast cancer and mutations in the BRCA-1 gene, efforts directed to investigating the contribution of environmental xenobiotics as epigenetic effectors of BRCA-1 are warranted. PAHs, ubiquitous pollutants known to induce mammary tumors in rodents [26], are found in tobacco smoke, industrial pollution and auto exhaust [27]. Among PAHs, B[*a*]P is a prototype known to act both as a tumor initiator and promoter, and to stimulate the expression of gene products of the cytochrome *P*450 family, which metabolize B[*a*]P to the ultimate carcinogen, BPDE [28]. The BPDE damages DNA by forming bulky adducts and apurinic sites that are degraded further to DNA strand breaks [29], thus contributing to inhibition of DNA synthesis [30] and cell cycle arrest [31].

To elucidate the function of BRCA-1, genetic models defective in BRCA-1 and other cell cycle regulators have been developed [11,13–15,32]. In previous work [22], we documented that B[*a*]P inhibited BRCA-1 expression in estrogen receptor-positive breast MCF-7 and ovarian BG-1 cancer cells in a dose- and time-dependent fashion. In this study, we hypothesized that one modality of PAH-mediated breast oncogenesis may involve the coordinate disruption of BRCA-1 and cell cycle regulation. The loss of BRCA-1 may abrogate normal defense mechanisms, such as cell cycle and growth control functions, thus predisposing to accumulation of DNA damage that may be critical for tumor development. Our findings provide novel evidence that non-cytotoxic doses of B[*a*]P (0.5 μM) reduce the levels of BRCA-1 protein in MCF-7 cells. The significance of this observation is that BRCA-1 may be a cellular target for repression by chronic exposure to PAHs, which may lower DNA repair functions and predispose to the fixation of mutations.

At cytotoxic concentrations (1 to 5 μM), nearly 20% to 30% of cells survived the treatment with B[*a*]P, but contained decreased or no BRCA-1 protein. Because cytotoxic damage and multi-drug resistance are hallmarks of cancer, we examined whether cells resistant to B[*a*]P, having reduced levels of BRCA-1, had acquired altered cell cycle characteristics. Our results indicated that acute exposure to cytotoxic levels of B[*a*]P lengthened the S-phase interval and led to G_2/M accumulation. The stabilization of p53 and p21 and dynamic fluctuations in mdm2 confirmed that surveillance mechanisms were alerted to halt cell cycle progression [16,33]. However, results obtained with MCF-7 cells synchronized with aphidicolin showed that the arrest in S-phase was transient. In the presence of B[*a*]P, cycling from S to G_2/M occurred with an approximate 12-hour delay compared with cells released into control medium. In fact, 24 hours after release from S-phase, a large fraction of cells treated with B[*a*]P occupied the G_2/M window with increased levels of p53, but reduced BRCA-1, whereas levels of p21 were unaffected. The failure of p21, which is required for maintaining the G_2 block [17], to increase under these conditions could be attributed, at least in part, to the downregulation of BRCA-1. The latter has been shown to contribute to *trans*-activation of the p21 promoter [13]. These findings suggest that B[*a*]P contributes to accumulation in G_2/M-phase of cells containing reduced levels of BRCA-1, and complement recent work [15] documenting that embryonic fibroblasts carrying an exon-11 BRCA-1 deletion are defective in G_2/M checkpoint. The prolonged arrest in G_2 of cells harboring DNA damage is known to cause aberrant mitosis [34]. Because BRCA-1 is a target for phosphorylation by the ATM protein

kinase [9] and is required for S-phase [13] and G_2/M regulation [15], one could envision that the carcinogenicity of B[*a*]P may stem, at least in part, from reduction of BRCA-1 expression and repair functions involving the ATM–BRCA-1 cascade.

Previous studies have reported on the inhibition of BRCA-1 by chemical agents, including adriamycin and ultraviolet radiation in breast cancer cells [19]. In this study, we provided evidence that disruption of cell cycle kinetics by B[*a*]P correlated with inverse expression patterns of BRCA-1 and p53 in MCF-7 cells. In fact, co-treatment with ANF restored normal cell cycle distribution and counteracted the loss of BRCA-1 expression, while preventing the accumulation of p53. Because embryonic death associated with disruption of the BRCA-1 gene was less pronounced in p53-null embryos [6], one could attribute the inverse expression patterns of BRCA-1 and p53 to antagonistic interactions among factors ubiquitous to the regulation of BRCA-1 and p53 [35].

With respect to loss of cell viability induced by B[*a*]P, one could expect that the coordinate loss of BRCA-1 and accumulation of p53 may lead to p53-dependent apoptosis of severely damaged cells. However, electron microscopy analysis of cells treated with B[*a*]P did not reveal changes in cellular architecture or morphology characteristic of apoptosis, such as chromatin condensation and membrane blebbing (unpublished data). Considering that approximately 30% of cells did not succumb to the treatment with cytotoxic levels of B[*a*]P, one could envision that B[*a*]P-resistant cells expressing lower levels of BRCA-1 may have acquired a cytotoxic drug resistance phenotype [36].

With regard to the mechanisms through which B[*a*]P may repress BRCA-1, one could extrapolate that through general inhibition of RNA synthesis, B[*a*]P may preferentially deplete the cellular levels of proteins with a rapid turnover, such as BRCA-1, as opposed to p53, which is regulated at the posttranscriptional level following DNA damage. This hypothesis is not supported by our earlier data [22], which document that in estrogen receptor-negative HBL-100 and MDA-MB-231 breast cancer cells, neither BRCA-1 nor cell growth was compromised by equimolar (5 μM) concentrations of B[*a*]P. Moreover, in the same study, we found that the dioxin-like TCDD, which exhibits high affinity for the AhR but is not metabolized, did not repress BRCA-1 mRNA and protein levels [22]. Therefore, we hypothesized that the responsiveness of MCF-7 cells to B[*a*]P may be a consequence of their ability to metabolize B[*a*]P to reactive end products. To test this contention, we treated MCF-7 cells with the metabolite BPDE. Consistent with this hypothesis, BPDE induced S-phase arrest and suppressed BRCA-1. These effects were observed at doses 10-fold lower (50 to 500 nM) than those used for B[*a*]P (0.5 to 5 μM). More importantly, we observed that the short-term exposure to BPDE reduced the potential to express BRCA-1, since removal of BPDE did not allow for complete reversal of the repression. In contrast, levels of p53 and p21 were increased in cells pre-treated with BPDE for at least 6 to 12 hours. We did not attribute the loss of BRCA-1 to residual BPDE since BPDE is hydrolyzed within minutes in cellular systems [25]. In addition, MCF-7 cells were cultured in fresh media after washing out of the pre-treatment medium. Rather, these cumulative observations suggested that the effects of BPDE were not transient. A possible interpretation of these data is that BPDE may repress transcription of BRCA-1 or alter the expression of other factors such as p53, which may contribute to repression of BRCA-1. For example, the functionality of cellular checkpoints may contribute to the deleterious effects of BPDE on BRCA-1 expression. MCF-7 cells have functional p53 and Rb, both of which complex with BRCA-1 [11]. Conversely, in SV40-transformed HBL-100 cells, which are refractory to B[*a*]P, both p53 and Rb are inactivated [37]. Investigations are currently in progress in our laboratory to clarify whether elevation of p53 is an absolute requirement for BPDE-dependent regulation of BRCA-1.

In summary, there are ample data suggesting that environmental factors can initiate and promote chemical carcinogenesis [38]. While maintenance of genome integrity is the result of balance between production and repair of DNA [39], this work provides novel evidence that B[*a*]P disrupts normal BRCA-1 expression and cell cycle kinetics. These effects are mediated, at least in part, by the reactive metabolite BPDE, which reduces the potential for BRCA-1 expression. The identification of a possible link between carcinogenicity induced by PAHs and loss of BRCA-1 offers an exciting opportunity to gain insights into the potential role of PAH–gene interactions in the development of sporadic breast cancer.

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References

[1] Gowen LC, Avrutskaya AV, Latour AM, Koller BH, and Leadon SA (1998). BRCA-1 required for transcription-coupled repair of oxidative damage. *Science* **286**, 804–810.

[2] Scully R, Chen J, Plug A, Xiao Y, Waver D, Feunteun J, Ashley T, and Livingston DM (1997). Association of BRCA-1 with Rad51 in mitotic and meiotic cells. *Cell* **88**, 265–275.

[3] Zhang H, Tombline G, and Weber BL (1998). BRCA-1, BRCA-2 and DNA damage response: collision or collusion. *Cell* **92**, 433–436.

[4] Zhong Q, Chen C-F, Li S, Chen Y, Wang C-C, Xiao J, Chen P-L, Sharp ZD, and Lee W-H (1999). Association of BRCA-1 with the hRad50–hMre11–p95 complex and the DNA damage response. *Science* **285**, 747–750.

[5] Hakem R, de la Pompa JL, Elia A, Potter J, and Mak TW (1997). Partial rescue of BRCA-1 (5–6) early embryonic lethality by p53 or p21 null mutation. *Nat Genet* **16**, 298–302.

[6] Ludwig T, Chapman DL, Papaioannou VE, and Efstratiadis A (1997). Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of BRCA-1, BRCA-2, BRCA-1/BRCA-2,

BRCA-1/p53, and BRCA-2/p53 nullizygous embryos. *Genes Dev* **11**, 1226–1241.

[7] Scully R, Chen J, Ochs RL, Keegan K, Hoekstra M, Feunteun J, and Livingston DM (1997). Dynamic changes of BRCA-1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell* **90**, 425–435.

[8] Thomas JE, Smith M, Tonkinson JL, Rubinfeld B, and Polakis P (1997). Induction of phosphorylation on BRCA-1 during the cell cycle and after DNA damage. *Cell Growth Differ* **8**, 801–809.

[9] Cortez D, Wang Y, Qin J, and Elelldge SJ (1999). Requirement of ATM-dependent phosphorylation of BRCA-1 in the DNA damage response to double-strand breaks. *Science* **286**, 1162–1171.

[10] Chen Y, Farmer A, Chen C-F, Jones DC, Chen P-L, and Lee W-H (1996). BRCA-1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner. *Cancer Res* **56**, 3168–3172.

[11] Ouchi T, Monteiro AN, August A, Aaronson SA, and Hanafusa H (1998). BRCA-1 regulates p53-dependent gene expression. *PNAS* **95**, 2302–2306.

[12] Li S, Chen P-L, Subramanian T, Chinnadurai G, Tonlinson G, Osborne CK, Sharp ZD, and Lee W-H (1999). Binding of CtIP to the BRCT repeats of BRCA-1 involved in the transcription regulation of p21 is disrupted upon DNA damage. *J Biol Chem* **275**, 11334–11338.

[13] Somasundaram K, Zhang H, Zeng Y-X, Houvras Y, Peng Y, Zhang H, Wu GS, Licht JD, Weber BL, and El-Deiry WS (1997). Arrest of the cell cycle by the tumor suppressor BRCA-1 requires the CDK inhibitor p21 WAF1/CiPI. *Nature* **389**, 187–190.

[14] Larson JS, Tonkinson JL, and Lai MT (1997). A BRCA-1 mutant alters G_2–M cell cycle control in human mammary epithelial cells. *Cancer Res* **57**, 3351–3355.

[15] Xu X, Weaver Z, Linke SP, Li C, Gotay J, Wang X-W, Harris CC, Ried T, and Deng C-X (1999). Centrosome amplification and a defective G_2–M cell cycle checkpoint induce genetic instability in BRCA-1 exon 11 isoform-deficient cells. *Mol Cell* **3**, 389–395.

[16] Hartwell LH, and Kastan MB (1994). Cell cycle control and cancer. *Science* **266**, 1821–1828.

[17] Bunz F, Dutriaux A, Lenauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, and Vogelstein B (1998). Requirement for p53 and p21 to sustain G_2 arrest after DNA damage. *Science* **282**, 1497–1501.

[18] Brugarolas J, and Jacks T (1997). Double indemnity: p53, BRCA and cancer. *Nat Med* **3**, 721–722.

[19] Andres JL, Fan S, Turlek GJ, Wang J-A, Twu N-F, Yuan R-Q, Lamszus K, Goldberg ID, and Rosen EM (1998). Regulation of BRCA-1 and BRCA-2 expression in human breast cancer cells by DNA-damaging agents. *Oncogene* **16**, 2229–2241.

[20] Vaziri C, and Faller DV (1997). A benzo[*a*]pyrene-induced cell cycle checkpoint resulting in p53-independent G_1 arrest in 3T3 fibroblasts. *J Biol Chem* **272**, 2762–2769.

[21] Shackelford RE, Kaufmann, and Paules RS (1999). Cell cycle control, checkpoint mechanisms, and genotoxic stress. *Environ Health Perspect* **107**, 5–24.

[22] Jeffy BD, Schultz EU, Selmin O, Gudas JM, Bowden GT, and Romagnolo D (1999). Inhibition of BRCA-1 expression by benzo[a]-pyrene and its diol epoxide. *Mol Carcinogen* **26**, 100–118.

[23] Romagnolo D, Annab LA, Lyon TT, Risinger JI, Terry LA, Barrett JC, and Afshari CA (1998). Estrogen upregulation of expression of BRCA-1 with no effect on localization. *Mol Carcinogen* **22**, 102–109.

[24] Gudas JM, Nguyen H, Li T, and Cowen KH (1995). Hormone-dependent regulation of BRCA-1 in human breast cancer cells. *Cancer Res* **55**, 4561–4565.

[25] MacLeod MC, and Selkirk JK (1982). Physical interactions of isomeric benzo[*a*]pyrene diol epoxides with DNA. *Carcinogenesis* **3**, 287–292.

[26] Ronai Z, Gradia S, el-Bayoumy K, Amin S, and Hecht SS (1994). Contrasting incidence of *ras* mutations in rat mammary and mouse skin tumors induced by anti-benzo[*a*]phenan-threne-3,4-diol-1,2-epoxide. *Carcinogenesis* **15**, 2113–2116.

[27] Batsch H, and Hietanen E (1996). The role of individual susceptibility in cancer burden related to environmental exposure. *Environ Health Perspect* **104**, 569–577.

[28] Nebert DW, Petersen DD, and Fornace AJ Jr (1990). Cellular responses to oxidative stress: the *Ah* gene battery as a paradigm. *Environ Health Perspect* **88**, 13–25.

[29] Chakravarti D, Pelling JC, Cavalieri EI, and Rogan EG (1995). Relating aromatic hydrocarbon-induced DNA adducts and c-H-*ras* mutations in mouse skin papillomas: the role of apurinic sites. *PNAS* **92**, 10422–10426.

[30] Yamanishi DT, Bowden GT, and Cress AE (1987). An analysis of DNA replication in synchronized CHO cells treated with benzo[*a*]pyrene diol epoxide. *Biochim Biophys Acta* **910**, 34–42.

[31] Black KA, McFarland RD, Grisham JW, and Smith GJ (1989). S-phase block and cell death in human lymphoblasts exposed to benzo[*a*]pyrene diol epoxide or *N*-acetoxy-2-acetylaminofluorene. *Toxicol Appl Pharmacol* **97**, 463–472.

[32] Cressman VL, Backlund DC, Hicks EM, Gowen LC, Godfrey V, and Koller BH (1999). Mammary tumor formation in p53- and BRCA-1-deficient mice. *Cell Growth Differ* **10**, 1–10.

[33] Orren DK, Petersen LN, and Bohr VA (1997). Persistent DNA damage inhibits S-phase and G_2 progression, and results in apoptosis. *Mol Biol Cell* **8**, 1129–1142.

[34] Johnson PA, Clements P, Hudson K, and Caldecott KW (1999). A mitotic spindle requirement for DNA damage-induced apoptosis in Chinese hamster ovary cells. *Cancer Res* **59**, 2696–2700.

[35] Koonin EV, Altschul SF, and Bork P (1996). BRCA-1 protein products: functional motifs. *Nat Genet* **13**, 266–268.

[36] Moore M, Wang X, Lu Y-F, Wormke M, Craig A, Gerlach JH, Burghardt R, Barhoumi R, and Safe S (1994). Benzo[*a*]pyrene-resistant MCF-7 human breast cancer cells. *J Biol Chem* **269**, 11751–11759.

[37] Aprelikova ON, Fang BS, Meissner EG, Cotter S, Campbell M, Kuthiala A, Bessho M, and Jensen RA (1999). BRCA-1-associated growth arrest is RB-dependent. *PNAS* **96**, 11866–11871.

[38] Minamoto T, Mai M, and Ronai Z (1999). Environmental factors as regulators and effectors of multistep carcinogenesis. *Carcinogenesis* **20**, 519–527.

[39] Kinzler KW, and Vogelstein B (997). Cancer susceptibility genes: gatekeepers and caretakers. *Nature* **386**, 761–763.

Transcriptional repression of BRCA-1: requirements for metabolism of benzo[a]pyrene to BPDE[1]

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Running Title: Repression of BRCA-1 transcription

Kew words: Polycyclic aromatic hydrocarbons, benzo[a]pyrene, BPDE, BRCA-1, sporadic breast cancer.

ABSTRACT

Reduction of BRCA-1 expression through non-mutational events may be a predisposing event in the onset of sporadic breast cancer. In this study, we investigated the mechanisms through which the tobacco carcinogen benzo[a]pyrene (B[a]P) lowered BRCA-1 mRNA levels in breast cancer MCF-7 cells. We report that B[a]P did not compromise the stability of BRCA-1 mRNA, but repressed in a dose-dependent fashion transcriptional activity of a 1.69-kb BRCA-1 (pGL3-BRCA-1) promoter fragment, containing both exon-1A and exon-1B transcription start sites. In cells treated with B[a]P, loss of BRCA-1 promoter activity was accompanied by accumulation of CYP1A1 and BAX-α mRNA, p53 and p21 protein, whereas levels of Bcl-2 mRNA were reduced. The aromatic hydrocarbon receptor (AhR)-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 induced transcription activity of a CYP1A1-like promoter containing an array of xenobiotic responsive elements (XRE). These data suggested that the activated AhR did not inhibit transcription at the BRCA-1 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 a dominant-negative p53 protein mutated at position 175 (R to H) counteracted the detrimental effects of BPDE on BRCA-1 promoter activity and protein levels. We conclude that activation of the AhR is not sufficient for down-regulation of BRCA-1

transcription, which however is inhibited by the B[a]P-metabolite BPDE through a p53-dependent pathway.

INTRODUCTION

The characterization of many germline 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), but no sporadic breast cancers (5), has 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 polycyclic aromatic hydrocarbons (PAHs[3]) 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) and base substitutions (14). We (15, 16), and others (17) have documented that cellular levels of BRCA-1 mRNA and protein were elevated by

[3]The abbreviations used are: PAHs, polycyclic aromatic hydrocarbons; AhR, aromatic hydrocarbon receptor; B[a]P, benzo[a]pyrene; BPDE, 7r,8t-dihydroxy-9t,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; XRE, xenobiotic responsive elements;

estrogen in breast and ovarian cancer cells. In contrast, our group has recently reported that acute and chronic exposure to benzo[a]pyrene, a prototype PAH, lowered both constitutive and estrogen-dependent expression of BRCA-1 in breast and ovarian cancer cells (18). 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 aromatic hydrocarbon receptor (AhR)-antagonist α-naphthoflavone restored normal cell cycle distribution and BRCA-1 expression (19), entailed the AhR pathway contributed to down-regulation of BRCA-1. In this study, we investigated the mechanisms through which B[a]P lowered BRCA-1 mRNA levels in breast cancer MCF-7 cells. We reported that B[a]P repressed transcription of the BRCA-1 promoter. However, activation of the AhR pathway by B[a]P was not sufficient for down-regulation of BRCA-1 promoter activity, which was inhibited by the metabolite BPDE through a p53-dependent pathway.

MATERIALS AND METHODS

Cell culture and chemicals. Estrogen receptor positive MCF-7 cells were obtained from the American Type Culture Collection (ATCC), (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories, Inc, Logan, UT) as described previously (15). B[a]P and actinomycin-D were obtained from Sigma

Chemical Co. (St. Louis, MO), BPDE and TCDD were obtained from Midwest Research Institute (Kansas City, MO).

Semi-quantitative RT-PCR and ribonuclease protection assay. For mRNA studies, MCF-7 cells were plated at a density of $2x10^6$ 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 semi-quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR) analysis of BRCA-1 and CYP1A1 mRNA are described elsewhere (18). Briefly, total RNA (400 ng) was incubated with random hexamer primers, Moloney murine leukemia virus-RT, RNase inhibitor (Life Technologies/Gibco BRL, Gaithersburg, MD), and RT 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 size and their authenticity to the GeneBank was confirmed by direct sequencing. Preliminary control experiments (data not shown) were carried out to assure RT-PCR conditions allowed for linear amplification of PCR products. For amplification of the internal standard 18S ribosomal RNA (488-bp) we used the Competimer™ oligonucleotide module from Ambion Inc. (Austin, TX). The expression levels of BRCA-1 were quantified by Alpha Imager (Alpha Innotech Inc, San Diego, CA) analysis and corrected for the expression of the control mRNA (BRCA-1/18S). Details for ribonuclease protection assay of BRCA-1 mRNA are described elsewhere (18). Briefly, a 162-bp BRCA-1 riboprobe encoding for a portion of exon 15 was transcribed in the antisense orientation from the transcription vector Triplescript (Ambion Inc.). As internal control for ribonuclease protection assay, we transcribed a riboprobe for human

cyclophilin from the pTRIcyclophilin vector (Ambion Inc.). Relative phosphorimage 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 (19). Cell extracts were normalized to protein content and separated by 4-12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblotting was carried out with antibodies raised against BRCA-1 (Ab-2), p53 (Ab-2), and p21 (Ab-1) obtained 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 (ATCC) was utilized for PCR amplification of a 1.69-Kb BRCA-1 promoter fragment using the forward DRPR-F-KpnI (5'-ATCGGTACCGCATTCTGA ACCACAGACTCT-3') and reverse LH9-R-BglII (5'-ACTAGATCTACCTCATGACC AGCCGACGTT-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 GeneBank (accession #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 cells using the Lipofectamine-Plus procedure, as described by the manufacturer (Life Technologies, Gaithersburg, MD). Variations in transfection efficiency were accounted for by co-transfection with plasmids encoding for the β-galactosidase or renilla gene. Internal standards for luciferase activity were the pGL3Control vector containing an SV40 promoter, and pGL3Basic (empty) (Promega). To control for the efficacy of treatments with B[a]P and TCDD, MCF-7 cells were transfected with plasmid p1A1-4X-LUC (a gift from Dr. Pasco, University of Mississippi) containing a CYP1A1 consensus sequence linked to an array of four GCGTG elements. Cell extracts were collected at 24 h after treatment with various concentrations of B[a]P, TCDD, or BPDE. Luciferase reporter activity was monitored using a Luminometer 20/20 and expressed as relative luciferase units (RLU) corrected for 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 upregulation of the CYP1A1 gene, which encodes for a member of the P450 family of metabolizing enzymes (20). The accumulation of CYP1A1 mRNA confirmed the functionality of the AhR pathway in MCF-7 cells, albeit B[a]P was more effective than BPDE in elevating the content of CYP1A1 transcripts. The 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. Neither Bax-α nor 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 revealed that B[a]P lowered BRCA-1 protein levels, while increasing p53 (Fig. 1B). Expectedly, the treatment with BPDE reduced significantly BRCA-1 protein, whereas the cellular contents of p53 and p21 were elevated. In contrast, treatment with 10 nM TCDD did not alter BRCA-1, p53 or p21 protein levels (Fig. 1C), but elicited the accumulation of CYP1A1 mRNA levels (data not shown) (18).

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 ribonuclease protection assay (Fig. 2A) experiments revealed that, compared with DMEM, the levels of BRCA-1 mRNA corrected for the cyclophilin mRNA were reduced 3.0-fold by treatment 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 control and B[a]P-treated cells. After MCF-7 cells were pre-cultured for 24 h in DMEM/F12-10% FCS or DMEM/F12-10% FCS plus B[a]P, culture media were replaced with fresh DMEM/F12-10% FCS plus 5 μg/ml actinomycin-D (ActD) to inhibit the production of new transcripts, in the presence or absence of B[a]P. Kinetics of disappearance were assessed by calculating at 6, 9, 12, and 18 h the relative

levels of BRCA-1 mRNA corrected for the cyclophilin mRNA. The temporal changes depicted in Fig. 2B documented that the rate of disappearance of the protected BRCA-1 fragment was not influenced by B[a]P. In fact, the half-life of the BRCA-1 transcript was of approximately 12 h in both control and B[a]P-treated cells.

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 pGL3-BRCA-1. However, upon 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 μg of the pGL3-BRCA-1 vector, respectively. The RLU detected upon transfection with 1 μg 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 μg of the pGL3-BRCA-1 vector. Concentrations of 0.5 μM B[a]P did not influence RLU, whereas doses of 1 and 5 μM 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 XRE, 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 illustrate 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 (18), increasing the concentration of TCDD from 10 nM up to 1000 nM affected neither BRCA-1 mRNA nor protein content in MCF-7 cells, although cell viability was reduced from 50 to 80% with 10 and 1000 nM TCDD, respectively. Because the affinity of TCDD for the AhR is approximately 100-fold higher than that of B[a]P, but is not metabolized (21), 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 downregulation of BRCA-1. To test this contention, we compared the effects of BPDE (100 and 500 nM) and TCDD (10 nM) on BRCA-1 promoter activity in cells transiently transfected with the pGL3-BRCA-1 construct. Other groups have used concentrations up to 1.2 μM BPDE to investigate repair of DNA damage (22). However, we used lower concentrations ranging from 100 to 500 nM BPDE, which in our hands have been effective in promoting cell cycle arrest and loss of BRCA-1 expression in MCF-7 cells (19). The results of Fig. 4A indicated 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, activity of the BRCA-1 reporter construct was not affected by treatment with 10 nM TCDD, which, nevertheless, increased (1.6-fold) the reporter activity of the positive control p1A1-4X-LUC (Fig. 4B). These observations suggested that the metabolite

BPDE, or factors regulated by BPDE, contributed al least in part to B[a]P-dependent inhibition of BRCA-1 transcription.

Repression of BRCA-1 promoter activity by B[a]P and BPDE require functional p53. Based on our published observation that the AhR-antagonist α-naphthoflavone restored normal cell cycle distribution and BRCA-1 expression, while preventing the accumulation of p53 (19), we questioned whether gain of p53 functions in MCF-7 cells treated with B[a]P contributed to reduce BRCA-1 promoter activity. To test this hypothesis, we co-transfected MCF-7 cells with a plasmid containing a cassette encoding for p53 mutated at position 175 (Arginine to Histidine) under the control of the cytomegalovirus promoter (pCVM53mut) subcloned into pCMV (plasmids were gifts from Dr. Bert Vogelstein and made available by Dr. J. Martinez). 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 with pCMV53mut encoding 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 DMEM basal medium, levels of p53 were low in cells non-transfected (NT) or 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 upon transfection with the pCMV53mut vector. The intensity of the p53 immunocomplex increased further upon treatment with B[a]P, presumably because of coincident immuno-detection of endogenous and recombinantly expressed p53.

Next, we 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, the cotransfection with pCMV53mut restored luciferase activity to control levels. Similar results were obtained upon cotransfection of a vector encoding for the papilloma virus E6 protein, which prevented the loss of reporter activity elicited by BPDE (data not shown). The treatment with BPDE reduced BRCA-1 protein, while 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 nearly 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.

DISCUSSION

The primary objective of this study was to shed some light into the mechanisms responsible for the reduction of BRCA-1 mRNA levels in breast cancer MCF-7 cells exposed to acute levels of B[a]P (18, 19). The cigarette smoke carcinogen B[a]P, a prototype PAH, has been implicated in the development of lung (13) and skin (22) 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 and induce transversions at mutational hot-spots (23-25). 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 is at least three-fold. First, no sporadic breast tumors have been shown to harbor mutations in the BRCA-1 gene (26), but express lower levels of BRCA-1 (27). This implies the existence of regulatory mechanisms that lower BRCA-1 expression, in the absence of mutational alterations. Second, B[a]P and BPDE repress constitutive and estrogen-induced expression of BRCA-1 in breast and ovarian cancer cells (19, 20). This effect is not unique to B[a]P since other PAHs hamper in a dose-dependent fashion BRCA-1 protein levels in MCF-7 cells in the order: 3-methylcholanthrene > B[a]P > benzo[e]pyrene (our unpublished data). Third, physiological rather than genotoxic stresses have been implicated in BPDE-dependent tumorigenesis (28).

In mammalian models, activation of the AhR-pathway elicits cell cycle arrest, apoptosis, and expression of genes encoding for enzymes of the cytochrome P450 family, which contribute to bioactivation of AhR-ligands (29-31). The AhR is a ligand-activated factor that modulates transcription through interactions with xenobiotic responsive elements (XRE), whose core recognition sequence (5'-GCGTG-3') is harbored in the 5' flanking region of several genes including the CYP1A1, CYP1A2, UDP-glucuronosyl-transferase, and the estrogen-inducible cathepsin-D (32, 33). The fact that CYP1A1 transcripts and the reporter activity of a PAH-inducible promoter (p1A1-4X-LUC) harboring a tandem of four XRE were greatly induced by B[a]P provided confirmatory evidence the AhR pathway was functional in MCF-7 cells under the current experimental conditions. This notion is also supported by earlier reports documenting regulation by AhR-ligands of XRE-containing promoter segments in MCF-7 cells (34).

With respect to XREs that 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 (unpublished observations). While we cannot discard the possibility that binding of the activated AhR to XREs in the BRCA-1 gene may contribute to its negative regulation, the data presented in this report are consistent with a model in which the metabolite BPDE contributes, at least in part, to inhibition of BRCA-1 transcription. Because TCDD failed to lower BRCA-1 promoter activity and protein levels, we concluded that effectors downstream of the AhR, such as BPDE, a product of B[a]P bioactivation (35), alerted cellular signals that repressed BRCA-1 promoter activity.

The tumor suppressor gene p53 encodes for one of such effectors, whose stability was increased significantly in cells treated with B[a]P or BPDE. The p53 gene product has been shown to elicit transcription of several genes including Bax, p21, and mdm2 (36), which are involved in cell cycle control and apoptosis (37). Based on published observations that expression of p53 and BRCA-1 may be regulated through a feedback loop (38-40), we tested whether inhibition of BRCA-1 transcription by B[a]P and BPDE resulted from gain of p53 functions. In keeping with this concept, transfection with a vector encoding for p53 mutated at amino acid position 175 (R to H) 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 wild-type p53 functions in a trans-dominant negative fashion. Class II mutations, such as that at position 175, alter the conformational stability of the p53 protein and suppress wild-type transcriptional activity (41). Similarly, transient transfection 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 (42).

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. Because it is known that levels of BRCA-1 vary during the cell cycle, with minimal expression in G0/G1 (43), 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 reputed this possibility unlikely since both B[a]P and BPDE induced accumulation of MCF-7 cells in S-phase (19), at which interval expression of BRCA-1 has been shown to peak (43). Also, 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 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 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 (44-47). Previous reports from our laboratory (18) indicated that in HBL-100 cells expressing the SV40 large T-antigen, which is known to inhibit the transcriptional transactivation functions of p53 (42), neither BRCA-1 expression nor proliferation was

affected by B[a]P. Conversely, in addition to lowering BRCA-1 expression, the acute exposure to B[a]P induced cell death of 70 to 80% of breast MCF-7 cells, which express wild-type p53 (17). The accumulation of Bax-α mRNA, and p53 and p21 protein were paralleled in this study by loss of Bcl-2 mRNA suggesting that pro-apoptotic 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 classic apoptosis (44). A significant scenario emerging from these observations is one in which cells resistant to the cellular stresses induced by PAHs (48), but with a reduced potential for BRCA-1 expression, may be more likely to undergo neoplastic transformation (49).

In summary, the current study provides novel insights into the mechanisms through which PAHs may adversely affect transcription activity of the BRCA-1 gene. Activation of the AhR appears to be not sufficient for transcriptional repression of BRCA-1, which however may be hampered upon bioactivation of AhR-ligands to reactive metabolites, such as BPDE, and gain 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 disregulation of the BRCA-1 gene, and their role as a risk factor in the etiology of sporadic breast cancer.

Table 1. *Primers for RT-PCR*

Gene	Primers	Primer sequence	Size (bp)	Accession number
BRCA-1	DR6	5'-AGCTCGCTGAGACTTCCT GGA-3'	712	U1460
	MDR6	5'-CAATTCAATGTAGACAGACGT-3		
CYP1A1	A1AF	5"-TAACATCGTCTTGGACCTCTTTG-3	397	K03191
	A1AR	5'-GTCGATAGCACCATCAGGGGT-3'		
Bax-α	BαF	5'-CTGACATGTTTTCTGACGGC-3'	289	L22473
	BαR	5'-TCAGCCCATCTTCTTCCAGA-3'		
Bcl-2	BC2F	5'-TGCACCTGACGCCCTTCAC-3'	293	M14745
	BC2R	5'-AGACAGCCAGGAGAAATCACAG-3'		

Fig. 1. Effects of B[a]P, BPDE, and TCDD on expression profiles. *A*) MCF-7 cells were cultured for 24 in basal DMEM/F12 plus 10% FCS, or basal medium 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 CYP1A-1 (397 bp), BRCA-1 (712 bp), Bax-α (289 bp), Bcl-2 (293 bp), and control ribosomal 18S RNA (488 bp) from input cDNA corresponding to 400 ng of total RNA. MW represents DNA molecular weight markers. *B*) Western blot analysis of BRCA-1 and p53 in cells cultured in basal DMEM/F12 plus 10% or basal medium plus 5 μM B[a]P. *C*) Bands are immunocomplexes for BRCA-1, p53, and p21 in cells cultured in basal medium or basal medium plus 500 nM BPDE or 10 nM TCDD. Bands for β-actin are control immunocomplexes.

Fig. 2. B[a]P does not reduce stability of BRCA-1 mRNA. MCF-7 cells were cultured for 24 h in DMEM/F12 plus 10% FCS or basal medium 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/μl actinomycin-D (ActD). Then, cells were harvested and levels of BRCA-1 mRNA measured by ribonuclease protection assay in 10 μg of total RNA as described in Material and methods. *A*) Bands are ribonuclease-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 represents RNA molecular weight standards (bp). *B*) Decay of BRCA-1 mRNA expressed as the percentage of BRCA-1 mRNA remaining at each time point corrected for cyclophilin mRNA.

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. Relative luciferase units (RLU) were measured after cells were cultured for 24 h in DMEM/F12 plus 10% FCS or basal medium 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 pGL3-BRCA-1. *C*) Induction by B[a]P (5 μM) of the promoter construct p1A1-4X-LUC (1 μg)containing four xenobiotic responsive elements (XRE). p1A1-LUC is the empty vector lacking the XREs. Bars represent mean RLU corrected for β-galactosidase $\pm$ standard deviations from two independent experiments performed in triplicate.

Fig. 4. BPDE, but not TCDD, inhibits BRCA-1 promoter activity. MCF-7 cells were transiently transfected with pGL3-BRCA-1 (10 μg) or p1A1-4X-LUC (1 μg). Cells were cultured for 24 h in DMEM/F12 plus 10% FCS or basal medium plus *A*) 500 nM BPDE or *B*) 10 nM TCDD. Bars represent mean RLU corrected for β-galactosidase $\pm$ standard deviations from two independent experiments performed in triplicate.

Fig. 5. Expression of mutant p53 counteracts B[a]P-mediated loss of BRCA-1 promoter activity. *A*) MCF-7 cells were transiently transfected with pGL3-BRCA-1 (10 μg) or pCMV53mut (3 μg). Cells were cultured for 24 h in DMEM/F12 plus 10% FCS or basal medium plus 5 μM B[a]P. Bars represent mean RLU corrected for β-galactosidase

± standard deviations from two independent experiments performed in triplicate. *B*) Western blot analysis of cells non-transfected (NT), transfected with the empty vector pCMV (3 μg), or pCMV53mut (3 μg), and cultured for 24 h in basal DMEM/F12 plus 10% FCS or basal medium plus 5 μM B[a]P. Bands are immunocomplexes for p53 and control β-actin.

Fig. 6. Expression of mutant p53 counteracts BPDE-mediated loss of BRCA-1 promoter activity. *A*) MCF-7 cells were transiently transfected with pGL3-BRCA-1 (10 μg) or pCMV53mut (3 μg). Cells were cultured for 24 h in DMEM/F12 plus 10% FCS or basal medium plus 500 nM BPDE. Bars represent mean RLU corrected for renilla ± standard deviations from two independent experiments performed in triplicate. *B*) Western blot analysis of cells transfected with the empty vector pCMV (3 μg), or pCMV53mut (3 μg), and cultured for 24 h in basal DMEM/F12 plus 10% FCS or basal medium plus 500 nM BPDE. Bands are immunocomplexes for BRCA-1, p53, p21, and control β-actin.

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REFERENCES

1. Shih, H.A., Nathanson, K.L., Seal, S., Collins, N., Stratton, M.R., Rebbeck, T.R., and Weber, B.L. BRCA1 and BRCA2 mutations in breast cancer families with multiple primary cancers. Clin. Cancer Res., *6*, 4259-4264, 2000.
2. Peto, J., Collins, N., Barfoot, R., Seal, S., Warren, W., Rahman, N., Easton, D.F., Evans, C., Deacon, J., and Stratton, M.R. Prevalence of BRCA1 and BRCA2 gene mutations in patients with early-onset breast cancer. J. Natl. Cancer Inst., *91*, 943-949, 1999.
3. Frank, T.S., Manley, S.A., Olopade, O.I., Cummings, S., Garber, J.E., Bernhardt, B., Antman, K., Russo, D., Wood, M.E., Mullineau, L., Isaacs, C., Peshkin, B., Buys, S., Venne, V., Rowley, P.T., Loader, S., Offit, K., Robson, M., Hampel, H., Brener, D., Winer, E.P., Clark, S., Weber, B., Strong, L.C., Thomas, A., et al. Sequence analysis of BRCA1 and BRCA2: Correlation of mutations with family history and ovarian cancer risk. J. Clin. Oncol., *16*, 2417-2425, 1998.
4. Merajver, S.D., Pham, T.M., Caduff, R.F., Chen, M., Poy, E.L., Cooney, K.A., Weber, B.L. Collins, F.S., Johnston, C. and Frank, T.S. Somatic mutations in the BRCA1 gene in sporadic ovarian tumours. Nature Genet., *9*:439-443, 1995.
5. Futreal, P.A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L.M., Haugen-Strano, A., Swensen, J., Miki, Y., Eddington, K., McClure, M., Frye, C., Weaver-Feldhaus ,J., Ding, W., Gholami, Z., Soderkvist, P., Terry, L., Jhanwar, S., Berchuck, A., Iglehart, J.D., Mark, J., Ballinger, D.G.,

Barrett, J.C., Skolnick, M.H., Kamb, A., and Wiseman, R. BRCA-1 mutations in primary breast and ovarian carcinomas. Science, *266*, 120-122, 1994.

6. Dobrovic, A. and Simpfendorfer, D. Methylation of the BRCA1 gene in sporadic breast cancer. Cancer Res., *57*, 3347-3350, 1997.
7. Andres, J.L., Fan, S., Turkel, G.J., Wang, J-A., Twu, N-F., Yuan, R-Q, Lamszus, K., Goldberg, I.D., and Rosen, E.M. Regulation of BRCA-1 and BRCA-2 expression in human breast cancer cells by DNA-damaging agents. Oncogene, *16*, 2229-2241, 1998.
8. Bianco, T., Chenevix-Trench, G., Walsh, D.C., Cooper, J.E., and Dobrovic, A. Tumour-specific distribution of BRCA1 promoter region methylation supports a pathogenetic role in breast and ovarian cancer. Carcinogenesis, *21*, 147-151, 2000.
9. Magdinier, F., Billard, L.M., Wittmann, G., Frappart, L., Benchaib, M., Lenoir, G.M., Guerin, J.F., and Dante, R. Regional methylation of the 5' end CpG island of BRCA1 is associated with reduced gene expression in human somatic cells. FASEB J, *14*, 1585-1594, 2000.
10. Maher, V.M., Patton, J.D., Yang, J.L., Wang, Y.Y., Yang, L.L., Aust, A.E., Bhattacharyya, N., and McCormick, J.J. Mutations and homologous recombination induced in mammalian cells by metabolites of benzo[a]pyrene and 1-nitropyrene. Environ. Health Perspect., *76*, 33-39, 1987.
11. Ronai, Z., Gradia, S. el-Bayoumy, K., Amin, S., and Hecht, S.S. Contrasting incidence of ras mutations in rat mammary and mouse skin tumors induced by anti-benzo[c]phenanthrene-3,4-diol-1,2-epoxide. Carcinogenesis, *15*, 2113-2116, 1994.

12. Leadon, S.A., Stampfer, M.R., and Bartley, J. Production of oxidative DNA damage during the metabolic activation of benzo[a]pyrene in human mammary epithelial cells correlates with cell killing. Proc. Natl. Acad. Sci. USA, *85*, 4365-4368, 1998.

13. Denissenko, M.F., Pao, A., Tang, M., and Pfeifer, G.P. (1996) Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in p53. Science, *274*, 430-432.

14. Shukla, R., Liu, T., Geacintov, N.E., and Loechler, E.L. The major, N2-dG adduct of (+)-anti-B[a]PDE shows a dramatically different mutagenic specificity (predominantly, G→A) in a 5'-CGT-3' sequence context. Biochemistry, *36*, 10256-10261, 1997.

15. Romagnolo, D., Annab, L.A., Lyon, T.T., Risinger, J.I., Terry, L.A., Barrett, J.C., and Afshari, C.A. Estrogen upregulation of expression of BRCA-1 with no effect on localization. Mol. Carcinogen., *22*, 102-109, 1998.

16. Gudas, J.M., Nguyen, H., Li, T., and Cowen, K.H., Hormone-dependent regulation of BRCA-1 in human breast cancer cells. Cancer Res., *55*, 4561-4565, 1995.

17. Spillman, M.A. and Bowock, A.M. BRCA1 and BRCA2 mRNA levels are coordinately elevated in human breast cancer cells in response to estrogen. Oncogene, *12*, 1639-1645, 1996.

18. Jeffy, B.D., Schultz, E.U., Selmin, O., Gudas, J.M., Bowden, G.T., and Romagnolo, D. Inhibition of BRCA-1 expression by benzo[a]pyrene and its diol epoxide. Mol. Carcinogen., *26*, 100-118, 1999.

19. Jeffy, B.D., Chen, E.J., Gudas, J.M., and Romagnolo, D.F. Disruption of cell cycle kinetics by benzo[a]pyrene: Inverse expression patterns of BRCA-1 and p53 in MCF-7 cells arrested in S and G2. Neoplasia, *2*, 460-470, 2000.

20. Nebert, D.W., Petersen, D.D., and Fornace, A.J. Jr, Cellular responses to oxidative stress: the Ah gene battery as a paradigm. Environ. Health Perspect., *88*, 13-25, 1990.

21. Piskorska-Pliszczynska, J., Keys, B., Safe, S., and Newman, M.S. The cytosolic receptor binding affinities and AHH induction potencies of 29 polynuclear aromatic hydrocarbons. Toxicol. Letters, *34*, 67-74, 1986.

22. Lloyd, D.R. and Hanawalt, P.C. p53-dependent global genomic repair of benzo[a]pyrene-7,8-diol-9,10-epoxide adducts in human cells. Cancer Res., *60*, 517-521, 2000.

23. Long, D.J. II, Waikel, R.L., Wang, X-J., Perlaky, L., Roop, D.R., and Jaiswal, A.K. NAD(P)H: Quinone oxidoreductase 1 deficiency increases susceptibility to benzo[a]pyrene-induced mouse skin carcinogenesis. Cancer Res., *60*, 5913-5915, 2000.

24. Slaga, T.J, Bracken, W.M., Viaje, A., Berry, D.L., Fischer, S.M., Miller, D.R., Levin, W., Conney, A.H., Yagi, H., and Jerina, D.M. Tumor initiating and promoting activities of various benzo[a]pyrene metabolites in mouse skin. Carcinogenesis, *3*, 371-382, 1978.

25. Minamoto, T., Mai, M., and Ronai, Z. Environmental factors as regulators and effectors of multistep carcinogenesis. Carcinogenesis, *20*, 519-527, 1999.

26. Zhang, H., Tombline, G., and Weber, B.L. BRCA-1, BRCA-2 and DNA damage response: collision or collusion. Cell, *92*, 433-436, 1998.

27. Thompson, M.E., Jensen, R.A., Obermiller, P.S., Page, D.L., and Holt, J.T. Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nat. Genet., *9*, 444-450, 1995.

28. Rodin, S.N. and Rodin, A.S. Human lung cancer and p53: The interplay between mutagenesis and selection. Proc. Natl. Acad. Sci. USA, *97*, 12244-12249, 2000.

29. Monteith, D.K., Novotny, A., Michalopoulos, G., and Strom, S.C. Metabolism of benzo[a]pyrene in primary cultures of human hepatocytes: Dose-response over a four-log range. Carcinogenesis, *8*, 983-988, 1987.

30. Shackelford, R.E., Kaufmann, W.K., and Paules, R.S. Cell cycle control, checkpoint mechanisms, and genotoxic stress. Environ. Health Perspect., *107*, 5-24, 1999.

31. Safe, S. and Krishnan, V. Cellular and molecular biology of aryl hydrocarbon (Ah) receptor-mediated gene expression. Arch. Toxicol. Suppl., *17*, 99-115, 1995.

32. Krishnan, V., Porter, W., Santostefano, M., Wang, X., and Safe, S. Molecular mechanism of inhibition of estrogen-induced cathepsin D gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in MCF-7 cells. Mol. Cell. Biol., *15*, 6710-6719, 1995.

33. Harper, N., Wang, X., Liu, H., and Safe, S. Inhibition of estrogen-induced progesterone receptor in MCF-7 human breast cancer cells by aryl hydrocarbon (Ah) receptor agonists. Mol. Cell. Endocrinol., *104*, 47-55, 1994.

34. Krishnan, V., Wang, X., and Safe, S. Estrogen receptor-Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells. J. Biol. Chem., *269*, 15912-15917, 1994.

35. Gelboin, H.V. Benzo[a]pyrene metabolism, activation and carcinogenesis: role and regulation of mixed-function oxidases and related enzymes. Physio. Rev., *60*, 1107-1166, 1980.

36. Thornborrow, E.C. and Manfredi, J.J. One mechanism for cell type-specific regulation of the bax promoter by the tumor suppressor p53 is dictated by the p53 response element. J. Biol. Chem., *274*, 33747-33756, 1999.

37. Sigal, A., and Rotter, V. (2000) Oncogenic mutations of the p53 tumor suppressor: The demons of the guardian of the genome. Cancer Res., *60*, 6788-6793, 2000.

38. MacLachlan, T.K., Dash, B.C., Dicker, D.T., El-Dairy, W.S. Repression of BRCA-1 through a feedback loop involving p53. J. Biol. Chem., *275:31869-31875*, 2000.

39. Arizti, P., Fang, L., Park, I., Yin, Y., Solomon, E., Ouchi, T., Aaronson, S., and Lee, S.W. Tumor suppressor p53 is required to modulate BRCA1 expression. Mol. Cell. Biol., *20*, 7450-7459, 2000.

40. Ouchi, T., Monteiro, A.N., August, A., Aaronson, S.A., and Hanafusa, H. BRCA-1 regulates p53-dependent gene expression. Proc. Natl. Acad. Sci. USA, *95*, 2302-2306, 1998.

41. Park, D.J., Nakamura, H., Chumakov, A.M., Said, J.W., Miller, C.W., Chen, D.L., and Koeffler, H.P. Transactivational and DNA binding abilities of endogenous p53 in p53 mutant cell lines. Oncogene, *9*, 1899-1906, 1994.

42. Mietz, J.A., Unger, T., Huibregtse, J.M., and Howley, P.M. The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. EMBO J., *11*, 5013-5020, 1992.

43. Chen, Y., Farmer, A., Chen, C-F., Jones, D.C., Chen, P-L., Lee, W-H. BRCA-1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner. Cancer Res. *56, 3168-3172*, 1996.

44. Janicke, R.U., Sprengart, M.L., Wati, M.R., and Porter, A.G. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J. Biol. Chem., *273*, 9357-9360, 1998.

45. Vogelstein, B. and Kinzler, K.W. p53 function and dysfunction. Cell, *70*, 523-526, 1992.

46. Lee, J.M. and Bernstein, A. p53 mutations increase resistance to ionizing radiation. Proc. Nat. Acad. Sci. USA, *90*, 5742-5746, 1993.

47. Levine, A.J. p53, the cellular gatekeeper for growth and division. Cell, *88*, 323-331, 1997.

48. Moore, M., Wang, X., Lu, Y-F., Wormke, M., Craig, A., Gerlach, J.H., Burghardt, R., Barhoumi, R., and Safe, S. Benzo[a]pyrene-resistant MCF-7 human breast cancer cells. A unique aryl hydrocarbon-nonresponsive clone. J. Biol. Chem., *269*, 11751-11759, 1994.

49. Kinzler, K.W. and Vogelstein, B., Cancer susceptibility genes: gatekeepers and caretakers. Nature, *386*, 761-763, 1997.

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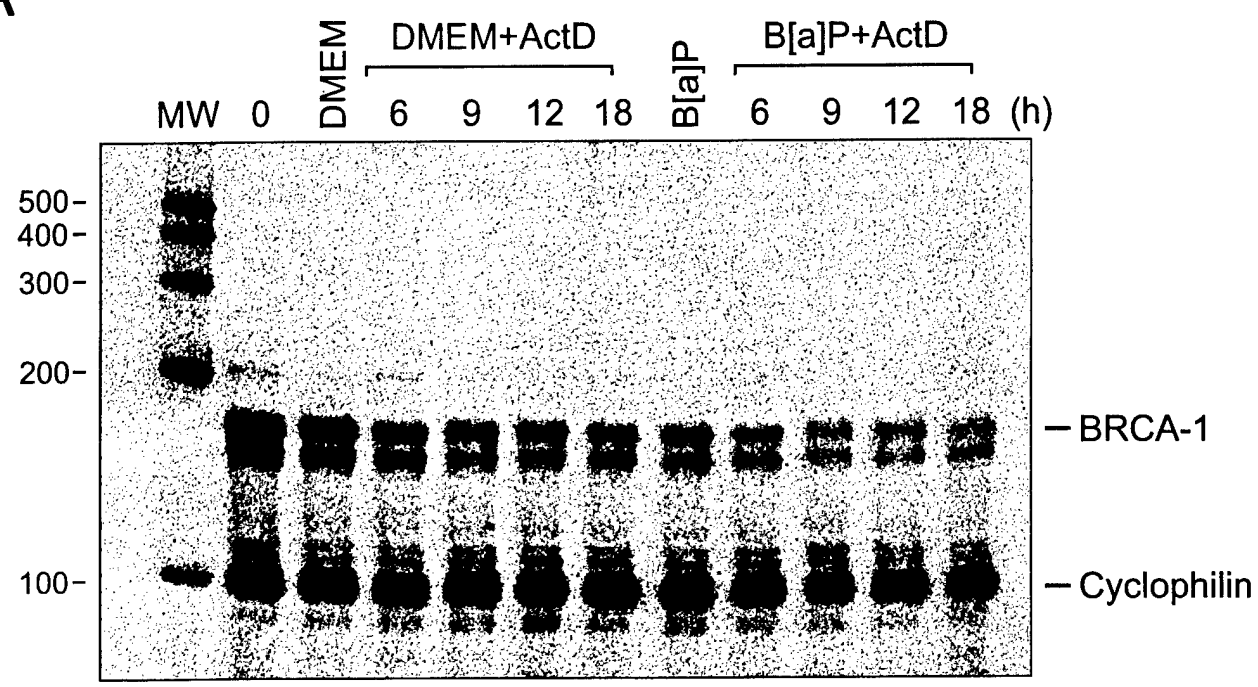
DMEM
DMEM+ActD
B[a]P
B[a]P+ActD
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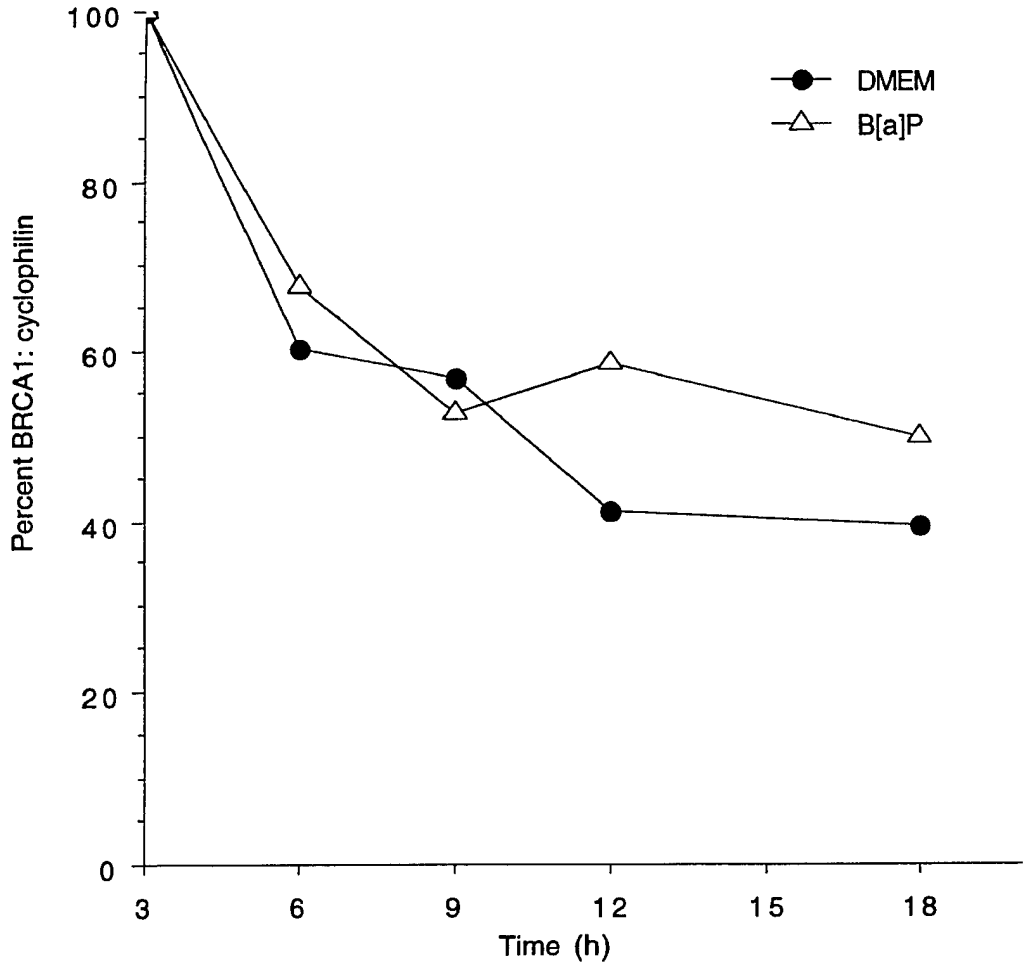
500-
400-
300-
200-
100-
BRCA-1
Cyclophilin

B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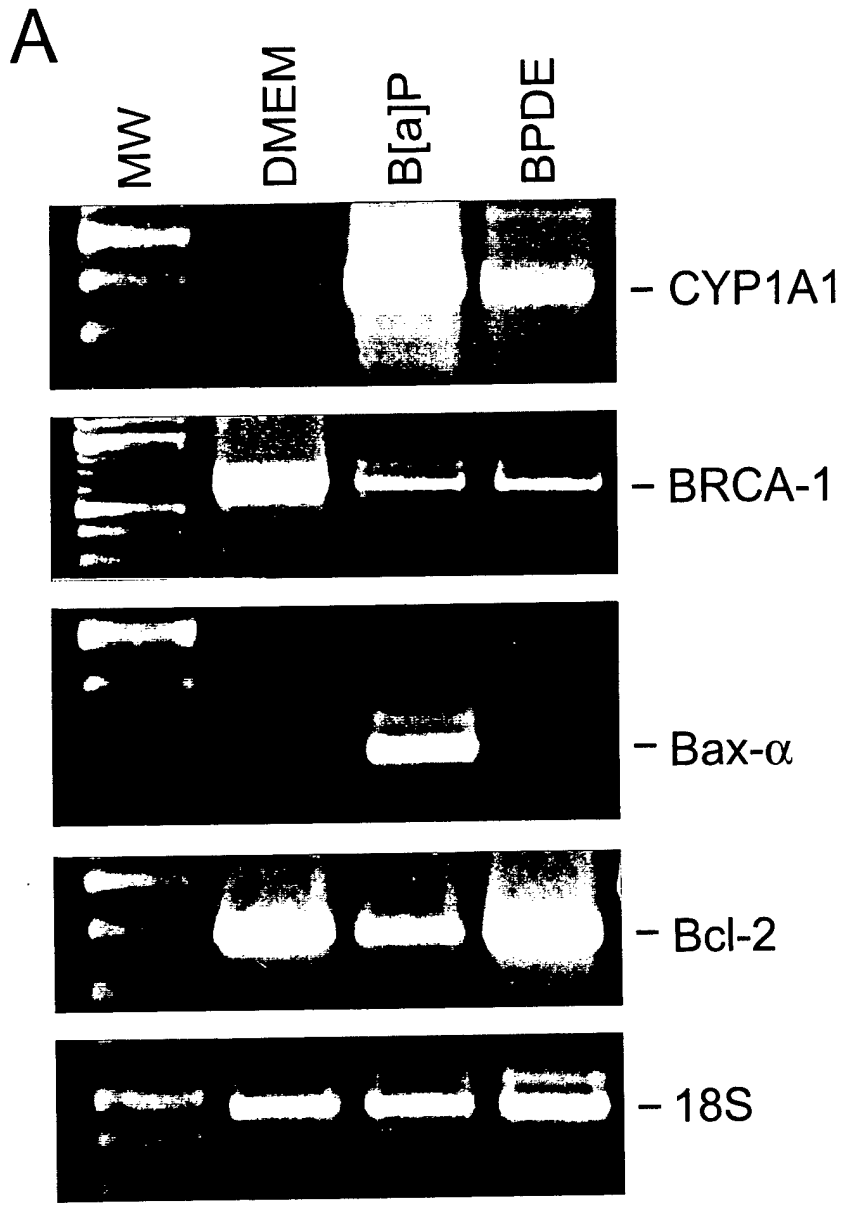
60
40
20
0
Percent BRCA1: cyclophilin
DMEM
B[a]P
3
6
9
12
15
18
Time (h)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– BRCA-1
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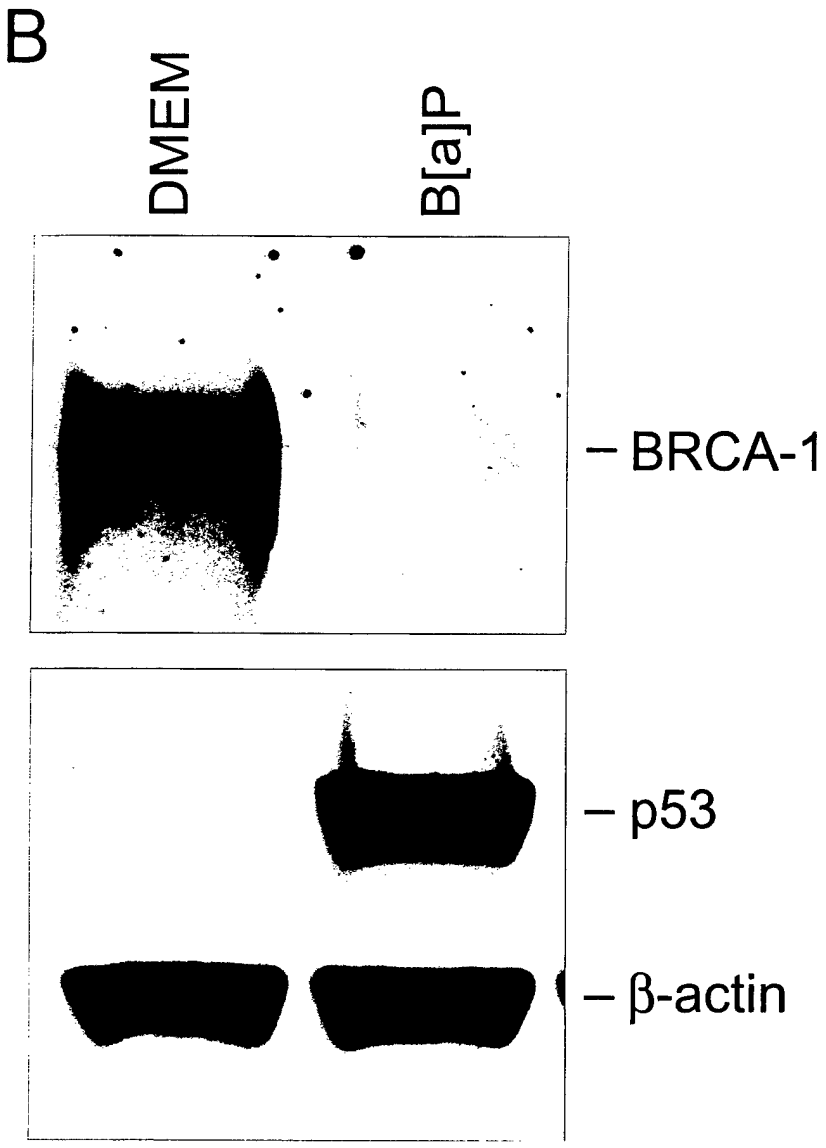
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– BRCA-1
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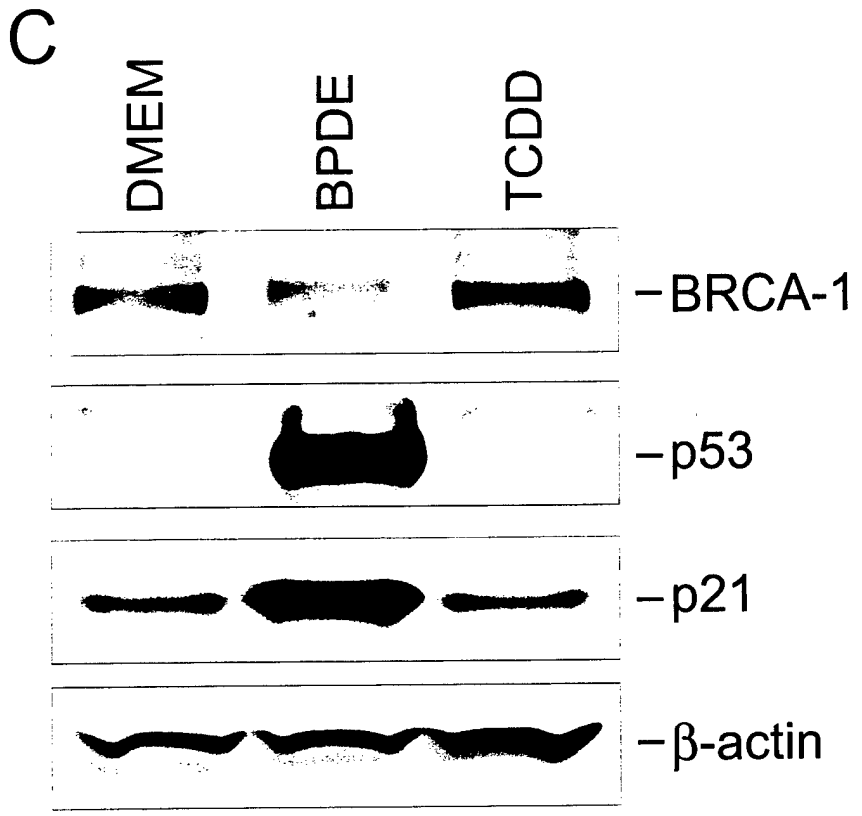
– β-actin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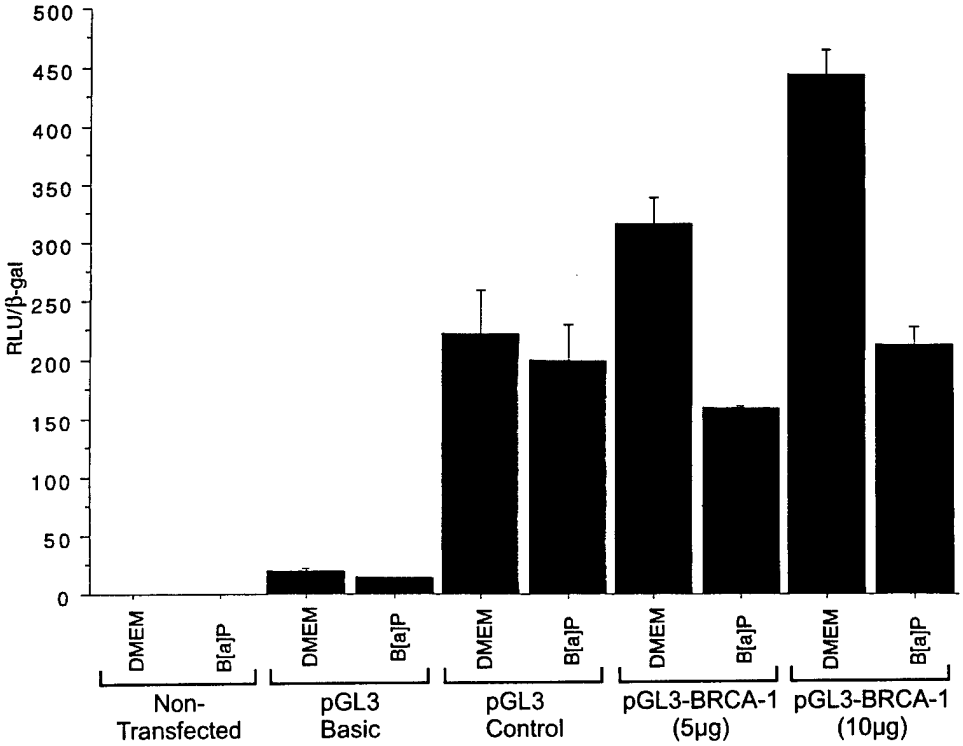
–p21
–β-actin

A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450
400
350
300
250
200
150
100
50
0
DMEM
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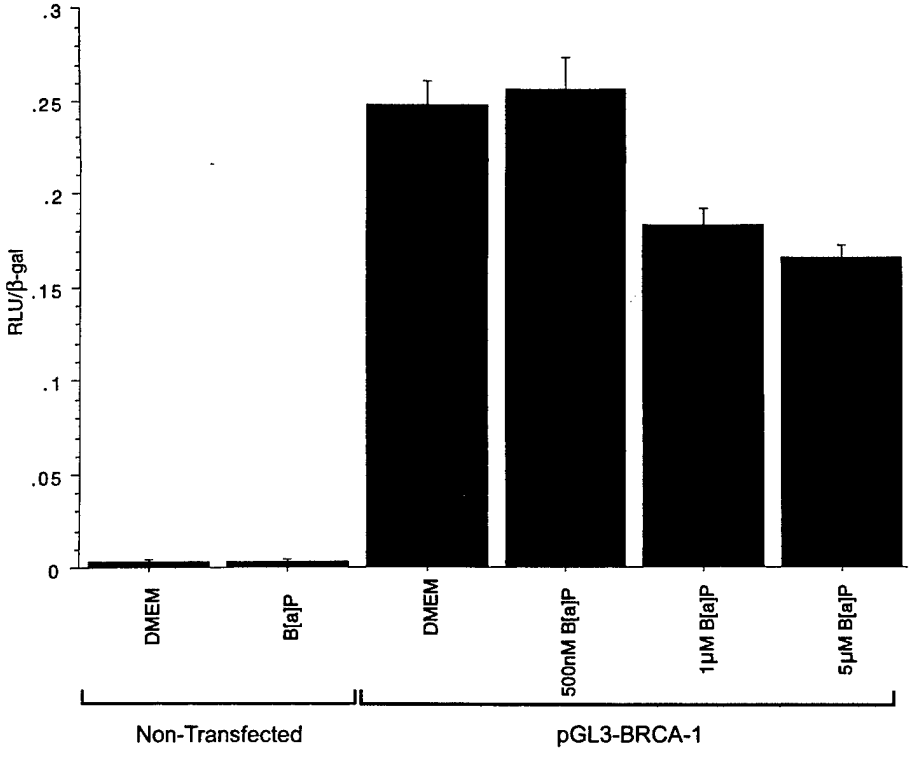
Non-Transfected
pGL3 Basic
pGL3 Control
pGL3-BRCA-1 (5µg)
pGL3-BRCA-1 (10µg)

B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.15
.1
.05
0
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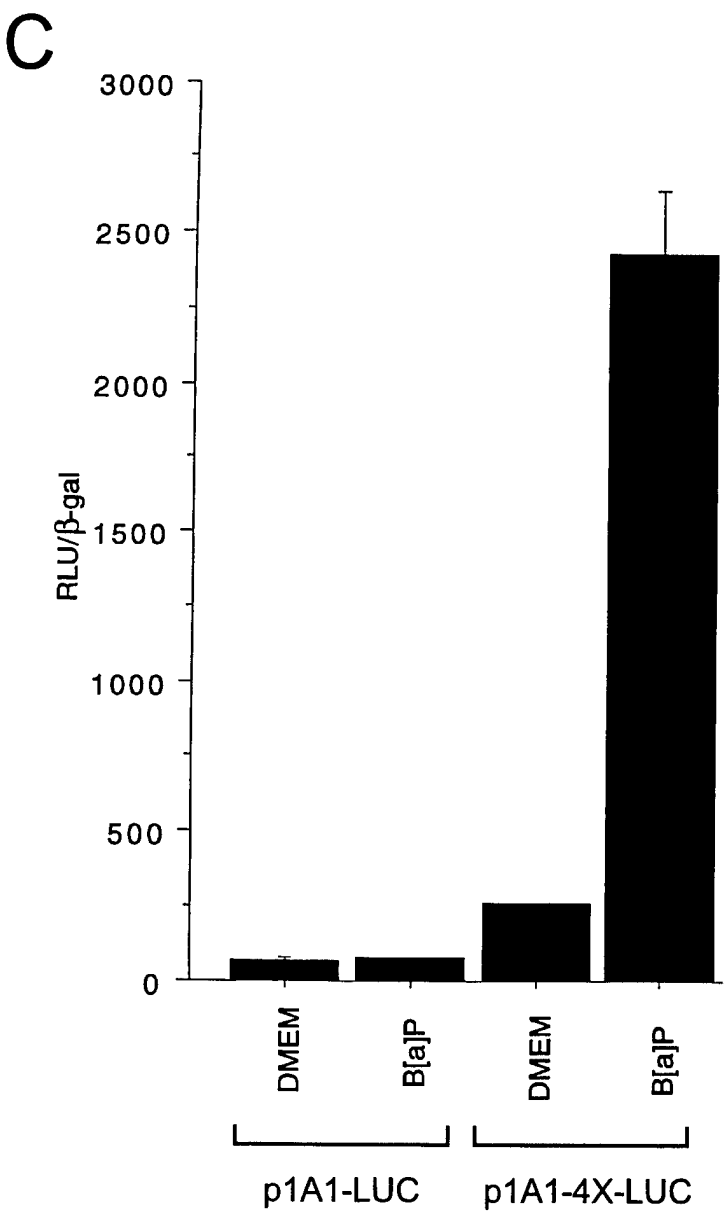
5µM B[a]P
Non-Transfected
pGL3-BRCA-1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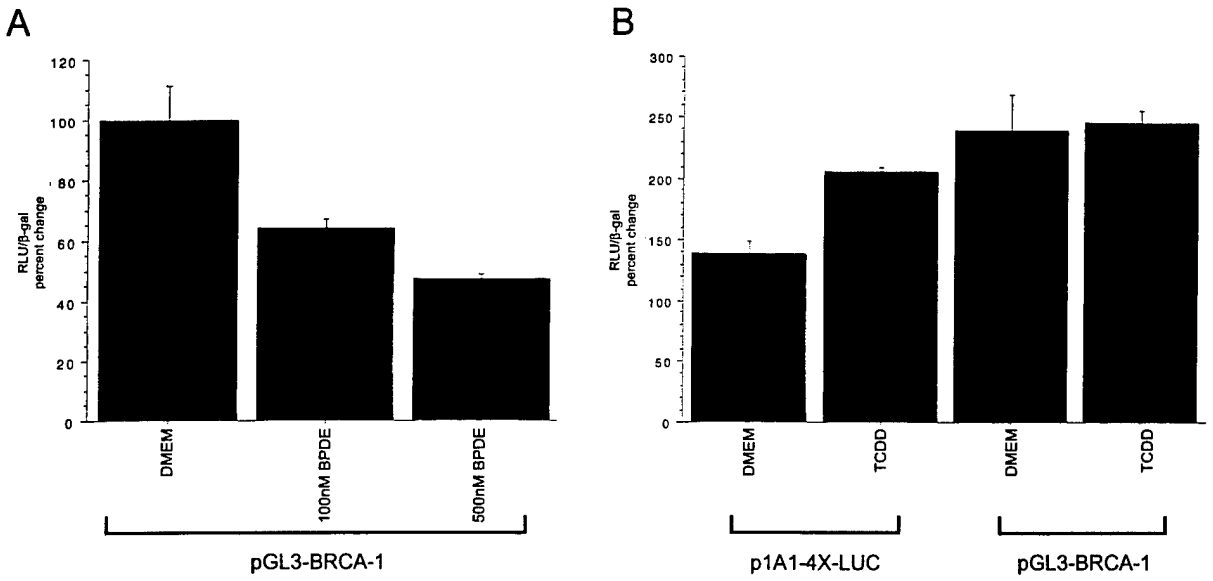
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100
80
60
40
20
0
RLU/β-gal
percent change
DMEM
100nM BPDE
500nM BPDE
pGL3-BRCA-1
B
300
250
200
150
100
50
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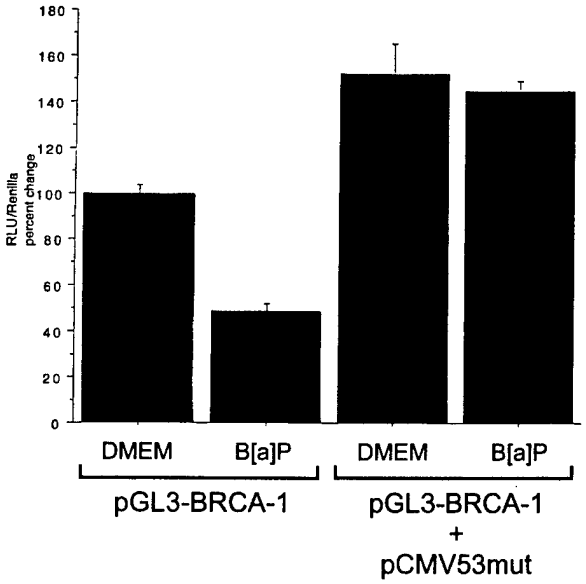
percent change
DMEM
TCDD
DMEM
TCDD
p1A1-4X-LUC
pGL3-BRCA-1

A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140
120
100
80
60
40
20
0
RLU/Renilla
percent change
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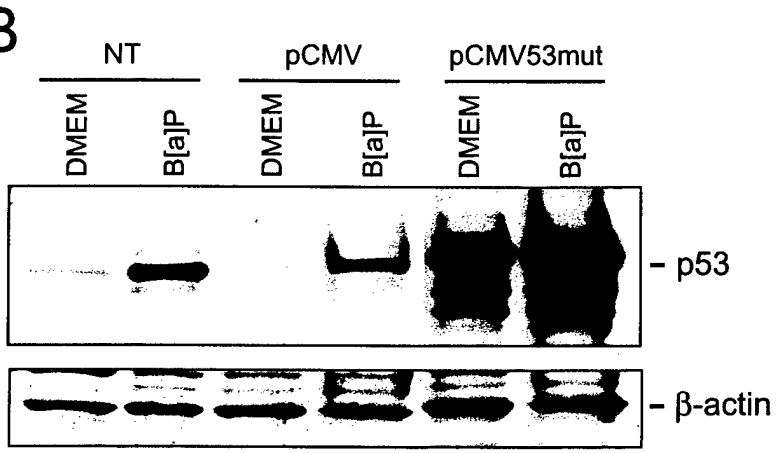
DMEM
B[a]P
pGL3-BRCA-1
pGL3-BRCA-1
+
pCMV53mut

B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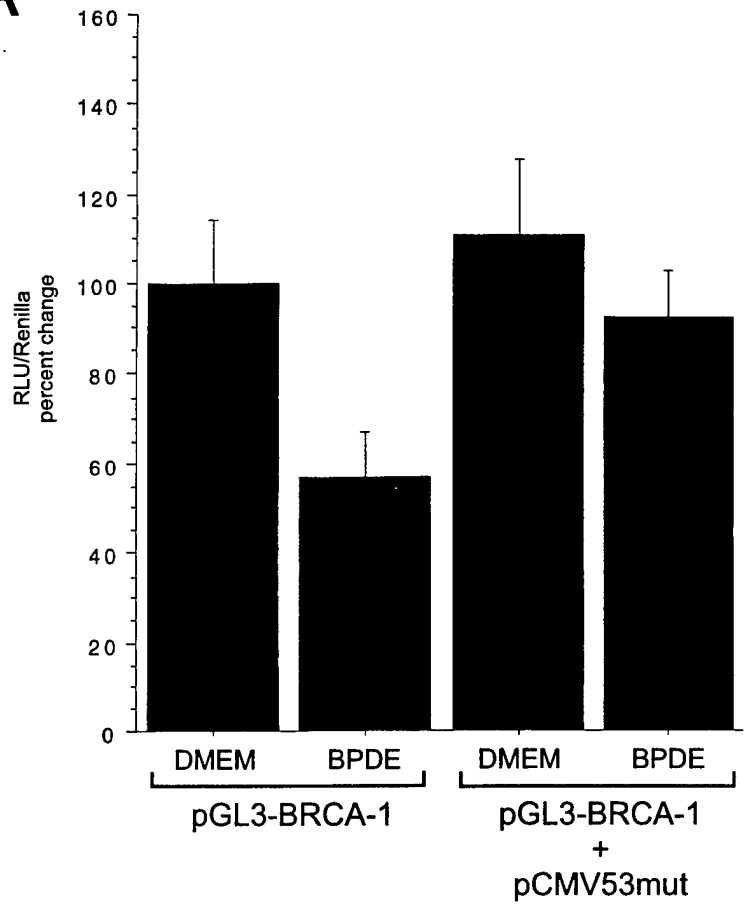
DMEM
B[a]P
DMEM
B[a]P
- p53
- β-actin

A


160
140
120
100
80
60
40
20
0
RLU/Renilla
percent change
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DMEM
BPDE
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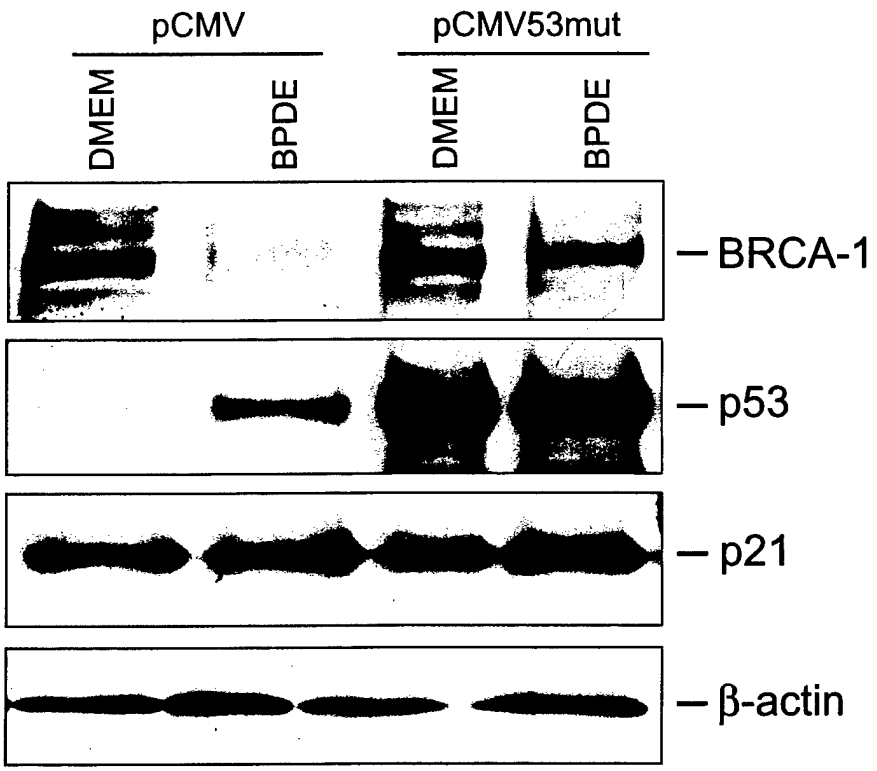
pGL3-BRCA-1
+
pCMV53mut

B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