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Cobaltous chloride and hypoxia inhibit aryl hydrocarbon receptor-mediated responses in breast cancer cells

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Abstract

The aryl hydrocarbon receptor (AhR) is expressed in estrogen receptor (ER)-positive ZR-75 breast cancer cells. Treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces CYP1A1 protein and mRNA levels and also activates inhibitory AhR-ERα crosstalk associated with hormone-induced reporter gene expression. In ZR-75 cells grown under hypoxia, induction of these AhR-mediated responses by TCDD was significantly inhibited. This was not accompanied by decreased nuclear AhR levels or decreased interaction of the AhR complex with the CYP1A1 gene promoter as determined in a chromatin immunoprecipitation assay. Hypoxia-induced loss of Ah-responsiveness was not associated with induction of hypoxia-inducible factor-1α or other factors that sequester the AhR nuclear translocation (Arnt) protein, and overexpression of Arnt under hypoxia did not restore Ah-responsiveness. The p65 subunit of NFκB which inhibits AhR-mediated transactivation was not induced by hypoxia and was primarily cytosolic in ZR-75 cells grown under hypoxic and normoxic conditions. In ZR-75 cells maintained under hypoxic conditions for 24 h, BRCA1 (an enhancer of AhR-mediated transactivation in breast cancer cells) was significantly decreased and this contributed to loss of Ah-responsiveness. In cells grown under hypoxia for 6 h, BRCA1 was not decreased, but induction of CYP1A1 by TCDD was significantly decreased. Cotreatment of ZR-75 cells with TCDD plus the protein synthesis inhibitor cycloheximide for 6 h enhanced CYP1A1 expression in cells grown under hypoxia and normoxia. These results suggest that hypoxia rapidly induces protein(s) that inhibit Ah-responsiveness and these may be similar to constitutively expressed inhibitors of Ah-responsiveness (under normoxia) that are also inhibited by cycloheximide.

Keywords: Hypoxia; Ah receptor; BRCA1; CYP1A1 inhibition

Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that is a member of the basic helix–loop–helix family of nuclear transcription factors (Swanson and Bradfield, 1993; Wilson and Safe, 1998). The AhR is primarily cytosolic and addition of ligand results in formation of a nuclear AhR complex containing both the AhR and the AhR nuclear translocator (Arnt) protein (Reyes et al., 1992). The AhR complex interacts with dioxin responsive elements (DREs) in Ah-responsive gene promoters and this activates recruitment of other nuclear coregulatory proteins and the pre-initiation complex resulting in induction of gene expression (Reyes et al., 1992; Swanson and Bradfield, 1993; Whitlock, 1999; Wilson and Safe, 1998). The AhR was initially identified as the intracellular receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally related halogenated aromatic pollutants (Poland and Knutson, 1982; Poland et al., 1976). However, the AhR binds structurally diverse synthetic compounds, combustion by-products, endogenous biochemicals, and chemoprotective phytochemicals (Denison et al., 1998; Denison and Nagy, 2003).

TCDD induces a broad spectrum of AhR-mediated toxic and biochemical responses in laboratory animal models, and induction of many of the toxic responses are species-, sex- and age-dependent. For example, TCDD causes a severe chloracne in human, monkeys, rabbits and some hairless strains of mice but not in most other species. The reasons for these differences in TCDD-induced toxicities are not yet fully understood. TCDD

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and related compounds also inhibit 17β-estradiol (E2)-induced responses in rat mammary tumors, the rodent uterus, and human breast and endometrial cancer cells (Safe and Wormke, 2003). Research in this laboratory has focused on the mechanisms of inhibitory AhR-estrogen receptor (ER) crosstalk and the development of selective AhR modulators (SAhRMs) for treatment of breast cancer (McDougal et al., 2001; Safe et al., 2001). The mechanisms of AhR-mediated inhibition of E2-induced responses are complex and due to several pathways which may be gene specific (Safe and Wormke, 2003). For example, inhibition of some E2-responsive genes involves direct interactions of the AhR complex with inhibitory dioxin response elements (iDREs) in the cathepsin D, heat shock protein 27, pS2 and c-fos gene promoters (Duan et al., 1999; Gillesby et al., 1997; Krishnan et al., 1995; Porter et al., 2001), whereas inhibition of other genes is iDRE-independent (Safe and Wormke, 2003). Moreover, the AhR ligand-dependent degradation of ERα may be due to the recently reported E3 ubiquitin ligase activity of the AhR (Ohtake et al., 2007).

Previous studies in this laboratory reported that TCDD induced proteasome-dependent degradation of ERα in breast cancer cells, and this AhR-dependent response may contribute to the observed antiestrogenic effects (Wormke et al., 2003). Hypoxia also induces proteasome-dependent degradation of ERα resulting in decreased E2-responsiveness (Stoner et al., 2002); however, we also observed that inhibitory AhR-ERα crosstalk was significantly decreased in breast cancer cells grown under hypoxic conditions (Stoner, 2002), and this is consistent with other reports showing that hypoxia decreases Ah-responsiveness (Chan et al., 1999; Gradin et al., 1996; Kim and Sheen, 2000; Pollenz et al., 1999; Prasch et al., 2004). However, the mechanisms of hypoxia-dependent loss of Ah-responsiveness are unclear. In this study, we show that hypoxia decreases AhR-mediated transactivation in ZR-75 breast cancer cells, and this was not due to decreased expression of AhR or Arnt or their interactions with the CYP1A1 promoter. In addition, decreased expression of induced CYP1A1 under hypoxia was not due to activation of proteasomes, induction of HIF-1α, limiting levels of Arnt, or inhibition by the p65 subunit of NFκB. ZR-75 cells maintained under hypoxia for 24 h decreased BRCA1 protein, an AhR enhancer, and this could contribute to loss of Ah-responsiveness. BRCA1 expression is not decreased in ZR-75 cells grown under hypoxia for only 6 h, whereas loss of Ah-responsiveness is observed at this time point. However, cotreatment of breast cancer cells with TCDD and cycloheximide under normoxic or hypoxic conditions for 6 h enhanced Ah-responsiveness, suggesting that decreased AhR-dependent transactivation after 6 h under hypoxia may be due to rapid induction of inhibitory factors.

Materials and methods

Cells, chemicals, biochemicals and plasmids. ZR-75 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and were cultured in RPMI 1620 medium with phenol red (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS; or Atlanta Biologicals, Inc., Norcross, GA), 1.5 g/l sodium bicarbonate, 2.38 g/l HEPES, 4.5 g/l dextrose, and 0.11 g/l sodium pyruvate. Cells cultured under normoxic conditions were maintained in 37 °C incubators under humidified 5% carbon dioxide/95% air. For hypoxia experiments, cells were cultured in a modular incubator flushed with a gas mixture containing 94% nitrogen, 5% carbon dioxide, and 1% oxygen. Dimethyl sulfoxide (DMSO), E2, $CoCl_2$, cycloheximide, and phosphate buffered saline (PBS) were purchased from Sigma. MG132 was purchased from Calbiochem (EMD Biosciences, Inc., CA). TCDD was prepared in this laboratory and was shown to be >99% pure by gas chromatographic analysis. Reporter lysis buffer and Luciferase Assay Reagent were purchased from Promega Corp. (Madison, WI) and/or Boehringer Mannheim (Indianapolis, IN). β-Galactosidase activity was measured using Tropix Galacto-Light Plus Assay System (Tropix, Bedford, MA, USA). Instant Imager and Lumicount micro-well plate reader were purchased from Packard Instrument Co. (Downers Grove, IL). β-Actin antibody was obtained from Sigma and all other antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Human ERα expression plasmid was originally provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX) and was recloned into pcDNA3 in this laboratory. The pVEGF1 construct contains the −2018 to +50 VEGF promoter insert as previously described (Stoner et al., 2004) and was kindly provided by Drs. Gerhard Seimeister and Gunter Finkenzeller (Institute of Molecular Medicine, Tumor Biology Center, Freiburg, Germany). Dioxin response element (DRE)-luciferase (DRE-luc) reporter construct was constructed in this laboratory and contained three tandem consensus DREs. The expression vector pBM5/NEO-M1-1 containing the 2.6-kb human *ARNT* cDNA was a gift from Dr. O. Hankinson (University of California at Los Angeles).

Transient transfection assays. Cells were seeded in DME/F12 medium supplemented with 2.5% charcoal-stripped serum overnight in 12-well plates. Transfection was carried out using GeneJuice (Novagen, EMD Biosciences, Inc., CA) according to the manufacturer's protocol. Cells were then treated for 24 h under normoxic conditions in the presence or absence of 500 μM $CoCl_2$ and harvested in 100 μL of cell lysis buffer (Promega Corp.). Luciferase activities in the various treatment groups were performed on 20 μL of cell extract using the luciferase assay system (Promega Corp.) in a luminometer (Packard Instrument Co., Meriden, CT), and results were normalized to β-galactosidase enzyme activity which was carried out on 20 μL of cell extract.

Northern blot analysis. Cells were seeded in DME/F12 medium supplemented with 2.5% charcoal-stripped serum overnight in 6-well plates. Cells were then treated with DMSO (D) or 10 nM TCDD for 6 h in the presence or absence of 500 μM $CoCl_2$ with or without cycloheximide (pretreatment for 45 min), and RNA was extracted using RNAzol B (Tel-Test) following the manufacturer's protocol; 15–20 μg of RNA were separated on a 1.2% agarose/1 M formaldehyde gel, and transferred to a nylon membrane for 48 h. RNA was crosslinked by exposing the membrane to UV light for 10 min, and the membrane was baked at 80 °C for 2 h. The membrane was then prehybridized for 18 h at 60 °C using ULTRAhyb-Hybridization Buffer (Ambion, Austin, TX) and hybridized in the same buffer for 24 h with the [γ^{32}P]-labeled CYP1A1 cDNA probe. The membrane was then washed in 2X SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7) and 0.5% sodium dodecyl sulfate (SDS) for 1 h, and then washed in 2× SSC for 6–8 h. β-Tubulin mRNA were used as an internal control.

Preparation and analysis of nuclear and cytosolic proteins. ZR-75 cells were seeded into 100 mm diameter plates in DME/F12 medium supplemented with 2.5% charcoal-stripped serum. Cells were treated with DMSO (D) or 10 nM TCDD (T) and grown in 21% O_2 with or without 500 μM $CoCl_2$ for varying times. Nuclear and cytosolic extracts were obtained using the NE-PER nuclear and cytoplasmic extraction kit (Pierce) according to the manufacturer's instructions. Protein samples were boiled in 1× sample buffer (50 mM Tris–HCl, 2% SDS, 0.1% bromphenol blue, 175 mM β-mercaptoethenol) for 5 min, separated on 7.5–10% SDS-PAGE gel for 3 h at 150 V, and Western blot analysis was performed.

Preparation of whole cell extract and Western blot analysis. ZR-75 cells were seeded into six-well plates in DME/F12 medium supplemented with 2.5% charcoal-stripped serum. Cells were exposed to normoxia (21% O_2), physiological hypoxia (1% O_2), or chemically induced hypoxia (500 μM $CoCl_2$) in the presence of DMSO or 10 nM TCDD for varying times. Cells were harvested