REGULATION OF TCDD-INDUCED DEGRADATION OF AH RECEPTOR BY CYCLOHEXIMIDE

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Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) represents the prototype for a class of structurally related halogenated aromatic hydrocarbons (HAHs), including polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls[1,2]. Animals exposed to TCDD exhibit a wide range of toxic and adaptive responses, including a wasting syndrome, tumor promotion in skin and liver, cleft palate, chloracne, immune and endocrine dysfunctions, and induction of drug metabolizing enzymes[1-6]. The health effect of TCDD on human beings remains a matter of debate.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor with a basic helix-loop-helix PAS (bHLH/PAS) modular structure[2,3,7,8]. Mouse genetic studies implicate AhR in most of the biological responses to TCDD, presumably by affecting the expression of target genes[9-11]. Studies on the induction of CYP1A1 gene expression by TCDD provided major mechanistic understanding of the mechanism of action and regulation of AhR[2,3]. In uninduced cells, AhR is localized in the cytoplasm, complexed with hsp90[12] and AIP, an immunophillin-type chaperon protein[13-15]. Binding with an agonist triggers the dissociation of AhR from the associated proteins and translocation into nucleus, where AhR dimerizes with Arnt, another bHLHPAS transcription factor[16]. The AhR/Arnt dimer binds to a specific nucleotide sequence termed DRE (dioxin responsive element) in the enhancer region of the CYP1A1 gene[17]; the transcription activation domains of AhR are essential for the subsequent transcriptional events, including alterations in chromatin structure, binding of general transcription factors to the promoter, and induction of transcription of the gene[2,3].

Several cellular mechanisms have been recognized for the regulation of the AhR activity during the induction of CYP1A1. For example, cycloheximide enhances the induction of CYP1A1 gene expression by TCDD, a phenomenon termed "superinduction". Early studies established that the superinduction involves an increase in the rate of transcription of the gene, requires functional DREs, but does not change several measurable properties of the TCDD-receptor complex such as the sedimentation velocity of the complex[18,19]. Since cycloheximide is known to inhibit protein synthesis, it is assumed that a labile, inhibitory protein factor regulates the AhR activity. However, the nature and the mechanism of action of the putative "labile" factor remain unknown. In another scenario, treatment with TCDD shortens the half-life of the AhR protein from 28 h to 3 h[20,21]. The TCDD-induced turnover of AhR is mediated through the 26S proteasome, involves ubiquitination of AhR, and requires the transcription activation domain of AhR[20]. Moreover, inhibition of the 26S proteasome by proteasome inhibitors increases the induction of CYP1A1 by TCDD; these findings implicate the agonist-induced AhR degradation in the regulation of AhR function.

To identify the molecular target of cycloheximide, we analyzed the TCDD-induced AhR turnover in the superinduction. We show here that cycloheximide blocks TCDD-induced degradation of

AhR. Inhibition of the TCDD-induced AhR degradation requires inhibition of protein synthesis and correlates with the superinduction in a time- and dose-dependent manner. Furthermore, cycloheximide is shown to increase the accumulation of the AhR and the functional AhR/Arnt complex in nucleus. In addition, we show that inhibition of the 26S proteasome superinduces CYP1A1 expression in a similar fashion to cycloheximide. To our knowledge, this report is the first study demonstrating that cycloheximide blocks the agonist-induced degradation of the AhR protein. Our findings provide a novel mechanism of superinduction of CYP1A1 in which a cycloheximide-sensitive, "labile" protein factor (designated as AhR Degradation Promoting Factor, or ADPF) negatively regulates the stability of agonist-activated, nuclear AhR.

Methods and Materials

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared according to published procedures[17]. EMSA was carried out using nuclear extract from hepa1c1c7 cells, as described[17], except that 6% polyacrylamide gels were used. The DNA probe contains the DNA recognition sequence for the AhR/Arnt heteromer designated as DRE D[22].

Immunoblot Analysis. For immunoblotting, total cell lysate or nuclear extract of 5 μg were fractionated on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes according to established procedures[23]. An affinity-purified Polyclonal antibody against AhR[13] was used for detection of AhR Signals were visualized by chemiluminescence using an ECL kit (Amersham). To ensure equal loading of the samples, the same blots were reprobed with a monoclonal anti-mouse actin antibody (Santa Cruz Biotechnology, Inc). For quantitation of the blotting results, the visualized results were scanned and analyzed by using the ImageQuaNT program (Molecular Dynamics).

RNA Analysis. For Northern blotting of CYP1A1, a cDNA fragment (~700 bp) encoding the 5'-untranslated region of the mouse CYP1A1 messenger RNA was used to generate a riboprobe for CYP1A1. To prepare an actin probe, a cDNA fragment of mouse actin was generated by RT-PCR with primers specific for mouse actin (Stratagene), subcloned into pCRII, and used as a template for riboprobe synthesis. Riboprobes were synthesized in the presence of DIG-UTP using a DIG RNA-labeling kit (Roche Molecular Biochemicals). Total RNA was isolated from cells using a Qiagen total RNA isolation kit (Qiagen). RNA samples of 5 μg each were electrophoresed in a 1% agarose-formaldehyde gel and transferred to a Nytran membrane. After cross-linking, the membranes were hybridized with the DIG-labeled riboprobes at 68 ^{0}C overnight; signals were visualized by chemiluminescence. For all samples analyzed, parallel blots were assayed at the same time for both CYP1A1 and actin mRNAs. Quantitation of the blotting results were performed by using the ImageQuaNT program as described above. All data were corrected for loading variations by comparing the amount of actin of each sample analyzed.

Pulse-chase labeling. Cells grown to near confluence were incubated in methionine-free medium with 10% dialyzed FBS for 1 h and incubated for another hour in fresh methionine-free medium with 10% dialyzed FBS plus ^{35}S-methionine. The cells were then incubated in αMEM with 10% FBS and treated with DMSO, cycloheximide (10 μg/ml), TCDD (1nM), or TCDD plus cycloheximide for various time periods. The cells were scraped into RIPA buffer. The ^{35}S-labled AhR was precipitated with the anti-AhR antibodies, fractionated by SDS-PAGE (10%), and visualized by fluorography.

Immunoprecipitation. AhR was precipitated with anti-AhR antibodies according to a standard method[24]. Briefly, cells grown in 6 well plates were scraped into RIPA buffer. Cell extracts were prepared by centrifugation at 13,000 xg for 10 min, followed by preclearing by incubation with

normal rabbit IgG and protein A-agarose for 30 min at 4°C. The extracts were then incubated with the anti-AhR antibodies[13] for 1 h and with protein A-agarose for an additional hour. The precipitated agarose beads were washed 3 times with the RIPA buffer and resuspended in a loading buffer for analysis by SDS-PAGE.

Immunofluorescence staining and concocal microscopy. Immunofluorescent staining of cells with anti-AhR IgG was performed according to standard procedures[24]. Briefly, cells grown on coverslips were washed with 1xPBS, fixed in 3.7% formaldehyde for 10 min, and permeabilized with methanol at –20°C for 6 min. The cells were then blocked in 1% BSA for 30 min with shaking, and blotted with an affinity-purified polyclonal anti-mouse AhR IgG (Biomol) in 1% BSA for 1 h, followed by incubation with a fluorescein-conjugated anti-rabbit IgG for an additional 1 h in the dark. The glass coverslips were mounted onto slides with Prolong, an anti-fade mounting medium. Fluorescence was visualized using a Sarastro 2000 laser scanning confocal microscope fitted with an argon-ion laser and an Optiphot-2 microscope. Confocal images were recorded through a 60x lens objective using a 488-nm laser line.

Results and Discussion

To investigate the molecular target for "superinduction", we analyzed the agonist-induced degradation of AhR. Whereas TCDD, a potent agonist of AhR, induces a rapid reduction of the AhR protein, cycloheximide blocks the down regulation of steady state AhR. Analyses of the turnover of AhR reveal that cycloheximide blocks the shortening of the half life of AhR by TCDD. Blocking of the TCDD-induced AhR degradation requires inhibition of protein synthesis as evidenced by the observations (a) that cycloheximide inhibits protein synthesis at the concentration at which it causes superinduction and inhibition of AhR degradation, and (b) that puromycin, an inhibitor of protein synthesis by mimicking aminoacyl tRNA, also blocks the TCDD-induced AhR degradation. The blocking of the TCDD-induced AhR degradation correlates with the superinduction of CYP1A1 gene expression in a time- and dose-dependent manner. Furthermore, cycloheximide is shown to increase the accumulation of the TCDD-activated AhR and the functional AhR/Arnt complex in nucleus. Collectively, our results reveal a mechanism of superinduction by cycloheximide by enhancing the stability of agonist-activated AhR. The finding that inhibition of protein synthesis blocks the TCDD-induced AhR turn over implicates a cycloheximide-sensitive, labile factor (designated as AhR Degradation Promoting Factor, or ADPF) in controlling the removal of agonist-activated AhR in nucleus.

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