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TITLE: A Molecular Model for Repression of BRCA-1 Transcription by the Aryl Hydrocarbon Receptor

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13. ABSTRACT (Maximum 200 Words)
The purpose of this project is to investigate whether or not 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 activated AhR alters BRCA-1 transcription through binding to several xenobiotic responsive elements (XRE) strategically located at -539 bp (CCGTFFAA=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 the experiments conducted in year 3 were: 1) Completed testing of truncation constructs for the BRCA-1 promoter region flanking XRE-3. 2) Investigated the effects of the Ahr ligands TCDD and alpha-naphthoflavone (ANF) on estrogen regulation of the BRCA-1 gene. 3) Investigated the cross-talk between the estrogen receptor and the AhR pathways. Results of these experiments indicate that XRE-3 is a negative regulator of BRCA-1 transcription. Treatment with TCDD or ANF represses estrogen stimulation of BRCA-1 transcription suggesting that AhR ligands exert negative and effect on BRCA-1 expression. We also have gained evidence that the ER-alpha is recruited at the XREs flanked in the BRCA-1 promoter.

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BRCA-1, Benzo [a] pyrene, aromatic hydrocarbon receptor

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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 altering transcription of the tumor suppressor gene, BRCA-1. The *purpose* of this project is to investigate whether or not changes in the expression of the BRCA-1 gene in breast epithelial cells 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 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.

Body

Synopsis

The tasks of year 3 of the project focused on 1) completing the testing of truncation constructs for XRE-3 and examine their transcriptional activity in the presence or absence of estrogen (E2), the AhR-ligand and dioxin-like TCDD, and their combination; 2) Investigate the effects of AhR and estrogen receptor (ER) antagonists, respectively alpha-naphthoflavone (ANF) and tamoxifen (TMX) on estrogen regulation of BRCA-1 transcription. 3) Investigated the recruitment of the ER-alpha at the XREs in the presence or absence of the AhR-ligand TCDD.

Truncation constructs

To investigate the role of XRE-3 (GCGTG) we developed a promoter-luciferase construct named pGL3-Ex1A1B comprising a 2.7 Kb segment of DNA extending from position -216 from the ATG at the 3' end and flanking at the 5' end the CYP1A1-like XRE. This template was used to develop truncation constructs deleted of 432 (T-432), 828 (T-828) or 933 bp from the -216 bp position (Fig.1). These promoter constructs were transfected into breast cancer MCF-7 cells and their activity examined based on luciferase readings. Fig. 2 indicates that activity of the full-length construct pGL3-Ex1A1B was induced by estrogen and to some extent (~30%) by the AhR ligand TCDD. However, TCDD repressed by a similar amount (~30%) the stimulatory effects of estrogen. These data confirmed those presented in earlier progress reports (2001 and 2002) that AhR ligands can have both negative and positive effects on transcription of the BRCA-1 gene. The construct T432 sustained a very low transcription activity suggesting that the fragment comprised between -216 and -432 is important for basal and estrogen regulation of BRCA-1. However, deletion of the region containing XRE-3 (T828) increased basal transcription by 3.0-fold compared with T432. These data suggested that XRE3 is a negative regulator of BRCA-1 transcription. This conclusion was confirmed in a subsequent experiment in which we compared the activity of the T432 construct with that of pGL3BRCA-1 (1.7Kb) (Fig. 3). As expected, BRCA-1 transcription was induced by estrogen, whereas luciferase activity was reduced by ~75% in cells transfected with T432, thus confirming the presence of a negative element in the DNA segment comprised between position -933 and -432. The luciferase activity was restored upon deletion of the DNA fragment containing XRE-3 (T828).

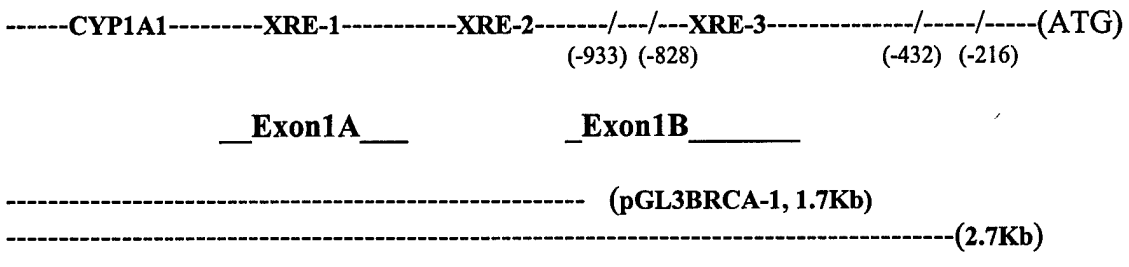

Fig. 1. Organization of BRCA-1 promoter truncation constructs. Numbers are base pairs 5' from the translation start site (ATG) in the BRCA-1 gene.

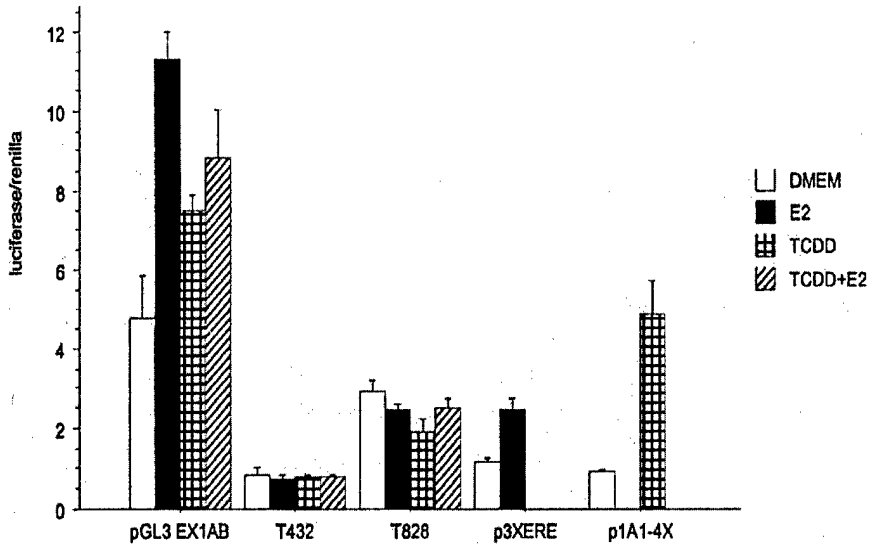

Fig. 2. Transcription activity of various BRCA-1 truncation constructs. Transfected MCF-7 cells were cultured in control medium DMEM, or DMEM plus 10 nM estrogen (E2), 10 nM dioxin (TCDD), or their combination for 24 h. Units are relative luciferase units corrected for the internal standard renilla.p3XERE and p1A1-4X are positive controls respectively for estrogen and TCDD treatments.

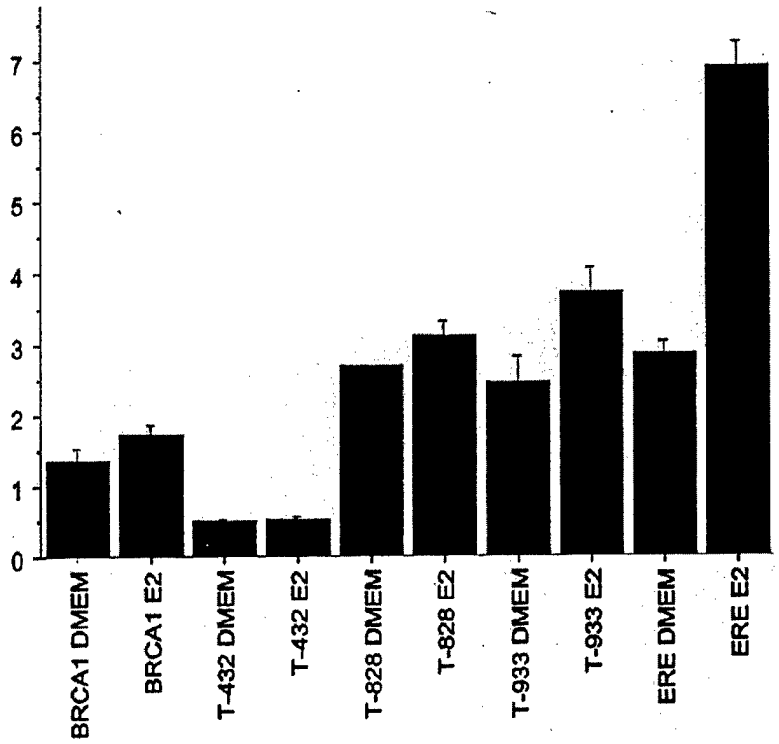

Fig. 3. Transcription activity of various BRCA-1 promoter fragments. Transfected MCF-7 cells were cultured in control medium DMEM, or DMEM plus 10 nM estrogen (E2) for 24 h. Units are relative luciferase units corrected for the internal standard renilla. BRCA-1 is the 1.7 Kb promoter fragment. T-43, T-828, and T-933 represent various BRCA-1 fragments differing in length (please see Fig. 1). ERE represents MCF-7 cells transfected with an estrogen-responsive (positive control) construct.

Effects of AhR and ER antagonists on BRCA-1 transcriptional activity

Having established that estrogen induces BRCA-1 transcription, but that cotreatment with the AhR ligand TCDD antagonized this effect, we wished to examine the effects of the antagonist ANF, which is known to bind to the AhR. We found that ANF did not influence basal transcription activity, but it abrogated completely estrogen stimulation of BRCA-1 transcription (Fig. 4). Similarly, the ER antagonist tamoxifen (TMX) prevented estrogen stimulation of BRCA-1. These are very interesting data because they establish a link between the AhR and ER pathways in the regulation of BRCA-1 expression. Specifically, our data imply that the AhR may contribute to estrogen regulation of BRCA-1 transcription. Previous studies have reported that the AhR physically interacts with the ER. Therefore, one possibility is that sequestration of the AhR by ANF, may interfere with binding of the AhR to ER and disrupt estrogen regulation of BRCA-1. This conclusion is supported by our finding that the CYP1A1, XRE-1 and XRE-2 are required for optimal stimulation of BRCA-1 transcription by estrogen. In fact, mutation of these elements reduced estrogen induction of BRCA-1 promoter activity (Fig. 5).

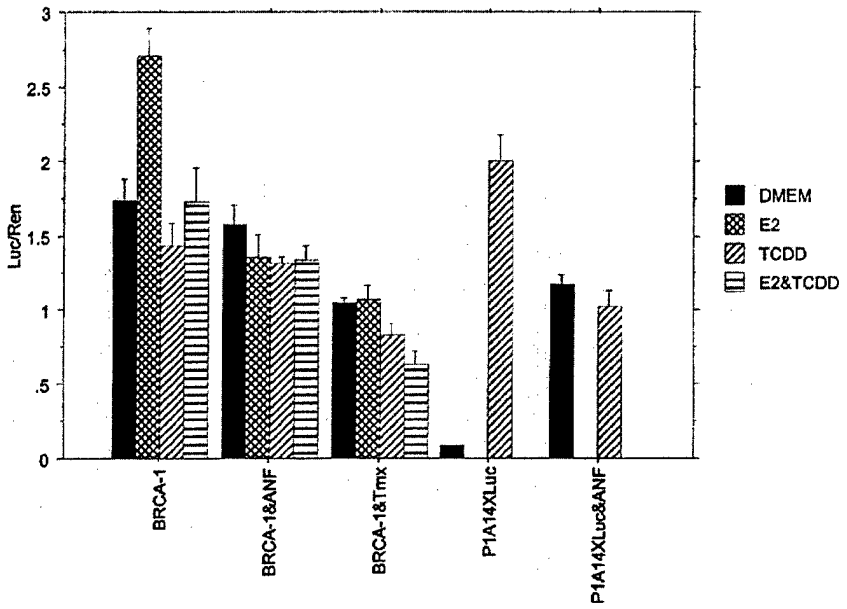

Fig. 4. Effects of the AhR- and ER-antagonists on BRCA-1 transcription activity. Transfected MCF-7 cells were cultured in control medium DMEM, or DMEM plus 10 nM estrogen (E2), 10 nM dioxin (TCDD), or their combination for 24 h, in the presence or absence of the the AhR-antagonist alpha-naphthoflavone (ANF) or ER antagonist tamoxifen (TMX). Units are relative luciferase units corrected for the internal standard renilla. p1A1-4X is a positive control for TCDD treatment.

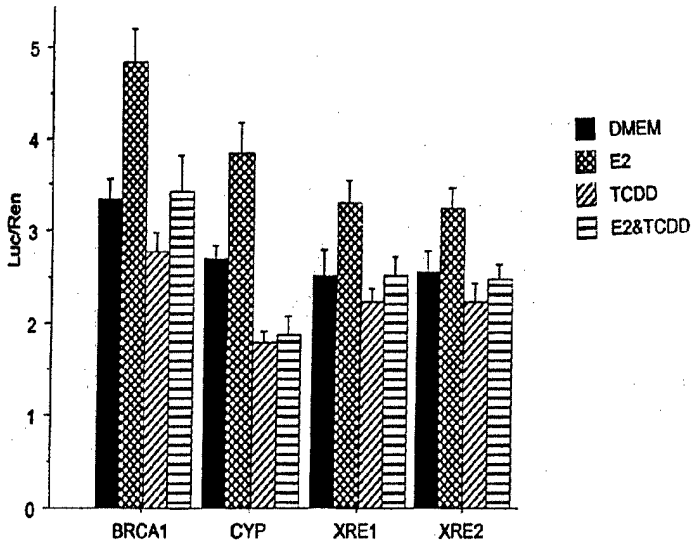

Fig. 5. Mutation of the GCGTG sequences of CYP1A1-like, XRE-1. and XRE-2 elements reduces estrogen induction of BRCA-1 transcription. Transfected MCF-7 cells were cultured in control medium DMEM, or DMEM plus 10 nM estrogen (E2), 10 nM dioxin (TCDD), or their combination for 24 h.

The ER-alpha contributes to formation of a transcription complex at the CYP1A1, XRE-1, and XRE-2 elements

To further examine the cross-talk between the ER and AhR pathways in the regulation of the BRCA-1 gene, we cultured MCF-7 cells in control medium (DMEM) or medium supplemented with TCDD. Nuclear extracts were prepared and used for electromobility shift assays. The results depicted in Fig. 6 indicate that the ER-alpha was bound to a protein complex present at the CYP1A1, XRE-1 and XRE-2 sites. A positive control dioxin-responsive element (DRE) for a consensus XRE was also included confirming that the experimental conditions were appropriate for examining the effects of TCDD. Interestingly, supershifted band corresponding to the ER-alpha was higher in intensity at the CYP1A1 and XRE-2 elements suggesting a more significant role of these elements in regulation of BRCA-1 transcription compared with XRE1. However, treatment with TCDD reduced proportionally the intensity of the supershifted bands representing the DNA/ER complexes. These data suggest that activation of the AhR pathway by the ligand TCDD may antagonizes the recruitment of the ER-alpha at the XREs in the BRCA-1 promoter, and explain at least in part why exposure to AhR ligands disrupt normal estrogen regulation of BRCA-1 expression.

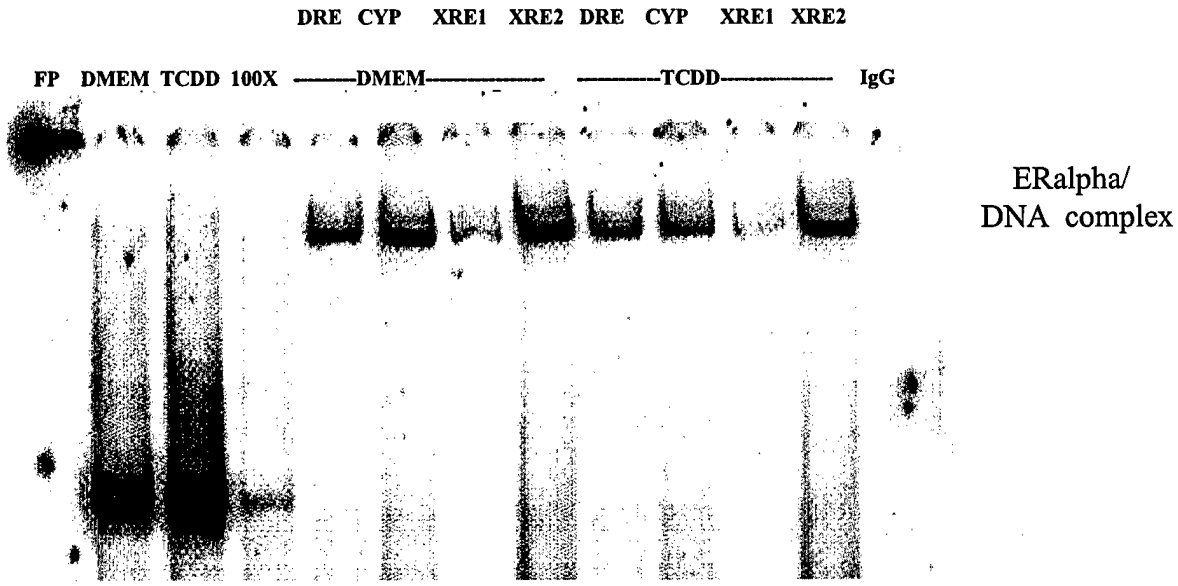

Fig. 6. The ERalpha participates in the formation of a transcription complex at AR-binding sites in the BRCA-1 promoter. FP=free probe; DMEM=control medium; TCDD, 10 nM, 100X=cold competitor oligonucleotide; DRE=dioxine responsive element (positive control); CYP, XRE-1, XRE-2= BRCA-1 oligonucleotides containing GCGTG core sequences; IgG negative control for antibody. Supershifted bands were generated following incubation of nuclear extracts with antibody raised against the ER-alpha.

Key research Accomplishments

- Completed testing of truncation contructs for XRE-3 and mutation constructs for CYP1A1, XRE1, XRE2
- Investigated the role of the AhR in estrogen regulation of BRCA-1.
- Obtained evidence in EMSA experiments that the candidate BRCA-1 XREs are targets by the ER alpha contained in nuclear extracts obtained from MCF-7 cells, and that TCDD antagonizes the recruitment of the ER at these XREs. These results may explain the mechanism through which AhR ligand may disrupt estrogen regulation of the BRCA-1 gene.

Reportable Outcomes

1. Romagnolo, D. et al. Mechanisms of BRCA-1 regulation. Nutrition and Cancer, 2003 (In press)

2. Ryan B. Chirnomas, Brandon D. Jeffy and Donato F. Romagnolo. Toxicogenomics of polycyclic aromatic hydrocarbons (Manuscript in submission: Nutrition and Cancer, Journal of Nutrition)

3. The activated aromatic hydrocarbon receptor regulates basal and estrogen-dependent activity of the BRCA-1 promoter. Jeffy, Ryan B. Brandon D. Jeffy, and Donato F. Romagnolo. Manuscript in submission (Journal of Biological Chemistry).

The support of the US Army Medical Research and Materiel Command has been acknowledged in the Acknowledgment section of these manuscripts:

1. Two Abstracts presented at the 2003 Meetings of the American Association for Cancer Research, July 2003, Washington, D.C.
2. Posters presented at the 2003 Arizona Cancer and Southwest Environmental Health Sciences Centers Science Fairs, The University of Arizona, Tucson, AZ.
3. The information being gathered through the execution of the experiments supported by this award is being used as the backbone of a Ph.D. project for Brandon Jeffy and Jennifer Ku, who are currently working on this project in the laboratory of the P.I. Mr. Jeffy is a Ph.D. 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 regulates transcription of the BRCA-1 gene, likely through XREs comprised in the BRCA-1 promoter. The mechanism being confirmed is that through binding to AhR-binding domains, the AhR regulates the expression of BRCA-1. As previously suggested at the end of year 1 and 2 the candidate responsive elements (CYP1A1 and XREs) appear to have distinct functions.

Importance and Implications

The results presented in this annual report confirm that the candidate XREs are target for binding by the estrogen receptor-alpha (ER). This observation offers new evidence that may explain how exposure to AhR-ligands disrupts estrogen induction of BRCA-1. We propose that the AhR and the ER contribute to formation of a transcription complex at the CYP1A1-like and XRE 1 and XRE2 sites. We conclude that ligands of the AhR compete with activation by estrogen of BRCA-1 transcription by interfering with recruitment of the ER.

Relevant 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. Brandon D. Jeffy, Ryan B. Chirnomas, Eddy J. Chen, Jean M. Gudas, and Donato F. Romagnolo. Activation of the Aromatic Hydrocarbon Receptor Pathway Is Not Sufficient for Transcriptional Repression of BRCA-1: Requirements for Metabolism of Benzo[*a*]pyrene to 7*r*,8*t*-Dihydroxy-9*t*,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. Cancer Res 2002 62: 113-121.
5. Brandon D. Jeffy, Ryan B. Chirnomas, and Donato F. Romagnolo. Epigenetics of breast cancer: polycyclic aromatic hydrocarbons as risk factors. Environ. Mol. Mutagenesis, Vol 39, 2-3, 2002.

Appendices

Abstracts presented at the 2003 Annuual Meetings of the American Society for Cancer Research, Washington, D.C., July 2003.