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The Role of Dioxin Receptor in Mammary Development and Carcinogenesis

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This research is testing the hypothesis that the dioxin receptor (AhR) plays a central role in breast carcinogenesis. Following on preliminary observations of the dramatic up-regulation of AhR in advanced human breast carcinoma (HBC) cell lines, we addressed whether the overexpression of the AhR alone is sufficient to induce carcinogenic transformation in mammary epithelial cells. Overexpression of AhR in clones correlated with decrease in population doubling times subsequent to abrogation to cell cycle, enhanced motility and increased migration. Furthermore, these clones acquired the ability to invade matrigel matrix and to form colonies in soft agar. Conversely, retrovirus vectors producing siRNAs targeted against AhR were used to generate stable clones with a knockdown of 75–90% in AhR expression. Although these clones exhibited subsequent suppression of AhR-transcriptional activity, they showed no change from the vector control clone or parent cells in population doubling times, cell cycle distribution, ability to invade matrigel matrix or to form colonies in soft agar. These results suggest that AhR alone is capable of inducing transformation of immortalized normal mammary epithelial cells into a malignant phenotype, but its depletion is insufficient to reverse the malignant phenotypes in metastatic breast cancer cells. More research is required to delineate the mechanisms of AhR involvement in breast cancer progression.

Toxicology, Tumor Biology, Pathophysiology
# Table of Contents

Cover .............................................................................................................................. 1  

SF 298 .............................................................................................................................. 2  

Introduction ..................................................................................................................... 4  

Body ................................................................................................................................. 5  

Key Research Accomplishments .................................................................................... 6  

Reportable Outcomes ..................................................................................................... 6  

Conclusions ...................................................................................................................... 8  

References ....................................................................................................................... 9  

Appendices list ............................................................................................................... 11
Introduction

The proposed research studies are based on our novel observation that in three different sets of human breast carcinoma (HBC) cell lines the expression of the Aryl hydrocarbon receptor (AhR) protein was upregulated in a direct correlation with the progression of tumorigenicity. The overall objective is to determine the role of AhR in human breast carcinogenesis. AhR which is well known for its mediation of the toxic responses to environmental polyhalogenated aromatic hydrocarbons (PAH) such as dioxin (1), is a cytosolic protein and binding of PAH leads to its activation to a nuclear transcription factor and subsequent down-regulation by proteolysis (2,3,4). Molecular cloning and characterization of AhR cDNA has identified it as a member of family of ligand-activated basic helix loop helix (bHLH) transcription factors (5). PAH-activated AhR heterodimerizes with its partner AhR nuclear translocation protein (ARNT) another bHLH transcription factor and induces the expression of a number of genes, including cytochrome P4501A1 (CYP1A1) and CYP1B1 (reviewed in 6 & 7). No endogenous ligand for the AhR has yet been identified, however, its constitutive activation via disturbing cellular adhesion to the extra-cellular matrix (8,9), increasing intracellular Ca2+ (10), and disturbing cytoskeleton (11) has provided evidence for physiologically activated pathways linked to adhesion. Furthermore, other lines of evidence are gathering to implicate the AhR in normal development and tissue homeostasis. For instance, TCDD exposure in animals induces teratogenesis, immunosuppression, reproductive defects and tumor promotion, in an AhR-dependent manner. The dioxin-dependent activation of the AhR has also been linked to inhibition of proliferation in mammary and uterine tumor cell lines (reviewed in 12), and enhanced terminal differentiation in keratinocytes and palatal epithelia (13,14). Moreover, the AhR null mice generated by two independent laboratories are normal and fertile, exhibiting a spectrum of hepatic and immune system defects (15,16), but are resistant to benzo(a)pyrene-induced skin and liver carcinogenicity (17). In cultured cells, TCDD shows marked effects on cell cycle progression, where it induced a cell cycle arrest at G1/S check point, an effect that is mediated through the AhR and it involves the induction of cyclin-dependent kinase (CDK) inhibitor p27kip1. The AhR in absence of dioxin or other ligands, was shown to influence cell cycle progression, cell shape and differentiation (18, 19). This effect on the cell cycle progression relies on a direct protein-protein interaction of AhR with retinoblastoma (Rb) through an LXCXE domain on the AhR (20). However, studies in MCF-7 showed that AhR associates with Rb only after receptor activation and nuclear translocation (21). Furthermore, this interaction with Rb protein is required for the maximal AhR transcriptional activity (22). Although TCDD acting through AhR is a potent tumor promoter in mouse skin and in rat liver, it has strong anti-mitogenic effect in estrogen-responsive tissues and exhibits a broad spectrum of anti-estrogenic activities in human breast carcinoma cells (reviewed in 12). The AhR-null mouse or normal mouse treated with TCDD, exhibited impaired development of mammary gland ductal branching (23). Taken together with the anti-mitogenic effect exerted by TCDD on these cells, and the fact that TCDD down-regulates the AhR subsequent to its activation, these findings suggests that AhR is involved in regulating the proliferative stage required for mammary gland development. Preliminary investigations in our laboratory have demonstrated the expression of high levels of AhR protein in human mammary carcinoma cell lines in direct proportion to their degree of tumorigenicity and metastatic potential (24). We hypothesize that the AhR plays a major role in regulating mammary epithelial network during mammary gland development, and its over-expression contributes to the development of
metastatic phenotypes in human breast carcinoma. The initial aim of this work has been to investigate the mechanisms of AhR involvement in regulating mammary epithelia both during development and tumorigenesis.

**Body**

**Progress Year 4**

A fourth year was requested (without cost) in the hope of finishing some of the proposed experiments on the knockout mice (Objective 1). However, the breeding difficulty of these mice made it impossible to accomplish this goal.

**Objective 1: Restoration of the normal mammary development in the AhR-KO mice by transfecting AhR cDNA into their mammary tissues in situ using retroviral expression vectors**

This objective was partially fulfilled, however the difficulty in breeding the AhR knockout mice hampered and prevented the accomplishment of this objective, as we outlined in progress reports for years two and three (June 2004 and June 2005). We have requested the last year extension in order to continue the breeding attempts to obtain enough number of AhR$^{-/-}$ pups for the reconstitution experiments. However these were futile attempts and we could not manage to get enough mice for the viral injection or the few that we injected have died before they reached the developmental stage for collecting mammary glands. As we reported previously (Report 2 in June 2004), we were successful in accomplishing task 1 by generating the retroviral expression vectors for both mouse and human cDNA and producing high titer viruses from each. These viruses are stored at –80°C and they will be one of the laboratory resources, which will be useful tools for other projects in our laboratory involving the murine and human AhR. Tasks 3, 4, 5, and 6 of this objective were all completed as outlined in Report 2 in June 2004.

**Objective 2: To determine the status of the AhR activation in the Sager’s cells in presence or absence of TCDD treatment.**

This objective was successfully completed and a manuscript containing the data was submitted to a cancer research journal, however the manuscript was returned requesting analysis of more human breast carcinoma cell lines used in the field. These analyses were finished and the results were presented at the annual meeting of the American Association for Cancer Research this past April (Abstract in appendix). A revised manuscript containing the new data is being prepared to be resubmitted in the near future.

**Objective 3: Over-expression of AhR in normal immortalized human mammary epithelial cells.**

**Objective 5: Characterization of transformed cell lines generated in Objectives 3 for their tumorigenic and invasive phenotypes.**

As we reported in last year progress report this objective was successfully finished. This last year a manuscript containing the data generated was submitted to the journal of “Breast Cancer Research” and a revised version which addressed the reviewers’ concerns was recently submitted and awaiting a response (pre-print is attached as an appendix).

**Objective 4: Blocking of AhR expression in highly metastatic Sager’s 21MT2 human breast carcinoma cell line by stably transfecting AhR siRNAs.**
Objective 5: Characterization of transformed cell lines generated in Objectives 4 for their tumorigenic and invasive phenotypes.

These objectives were accomplished, as reported in last year progress report (June 2005). The details of the experiments and the results of the analyses were explained extensively in that report.

Key Research Accomplishments
- Obtaining of data to support our hypothesis that over-expression of AhR is sufficient for transformation of human mammary epithelial cells to malignant stages.
- Data obtained point to AhR as a potential target for therapeutic intervention for breast cancer.

Reportable Outcomes
- Development of retroviral expression vectors and production of retroviruses expressing genes for both human and mouse AhR.
- Development and banking of Phoenix packaging cells stably producing high titer AhR viruses
- Demonstration of the capability of these viruses to stably express high levels of both AhR gene and GFP marker gene in cultured cells and in mice mammary glands.
- Successfully establishing for the first time, a method for in situ viral infection to mouse mammary glands, as a means of introducing genes in a mammary gland.
- Design and synthesis of four siRNA for human AhR
- Development and banking of AhR-KO mouse embryo fibroblasts.
- Abstract-poster was presented at the 96th AACR meeting (Appendix 1).
- Abstract-poster was presented at the Era of Hope Department of Defense Breast Cancer Research Program meeting (Appendix 2)
- Two manuscripts are published form work related to this project and was running parallel to this project (appendix 7 &8)
- A manuscript is submitted and provisionally accepted (Appendix 9).
- A manuscript is in preparation to be submitted.
- Two dissertations were submitted to the graduate school at Meharry Medical College in partial fulfillment of the requirement for doctorate of philosophy (Ph.D.) degrees in Pharmacology (Dr. Joann Brooks 2005, who is currently a FIRST fellowship awardee
Postdoctoral Associate at the Winship Cancer Institute, Emory University, GA; and Dr. Yolanda Dale 2006, who is currently a Sallie Kaplan postdoctoral fellow at the National Cancer Institute, NIH).

- Data from this grant will be used as preliminary data for a research project grant from NIH.

**Bibliography of manuscripts and meeting abstracts submitted in connection to this research grant:**


4. **Eltom, SE**, Gasmelseed, AA and Saoudi-Guentri D. The aryl hydrocarbon receptor is over-expressed and constitutively activated in advanced breast carcinoma. Presented at the 97th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 1-6, 2006.

5. Dale YR and **Eltom SE**. Calpain mediates the dioxin-induced activation and down-regulation of the aryl hydrocarbon receptor in metastatic breast cancer cell lines. Presented at the 97th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 1-6, 2006.


9. **Eltom, S.E**, C.R. Jefcoate, A.A. Gasmelseed and D. Saoudi-Guentri. The aryl hydrocarbon receptor is over-expressed and constitutively activated in advanced breast carcinoma Cell lines. *In preparation to be submitted to Cancer Research.*
Personnel received pay from the grant:

2. Jaikun Wang, MD, PhD- Postdoctoral Associate, February 2002-June 2004
3. Dajla Saoudi-Guentry, MD- Postdoctoral Associate, July 2004-June 2005

Conclusions

A role for AhR in breast cancer initiation and promotion is becoming more definitive. In this study we have shown that AhR alone is capable of transforming immortalized normal mammary epithelial cells into a malignant phenotype. The results of the addressed aims that help to prove our hypothesis are: (1) We have shown that AhR over-expression leads to increased AhR activity upon activation as well as constitutive activation in the absence of agonist stimulation. The constitutive expression of CYP1A1 seen by over-expressing AhR is an observation seen also in metastatic breast cancer cells, which exhibit high AHR expression. In both the H16-overexpressing AhR cells and the metastatic MT2, this constitutive expression is a product of AhR being localized in the nucleus. (2) We have also shown that increase in AhR expression in the normal mammary epithelial cells also increases their proliferation to a rate comparable to the metastatic cell line. This is also reflected in an increase of cells transitioning through the cell cycle. An increased rate of transition out of G0/G1 may not provide the cells with the appropriate amount of time necessary to prevent DNA mutations from entering the synthesis phase. This is another possible mechanism by which increases in AhR expression may lead to carcinogenesis. In addition, we have been able to show that increases in AhR expression can lead to the ability of a cell to gain invasive potential. Invasiveness is an essential characteristic of transformed cells and certainly defines a cell as malignant.

Our results indicate that AhR is capable of transforming normal mammary epithelial cells. However, some of the data derived in this study does not support the notion that AhR is responsible for the malignant state of the metastatic mammary epithelial cells. However, the inability of decreased AhR expressions to revert the metastatic phenotype to a more normal phenotype may be a consequence of increasing mutations. Cancer cells readily acquire defects in the repair process that accelerate the mutation rate and genetic instability is a consequence of defective repair mechanisms. It is feasible to speculate that even if AhR is capable of inducing
transformation in normal mammary epithelial cells, increased mutations following the initial event may be too extensive to reverse that transformation by merely removing the causative agent. A large advantage to this study is the acquisition of both the normal and metastatic cell lines being derived from a single individual. This eliminates biological variations between cell lines and more definitely implicates AhR as the causative factor in the transformation observed. An obvious step in determining the mechanism behind AhR ability to transform the normal mammary epithelial cells is to determine what other proteins are affected by the increase in AhR expression. Several pathways proven to play an important role in carcinogenesis have already been implicated in interacting with the AhR pathway. Determining whether the expression of genes within these implicated pathways is regulated by AhR would be a major step toward understanding the mechanism by which AhR contributes to carcinogenesis. Microarray analyses of differential gene expression are underway as the next step to investigate these pathways, which are directly regulated by AhR.

References


Appendices

Appendix 1: Abstract presented at the 96th Annual Meeting of the American Association for Cancer Research (2005)- 1 page.


Appendix 3: Abstract presented at the 45th Annual Meeting of Society of Toxicology (2006)-1 page.


Appendix 5: Manuscript published in Molecular Pharmacology (7 pages)

Appendix 6: Manuscript published in Toxicology Letters (10 pages)

Appendix 7: Manuscript provisionally accepted in Breast Cancer Research (35 pages).
Overexpression of the aryl hydrocarbon receptor in mammary epithelia cells increases proliferation

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There is a growing interest surrounding the role the Aryl hydrocarbon receptor (AhR) in carcinogenesis; however, little is known about the mechanism by which AhR may exert its carcinogenic effect. AhR is a cytosolic basic helix-loop-helix (bHLH) protein that upon activation by polyaromatic hydrocarbons (PAH), translocates to the nucleus and heterodimerizes with another bHLH, the aryl hydrocarbon nuclear translocator (ARNT) to increase transcription of certain genes including CYP1A1 and CYP1B1. Biochemical and molecular biology studies have revealed that the AhR mediates the toxic and biological effects of environmentally persistent PAHs, the most potent of which is 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD). Activation of AhR by TCDD in cultured cell lines has been shown to cause a block in G1 to S-phase cell cycle transition. Also, several lines of evidence suggest that AhR is involved in regulating cell proliferation. Experiments were designed to address the question of whether AhR over-expression alone is sufficient to increase proliferation and alter cell cycle distribution. High levels of the AhR were expressed in immortalized normal mammary epithelial cells by stably transfecting human AhR cDNA using retroviral gene expression vectors. The level of AhR protein was determined by immunoblotting. The generate clones over-expressing AhR were shown to have a significant increase in the rate of proliferation. Cells over-expressing AhR showed a 95% increase in proliferation over normal cells during a 24h period. Also fluorescence activated cell sorting (FACS) analysis demonstrated that the increased levels of AhR result in a decrease in the percent of cells remaining in G0/G1. Normal cells contained 76% of cells in G0/G, while cells over-expressing AhR resulted in 36% remaining in G0/G1. However the decrease in G0/G1 is reflected by an increase in both S-phase and G2/M distribution. Together, this data suggest a mechanism by which AhR can enhance mutations by decreasing the time allotted for checkpoints thus leading to increase carcinogenesis.
P29-4: OVEREXPRESSION OF THE ARYL HYDROCARBON RECEPTOR IN MAMMARY EPITHELIAL CELLS INCREASES PROLIFERATION BY INCREASING G1/S PHASE TRANSITION

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There is a growing interest surrounding role the Aryl hydrocarbon receptor (AhR) in exerting the carcinogenic effect of polyaromatic hydrocarbons (PAHs); however, little is known about the mechanism by which AhR may exert its carcinogenic effect. AhR is a cytosolic basic helix-loop-helix (bHLH) protein that upon activation by PAHs, translocates to the nucleus and heterodimerizes with another bHLH, the aryl hydrocarbon nuclear translocator (ARNT) to increase transcription of certain genes including CYP1A1 and CYP1B1. Biochemical and molecular biology studies have revealed that the AhR mediates the toxic and biological effects of environmentally persistent PAHs, the most potent of which is 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD). Activation of AhR by TCDD in cultured cell lines has been shown to cause a block in G1 to S-phase cell cycle transition. Also, several lines of evidence suggest that AhR is involved in regulating cell proliferation. Experiments were designed to address the question of whether AhR over-expression alone is sufficient to increase proliferation and alter cell cycle distribution. High levels of the AhR were expressed in immortalized normal mammary epithelial cells by stably transfecting human AhR cDNA using retroviral gene expression vectors. The level of AhR protein was determined by immunoblotting. The generated clones over-expressing AhR were shown to have a significant increase in the rate of proliferation. Cells over-expressing AhR showed a 3-fold increase in proliferation over normal cells during a 24h period. Also fluorescence activated cell sorting (FACS) analysis demonstrated that the increased levels of AhR result in a decrease in the percent of cells remaining in G0/G1. Normal cells contained 76% of cells in G0/G1, while cells over-expressing AhR resulted in 36% remaining in G0/G1. However the decrease in G0/G1 is reflected by an increase in both S-phase and G2/M distribution. Together, this data suggest a mechanism by which AhR can contribute to carcinogenesis.

The U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0458 and DAMD17-02-1-0483 supported this work.
treated AhR null: 26 ± 1.3 nmol/ml, p=0.050), a non-specific assessment of ROS production. Further, BQ-123 reduced both cardiac lucigenin chemiluminescence (AhR null: 7.3 ± 0.4; BQ-123-treated AhR null: 4.1 ± 0.2; AhR wildtype: 3.7 ± 0.2 RLU/mg tissue/5 min, p<0.001) and cardiac mRNA expression of NAD(P)H oxidase subunits gp91phox, p47phox, and p67phox, in AhR null mice, compared to wildtype. These findings demonstrate that ET-1 activation of ET receptors mediates an increase in ROS which is associated with cardiac hypertrophy in AhR null mice. Further, the ET-1-mediated increase in ROS appears to be a result of increased NAD(P)H oxidase activation in AhR null mice. Supported by ES010433 to MKW; 530 ES12072 to UNM.

356 CHARACTERIZATION OF HSP90-BINDING TO THE AH RECEPTOR. CENTRAL ROLE OF THE PASB DOMAIN

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The Ah receptor (AhR) is a ligand-dependent transcription factor, which regulates the biochemical and toxic effects of structurally diverse chemicals. Hsp90, one subunit of the AhR complex, appears to direct proper folding and maintenance of the high affinity ligand binding conformation of the AhR in some species. Using a structural homology model we developed for the ligand- and hsp90-binding PASB domain of the AhR to guide our mutagenesis, we have examined the physical interactions of hsp90 with AhR and subsequent effects on AhR signaling (hsp90, ligand and DNA binding and transcriptional activation). Deletion of the PASB domain resulted in the complete loss of hsp90-binding and constitutive ligand-independent activation of DNA-binding and reporter gene transcription, suggesting that documented interactions of hsp90 with the AhR hLH domain are insufficient on their own for hsp90 binding. Swapping of the PASB domain of Arnt (which does not bind hsp90) for that of the AhR did not completely eliminate hsp90 binding suggesting that the AhR PASB domain can confer and/or maintain weak hsp90 binding in the context of the full-length AhR; binding through the hLH site is dependent on the presence of the intact PASB domain. Deletions within the AhR PASB domain resulted in intermediate levels of hsp90 binding and revealed that the bulk of the central PASB 5 strand α-sheet is required for the optimal hsp90-binding. PASB deletions excluding amino acids 339-362 resulted in ligand-independent transformation and DNA-binding, similar to full PASB deletion. Based on the AhR PASB model, these results not only indicate that two α-strands with the connecting flexible loop are essential for ligand-induced activation of AhR transformation, but they suggest that ligand binding to the PASB domain may induce a conformational change in this region that weakens its association with hsp90 exposing the AhR hLH-PASα dimerization domain for Arnt. (NIH ES07685).

357 THE ROLE OF ENDOGENOUS XAP2 IN THE SUBCELLULAR LOCATION AND NUCLEOCYTOSPLASMIC SHUTTILING OF THE ENDOGENOUS AHα RECEPTOR

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The endogenous Ahα1 receptor expressed in the murine Hepa-1 cell line is primarily localized to the cytoplasm and does not accumulate in the nucleus when nuclear export is blocked by leptomycin B (LMB). Studies using transient expression systems have implicated the immunophilin-like XAP2 protein in the control of the localization of the Ahα1 receptor and suggest that XAP2 dissociates prior to ligand-mediated nuclear localization. To evaluate the role of endogenous XAP2 on the localization of the endogenous Ahα1 receptor, Hepa-1 cells were exposed to TCDD for 0, 15, 30, 45 and 60 minutes and harvested for immunoprecipitation studies (IP) or fixed and immunostained. TCDD exposure resulted in the translocation of the AhR to the nucleus within 30 minutes, yet equal amounts of XAP2 were precipitated with the AhR at all time points. XAP2 was also detected in association with the non-degraded AhR fraction that remained in the cells following 4 hours of TCDD exposure. Reduction of endogenous XAP2 in Hepa-1 cells with siRNA, resulted in an Ahα1 receptor that was not associated with significant levels of XAP2, yet retained a cytoplasmic localization in the absence of ligand. However, in these cells, the endogenous Ahα1 receptor could be detected in the nucleus following exposure to LMB. Truncation of the COOH-terminal 305 amino acids of the Ahα1 receptor resulted in an AhR protein that exhibited a predominant nuclear localization and was associated with the same level of XAP2 as full-length AhRs that exhibited a cytoplasmic localization. These studies support a role of the XAP2 protein in the cytoplasmic retention of the unliganded full-length Ahα1 receptor. However, in the absence of the COOH-terminal domain, XAP2 remains associated with the AhR but does not function to retain the receptor in the cytoplasm. In addition, the studies indicate that XAP2 does not dissociate from the endogenous Ahα1 receptor following ligand binding. Supported by NIH grant ES010991 (RSP).

358 3-METHYLCOLANTHRENE AND OTHER ARYL HYDROCARBON RECEPTOR AGONISTS DIRECTLY ACTIVATE ESTROGEN RECEPTOR ALPHA

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3-Methylcholanthrene (3MC) is an aryl hydrocarbon receptor (AhR) agonist, and it has been reported that 3MC induces estrogenic activity through AhR-estrogen receptor (ER) interactions. In this study, we used 3MC and 3,5,4′,4′-pentachlorophenyl (PCP) as prototypical AhR ligands, and both compounds activated estrogen-responsive reporter genes/products (cathepsin D) in MCF-7 breast cancer cells. The estrogenic responses induced by these AhR ligands were inhibited by the antiestrogen ICI 182780 and by transfection of a small inhibitory RNA (siRNA) for ER but were not affected by an siRNA for AhR. These results suggest that 3MC and PCP directly activate ERα, and this was confirmed in a competitive ERα binding assay and in a fluorescence resonance energy transfer (FRET) experiment where PCB and 3MC induced CFP-ERα/YFP-ERα interactions. In a chromatin immunoprecipitation assay, PCB and 3MC enhanced ERα and 3MC directly activate ERα-dependent transactivation and extend the number of ligands that activate both AhR and ERα.

359 MALIGNANT TRANSFORMATION OF MAMMARY EPITHELIAL CELLS BY OVER-EXPRESSION OF THE ARYL HYDROCARBON RECEPTOR

J. Brooks and S. E. Elenius, Biomedical Sciences, Division of Cancer Biology, Meharry Medical College, Nashville, TN.

The aryl hydrocarbon (Ah) receptor, which is well known for its mediation of the toxic responses to the environmental poly-halogenated aromatic hydrocarbons (PAH), is a ligand activated basic helix-loop-helix transcription factor. Many reports have documented the role of the Ah receptor (AhR) in PAH-induced carcinogenicity; however in this report we addressed whether the over-expression of the AhR alone is sufficient to induce carcinogenic transformation in mammary epithelial cells. Retroviral expression vectors were used to stably express high levels of AhR proteins in an immortalized normal human mammary epithelial cell line (HMEC) with low background AhR expression. Series of HMEC stables with varying expression levels of AhR were generated. Clones overexpressing AhR by more than 3-fold showed a two fold decrease in the population doubling time compared to vector-control cells. Cell cycle analysis revealed that this enhancement in proliferation rate was mainly due to an increase in the percentage of cells transiting from G0/G1 to S- and G2/M phases. The overexpression of AhR has resulted in morphological transformation of these HMEC into a fibroblast-like cells with acquired motility and increased in their migration by more than two folds compared to their vector-expressing clones. Most significantly, these transformed cells have acquired the ability to invade matrigel matrix, where more than 80% of plated cells invaded the matrix and crossed the membranes within 24 h. In contrast, none of the vector controls or the parent HMEC were able to invade matrigel. Furthermore, these malignant phenotypes were coupled with the ability of the clones to form colonies in soft agar, reflecting their malignant transformation. Collectively, these data provide the first evidence for the direct role of AhR in progression of breast carcinoma. Funded by G12-RR03032, U54-CA91408-03, DAMD17-02-1-0483, DAMD17-02-1-0458.

360 THE DIFFERENTIAL RECRUITMENT OF ERα TO CYP1A1 BY BNF, ICZ, AND DM AND THE IMPORTANT ROLE OF ERα IN AHR TRANSCRIPTION

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We have recently described the TCDD-dependent recruitment of estrogen receptor alpha (ERα) to AhR-regulated target genes CYP1A1 and CYP1B1. However, whether and to what extent other AhR agonists induce the recruitment of ERα to CYP1A1 is not known. Using ChIP assays in T47D breast cancer cells we demonstrate that beta naphthoflavone (BNF), diindolylmethane (DIM) and indol-3,2-b-carbazole (ICZ) induce the recruitment of ERα to CYP1A1, although the level of promoter occupancy of ERα varied among the different ligands. BNF, TCDD and ICZ induced a 10-fold promoter enrichment of ERα to CYP1A1 compared to solvent controls, whereas DIM induced an 80-fold enrichment.

SOT 2006 Annual Meeting 73
Cellular and Molecular Biology 27: Transcription Factors

Abstract #1730

The aryl hydrocarbon receptor is over-expressed and constitutively activated in advanced breast carcinoma

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The aryl hydrocarbon receptor (AhR), a ligand-activated helix loop helix transcription factor, binds environmental poly aromatic hydrocarbons (PAH) such as dioxin, and mediates their toxicity, including carcinogenesis. This study was designed to investigate the functional significance of AhR in breast carcinogenesis. The AhR expression and its transcriptional activity was analyzed in a battery of human breast carcinoma (HBC) cell lines with varying degrees of malignancy in comparison to immortalized normal and primary human mammary epithelial cells (HMEC). Western immuno-blotting revealed dramatic elevated levels of AhR proteins in tumorigenic HBC of advanced malignancy (MD231, MDA468, ZR-75, MDA435, MCF-7), while less levels were expressed in HBC typically categorized as early stages of malignancy (T-47D, HBL100, BT-549), normal immortalized (H16N2 and MCF10A) and primary HMEC. RT-PCR analysis of mRNA revealed a similar trend although not as dramatic high levels, suggesting a role for protein stabilization in these elevated levels. These results were further confirmed in Sager’s 21T series, which are closely matched pairs of HBC derived from a single patient and are characterized by exhibiting a gradient order of malignancy (21MT2>21NT>21PT). The 21MT-2 lines showed the highest expression of protein and mRNA and 21NT was medium between 21MT-2 and 21PT lines. This high expression of AhR is independent of estrogen receptor (ER) status, as verified in MDA 231 (ER negative) and its variant S30 (ER positive), as well as T47D-A18 (ER positive) and its variant T47D-C4:2W (ER negative), as well as in 21T series which are all ER negative. The AhR is usually localized in the cytoplasm of most normal cells including HMEC and is translocated to the nucleus upon ligand (dioxin) activation. However, both sub-cellular fractionation experiments and fluorescence immuno-cytochemical staining has shown that the AhR in the HBC are predominantly localized to the nuclear compartments in absence of ligand treatment. Similarly, immuno-histochemical analysis of human breast tumors has shown an increase in AhR expression in the invasive carcinomas, where the AhR staining was predominantly or exclusively nuclear. Notably, this nuclear accumulation of AhR in untreated HBC was transcriptionally active as evidenced by the substantial expression of CYP1A1 mRNA, which is exclusively regulated transcriptionally by the activated AhR. In conclusion, this study reports a novel finding of elevated levels of AhR in human breast carcinomas in direct proportion to their degree of malignancy. These data identify the AhR as a possible regulator of breast cancer progression and its possible consideration as a candidate prognostic factor for survival as well as its potential as a target for breast cancer therapeutic intervention.
ACCELERATED COMMUNICATION

Calpain Mediates the Dioxin-Induced Activation and Down-Regulation of the Aryl Hydrocarbon Receptor

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ABSTRACT

The aryl hydrocarbon receptor (AhR) is a ligand-activated basic-helix-loop-helix transcription factor that binds polyaromatic hydrocarbons (PAH), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and mediates their toxicity. Binding of PAH to AhR in the cytoplasm triggers a poorly defined transformation step of the receptor into a nuclear transcription factor. In this study, we show that the calcium-dependent cysteine protease calpain plays a major role in the ligand-induced transformation and signaling of AhR. Fluorescence imaging measurements showed that TCDD treatment elevates intracellular calcium, providing the trigger for calpain activation, as measured toward t-butoxy-carbonyl-Leu-Met-chloromethylaminocoumarin, a calpain-specific substrate. Inhibition of calpain activity by the N-benzyloxycarbonyl-Val-Phe-aldehyde (MDL28170) blocked the TCDD-induced nuclear translocation of AhR in Hepa1c1c7 mouse hepatoma cell line. Treatment of the human metastatic breast carcinoma cell line MT-2 with MDL28170 and 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid (PD 150606), two calpain-selective inhibitors, completely abolished the TCDD-induced transactivation of AhR as assessed by transactivation of CYP1A1 gene. Previous studies have established that after TCDD-induced transactivation, the AhR undergoes a massive depletion; we show here that selective calpain inhibitors can block this step, which suggests that the ligand-induced down-regulation of the AhR is calpain-dependent. The data presented support a major role for calpain in the AhR transformation, transactivation, and subsequent down-regulation, and provide a possible explanation for many of the reported phenomena of ligand-independent activation of AhR.

The AhR is a ligand-activated basic helix-loop-helix transcription factor that regulates the adaptive and toxic responses to a variety of environmental carcinogens, including polyaromatic hydrocarbons (PAH), such as TCDD (Poland and Knutson, 1982). In the absence of ligand, the AhR resides predominantly in the cytoplasm in a conformation stabilized by chaperone proteins hsp90, XAP2, and p23 (Carver and Bradfield, 1997; Ma and Whitlock, 1997; Meyer and Perdew, 1999). The current working model for the AhR signaling proposes that ligand binding to AhR facilitates the dissociation of the chaperone proteins and AhR transformation into a form that readily translocates to the nucleus. In the nucleus, AhR dimerizes with the related bHLH aryl hydrocarbon receptor nuclear translocator (ARNT) protein (Kazlauskas et al., 2001), and binding of this heterodimer to DNA recognition motifs designated as xenobiotic-responsive elements results in enhanced transcription of the Ah-responsive genes (Jones et al., 1985), typified by CYP1A1 and CYP1A2 (Gonzalez et al., 1984). The protein products of these cytochrome P450 genes are catalytically active in metabolizing not only many endogenous compounds such as β-estradiol, but also many drugs, dietary components, mutagens, carcinogens,

ABBRVIATIONS: AhR, aryl hydrocarbon receptor; PAH, polyaromatic hydrocarbons; hsp90, 90-kDa heat shock protein; ARNT, aryl hydrocarbon receptor nuclear translocator; MDL 28170, and CA91408 and Department of Defense grant DAMD17-02-1-0483 (to S.E.E.). Y.D. was supported through NIH grants R25-GM59994 and T32-CA09592.

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and environmental pollutants (Conney, 1982). After transcriptional activation, the liganded-AhR undergoes a rapid degradation (Prokipcak and Okey, 1991; Reick et al., 1994; Pollenz, 1996). Studies have suggested that this ligand-induced down-regulation of AhR is proteasome-dependent (Davarinos and Pollenz, 1999) and that the nuclear export is required for this process (Song and Pollenz, 2002).

Although forced dissociation of the chaperone proteins, especially hsp90 by geldanamycin, allows for the nuclear translocation of the receptor, it is not sufficient for its trans-activation (Song and Pollenz, 2002). In vitro studies have shown the AhR to be a substrate for calpain (Poland and Glover, 1988), a member of a family of cytosolic calcium-dependent cysteine proteases. Calpain is involved in regulating many cellular processes, including proliferation, differentiation, cell motility, and metastasis through regulation of signal transduction and cleavage of many target cellular regulatory proteins (Wang, 1990; Potter et al., 1998). Of the several calpain isoforms, calpain I (μ-calpain), and calpain II (m-calpain) are ubiquitous enzymes, activated with low and high calcium concentrations, respectively. Because the treatment of cells with PAH can elicit a rapid increase in intracellular calcium (Hanneman et al., 1996; Tanneheimer et al., 1997), we hypothesize that this increase in intracellular calcium could provide the trigger to activate calpain. In this study, we have examined the involvement of calpain in the transformation process required for the nuclear translocation, transactivation, and subsequent degradation of AhR.

**Materials and Methods**

**Materials.** Calpain inhibitor III (MDL 28170), PD 150606, MG-132, epoxomicin, and ionomycin were purchased from Calbiochem (San Diego, CA). Dioxin (TCDD) was purchased through NCI Chemical Carcinogen Repository–Midwest Research Institute (Kansas City, MO). The calpain substrate BOC-LM-CMAC and Fluo-4-AM were purchased from Invitrogen (Carlsbad, CA). Real-time PCR kit was purchased from Bio-Rad Laboratories (Hercules, CA). The rabbit polyclonal anti-AhR antibodies (Poland and Glover, 1990; Pollenz et al., 1994) were a kind gift from Dr. Christopher Bradfield (University of Wisconsin, Madison, WI). The Sager MT-2 metastatic cell line was a kind gift from Dr. Vilma Band (Northwestern University, Chicago, IL).

**Cell Culture and Stimulation of MT-2 Cells.** The metastatic MT-2 cell line was grown in DFCI-1 medium as described previously (Band et al., 1990). For experiments, MT-2 cells were seeded in six-well plates at a density of 5 × 10⁵ cells per plate and grown for 24 h. Cells were preincubated with inhibitors MG-132, epoxomicin, or MDL 28170 for 2 h and maintained during 3-h TCDD treatment. TCDD, MG-132, MDL 28170, and epoxomicin were solubilized in DMSO, with an equivalent volume added to control cells [maximum of 0.1% (v/v)].

**Intracellular Calcium Measurements.** MT-2 cells cultured in 24-well plates (2 × 10⁵) in DFCI-1 medium were loaded with 5 μM Ca²⁺-sensitive dye Fluo-4-AM (Invitrogen) for 45 min at 37°C. After preincubation, cells were rinsed three times with DFCI medium to remove free dye and continued to incubate for 30 min in medium alone to allow complete de-esterification of AM esters. Fluo-4-loaded cells were then stimulated with 1 or 10 nM TCDD, 10 μM ionomycin, or vehicle alone for 25 min. Changes in intracellular calcium were measured as captured fluorescence images of cells using a fluorescence microscope (excitation at 385 nm, emission at 512 nm; IX50; Olympus, Tokyo, Japan).

**Calpain Activity Assay.** Calpain activity in MT-2 cells was assessed by fluorescence microscopy using the calpain substrate BOC-LM-CMAC (Invitrogen). The nonfluorescent cell-permeable substrate is conjugated by intracellular thiolos into a membrane impermeable form, allowing substrate accumulation within the cell (Carragher et al., 2004). Proteolytic cleavage of BOC-LM-CMAC by calpain results in blue fluorescence. Cells in 24-well plates were pretreated with 15 μM calpain inhibitor MDL28170, MG-132, or epoxomicin followed by TCDD or ionomycin treatment. In brief, cells were incubated with 50 μM BOC-LM-CMAC for 20 min at 37°C. Fluorescence intensity corresponding to calpain activity was visualized, and images were captured with an Olympus IX50 fluorescence microscope using a digital camera with MagnaFire software, and quantified by ImageJ software (http://rsb.info.nih.gov/j/). The image exposure settings were identical within each experiment. Data for each experiment were normalized to ionomycin values (set as 100%).

**Immunocytochemical Staining and Fluorescence Microscopy.** Cells growing on cover slips in six-well plates were washed in phosphate-buffered saline and then fixed by incubation in a (1:1) methanol/aceton solution at 4°C for 30 min and subsequently air-dried. For staining, cells were rinsed and hydrated with TBST (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20) and transferred to clean six-well plates. The cover slips were incubated at room temperature for 1 h in 4% milk solution in TBST to block nonspecific binding. The cover slips were then incubated at room temperature for 1 h in 1 μg/ml anti-AhR polyclonal antibody (BEAR-4) in 2% milk solution in TBST while rocking. Cover slips were then washed three times (15 min each) with TBST. A 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies in 2% milk with TBST was added to the cover slips in reduced light and incubated at room temperature for 1 h. The cover slips were then washed extensively and mounted onto glass slides using mounting solution containing 1,4-diazabicyclo[2.2.2]octane as an anti-fading agent.

**Preparation of Total Cell Lysates and Immunoblotting.** After treatments, cell monolayers were lysed in 1 ml of TRIzol, which allowed for simultaneous isolation of RNA and protein. After lysis, both RNA and protein were isolated according to the vendor’s instructions. The protein pellets were resuspended in 2% SDS and sonicated briefly to dissolve. The protein concentration in cell extracts was determined using a BCA assay kit, per the manufacturer’s instructions. Equivalent amounts of protein (10 μg) were separated by SDS polyacrylamide gel electrophoresis and transferred for 2 h at 175 V to polyvinylidene difluoride membranes. The membranes were blocked at room temperature for 1 h in 4% nonfat milk in TBST with subsequent incubation with BEAR-3 anti-AhR (1 μg/ml). After brief washing, blots were incubated with the corresponding horseradish peroxidase-coupled anti-rabbit or anti-mouse secondary antibody (1:20,000) for 1 h followed by additional washing in TBST and TBS. Reactive protein bands were visualized using enhanced chemiluminescence reagents. Band density was quantified by UV Bio-Imaging System using LabWorks Image Acquisition Analysis Software (UVP Inc., Upland, CA). Thereafter, blots were probed with actin monoclonal antibodies (1:4000) for normalization of protein loading. The relative levels of AhR protein were then normalized to the level of β-actin to generate normalized values for the relative concentration of AhR in each sample.

**Reverse Transcriptase Polymerase Chain Reaction.** After lysis with TRIzol, total RNA was isolated according to manufacturer’s protocol. cDNA was prepared from 2 μg of mRNA using random hexamer primers and Moloney murine leukemia virus reverse transcriptase. CYP1A1 PCR amplification was done using forward primer sequence 5’-TAG ACA CTG ATC TGG CTG CAG’-3’ and the reverse primer sequence 5’-GGG AGA CTC TCA CTA CGC TCC-3’. Housekeeping gene glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for forward primers 5’-TGG GAC GTC CAT GCC ATC AC-3’ and reverse primer 5’-TCC ACC CTG TGT CTG TA-3’. PCR products were visualized in agarose gels.
stained with ethidium bromide, and bands were quantified by densitometric scanning, as described previously (Eltom et al., 1999).

**Real Time RT-PCR.** Real-time quantitative PCR was performed using an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories). cDNA generated from TCDD-treated MT-2 cells was serially diluted to establish a standard curve (20,000–0.2 pg). Reactions were performed in triplicates using 2.5 μl of cDNA per 25-μl reaction containing iQ SYBR green super mix and CYP1A1 primers (5' -CTA TGA CCA CAA CCA CCA AGA ACT G-3', forward primer and 5'-AGG TAG CGA AGA ATA GGG ATG AAC TC-3' reverse primer) with the following PCR parameters: 95°C for 5 min followed by 45 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 15 s. Reactions for detection of the endogenous control gene, ribosomal 18s rRNA, were run in parallel for each cDNA template as a reference for normalization using the following primers: 5'-CGG AGA GTA TGG ACA GAT TAG CGA TAG C-3' forward primer and 5'-TGC CAG AGT CTC GTT COT TAT CG-3' reverse primer. A melting curve analysis was performed for each amplification run to ensure the specificity of product amplification.

**Statistical Analysis.** The data from different experiments were analyzed using Prism software (GraphPad Software, San Diego, CA) by one-way analysis of variance and Tukey-Kramer multiple comparison tests of values from control versus treated samples.

**Results**

**TCDD Mobilizes Calcium in MT-2 Cells.** Exposure of human T cells and primary human epithelial cells to PAH leads to the mobilization of intracellular calcium. Because this phenomenon was not observed in the MCF-10A mammary epithelial cell line, which has low concentrations of AhR (Tannheimer et al., 1997), it was important to determine whether TCDD is capable of evoking an increase in intracellular calcium in the MT-2 human metastatic breast cancer cell line, which has high concentrations of AhR. We measured [Ca\(^{2+}\)]\(_i\) in MT-2 cells in the presence of TCDD, using Fluo-4 AM as a probe. In MT-2 cells, TCDD resulted in an increase in [Ca\(^{2+}\)]\(_i\) that was comparable with values of the calcium ionophore ionomycin, which results in a profound increase in [Ca\(^{2+}\)]\(_i\), compared with the solvent control (DMSO) (Fig. 1).

**Exposure of Cells to TCDD Activates Calpain.** Calpain is activated by elevated intracellular calcium, resulting in auto-proteolytic cleavage to further enhance its activity (Mathiasen et al., 2002). To explore whether TCDD-induced changes in intracellular calcium could lead to calpain activation, whole-cell calpain activity assay was preformed in MT-2 cells using the cell-permeable substrate BOC-LM-CMAC. TCDD exposure resulted in a robust increase in calpain activity, comparable with the levels induced by ionomycin, the ionophore that was used as a positive control (Fig. 2). These data indicate that TCDD could activate calpain. The activation of calpain by TCDD was strongly inhibited by MDL 28170, a potent calpain inhibitor. Figure 2 also shows that MG-132, which has previously been reported to inhibit both proteasomes and calpain (Mathiasen et al., 2002), mimicked the effect of MDL 28170 on TCDD-induced calpain activity. Epoxomicin, which inhibits proteasomes only (Meng et al., 1999), had no effect on calpain activity. These results support the conclusion that TCDD is involved in the activation of calpain in MT-2 cells. The data further establish the selectivity of both MG-132 and MDL 28170 in inhibiting calpain, in agreement with published reports (Potter et al., 1998; Mathiasen et al., 2002).

**TCDD-Induced Nuclear Accumulation of AhR Is Calpain-Dependent.** To explore whether calpain might be involved in the transformation of AhR leading to its translocation from the cytoplasm into the nucleus, subcellular localization of AhR was analyzed in Hepa-1, the murine hepatoma cell line after treatment with TCDD. Hepa-1 cells were used here because they have no detectable nuclear AhR levels under basal conditions, unlike the human metastatic MT-2 cells, which have substantial nuclear AhR levels in the absence of ligand treatment. TCDD treatment results in enhanced fluorescence nuclear accumulation accompanied by reduction in the cytoplasmic staining, indicative of the receptor nuclear translocation. As shown in Fig. 3, AhR is predominantly localized within the cytoplasm in DMSO-treated control cells, as shown by fluorescence immuno-staining (Fig. 3A). Treatment with MDL 28170 before TCDD exposure resulted in predominant cytoplasmic staining, indicating that calpain influences the localization of the AhR after treatment with TCDD.

**Inhibition of Calpain Blocks Transcriptional Activity of AhR.** The expression of CYP1A1, a gene that is transcriptionally regulated by AhR, is induced by increases in
intracellular calcium but the mechanism of this induction is poorly defined (Le Ferrec et al., 2002). It is noteworthy that the disruption of hsp90-AhR complex facilitates nuclear localization of the AhR yet fails to induce gene expression. Based on the data presented in Fig. 3, we presume that calpain-mediated events may be important for AhR-induced transactivation. To this end, we measured CYP1A1 gene transcription in MT-2 cells treated with MDL 28170 to specifically block the actions of calpain. As shown in Fig. 4A, MT-2 cells have low constitutive levels of CYP1A1 mRNA, and TCDD treatment resulted in a significant increase in CYP1A1 mRNA that was suppressed to basal levels by the calpain inhibitors MDL 28170 and PD 150606.

Real-time RT-PCR was also used to provide more quantitative analysis of CYP1A1 mRNA expression. The results in Fig. 4B show that treatment with TCDD for 3 h resulted in an increase in CYP1A1 expression of approximately 40-fold over DMSO-treated control. MDL 28170 completely blocked the TCDD ligand-induced CYP1A1 gene transcription. Likewise, MG-132, which also inhibits calpain, inhibited TCDD-induced CYP1A1 gene expression to the same degree as MDL28170, whereas epoxomicin, a proteasome inhibitor, had no effect on the TCDD-induced CYP1A1 expression. Therefore, we conclude that inhibition of calpain preferentially reduces the ability of TCDD to induce the expression of CYP1A1.

**AhR Degradation after TCDD Treatment.** The data presented thus far demonstrate that TCDD mobilizes calcium, triggering calpain activation, which could transform the AhR into a transcription factor, possibly by limited cleavage of the receptor. After enhancing CYP1A1 transcription, AhR is shuttled from the nucleus to the cytoplasm, where it is degraded. To assess whether calpain contributes to this degradation, TCDD-induced AhR degradation was analyzed in the presence and absence of the calpain inhibitor MDL 28170. Treatment of MT-2 cells with TCDD resulted in greater than 75% reduction in AhR protein levels (Fig. 5A), and pretreatment with MDL 28170 completely blocked this degradation. However, although these treatments with ligand and protease inhibitors had an effect on the AhR levels, they had no effect on the levels of ARNT, the AhR partner for transcriptional activation (Fig. 5A).

Recent reports have implicated proteasomes in the degradation of the AhR; therefore, the proteasome inhibitors MG-132 and epoxomicin were tested. Epoxomicin, a potent and selective proteasome inhibitor, has no cross-activity against nonproteasomal proteases such as calpain (Meng et al., 1999), whereas MG-132 interferes equally with both proteasomes and calpain (Davarinos and Pollenz, 1999; Mailhes et al., 2002). As shown in Fig. 5B, epoxomicin treatment did not protect the receptor from this TCDD-induced down-regulation, whereas MDL 28170 effectively protected the AhR from TCDD-induced down-regulation (Fig. 5B). Unlike epoxomicin, MG-132 inhibited the TCDD-induced degradation of the AhR, to a comparable level of the MDL 28170 (Fig. 5C). These

**Fig. 2.** Activation of calpain in MT-2 cells after exposure to TCDD and ionomycin in the absence and presence of calpain and proteasome inhibitors. Fluorescence intensity corresponding to calpain activity was captured at 20 min by fluorescence microscopy (magnification, 200×). Blue fluorescence is indicative of calpain activity. The average fluorescence units of the images were quantified using the ImageJ software from several fields, and plotted values are the average of n = 3 independent experiments. *, p < 0.05; comparing drug treatments to vehicle control.
results demonstrate that the degradation of the AhR is a calpain-dependent process.

**Discussion**

PAHs, such as TCDD, elicit a wide range of toxic effects, including carcinogenesis. One way that AhR mediates the toxic responses of these chemicals is through its ability to enhance the transcription of CYP1A1 gene in many tissues. The protein product of CYP1A1 catalyzes the bioactivation of these chemicals as well as some endogenous hormones producing reactive metabolites that cause DNA damage and initiate neoplasia. Data presented in this report provide compelling evidence that the Ca\(^{2+}\)-dependent protease calpain is a critical player in driving this AhR-mediated process.

For AhR to direct the ligand-induced CYP1A1 transcription, it has been established that ligand binding to AhR results in a sequence of events starting with the receptor dissociation from chaperone proteins, such as hsp90, and adoption of conformational changes that allow the AhR to translocate into the nucleus to bind DNA and activate the transcription of CYP1A1 (Whitlock, 1999). However, the mere dissociation of the hsp90 from the receptor complex, although allowing for AhR nuclear translocation, is insufficient to induce the receptor transactivation (Song and Pollenz, 2002). This observation suggests that additional processing of AhR is required after its dissociation from chaperone proteins. Our data clearly implicate calpain in the transformation required for the AhR nuclear translocation and subsequent transactivation. Inhibition of calpain com-
completely blocked the transcription of CYP1A1, indicating that calpain is required for the transformation of AhR into a transcriptional factor. We demonstrate that the trigger for activation of calpain is provided by elevation in intracellular calcium by AhR ligands such as TCDD, which was previously reported in other cell systems.

Previous reports have shown that deletion of the P/S/T domain in the carboxyl terminal of AhR leads to an increase in its transcriptional activity (Kumar et al., 2001). Although calpain prefers Leu or Val as a second residue on the N-terminal side of cleavage site (Wang, 1990), it also recognizes hydrophilic sequences enriched in Pro, Glu, Asp, Ser, and Thr (or PEST sequences) near cleavage sites (Wang et al., 1989). Therefore, it is conceivable that activated calpain could cleave the carboxyl-terminal P/S/T domain of AhR resulting in its transcription activation. This truncated form of AhR may be recognized by the importin receptors of the nuclear membrane in a ligand-dependent or -independent manner. Intriguingly enough, such a truncated form of AhR (∼90 kDa) was recovered from nuclei of TCDD-treated Hepa-1 cells, which was slightly smaller than the cytosolic AhR in these cells (∼95 kDa) (S. E. Eltom, unpublished data).

After transcriptional activation, the liganded-AhR undergoes a rapid degradation leading to a massive depletion (Prokipcak and Okey, 1991). Some studies have suggested that this process is proteasome-dependent (Davarinos and Pollenz, 1999). On the other hand, Poland and Glover (1988) reported fragments of AhR after increases in calcium concentration, which they attributed to calpain activation. To distinguish the role of the two protease systems, the current study used highly selective inhibitors of both calpain and proteasomes, with no overlapping reactivity. Although the inhibitor MG-132 was used as a proteasome inhibitor to implicate proteasomes in the degradation of the AhR, this report, in agreement with others, has identified MG-132 as a strong inhibitor of calpain (Mailhes et al., 2002). Moreover, classic calpain inhibitors, which were used to discredit calpain in the AhR degradation process, have been shown to have less affinity for calpain I and II (Bang et al., 2004). In our study, MDL 28170, the most potent inhibitor of calpain, demonstrated that calpain is responsible for the transcriptional activation of AhR and subsequent degradation. In addition, MG-132, which exhibits a cross-specificity for calpain and proteasomes, has blocked the TCDD-induced degradation to the same extent as MDL 28170. On the other hand, the proteasome-selective inhibitor epoxomicin failed to prevent TCDD-induced degradation of the receptor. These lines of evidence suggest that calpain is also responsible for the agonist-dependent degradation of AhR.

Thus, the activation of calpain by TCDD contributes to each of the sequential steps of the AhR-mediated transcriptional activation of CYP1A1 and the subsequent AhR degradation. We propose a model in which TCDD elevates intracellular calcium, triggering calpain activation to both initiate and terminate the AhR signaling. In such a model, calcium is the second messenger subsequent to TCDD exposure, and calpain is the downstream effector molecule. Transcriptional activation of CYP1A1 by AhR has been reported in response to agents that do not bind AhR, such as caffeine (Goasduff et al., 1996), olitipraz (Le Ferrec et al., 2002), and omeprazole (Quattrochi and Tukey, 1993). AhR is also activated by disturbing cellular adhesion to extracellular matrix (Sadek and Allen-Hoffmann, 1994), increasing intracellular Ca2+ (Riechers et al., 1990), or disturbing cytoskeleton (Scholler et al., 1994). The common factor among all these phenomena of ligand-independent activation of AhR is the increase in the intracellular calcium, providing the second messenger to activate AhR through the effector enzyme, calpain. Further work is required to delineate the exact mechanisms by which calpain activates AhR.

**Fig. 5.** Inhibition of calpain suppresses the TCDD-induced degradation of the AhR. A, triplicate culture plates of MT-2 cells were pre-exposed to 15 μM MDL28170 or the vehicle (DMSO) for 2 h at 37°C followed by 1 nM TCDD or DMSO for additional 3 h. A representative Western blot image of one experiment is presented at the top for illustration. Bars represent the average ± S.D. values from three independent experiments. *, p < 0.05, comparing TCDD-treated cells to DMSO controls. †, p < 0.05, comparing MDL 28170-TCDD-treated cells to DMSO controls. B, MT-2 cell cultures were pretreated with 15 μM MDL28170, 25 μM MG-132, or vehicle (DMSO) for 2 h followed by a 3-h TCDD treatment. †, p < 0.05, comparing TCDD to DMSO. †, p < 0.05, comparing TCDD to MDL28170-TCDD and MG-132-TCDD. C, Western blot analysis of AhR after calpain and proteasome inhibition. Duplicate cultures of MT-2 cells were pretreated with proteasome inhibitor epoxomicin (25 μM) or calpain inhibitor MDL28170 (15 μM) followed by 3 h of TCDD (1 nM) or DMSO. *, p < 0.05, compared with DMSO control. †, p < 0.05, comparing TCDD to MDL28170-epoxomicin-TCDD. The bars represent the average ± S.D. for three independent experiments.
Recent studies have established that AhR, independent of PAH ligands, is directly responsible for inducing cancers of the stomach (Andersson et al., 2002) and pancreas (Koliopanos et al., 2002) and the progression of breast carcinoma (S.E. Eltorn, unpublished data). The involvement of calpain in the activation of AhR identifies calpain as a potential therapeutic target for AhR associated cancers. The advantage of this strategy is that calpain exists in a latent form requiring activation of AhR associated cancers. The advantage of this strategy is that calpain exists in a latent form.

References


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The induction of CYP1A1 by oltipraz is mediated through calcium-dependent-calpain

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Abstract

The induction of CYP1A1 expression by oltipraz, a synthetic chemo-preventive agent, which increases intracellular calcium concentration, has previously been shown to result from transcriptional activation of CYP1A1 gene mediated by the Ah receptor (AhR), although oltipraz does not bind the receptor. The present study investigated the possible mechanisms of oltipraz-induced activation of AhR and the subsequent induction of CYP1A1 transcription. Treatment of the human metastatic breast cancer cell line MT-2 with oltipraz results in a concentration-dependent increase in the activity of the calcium-dependent calpain, as measured towards the BOC-LM-CMAC fluorescent substrate. This increase in calpain activity was coupled with the AhR activation, as evidenced by its nuclear localization and increased transcription of CYP1A1 gene. Treatment of cells with calpain specific inhibitor MDL 28170 completely blocked the oltipraz-induced nuclear translocation of AhR and subsequent CYP1A1 expression. Furthermore, treatment with oltipraz resulted in the classical ligand-dependent down-regulation of AhR protein, in a concentration dependent manner. The presented data established for the first time a mechanism of activating AhR and its transcription of CYP1A1 by oltipraz through activation of calcium-dependent calpain.

Keywords: Ah receptor; CYP1A1; Oltipraz; Intracellular calcium; Calpain

1. Introduction

Cytochrome P450 1A1 (CYP1A1) catalyzes the metabolic activation of polycyclic aromatic hydrocarbons (PAH) resulting in generation of genotoxic metabolites that cause DNA damage and subsequent carcinogenesis (Marston et al., 2001; Shields et al., 1993). CYP1A1 expression is regulated transcriptionally by PAH through the aryl hydrocarbon receptor (AhR), by a signaling pathway that is well characterized (Denison et al., 1988; Jones et al., 1985). PAH binding to the AhR, which is anchored in the cytoplasm by chaperone proteins, leads to the AhR undergoing a transformation step in which the chaperone proteins dissociate. Upon dissociation, the receptor undergoes a conformational change resulting in exposure of the nuclear localization signal (NLS). The NLS is recognized by the α-importin transporter within the nuclear envelope or pore and facilitates nuclear translocation of the AhR (Ikuta et
CYP1A1 gene expression (Le Ferrec et al., 2002), how-
of CYP1A1, identifying calcium as a key regulator of completely abolished the oltipraz-induced expression 2002). Chelating intracellular calcium by BAPTA-AM (H9262)

This ligand-dependent depletion both in vivo and in vitro (Giannone et al., 1999; Harper et al., 1994; Prokipcak and Okey, 1991; Roberts and Whitelaw, 1999). This ligand-dependent down-regulation of AhR have been suggested to be a proteasome-dependent process (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000).

Oltipraz is a synthetic derivative of 1,2-dithiole-3-thione, a constituent of cruciferous vegetables, and it is being developed as a chemopreventive agent (Boone et al., 1990; Liu et al., 1988; Rao et al., 1993; Kensler et al., 1987; Talalay et al., 1995). Oltipraz was reported to induce the transcription of CYP1A1 in rat tissues and in human colorectal cancer cell line Caco-2 cells, without physically binding the AhR (Le Ferrec et al., 2002). Chelating intracellular calcium by BAPTA-AM completely abolished the oltipraz-induced expression of CYP1A1, identifying calcium as a key regulator of CYP1A1 gene expression (Le Ferrec et al., 2002), however, no direct mechanism has been proposed.

On the other hand, in vitro studies have shown the AhR to be a substrate for the calcium-dependent protease calpain (Poland and Glover, 1988). Calpain, which is a family of cytosolic calcium-dependent cysteine proteases, is involved in regulating many cellular processes including proliferation, differentiation, cell motility and metastasis through regulation of signal transduction and cleavage of many target cellular regulatory proteins (Schoenwaelder et al., 1997; Potter et al., 1998; Carragher et al., 2002; Rios-Doria et al., 2004; Harwood et al., 2005). There are several calpain isoforms, however calpain I (μ-calpain), and calpain II (m-calpain) are ubiquitous isoforms, which requires either low or high calcium concentrations for activation, respectively. In this study a hypothesis was tested that activation by oltipraz of the AhR to induce CYP1A1 is mediated through the calcium-activated calpain.

2. Materials and methods

2.1. Materials

Calpain inhibitor III (MDL 28170) was purchased from Calbiochem (San Diego, CA). Tissue culture media and high-grade reagents were purchased from Sigma–Aldrich (St. Louis, MO). Dioxin (TCDD) was purchased through NCI Chemical Carcinogen Repository—Midwest Research Institute (Kansas City, MO). Epidermal growth factor, cholera toxin, fetal bovine serum, bovine pituitary extract, and Trizol reagent were purchased from Invitrogen (Carlsbad, CA). The random primers, Moloney murine leukemia virus (M-MLV) reverse transcriptase, RNAsin, Taq polymerase, deoxyribonucleotide triphosphates (dNTPs), horseradish peroxides (HRP)-conjugated goat anti-mouse immunoglobulin (IgG) and HRP-conjugated goat anti-rabbit IgG were purchased from Promega (Madison, WI). FITC-conjugate donkey anti-rabbit secondary antibody was purchased from Jackson Immunoresearch laboratories (West Grove, PA). The BCA Protein Quantification Kit was purchased from Pierce Chemicals (Rockford, IL). The calpain substrate t-butoxycarbonyl-Leu-Met-7-amino-4-chloromethylaminocoumarin (BOC-LM-CMAC) and Fluo-4-AM were purchased from Molecular Probes (Eugene, OR). Real time PCR kit was purchased from Bio-Rad (Hercules, CA). The rabbit polyclonal anti-AhR (BEAR 3 and BEAR 4) antibodies (Poland et al., 1990; Pollenz et al., 1994) were a kind gift from Dr. Christopher Bradfield, University of Wisconsin (Madison, WI). The Sager MT-2 metastatic cell line was derived from a patient with an infiltrating and intraductal carcinoma medium (Band et al., 1990), and was kindly provided by Dr. Vilma Band from Northwestern University (Chicago, IL). The mouse hepatoma cell line, Hepa-1c1c7 was provided by Dr. Lynn Allen-Hoffmann (University of Wisconsin Madison, WI).

2.2. Cell culture and treatments

The human breast cancer cell line MT-2 cells were cultured in DFCI media containing 1% FBS medium (Band et al., 1990), and the mouse hepatoma cell lines, Hepa-1c1c7 were grown in DMEM-F12 supplemented with 5% heat-inactivated FBS. For experiments, cells were seeded in six-well plates at a density of 5 × 10^5 and grown for 24 h. Cells were pre-incubated with calpain inhibitor MDL 28170 or dimethyl sulfoxide (DMSO) (0.1%) for 1 h and then simultaneously treated with either oltipraz (50 μM) or TCDD (1 nM) for an extra 3 h. The vehicle used for MDL 28170, TCDD and oltipraz was DMSO, at 0.1% final concentration.

2.3. Immunocytochemical staining and fluorescence microscopy

Most of the experiments in this research were done on the MT-2 human breast cancer cell lines. However, due to the high levels of AhR associated with nuclear sites in the metastatic...
MT-2 cells, the experiments investigating the ligand-induced AhR nuclear translocation were performed on Hepa-1c1c7, a murine hepatoma cell line, which has no constitutive nuclear AhR, and is well characterized for studying the AhR signaling. Hepa-1c1c7 cells were seeded at 10^4 on cover slips in six-well plates and allowed to grow for 24 h, then were pre-treated with calpain inhibitor MDL 28170 or DMSO (0.1%) for 1 h and then simultaneously treated with either oltipraz (50 μM) or TCDD (1 nM) for an extra 1 h. Treatment media were removed and cells growing on cover slips were washed in cold PBS and then fixed by incubation in a (1/1) methanol/acetone solution at 4 °C for 30 min and subsequently air-dried and stored frozen at −20 °C air-tight, until immunostaining.

For immunostaining, cells grown on cover slips were first brought to room temperature, rinsed with TBST (10 mM Tris–HCl, 150 M NaCl, 0.05% Tween-20) and transferred to clean six-well plates. The cells on cover slips were incubated at room temperature for 1 h in 4% milk solution in TBST to block non-specific binding. The cover slips were then incubated at room temperature while rocking for 1 h in 1 μg/ml anti-AhR polyclonal (BEAR-4) antibody in 2% milk solution in TBST. Cover slips were then washed three times (15 min each) with TBST. A 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibodies in TBST containing 2% milk was added to the cover slips in reduced light. The cover slips were allowed to incubate with this secondary antibody at room temperature for 1 h. The cover slips were then washed extensively (3× for 15 min each in TBST; 3× for 10 min each in TBS). The cover slips were mounted on glass slides using mounting solution containing DABCO as a fluorescence anti-fading agent.

AhR immunostaining was scored at 200× magnification on the basis of the approximate number of cells with cytoplasmic or nuclear AhR staining relative to the total number of cells in the optical field. The percentages of cells with strong cytoplasmic or nuclear AhR staining were recorded in all optical fields and the mean percentage value was used to characterize the AhR subcellular localization as cytoplasmic or nuclear, respectively. Six consecutive microscope fields were analyzed for each slide. The percentages from different experiments were calculated and the mean of two independent experiments was plotted.

2.4 Preparation of total cell protein lysates and immunoblotting analysis

Following different treatments, cell monolayers growing in six-well plates were lysed in Trizol, which allowed for simultaneous isolation of RNA and protein as described previously (Eltom et al., 1999). The protein pellets were resuspended in 2% SDS and sonicated briefly to dissolve protein pellets and form lysates of total cellular extract. The protein concentration in cell extracts was determined using the BCA kit, per the manufacturer instructions (Pierce, Rockford, IL). Equivalent amounts of protein (10 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Eltom et al., 1999). The membranes were blocked at room temperature for 1 h in 4% non-fat milk in TBST with subsequent incubation with 1 μg/ml anti-AhR (BEAR-3). After brief washing the blots were incubated with the corresponding HRP-coupled anti-rabbit (1:20,000) for 1 h followed by additional washing in TBST and TBS. Reactive protein bands were visualized using enhanced chemiluminescence reagents (Pierce Chemicals, Rockford, IL). Immunoreactive bands of AhR were quantified by AlphaImager 2000 Digital system using Alpha Image Acquisition Analysis Software (Alpha Innotech Corp., San Leonadro, CA). Subsequently, blots were probed with actin monoclonal antibody (1:4000) followed by anti-mouse secondary antibody (1:20,000) for normalization of protein loading. The levels of AhR protein were then divided by the corresponding levels of actin to generate normalized values for the concentration of AhR in each sample.

2.5 Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA isolated from Trizol lysates was used to synthesize first strand cDNA using random hexamer primers and MMLV reverse transcriptase. To avoid genomic DNA contamination during PCR, all PCR primers were designed to overlap more than two exons. Human CYP1A1 PCR amplification was done using forward primer sequence 5′-CTG TCA GAC CAA GCC CCA CCA AGA ACT G-3′ and the reverse primer sequence 5′-AGG TAG CGA AGA ATA GGG ATG AAC TC-3′, which yield a product of 111 bp between exon 5 and 6. The mouse CYP1A1 was amplified using primers (forward primer, 5′-GCC TTC ATT CTG GAG ACC TTC C-3′; reverse primer, 5′-CAA TGG TCT CTC CGA TGC AC-3′) and the reverse primer sequence 5′-TCC ACC ACC CTG TTG CTA-3′ while the housekeeping gene 18S was amplified using 5′-CCG ACA GGA TTG ACA TAG TGA TAG C-3′ forward primer and reverse primer 5′-TGC CAG AGT CTC GTT CGT TAT CG-3′. CYP1A1 was amplified for 30 cycles, and GAPDH and 18S for 20 cycles, each cycle with the following segments: 1 min at 95 °C, 1 min 30 s at 60 °C, and 2 min at 72 °C. PCR products were separated on agarose gels and visualized with ethidium bromide. The bands were quantified using the NIH ImageJ software (Version 1.36) for image analysis.

2.6 Intracellular calcium measurements

MT-2 cells cultured in 24-well plates (2×10^5) in DFCI media were loaded with 5 μM Ca^{2+}-sensitive dye Fluo-4-AM (Molecular Probes, Eugene, Oregon) for 45 min at 37 °C. Following the pre-incubation, the cells were rinsed three times with DFCI media to remove any free dye and then incubated for a further 30 min period in media alone to allow complete
de-esterification of AM esters. Fluo-4-loaded cells were then stimulated with 10 nM TCDD, 50 μM oltipraz, or their vehicle DMSO. Changes in intracellular calcium were measured as captured fluorescence images of cells at 25 min, using the IX50 Olympus Fluorescence microscope (excitation at 385 nm, emission at 512 nm). Relative fluorescence values were quantified using the NIH ImageJ software for image analysis (Version 1.36) and translated into calcium units.

2.7. Calpain activity assay

Calpain activity in intact MT-2 cells was determined by measuring the hydrolysis of the calpain specific peptide substrate BOC-LM-CMAC, which was assessed by fluorescence microscopy as described previously (Glading et al., 2001) with the excitation and emission wavelengths 350 and 430 nm, respectively. The non-fluorescent cell-permeable substrate is conjugated by intracellular thiols into a membrane impermeable form allowing substrate accumulation within the cell (Carragher et al., 2004; Rosser et al., 1993), and proteolytic cleavage of this peptide by calpain produces chloromethyl-laminocoumarin (CMAC) which possesses blue fluorescence. MT-2 cells were treated with either 50 μM or 100 μM oltipraz for 1 h. Cells were then incubated at 37 °C for 20 min in the presence of 50 μM BOC-LM-CMAC. The cells were observed for CMAC fluorescence corresponding to calpain activity with an Olympus IX50 fluorescence microscope. The image exposure settings were identical within each experiment. Fluorescence of the images was quantified by using the NIH ImageJ software (Version 1.36), a Java image-processing program, which analyzes the pixel value of the fluorescent images.

2.8. Statistical analysis

The data generated from different experiments were analyzed using GraphPad Prism Software (San Diego, CA) by one-way analysis of variance ANOVA and Tukey-Kramer multiple comparison tests of control values with treated. Changes were deemed significant if \( p \leq 0.05 \).

3. Results

3.1. Oltipraz-induced calcium mobilization

Previous reports have demonstrated the ability of oltipraz to increase intracellular calcium in human colorectal carcinoma cells (Le Ferrec et al., 2002) in a concentration dependent manner. Therefore, it was important to first determine if oltipraz would affect the intracellular calcium in our study cell system, the metastatic MT-2 human breast cancer cell line. The data presented in Fig. 1. A illustrate the ability of oltipraz to mobilize calcium in a concentration-dependent fashion. The increase in calcium occurs instantaneously after the addition of the drug. Thus, oltipraz rapidly increases intracellular calcium in MT-2 cells to a comparable extent to the effect of Ionomycin, the calcium ionophore.

3.2. Calpain activity analysis following increases in \([Ca^{2+}]_i\)

To further assess the oltipraz-induced change in intracellular calcium on calpain activation a cell-permeable highly specific calpain substrate, BOC-LM-CMAC was utilized. The blue fluorescence of the calpain-cleaved product detected with the Olympus IX50 wide field fluorescence microscope is indicative of calpain activation. Oltipraz exposure for 1 h resulted in a significant increase in calpain activity indicated by the intense blue fluorescence (Fig. 1B) when compared to the DMSO control group, suggesting that oltipraz has the capacity to activate calpain. More importantly, calpain activation seen with oltipraz is concentration dependent. These results suggest that oltipraz is responsible for the activation of calpain through its ability to increase intracellular calcium concentrations.

3.3. Ligand-independent activation of the AhR by oltipraz

3.3.1. Nuclear translocation

Since it was previously shown that oltipraz could activate AhR to induce CYP1A1 expression without binding the AhR, we set to investigate the effect of oltipraz on the signaling pathway of the AhR activation. Following binding of ligand (such as TCDD) to AhR, the receptor is activated and it acquires the ability to translocate from the cytoplasm into nuclear sites where it functions as a transcriptional enhancer for many genes including CYP1A1. Therefore, the ability of oltipraz to facilitate the nuclear translocation was investigated here in comparison to TCDD, the most potent agonist for AhR. The data in Fig. 2 remarkably have shown that oltipraz was capable of evoking nuclear translocation of AhR within 1 h to almost the same extent as TCDD, resulting in nuclear translocation of AhR in approximately 70% of Hepa-1c1c7 cells, compared to 75% in TCDD-treated cells.

To investigate the role of oltipraz-induced calpain activation in this process, the potent calpain-specific inhibitor, MDL 28170 was used to block calpain and the effect of oltipraz was further examined. Pre- and co-treatment of Hepa-1c1c7 cells with MDL 28170 has resulted in a complete inhibition of oltipraz-induced nuclear translocation of AhR (Fig. 2). Similarly, MDL 28170 was capable of blocking TCDD-induced nuclear translocation of AhR, indicating that calpain is probably
Fig. 1. (A) The effect of oltipraz on intracellular free calcium concentration in MT-2 cells. MT-2 cells were pre-loaded with 5 μM Fluo-4-AM for 45 min at 37 °C (as described in Section 2). The Fluo-4 loaded cells were then stimulated with 50 μM or 100 μM oltipraz, 10 μM Ionomycin (positive control), or their vehicle DMSO. Changes in intracellular calcium were measured as captured fluorescence images of cells over a period of 40 min (every 5 min), using the IX50 Olympus Fluorescence microscope (excitation at 385 nm, emission at 512 nm). Relative fluorescence values for the peak calcium concentration at 25 min were quantified from several fields using the ImageJ software, and graphed values are means and standard deviations of three independent experiments. The lower panel is a representative of the images quantified. *p < 0.05, comparing drug treatments to vehicle control.

(B) Activation of calpain in MT-2 cells following exposure to different concentrations of oltipraz. Calpain activity was analyzed in MT-2 cells exposed for 1 h to 50 and 100 mM oltipraz or its vehicle DMSO using peptide substrate BOC-LM-CMAC. Blue fluorescence of the calpain-cleaved substrate, corresponding to calpain activity was captured over time (20 min) by fluorescence microscopy (100 × magnification). The fluorescence intensity of the images were quantified using the ImageJ software from several fields, and plotted values are the mean and S.D. of n = 3 independent experiments. *p < 0.05, comparing oltipraz treatments to vehicle control.
mediating this step for both chemicals, in spite of the difference in their mode of action on the AhR activation.

3.3.2. Enhanced transcription of CYP1A1

To assess the ability of oltipraz to increase the transcriptional activity of AhR, the expression of CYP1A1 mRNA as an endpoint was analyzed by RT-PCR. Data in Fig. 3 demonstrate that treatment of MT-2 cells with oltipraz for 6 h has resulted in a concentration dependent induction of CYP1A1 mRNA, in agreement with the previous reports (Le Ferrec et al., 2002).

3.4. Inhibition of oltipraz-induced CYP1A1 expression by the calpain inhibitor, MDL 28170

3.4.1. In the metastatic MT-2 human breast carcinoma cell line

To investigate whether calpain is involved in the oltipraz-induced expression of CYP1A1 in MT-2 cells, they were pre- and co-treated with the calpain potent inhibitor, MDL 28170 then challenged with oltipraz for 3 h. The data collected from multiple experiments on this cell line have clearly established that although...
oltipraz alone has induced CYP1A1 substantially over the vehicle-treated control, the co-treatment with the calpain inhibitor completely blocked this induction and brought the CYP1A1 to the DMSO-treated control levels (Fig. 4).

3.4.2. In Hepa-1c1c7 murine hepatoma cell line

To eliminate the possibility that this phenomenon is cell-specific, the same experiment was repeated using the mouse hepatoma cell line Hepa-1c1c7. Similarly, oltipraz alone has induced CYP1A1 substantially over the DMSO-treated control to a similar extent as TCDD, the positive control, and co-treatment with the calpain inhibitor MDL 28170 completely blocked this induction (Fig. 5).

3.5. Down-regulation of the AhR by oltipraz

In the well-established AhR signaling cascade, binding of a ligand to AhR will activate it to translocate to the nucleus, where it dimerizes with its partner ARNT to bind XRE of the enhancer region of AhR-responsive genes, such as CYP1A1 gene and enhances their transcription. Following the transcriptional activation, the AhR undergoes a massive depletion (Prokipcak and

Fig. 3. RT-PCR analysis of the CYP1A1 expression following treatment of MT-2 cells with increasing doses of oltipraz. MT-2 cells were treated with 25, 50 and 100 μM oltipraz for 6 h. RNA was isolated from the treated cells, cDNA was prepared by reverse transcriptase reaction (RT) and CYP1A1 mRNA expression was measured using conventional PCR. Ribosomal 18S was amplified as an internal control and their values were used to normalize CYP1A1 values. The bars represent the average ± S.D. for three independent experiments assayed in duplicates. A *p < 0.05, relative to DMSO control group. A representative of a gel of one PCR analysis is presented as an illustration in the top panel.

Fig. 4. Inhibition of oltipraz-induced CYP1A1 expression by MDL 28170, Calpain inhibitor in MT-2, human breast cancer cell line. MT-2 cells were pre-treated with calpain inhibitor, MDL 28170 (30 μM) or its vehicle DMSO for 1 h, then exposed for 3 h to 50 μM oltipraz, 1 nM TCDD (a positive control) or their vehicle DMSO. RNA was isolated from the treated cells and analyzed by RT-PCR as described in Section 2. GAPDH was amplified as an internal control and its value was used to normalize CYP1A1 values. The bars represent the average ± S.D. for three independent experiments of duplicate cultures, which were PCR-assayed in duplicates. A *p < 0.05, relative to DMSO control group. A representative gel image of one PCR analysis is presented in the lower for illustration.
Oltipraz has been developed as a chemopreventive agent for many malignancies, including liver and colorectal cancers, on the basis of its in vivo protective activity against chemically induced tumors, in a variety of animal models. This protection has been associated with an enhanced capacity to detoxify reactive carcinogens and, more recently, with increased DNA repair (Kensler et al., 1987; Talalay et al., 1995; O’Dwyer et al., 1997). The ability of oltipraz to induce both phase I and phase II drug metabolizing enzymes has been reported, however CYP1A1 seems to be among the most sensitive of all these enzymes. While most enzymes in Phase II group are detoxifying enzymes, which underlies the protective effect of oltipraz, CYP1A1 is involved in bioactivation of a number of carcinogens, including PAH and endogenous hormones such as estrogen.

Previous reports and our present study have established that oltipraz induces CYP1A1 transcription in an AhR- and calcium-dependent fashion (Le Ferrec et al., 2002). In this report we present evidence that calcium-dependent calpain is involved in mediating this induction by coupling the oltipraz-induced elevation of intracellular calcium and the activation of AhR. Although the increase in the intracellular calcium by oltipraz is the trigger that was identified previously to cause the activation of AhR to induce CYP1A1, the steps leading to the activation were not identified. It was proposed that \([Ca^{2+}]_i\) could mediate such a response by activating protein kinase C or other kinases to activate the AhR,
Fig. 6. Stimulation of down-regulation of the AhR protein by treatment with increasing doses of oltipraz. (A) MT-2 cells were exposed to varying concentrations of oltipraz for 6 h and total cellular proteins were analyzed for AhR protein levels. The relative expression of AhR was quantified by densitometric quantitation of AhR band and normalized to the level of actin. The bars represent the average ± S.D. for three independent experiments. (B) Western blot analysis of AhR protein levels in MT-2 cells exposed to TCDD. Triplicate cultures of MT-2 cells were exposed to either TCDD (1 nM) or its vehicle (DMSO) for 3 h. The relative expression of AhR was quantified by densitometric quantitation of AhR band and normalized to the level of actin. The bars represent the averages for two independent experiments. *p < 0.05, comparing treatment groups to DMSO control group.

however, previous studies have demonstrated that AhR is constitutively phosphorylated and no additional phosphorylation was observed upon its activation (Berghard et al., 1990; Pongratz et al., 1992).

The present study provides evidence that activation of calpain by oltipraz contribute to each of the sequential steps of the AhR signaling pathway leading to its transcriptional activation of CYP1A1. Previous studies have already established that oltipraz does not bind to the AhR, however, the next step following the ligand binding is the receptor transformation, which is the acquisition by the receptor of the ability to translocate from the cytoplasm to the nucleus and bind DNA and enhance transcription. Data presented here, clearly demonstrate that oltipraz is capable of inducing AhR nuclear translocation, through activation of calpain by means of increasing the intracellular calcium. It is given that the accumulation of AhR in the nucleus requires the tight binding of AhR (and its partner ARNT) to the XRE of the responsive genes.

Subsequently, oltipraz treatment has induced the expression of CYP1A1 within 3 h, and in a concentration-dependent manner, with concentrations ranging from 25 to 100 µM. Remarkably, blocking of calpain by MDL 28170 has completely abolished this induction in both the human breast cancer cell line and the murine hepatoma cell line, indicating that this phenomenon is not cell type- or species-specific.

In the final step in the AhR signaling pathway, following the transcriptional activation, the AhR exits the nucleus and undergoes a massive depletion within hours of ligand binding (Giannone et al., 1998; Harper et al., 1994; Prokipcak and Okey, 1991; Pollenz, 1996; Roberts and Whitelaw, 1999). The data presented in this report indicate that oltipraz through activation of calpain does indeed down-regulate AhR in a concentration-dependent fashion, and in a comparable magnitude to the most potent AhR agonist, TCDD.

Collectively, the data presented in this report provide evidence for the first time for involvement of calpain in mediating the oltipraz-induced expression of CYP1A1 and in activating AhR signaling pathways.

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Overexpression of the Aryl Hydrocarbon Receptor Induces Malignant Transformation in Mammary Epithelial Cells

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Abstract

Introduction

The aryl hydrocarbon receptor (AhR), which mediates toxic and carcinogenic responses to environmental polyaromatic hydrocarbons (PAH), is a ligand activated basic helix-loop-helix transcription factor. The AhR has been shown to interact with a number of proteins known to regulate the cell cycle, which provides further implications for its role in cancer. There is ample documentation of the role of AhR in PAH-induced carcinogenicity. However, in this report we addressed whether overexpression of AhR alone is sufficient to induce carcinogenic transformation in human mammary epithelial cells (HMEC).

Methods

Retroviral expression vectors were used to develop a series of stable cell lines expressing varying levels of AhR protein in an immortalized normal HMEC with relatively low endogenous AhR expression. Each cell line was characterized for AhR expression and transcriptional activity as well as phenotypic changes associated with malignancy. The increase in AhR activity and expression was correlated with the development of malignant phenotypes.

Results

Clones overexpressing AhR by 3-fold manifested a 50% decrease in population doubling time compared to vector-control cells. Cell cycle analysis revealed that this enhancement in proliferation was mainly due to an increase in the percentage of cells transiting from G0/G1 to S- and G2/M phases. Overexpression of AhR enhanced the motility of HMEC and increased their migration by ~60% compared to their vector-expressing clones. More importantly, these cells acquired the ability to invade matrigel matrix, where more than 80% of plated cells invaded the matrix and crossed the membranes within 24 h, whereas none of parental or the vector control HMEC was able to invade.
matrigel. These malignant phenotypes were also coupled with the ability of the clones to form colonies in soft agar.

**Conclusion**

Collectively, these data provide the first evidence for a direct role of AhR in the progression of breast carcinoma. The results suggest a novel therapeutic target that should be considered for treatment and prevention of progression of this disease.
**Introduction**

The aryl hydrocarbon receptor (AhR) is a basic helix-loop-helix (bHLH) transcription factor [1] that is well characterized for mediating the carcinogenic responses to environmental polyaromatic hydrocarbons (PAH), such as 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD) [2, 3]. Binding of PAH to AhR in the cytoplasm leads to its activation and subsequent translocation into the nucleus, where it dimerizes with another bHLH protein, the AhR nuclear translocation protein (ARNT). This heterodimer binds to xenobiotic responsive elements (XRE) in the enhancer region of PAH-responsive genes such as cytochrome P450 1A1, *CYP1A1*, and enhances their transcription [4-6]. No physiological function has been established for any of the identified endogenous ligands of AhR [7, 8]. However constitutive activation of AhR via disturbing cellular adhesion to extracellular matrix [9], increasing intracellular calcium [10] or disturbing cytoskeleton [11] has suggested that pathophysiologically-activated pathways are operating to regulate the receptor.

Exposure to TCDD in animals induces teratogenesis, immuno-suppression, reproductive defects and tumor promotion in an AhR-dependent manner [12-16]. TCDD-dependent activation of AhR has also been linked to enhanced-terminal differentiation in keratinocytes and adipocyte [17, 18]. Moreover, AhR null mice generated by two independent laboratories exhibit a spectrum of hepatic and immune system defects [19, 20] and are resistant to benzo[a]pyrene-induced carcinogenicity [21], providing evidence to link AhR to normal development and tissue homeostasis.

In cultured cells activation of AhR by TCDD inhibits cell cycle progression arresting cells at the G1/S checkpoint through induction of cyclin-dependent kinase inhibitor p27kip1 [22]. This effect on cell cycle progression relies on a direct protein-protein interaction between AhR and retinoblastoma (Rb) through two LXCXE domains on the AhR [23]. This association with Rb was shown to require
AhR activation and nuclear translocation [24]. Interestingly, the AhR in the absence of a ligand was shown to influence the cell cycle progression, cell shape and differentiation [25, 26].

A series of previous findings prompted the present studies. First, TCDD acting through AhR has been shown to exert a strong anti-mitogenic effect in estrogen-responsive tissues and to exhibit a broad spectrum of anti-estrogenic activities in human breast carcinoma cell lines [27-29]. Second, the AhR null mouse exhibits impaired development of mammary gland ductal branching [30], suggesting a role for AhR in regulating proliferative stages required for mammary gland development. Our own data have demonstrated that the expression of the AhR is elevated in direct proportion to the tumor progression in both human breast carcinoma cell lines and breast tumors [31]. This study was undertaken to investigate whether induced overexpression of AhR could be both necessary and sufficient for development of transformed phenotypes in human breast epithelial cells.

**Material and Methods**

**Cells and culture conditions**

The H16N2 immortalized human mammary epithelial cell line and the metastatic MT2 breast cancer cell line were kindly provided by Dr. Vilma Band (Northwestern University, Chicago, IL). MT2 cells, H16N2 cells and the subsequently generated clones were grown in DFCI-1 media as described [32] and were maintained in culture at 37°C and 5% CO₂.

**Recombinant retroviral expression vector and virus production**

The human AhR cDNA was provided by Dr. Chris Bradfield (McArdle Laboratory for Cancer Research, Madison, WI). Bgl II (5’) and Cla I (3’) restriction sites were added to the full length AhR by PCR to generate a fragment with compatible cloning sites for ligation into the pLNCX2 retroviral vector (Retro-X-System from BD Biosciences), under control of the human cytomegalovirus early promoter (pCMV). This vector also contains a neomycin resistance gene under the viral LTR
promoter to allow for selection in mammalian cells. The sequences of retroviral vectors containing the human AhR cDNA (ret-AhR) or the corresponding empty vector control (EV), were confirmed by DNA sequencing at the Vanderbilt-Ingram Cancer Center Molecular Biology Core Facility (Nashville, TN). The two vectors were transfected independently into the Phoenix packaging cell line by calcium phosphate method to produce infectious viral particles, following the manufacturer’s instructions. Equal amounts of the pBMN-I-GFP retro vector DNA were co-transfected with each vector to provide a visible screenable marker to assess the transfection efficiency. The titer of the viral stock was assayed using mouse embryo fibroblasts (MEF) generated in this laboratory from AhR-null mice.

Development of stable cell lines

H16N2 cells were plated at 2x10^6 per 6-cm plate and allowed to incubate overnight. Viral supernatants were mixed with culture media and added to the H16N2 cells in the presence of polybrene (4μg/ml) with gentle and thorough mixing. Cells were incubated at 37°C for 12-18 hours, viral medium was then removed and fresh medium was added to cells for an extra 24-48h, when the fluorescence of GFP was observed under fluorescence microscope.

Selection, cloning and expansion of clones:

H16N2 stable transfectants harboring the retro-AhR or EV control vector were isolated by G418 antibiotic selection. The optimal antibiotic concentration was determined as 800 μg G418/ml of medium and was added to the cells for one week, with fresh medium and antibiotic replenished every two days. Surviving cells were expanded and cloned by limited dilution. Briefly, cells were plated at 100 cells per 150mm plate surface. The position of individual attached cells was pinpointed on the under side of the culture dish using a marker. The cells were allowed to grow and expand for one week. Once the individual cells divided to form small colonies, sterile cloning discs pre-soaked in
Appendix 9

trypsin-EDTA were placed on each isolated colony. Cells from each disc were placed in an individual well of a 24-well plate and allowed to reach confluence. Each clone was then expanded and characterized for AhR protein expression by Western immunoblotting. Five clones, with varying AhR expression, and a clone of EV-expressing cell lines were selected for subsequent characterization.

**Western Immunoblotting**

Total proteins were isolated from Trizol (Invitrogen, Carlsbad, CA) lysates of cells per manufacturer’s instructions and protein concentration was determined using BCA protein assay (Pierce, Rockford, IL). Proteins were separated on 7.5% SDS-PAGE and transferred to PVDF membranes. Membranes were first stained with Ponceau S (Sigma Chemicals, St Louis MO) to visualize protein bands to ensure equal protein transfer. Membranes were blocked with 4% milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 hour. Membranes were then incubated with affinity-purified rabbit anti-AhR polyclonal antibody (BEAR-3) from Dr. Chris Bradfield (McArdle Laboratory for Cancer Research, Madison, WI) in 2% milk in TBST (1 µg/ml) for 1 hour. Membranes were rinsed 3x in TBST and followed by incubation for 1 hour in 1:20,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG in 2% milk solution in TBST (Promega, Madison, WI). Immunoreactive proteins were visualized using chemiluminescence reagents (Pierce Chemicals, Rockford, IL). To determine the relative expression of AhR protein among samples, band density was quantified by AlphaImager 2000 Digital system using Alpha Image acquisition analysis software (Alpha Innotech Corp, San Leonardo, CA). Subsequently, blots were re-probed with Actin monoclonal antibodies (1:4000) to normalize for protein loading.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

Following cell lysis with Trizol, total RNA was isolated according to manufacturer’s protocol. The cDNA was prepared from 2 µg of RNA using random hexamer primers and Moloney murine leukemia virus reverse
transcriptase. CYP1A1 PCR amplification was done using forward primer sequence 5’TAG ACA CTG ATC TGG CTG CAG3’ and the reverse primer sequence 5’GGG AAG GCT CCA TCA GCA TC3’. Glyceraldehyde 3-phosphate dehydrogenase, amplified using forward primer 5’ACC ACA GTC CAT GCC ATC AC3’ and reverse primer 5’TCC ACC ACC CTG TTG CTG TA3’, was used as an internal control.

**Immunocytochemical staining and fluorescence microscopy**

Cells growing on glass coverslips in 6-well plates were washed in cold PBS and fixed by incubation in a 1:1 methanol: acetone solution at 4°C for 30 minutes and then air dried. Fixed cells on coverslips were used for either staining immediately or stored airtight at –20°C until staining.

For immuno-staining, cells were rinsed and hydrated with TBST and transferred to a clean 6-well plate. The cells were incubated at room temperature for 1 hour in 4% milk solution in TBST to block nonspecific binding, followed by incubation at room temperature for 1 hour with affinity-purified rabbit anti-AhR polyclonal antibody (BEAR-4) in 2% milk solution in TBST (1 μg/ml) while rocking. Cells were then washed three times (15 min each) with TBST. Cells were incubated with a 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibodies (Jackson Immunoresearch laboratories, West Grove, PA) in 2% milk at room temperature for 1 hour. The cells were then washed extensively (4x for 15 min each in TBST, 3x for 10 min each in TBS, 2x for 10 min each in ddH₂O) and mounted on glass slides using PBS-glycerol supplemented with anti-fading agent (DABCO).

**Cell proliferation studies**

Proliferative capacity of the clones was determined using the FluoReporter Blue Fluorometric dsDNA Quantitation kit from Molecular Probes (Eugene, OR). This method measures cellular proliferation based on quantitation of cellular DNA using the blue-fluorescent Hoechst 33258 nucleic acid stain, with all manipulations carried out in 96-well plates. Serial dilutions of cells in 100ul
culture media were plated in microplate wells, ranging from 1000 to 100,000 cells per well. The plates were allowed to incubate at 37°C for 3, 12, 24, 48, and 72 hours at which times the medium was removed and the plates were placed at −80°C. The plates were allowed to thaw and 100ul of distilled water was added to each well and the plate was incubated at 37°C for 1 hour. The plate was then placed at −80°C until frozen and then thawed to room temperature. A 100ul aliquot of aqueous Hoechst 33258 was added to each well. Fluorescence of Hoechst 33258 dye was measured on a Cytoflour using excitation and emission filters 360nm and 460nm, respectively.

**Cell cycle analysis**

Cells were trypsinized and single cell suspensions were fixed in cold 70% ethanol. Fixed cells were stored at −20°C until staining. Cells were harvested by centrifugation at 700 rpm for 5 minutes and resuspended in PBS. The cells were checked microscopically to ensure no clumps persisted. If clumps were observed, cells were passed 3-5 times through a 25-gauge syringe needle. RNase in PBS (0.1mg/ml) and propidium iodide (40 μg/ml) was added to the suspension. The cells were incubated at 37°C for 30 minutes. The fluorescent cells were then analyzed in a FACS Caliber BenchTop Analyzer (Becton-Dickson). The percentage of cell cycle distribution was determined using ModFit analysis software.

**Invasion and migration studies**

The invasive potential of the generated clones was measured by using a fluorescence-based tumor cell invasion assay (FluoroBlok invasion assay kits, BD Biosciences, Franklin Lakes, NJ). The assay has coupled a multi-well insert device containing a micro-porous membrane with a BD Matrigel coating process. The micro-porous membrane allows separation of fluorescence readings of the top and bottom compartment of the chamber. The BD Matrigel coat functions as a barrier to the passage of non-invasive cells analogous to the *in vivo* extracellular basement membrane.
Appendix 9

Cell suspensions were prepared by trypsinizing cell monolayers and resuspending the cells in serum-free medium at $5 \times 10^4$ cells/ml. Medium (750ul) containing 5% fetal calf serum was added to the bottom of each well as a chemo-attractant. A 500ul aliquot of the cell suspension ($2.5 \times 10^4$ cells) was added to the top chamber. The cells were incubated at 37ºC for 24 hours. Following incubation, the medium from the top chamber was carefully removed by aspiration and the insert was transferred to a second plate containing 0.5ml/well of 4µg/ml Calcein AM (Molecular Probes, Eugene, OR). The plates were allowed to incubate for 1 hour at 37ºC. Calcein fluorescence of the invaded cells was read from the bottom at excitation and emission wavelength of 485 and 530 nm, respectively. The migration measurements were performed similar to the invasion assay procedure, with the exception that inserts without a matrigel coating were used, and migration was measured at three different cell densities.

Cloning in soft agar

A 0.6% agar underlay was prepared by combining an equal volume of 2X DFCI medium and 1.2% agar. The 0.6% agar was added to the bottom of 6-well culture plates and allowed to gel at room temperature. Cell suspensions of $1 \times 10^4$ cells/ml and $3.5 \times 10^3$ cells/ml were prepared and 20ul of each dilution were added to 2ml of 0.3% agar medium. The agar and cell suspensions were mixed and 1ml of each mixture was added to the respective wells. The solution was allowed to gel at room temperature. The culture plates were then stored in a 37ºC incubator for 2 weeks and were fed every three days. Cells were plated in triplicate at each cell density.

Statistical analysis:
Appendix 9

A two-tailed Student’s t-test was used to compare the mean values of cell doubling times, migration, invasion or soft agar colonization between empty vector (EV)-carrying control cells and different clones overexpressing the AhR. A $p$-value of 0.05 or less was deemed significant.

**Results**

**Overexpression of AhR is paralleled by transformation to a fibroblastic morphology**

We developed five independent clonal cell lines over-expressing the AhR to varying degrees when compared to the endogenous level in the parental H16 HMEC. The AhR expression levels in these clones range from a 30% to an ~20-fold increase compared to the vector control (Figure 1). Two of these clones (clones A & E) exhibited a remarkable change in their morphology, with a loss of their typical epithelial shape and adoption of a more elongated fibroblastic-like pattern (Figure 2). Western blot analysis (Figure 1) revealed that clone A and clone E also have the highest increase in AhR expression. Interestingly these clones also expressed the highest levels of vimentin, which is a fibroblast-specific marker (Figure 2).

**Increased AhR expression is paralleled by enhanced nuclear translocation and transcriptional activity**

Since Cytochrome P4501A1 (CYP1A1) expression requires an activated nuclear AhR and the expression of CYP1A1 is a direct measurement of AhR transcriptional activity, we utilized nuclear translocation of AhR and AhR-dependent CYP1A1 expression as two independent responses of AhR functional activity. Immunocytochemical staining revealed that substantial levels of AhR are localized in the nucleus in the absence of ligand treatment when AhR is overexpressed (Figure 3B). Similarly, clones overexpressing AhR by ~ 20-fold (clones A, D, E), showed substantial levels of CYP1A1 mRNA in the absence of ligand treatment (Figure 3A). These findings are consistent with ligand-independent AhR nuclear localization, also observed in these clones, thus explaining the
Appendix 9

constitutive activity of AhR in inducing CYP1A1 expression. Not surprisingly, TCDD treatment
induced CYP1A1 expression by only two-fold in these clones, despite the 20-fold higher AhR levels.
This minimal induction is expected since basal levels of CYP1A1 have been so dramatically elevated
by AhR overexpression alone.

AhR overexpression increases transition to S- and G2/M phases of cell cycle
To determine the impact of AhR overexpression on the cell cycle, we evaluated the cell cycle
distribution of clonal cell lines. Figure 4A provides a comparison of control cells (expressing empty
vector, EV) with clone A cell line, which expresses 20-fold higher AhR level. While 80% of the cells
remained in G0/G1 phase of the cell cycle in the EV control cells, only about 30% remained in
G0/G1 in clones that overexpressed AhR (Fig 4.A). Panel B reports the findings for all clonal cell
lines.

AhR overexpression correlates with reduced cell doubling time
In order to determine what effect the AhR-induced abrogation of cell cycle transition might have on
overall cellular proliferation, doubling times for each clone compared to the EV control were
determined. Only the clones with the highest AhR overexpression (clones A, D, and E) exhibited a
statistically significant decrease in doubling time. These clonal cell lines had a calculated doubling
time of about 25 hours based on nonlinear regression analysis of Hoechst 33258 staining data for
DNA content (see Methods). In contrast the proliferation rates of clonal cell lines with no (clone C)
or modest (clone B) increases in AhR expression, had a doubling time that was not significantly
different from the EV-control, i.e. a calculated doubling time of about 50 hours (Figure 5).

Increased AhR expression promotes mammary epithelial cell migration and invasion
Transformed cells possess a number of functional changes that distinguish them from untransformed
cells. In addition to increased proliferation and altered cell cycle regulation, transformed cells also
Appendix 9

could acquire the ability to invade the extracellular matrix, in part due to their enhanced migration and proteolytic activities. In order to assess these characteristics of transformation, we measured the invasive potential of each clonal cell line by determining its ability to invade matrigel matrix in vitro. Matrigel, by occluding the pores of a semi-porous membrane that bisects a cell culture well, provides a barrier that is sufficient to preclude non-invasive cells from moving from the upper to lower cell culture compartment. In contrast, invasive cells have the ability to degrade the matrigel and migrate to the underside of the membrane. As shown in figure 6, clones A, D & E manifest invasive potential that is comparable to the metastatic MT2 breast cancer cell line, which is included as a positive control (Fig 6A). Thus, approximately eighty to ninety percent of the cells from clonal lines A, D or E were invasive. Microscopic images of the underside of the membrane correlate with the percentage of invasive cells calculated (Figure 6B).

Preliminary studies using cells overexpressing AhR prior to their subcloning to individual clonal cell lines revealed that the degree of cellular migration also correlates with overall AhR expression (Figure 7) and -as expected, has a characteristic dependence on cell density. Cells overexpressing AhR exhibited an average of 1.5-fold enhanced migration compared to their empty vector-carrying controls, indicating that AhR significantly (p <0.05) increased the migration ability of H16 mammary epithelial cell lines (Figure 7).

**Increased AhR expression correlates with anchorage-independent growth**

In order to assess the ability of the clonal cell lines to grow detached from the surface, we grew the clones in soft agar for 2 weeks and determined the extent of colony formation microscopically. As shown in Figure 8, clonal lines A, D, and E formed colonies comparable in size and number to those colonies formed by the metastatic MT2 cell line. In addition, each formed 20-25 colonies per field
Appendix 9

compared to approximately 5 visibly single viable cells in each field for clones expressing no (clone C) or small (clone B) enhancement of AhR expression (figure 8).

**Discussion**

In this study we provide the first evidence that the AhR is capable of inducing tumorigenic transformation in immortalized human mammary epithelial cells. Classically, transformation is associated with genetic instability and three major classes of phenotypic changes: immortalization, aberrant growth control, and malignancy. While the parental cell line used in our studies was previously immortalized, increased AhR expression in this cell line was both necessary and sufficient to induce aberrant growth and malignant phenotypes, such as increased proliferation, changes in cell cycle regulation, enhanced migration, invasion of matrigel matrix and anchorage-independent growth.

Immortalization does not automatically result in a loss of growth control, since our cell cycle analysis showed that the immortalized parent human mammary epithelial cells (H16 or the empty vector-carrying control EV) are under stringent G1→S transition control. However, increased AhR expression appeared to release these mammary epithelial cells from the G1→S block towards a much dysregulated cell cycle leading to an accumulation of cells in the S and G2/M phases. Interestingly, the low to moderate increases in AhR expression (clones B and C) have resulted only in accumulation of cells in the S-phase, and only high AhR expressing clones (clones A, D and E) managed to transition S-phase and accumulate in the G2/M phase, suggesting an apparent requirement of a threshold level of AhR expression for S-phase transition. The failure of clones B and C to transition through S-phase was reflected in their growth rate, where their doubling times were not different from the parent cells. It is plausible that overexpressed-AhR have mediated transcriptional upregulation of the required factors for the S-phase transition, such as cyclin D/Cdk4.
and Cdk6 and cyclin E/Cdk2, resulting in hyper-phosphorylation of Rb and the subsequent release of E2F transcription factor to induce the transcription of S-phase genes. In support of such a proposal is a recent report that depletion of AhR by siRNA has resulted in a significant decrease in expression of cyclin D1, cyclin E, Cdk4, Cdk6 and Cdk2 in HepG2 cells [33], contributing to the growth inhibitory effect of AhR depletion on these cells.

The data we are reporting here on the effects of AhR in transforming HMEC do not involve its ligand-dependent activation, nevertheless the high expression of AhR in clones A, D and E was accompanied by constitutive activation of AhR as evident by its nuclear localization and transcriptional activation of CYP1A1 in absence of ligand treatment. It is not currently clear what mechanisms govern this ligand-independent activation and nuclear accumulation process, however it is conceivable that the activated nuclear species of AhR account for the difference in response between these high AhR-expressing clones and the other two clones with lower AhR expression. On the other hand, the AhR activation and nuclear localization [24] is required for the association between Rb and AhR, which is deemed crucial in the cell cycle arrest induced by the AhR agonist, TCDD [23]. To reconcile these two contradicting roles for the activated AhR in promoting cell cycle arrest and enhancing progression, two models were proposed. In one model, AhR-Rb interaction functions to repress E2F activity causing G1/S arrest, and the in other model AhR-Rb interaction acts as a transcriptional co-activator for genes encoding G1/S regulatory proteins [34]. This would entail a difference between the ligand-activated and ligand-independent-activated nuclear AhR species, where the interaction of the former with Rb will behave as a repressor and the complex of the latter with Rb will function as a co-activator. Alternatively, AhR has also been shown to directly interact with the transcription factor NF-κB, which is known to regulate many genes involved in proliferation, transformation, and apoptosis. In human breast epithelia and breast cancer cell lines,
there is a constitutive physical interaction between NF-κB and AhR leading to enhanced transcription of c-myc oncogene [35]. This enhanced transcription thereby might lead to increased proliferation and transformation.

Our data are important extension of previous studies that have explored the impact of relative AhR density on cellular growth [25, 26]. For instance, a lung cancer cell line (A549) manipulated to overexpress AhR grew at a faster rate in proportion to the extent of AhR expression [26]. Conversely, Hepa-1 variant cells that express lower amount of AhR when compared to wild-type Hepa-1 cells, exhibited a delayed cell growth and longer doubling time [25]. Our data are consistent with these findings, although they are on tumor cells, as they affirm that AhR expression level correlates with enhanced cellular proliferation. In addition, we have shown that AhR overexpression alone is sufficient to induce other malignant phenotypes in addition to enhanced cellular proliferation.

Invasion potential is a good indicator for malignancy, and our invasion experiments showed that overexpression of AhR by 20-fold was capable of inducing invasiveness in immortalized HMEC, to a level comparable to the metastatic cell line. Clones overexpressing AhR but to a lesser level failed to invade the matrigel matrix in vitro, suggesting that there may be a threshold for AhR expression level required for this phenotype, as well. Noteworthy, TCDD, an AhR potent agonist which activates and subsequently down-regulates the receptor [36], was reported to inhibit the invasion activity of a metastatic breast cancer cell line [37]. The ability of AhR to induce the invasiveness in HMEC might be due to previously reported affects on matrix metallo-proteinases (MMPs) and urokinase-plasminogen activator proteases (uPA) systems [38-42].

The most striking effect of the ectopic overexpression of AhR in these HMEC was the transformation of two of the clones from their typical epithelial shape into fibroblastic-like morphology. This epithelial-fibroblast transformation was accompanied by profuse expression of the fibroblast-
associated intermediate cytoskeleton protein, vimentin. This epithelial-fibroblast transformation resembles the epithelial-mesenchymal transition (EMT), one of the hallmarks of cancer progression. Transforming growth factor beta (TGF-β) is a major player in EMT, and it is well-documented that AhR has a direct effect on TGF-β expression [38, 41, 43-46].

Classically this receptor was viewed in the context of it role in mediating the toxic and biochemical responses to environmental PAH [3, 47]. The contribution of AhR to PAH-induced carcinogenesis is well characterized through its role in the transcriptional activation of a battery of genes including CYP1A1 and CYP1A2 [48]. The protein products of these genes catalyze the bioactivation of some endogenous and exogenous chemicals into reactive metabolites that form DNA adducts thus contributing to the initiation of pre-neoplastic lesions [49]. Our research investigated and provided evidence for a role for AhR in the progression of breast carcinoma. This novel role for AhR is independent of ligand activation, therefore this work is distinct from, although it complements the studies using constitutively active AhR [50]. These studies report that transgenic mice expressing constitutively active AhR exhibited severe tumors of the glandular part of the stomach [50] and acquired higher susceptibility to development of hepatocarcinoma in response to treatment with a liver carcinogen [51]. Although no ligand for AhR was involved, the constitutively active AhR was generated by deletion of the ligand-binding domain to mimick the agonist-activated receptor [52], therefore it is likely that the pathway involved is via the transcriptional induction of CYPs and bioactivation with the subsequent cancer initiation. Our study, on the other hand addressed the basic question of whether AhR itself has the potential of acting as a proto-oncogene and as a factor capable of advancing the progression of breast carcinoma and our data have provided some support for this claim.

**Conclusion**
Although the precise molecular mechanism requires further investigation, the present data suggest that AhR has an oncogenic potential and contributes to the progression of breast cancer from precancerous cells to malignant invasive carcinoma. Such a property might not be unique for the breast and might accompany tumor progression in other target organ/system. Thus the data identify AhR as a relevant therapeutic target for cancer treatment.

**Abbreviations**

AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocation protein; CYP1A1, cytochrome P450 1A1; CYP1B1, cytochrome P450 1B1; bHLH, basic helix-loop-helix; HMEC, human mammary epithelial cell line; PAH, polyaromatic hydrocarbons; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; FACS, fluorescence activated cell sorter; Rb, retinoblastoma; cdk, cyclin-dependent kinase; EV, empty vector.

**Competing interest**

The authors declare that they have no competing interest.

**Authors’ contributions**

SEE conceived the study. JB and SEE designed the study. JB performed collection of data and statistics. JB and SEE drafted the manuscript. All authors read and approved the final version of manuscript.

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FIGURE LEGENDS

FIGURE 1.

Analysis of AhR protein expression in AhR-transformed H16N2 clones compared to their empty vector (EV) control.

A. Western immunoblotting analysis. Five clones (A-E) with varying levels of AhR-expression and a clone expressing only the empty vector (EV) were isolated by limiting dilution from G418-selected transformed H16N2 cell lines. Ten μg of protein from each clone was analyzed by Western immunoblotting. Upper panel is a representative blot and lower panel is the densitometric quantitation of AhR protein (apparent molecular mass of 108 kDa) in the clones. Values in the graph are means and standard deviations of n=3 independent cultures of different analyses. Values from different experimental analyses were each normalized internally to the respective EV control before the mean and SD was calculated.

B. Immunocytochemical staining of AhR protein expression in H16 clones. Clones were grown on coverslips and fixed with methanol: acetone. AhR was visualized by staining with rabbit anti-AhR polyclonal antibodies followed by a rhodamine-conjugated donkey anti-rabbit. Images were captured on an Olympus fluorescence microscope (200x magnification). Lower panels are the phase contrast images of the cells.

FIGURE 2.

Immunocytochemical staining for vimentin in clones of H16 mammary epithelial cells

A. Western immunoblotting analysis

Ten μg of protein from each clonal cell line was analyzed by Western blotting. Illustrated is a representative blot of vimentin protein expression (apparent molecular mass of 58 kDa) in the clones.

B. Immunocytochemical staining for Vimentin expression in H16 clones
Clonal cell lines were grown on coverslips and fixed with methanol: acetone. Cells were immunostained for vimentin by incubating with a 1:50 dilution of mouse anti-vimentin monoclonal antibodies (clone V9, from Sigma Chemicals) followed by FITC-conjugated donkey anti-mouse antibodies. The nuclei were counter-stained with DAPI fluorescence dye. Cell images were captured by an Olympus fluorescence microscope (1000x magnification), and images from DAPI and FITC-fluorescence channels were merged. As a positive control, human mammary fibroblasts (HMF) were stained. Clone A cell line was stained as a negative control by eliminating primary antibody.

FIGURE 3.

A. RT-PCR analysis of CYP1A1 mRNA expression in H16 Clones overexpressing AhR. Cells were treated with 1 nM TCDD or vehicle (DMSO) for 24h and total RNA was isolated and subjected to reverse transcriptase (RT) reaction, as described in the methods section. RT products were subsequently used for semi-quantitative PCR amplification of CYP1A1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified simultaneously as an internal control. Upper panel is a representative image of ethidium bromide-stained gel of PCR products of clones. The intensity of bands was quantified by digital imaging and normalized to GAPDH signals. The means and standard deviations of duplicate PCR of three independent experiments were plotted in graph in lower panel.

B. Subcellular localization of AhR in H16 clones by immuno-cytochemical staining. Clone were grown on coverslips and fixed with methanol: acetone. AhR was visualized by staining with rabbit anti-AhR polyclonal antibodies followed by a rhodamine-conjugated donkey anti-rabbit. Images were captured on an Olympus fluorescence microscope (500x magnification). Clones with the highest
AhR-overexpression have substantial AhR levels localized to the nucleus in absence of ligand treatment.

FIGURE 4

Cell Cycle Distribution of AhR-overexpressing clonal cell lines of H16N2. Cells were fixed in 70% ethanol, stained with propidium iodide, and analyzed by fluorescence-activated cell sorting (FACS) analysis as described in the methods section. The percentage of cell cycle distribution was determined using ModFit analysis software. The upper panel is a representative histogram, comparing clone A to the EV control clone. The lower panel is a table summarizing the percentage distribution of cells among different phases of cell cycle. The values in table are means and standard deviations of two independent cultures, each analyzed in duplicates.

FIGURE 5

Population doubling times of AhR-overexpressing clones compared to their vector control. Cells were grown in 96 well plates for 3, 12, 24 and 48 hours. They were then lysed and the DNA stained with Hoechst 33258 nucleic acid dye. Fluorescence was measured (in Artificial Fluorescence Units AFU, which were correlated to cell numbers) using excitation and emission wavelength 360nm and 460nm, respectively. Cell numbers were plotted against time using Graphpad statistical software and doubling times were calculated by non-linear regression curve analysis (for exponential growth). Values in graph are average population doubling times of the clonal cell lines. Values are means and standard deviations of triplicate determination of three independent experiments. (*) denotes significant difference from the EV control ((p<0.05).
A. **Analysis of invasive potential of AhR-transformed clones of H16N2 cells.** Cell suspensions (2.5 x 10⁴) of normal or AhR over-expressing cells were plated in BD FluoroBlok invasion chambers containing matrigel, and incubated for 24 hours. Cells passing to the underside of the insert were stained with calcein AM fluorescence dye. Calcein fluorescence of invasive cells was measured on Cytofluor at excitation/emission wavelengths 485/530nm respectively. The fluorescence of the invasive cells was calculated as a percentage of the fluorescence of total cells. A. Values plotted are the means and standard deviations of three independent experiments, where each experiment was run in duplicate. (*) denotes significant difference from EV control (p< 0.05)

B. **Microscopic images of invasive cells.** Representative microscopic images of invasive cells were taken at 10X magnification following staining with calcein AM of the underside of the insert.

**FIGURE 7.**

**Effects of overexpressed AhR on migration of H16 mammary epithelial cells.**

The migration measurements were done using the BD FluoroBlok chambers without matrigel. Cell suspensions were prepared by trypsinizing cell monolayers and resuspending the cells in serum free media at 5x10⁴ cells/ml. Medium (750ul) containing 5% fetal calf serum was added to the bottom of each well as a chemo-attractant. A 500ul aliquot of the cell suspension (at three cell densities) was added to the top chamber. The cells were incubated at 37°C for 24 hours. Following incubation, the media from the top chamber was carefully removed by aspiration and the insert was transferred to a second plate containing 0.5ml/well of 4µg/ml Calcein AM (Molecular Probes, Eugene, OR). The plates were allowed to incubate for 1 hour at 37°C. Calcein fluorescence of the migrating cells was measured...
read at excitation and emission wavelength of 485 and 530 respectively. Symbols denote significant difference (p<0.05) from the respective EV control of different cell density.

FIGURE 8.

**Anchorage-independent growth of AhR-over-expressing clones of H16N2 in soft agar.**

Cell suspensions of different clones were prepared in 0.3% agar media and overlaid onto 0.6% agar layer coated on each well of the six-well plates. Cells were plated in triplicate at two cell densities. Individual cells were visualized microscopically and colony formation was monitored for up to 2 weeks. The numbers of colonies were counted at low magnification (20x) and the scoring was done by three independent observers. Values are means and standard deviations of two independent experiments. (*) is significantly different from EV control (p<0.05).
Figure 1

A.

AhR

β-actin

Fold AhR Level relative to EV Level (normalized to Actin)

B.

AhR Immunostain

Phase Contrast
Figure 2

A. Western

<table>
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<tr>
<th></th>
<th>EV</th>
<th>H16</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
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B. ICF images

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- H16
- EV
- HMF-positive C
- A-negative C
Figure 3

A. 

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GAPDH →

CYP1A1 →

Relative CYP1A1 mRNA levels (Normalized to values of untreated EV)

B.

B. A B C D E EV
Figure 4

<table>
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<tr>
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<td>32.44 +/- .46</td>
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<td>27.25 +/- 1.2</td>
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<tr>
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<td>80.37 +/- .75</td>
<td>10.775 +/- .785</td>
<td>8.86 +/- .05</td>
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</table>
Figure 5
Figure 7

![Bar graph showing average cell numbers for different conditions.](image)

- **Y-axis:** Average cell numbers
- **X-axis:** Conditions: EV, +AhR
- **Legend:** Grey bars: 5x10^4, Dark grey: 2.5x10^4, Black: 1.25x10^4
- **Significance markers:** †, ‡, *

![Western blots showing AhR and β-actin protein levels.](image)

- **Y-axis:** Relative AhR levels
- **X-axis:** Conditions: H16, +AhR, EV

Note: The graphs and images are placeholders for actual visual data representation.
Figure 8

Average number of Colonies per field

Clonal Cell lines

* \( p < .05 \)