Development and Application of a Novel Recombinant Cell Line to Detect and Characterize Chemicals that Affect Ah Receptor Nuclear Localization

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Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcriptional factor that mediates the biological and toxicological effects of halogenated aromatic hydrocarbon (HAHs), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related dioxin-like chemicals (DLCs), and nonhalogenated polycyclic aromatic hydrocarbon (PAHs). [1,2] More recently, the AhR has been shown to bind and be activated by a wide variety of synthetic and natural chemicals, many of which have structures and physiochemical characteristics that are dramatically different from the prototypical HAH and PAH ligands for the AhR.[1,2] Given its recently established role in key endogenous physiological processes, the AhR has become an important new target for the development of human therapeutic agents.[1-3] Accordingly, mechanism-based bioassays that can be used to identify and characterize AhR agonists and antagonists would be particularly useful for screening purposes.

In the absence of exogenous ligands, the AhR is predominantly found in the cytoplasm in a multiprotein complex and following binding of ligand, the AhR undergoes a conformational change that facilitates its translocation into the nucleus, where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) protein, converting the AhR into its high affinity DNA binding form. The binding of the ligand:AhR:Arnt complex to its specific DNA recognition sequence, the dioxin responsive element (DRE), stimulates transcription of adjacent genes.[1] While aspects of the AhR mechanism of action have been used to develop novel high throughput AhR-based gene expression bioassays (i.e. CALUX) for the detection of AhR agonists/antagonists in chemical libraries, additional bioassays that can confirm and evaluate AhR activation would provide useful second tier screening assays. In vitro ligand and DNA binding bioassays can be used to directly examine the ability of a chemical to bind to and stimulate AhR transformation into its DNA binding form and they have proven to be very widely accepted methods to confirm direct activation of the AhR by a chemical(s). However, these assays can't perfectly mimic AhR activation events that occur in cells. The availability of a simple method to evaluate changes in AhR cellular localization provides an avenue in which to facilitate identification/characterization of agonists and/or antagonists of the AhR signal transduction pathway.

Current methods to detect AhR nuclear accumulation involve isolation of nuclear extracts from ligand-exposed cells and DNA binding analysis of ligand activated AhR:ARNT complexes or quantitative Western blotting of AhR. Alternatively, detection of AhR in fixed cells by immunohistochemical methods and fluorescent microscopy has allowed visualization of ligand-dependent AhR translocation.[4,5] However, while the above methods allow detection of AhR nuclear translocation, they can be costly and don't easily allow evaluation or visualization of the time course of this event. Transient transfection of an AhR-green fluorescent protein (GFP) fusion protein or one of its variant has been used to examine AhR subcellular localization.[6,7] However, the transfection efficiency and expression level of AhR can vary dramatically between individual cells, and more importantly, large differences in AhR cellular distribution in the absence of ligand are commonly observed in cells with AhR transiently overexpressed. Thus there remains no useful and simple method to rapidly and inexpensively monitor ligand-dependent AhR nuclear translocation. Accordingly, here we describe the generation of a cell line containing a stably transfected AhR-yellow fluorescent protein (AhR-YFP) fusion protein expression plasmid. This novel recombinant cell line provides a simple method to examine AhR cellular localization and ligand-dependent nuclear translocation in intact cells following exposure to AhR ligands.

Materials and Methods

AhR expression constructs and transfection. N- and C-terminally YFP-tagged murine AhR (yAhR/pcDNA3 and AhRy/pcDNA3, respectively) were constructed using standard PCR cloning procedures and fusion protein constructs verified by sequencing. Cos-1 cells and AhR defective mouse hepatoma cell line, TAOc1BP^{r}c1 (TAO), were grown and maintained in α-Minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS). Cells were transfected with AhRy/pcDNA3 or yAhR/pcDNA3 expression vectors in the absence or presence of a AhR-responsive luciferase reporter gene plasmid (pGudLuc6.1) using Transfectol. For

establishing stably transfected cell lines, TAO cells were transfected with yAhR/pcDNA3 and the cells were grown in media supplemented with 500 mg/l G418. Stable clones, referred to yAHAY cells, were identified by fluorescent microscopy and isolated.

Gel retardation analysis and luciferase reporter gene assay. Ligand (TCDD) stimulated AhR transformation and DNA binding of in vitro expressed wt AhR, or YFP-tagged AhR was determined by gel retardation analysis as previously described.[8] AhR:Arnt:[^{32}P]-DRE complexes were resolved by gel retardation analysis, and protein-DNA complexes visualized and quantitated by phosphoimager analysis. yAHAYc6 cells plated in 24 well culture plates were transfected with pGudLuc6.1 using Lipofectamine2000 and after 24 h, cells were treated with DMSO 0.1% (v/v) or 1nM TCDD and further incubated for 24 h. Renilla and/or firefly luciferase activities were measured using Promega luciferase assay systems.[9]

Fluorescent microscopy. The stably transfected YFP-AhR (yAhR) expressing yAHAYc6 cell lines were plated in 8 well Lab-Tek Chambered #1.0 borosilicate coverglass plate (2.2-2.5 × 10^4 cells in 300 µl of α-MEM/FBS) and grown for 24-36 h before chemical treatment. Chemicals were added and the cells incubated chemical at 37 °C for the indicated time, followed by visualization using an Olympus IX71 fluorescence microscope with a YFP filter (excitation: HQ500/20, emission: HQ520lp, beam splitter: Q515lp) and UPlanApo 40× objective. Live cell images were obtained using a Hamamatsu Orca camera with a Lambda automated shutter (Sutter instrument, Novato, CA), controlled by Slidebook 4.2 imaging software.

Results and Discussion

Characterization of YFP-AhR Fusions. Given the potential for the fusion of YFP onto the AhR to interfere with its functionality (DNA binding, hsp90 binding, Arnt dimerization and other aspects), we examined the ability of the N- and C-terminal YFP-AhR fusion proteins to undergo ligand-dependent transformation and DNA binding, and stimulation of gene expression. Gel retardation analysis was used to determine the impact of YFP on ligand (TCDD)-dependent transformation and DNA binding (which also indirectly assesses the ability of the AhR to bind hsp90, ligand and Arnt). Both of the YFP-AhR fusion proteins could form TCDD-dependent AhR:Arnt:DRE complexes similar to that of the wild type AhR suggesting that the presence of YFP does not impair the functionality of the AhR (data not shown). To evaluate the effect of YFP fusion on the transactivation activity of the AhR, we examined the ability of each fusion protein to stimulate ligand (TCDD)-dependent activation of DRE-driven reporter gene induction. Cos-1 cells were transiently transfected with the AhR constructs (wtAhR/pcDNA3, AhRy/pcDNA3 or yAhR/pcDNA3), and pGudLuc6.1[10], incubated with DMSO (0.1% (v/v)) or TCDD (1 nM) for 24 h, followed by measurement of luciferase activity (Figure 1). Similar to wtAhR, the YFP-tagged AhRs supported TCDD-dependent induction of firefly luciferase reporter gene activity, with the yAhR exhibiting a greater overall magnitude of luciferase gene induction. To demonstrate their potential utility for an AhR translocation assay, we examined each YFP-AhR for measureable expression in cells (based on intracellular fluorescence levels) and ligand-dependent nuclear translocation in transient transfection experiments and visualized by fluorescence microscopy. For both of the constructs, YFP signals were observed predominantly in the cytoplasm, with some fluorescence present in the nucleus, and incubation with TCDD stimulated comparable nuclear accumulation of both YFP-AhR fusion proteins. Given the slightly greater activity of the yAhR, its somewhat lower cytosol to nuclear signal ratio and significantly greater expression and/or fluorescence compared to AhRy, yAhR was used for development of a stable cell line.

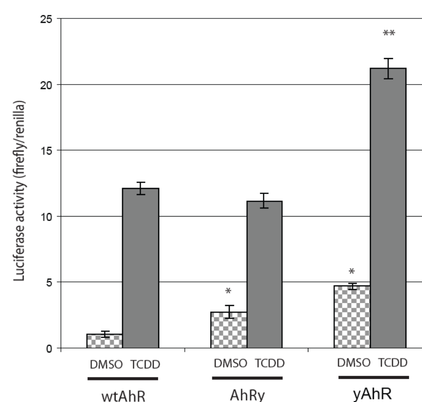

Figure 1. Wt and YFP-AhR fusions stimulate DRE-dependent luciferase gene expression

Stable Transfection of YFP-AhR Fusions. The mouse yAhR fusion construct was stably transfected into AhR-defective mouse hepatoma (TAOc1BP^{r}c1 (TAO)) cells which contain low levels of constitutive AhR expressions (~10% of wild type Hepa1c1c7 cells).[11] yAhR transfection resulted in ~20-30 individual TAO stable cell clones, but following 8 weeks of G418 selection, only 5 positive TAO cell clones remained. yAhR expression levels

and subcellular localization in the five TAO cell clones (c2, c5, c6, c10, and c13) were examined in the absence and presence of TCDD by fluorescent microscopy and except for c13, where yAhR expression levels varied significantly vary between individual cells, each of the clones expressed similar levels of yAhR fluorescence among the individual cells. In addition, while expression of yAhR varied somewhat between individual cell clones, nuclear accumulation of yAhR occurred in all cell clones following TCDD treatment, with a greater degree of nuclear translocation observed with clones c2 and c6. Additional transient transfection experiments in these two clones with the AhR- and TCDD responsive luciferase reporter plasmid pGudLuc6.1 and pRL-TK (for transfection normalization), followed by incubation with 1 nM TCDD or DMSO for 24 h revealed that the c6 clone had a greater overall induction response and as such, the clone c6 cell line (referred to as yAHAYc6 cells) was used further. The time course of TCDD stimulated nuclear accumulation of yAhR as well as the TCDD concentration dependence of this translocation were determined. Quantitation of nuclear translocation was determined by specifically quantitating the mean yAhR fluorescent intensities in the nucleus and dividing by the mean fluorescent intensities in cytoplasm. These analyses revealed both a TCDD concentration- and time-dependent increase in yAhR nuclear translocation (i.e., an increased nuclear/cytoplasmic ratio), with maximal nuclear accumulation occurring at ~70 minutes and maintained for up to 180 minutes.

Utilization of the yAHAYc6 Nuclear Translocation Assay. To evaluate the utility of the yAhR cell translocation assays, we examined the effect of AhR agonists, antagonists and modulators of the AhR signal transduction pathway in yAHAYc6 cells. Exposure of yAHAYc6 cells to the AhR agonists TCDD and beta-naphthoflavone (BNF) stimulated AhR nuclear accumulation (Figure 2). Similar results were observed with other AhR agonists, including PCB126. These results demonstrate the utility of the translocation bioassay to detect agonist-dependent yAhR nuclear localization.

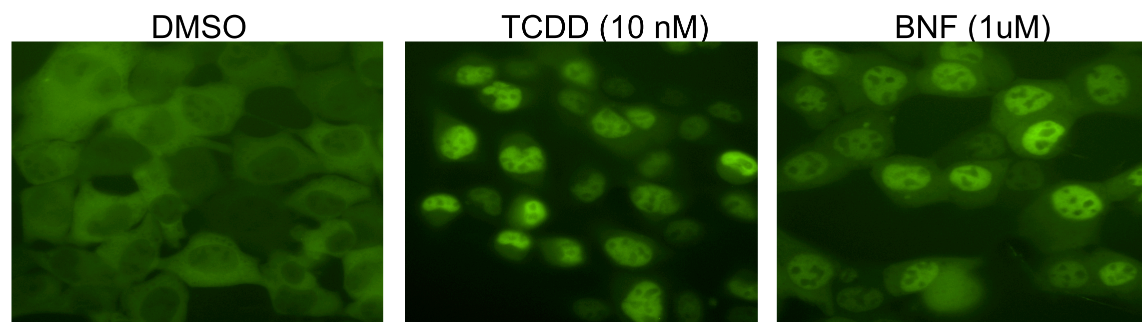

Figure 2. yAhR nuclear accumulation in yAHAYc6 cells incubated with TCDD and BNF.

A variety of AhR antagonists have also been identified and characterized with regards to their mechanism of action. A complication with most AhR antagonists is that they are not pure antagonists, but partial agonists and exposure to these compounds result a mixed response of induction and inhibition.[12] Recently, a novel pure AhR antagonist (CH223191) was identified that could not only completely block TCDD-dependent induction of AhR-dependent gene expression, but it also reduced AhR nuclear accumulation.[13] Although the authors concluded that CH223191 decreased the nuclear accumulation of AhR (at least by TCDD), their results revealed CH223191-dependent nuclear localization of AhR as determined by AhR immunohistochemistry. This difference in localization could suggest that while CH223191 could still allow nuclear localization of AhR, it fails to allow AhR dimerization with Arnt and thus, no DNA binding form of the AhR would be present in the nuclear extract. To test whether CH223191 exerts its antagonist effect on ligand inducible gene expression by blocking AhR translocation, yAHAYc6 cells were incubated with DMSO, TCDD (10 nM) or BNF (10 μM) in the absence or presence of 10 μM CH223191 for one hour and nuclear translocation of yAhR determined (Figure 3). Interestingly, although CH223191 significantly reduced (antagonized) TCDD-dependent nuclear translocation of yAhR, it had no apparent effect on nuclear localization of AhR activated by the flavonoid ligand BNF. Subsequent studies revealed that CH223191 is a ligand-specific antagonist, inhibiting binding and activation of the AhR by HAHs, such as TCDD, TCDF and PCBs, but not PAHs and PAH-like ligands.[14]

Overall, we describe the development of a novel cell line (yAHAYc6 cells) that respond to AhR agonists with nuclear accumulation of a YFP-AhR fusion protein that occurs in a chemical-, dose-, and time-dependent manner. The utility of this new cell line for characterization of AhR agonists/antagonists was demonstrated and

these cells will be useful for further investigating the effect of chemicals and extracts on AhR nuclear localization and its complements existing CALUX-type AhR-dependent gene expression screening bioassays.

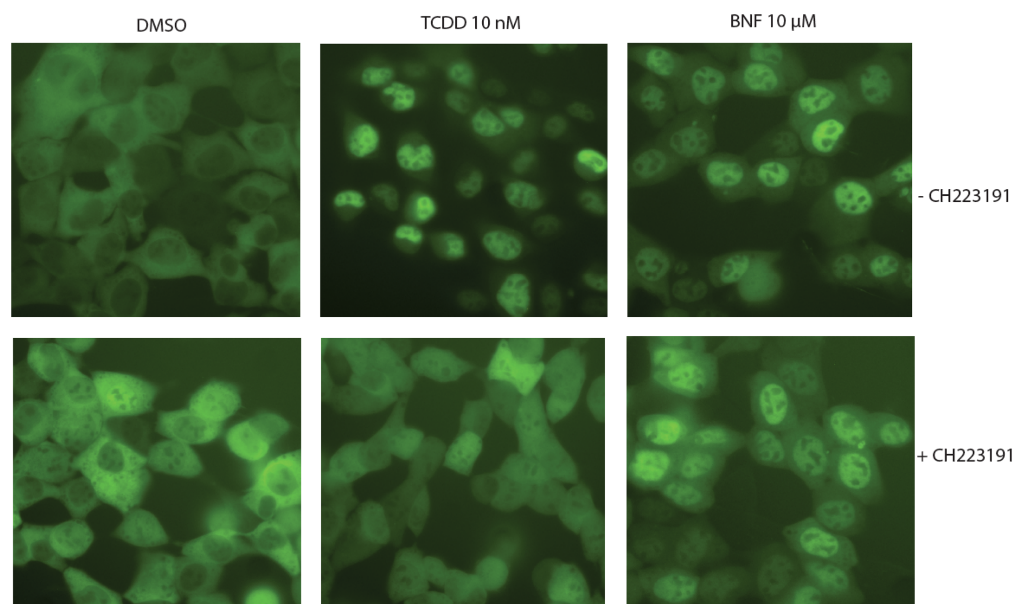

Figure 3. CH223191 inhibits AhR nuclear translocation stimulated by TCDD but not BNF.

Acknowledgements
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