INTERACTIONS OF THE AH RECEPTOR AND OXIDATIVE SIGNALING

Qiang Ma, Krista De Fede, Kimberly T. Baldwin, and Hayley E. Burdette

Receptor Biology Laboratory, Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, WV, USA

Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) represents the prototype for a class of structurally related halogenated aromatic hydrocarbons (HAHs)[1-3]. These man-made compounds are mostly by-products of industrial processes involving chlorine chemistry and combustion of fuels. Many such chemicals are widespread and persistent environmental contaminants. TCDD is the most potent among the chemicals; animals exposed to TCDD exhibit a wide range of toxic and adaptive responses. Humans exposed to TCDD exhibit certain skin lesions such as chloracne; the possibility that TCDD exposure causes certain neuro- and psycho-pathological alterations, some forms of cancers and diabetic conditions, and reproductive lesions is a particular concern of public health.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor with a basic helix-loop-helix PAS (bHLH/PAS) modular structure[1,4,5]. Mouse genetic studies implicate AhR in most of the biological responses to TCDD, presumably by affecting the expression of target genes. Recent observations also imply that AhR plays certain roles in embryonic development, liver and immune functions, and vascular development in mice[6-8], and modulates the growth, differentiation, and apoptotic processes in certain cell lines and mouse liver[9-11]; these functions of AhR were observed in the absence of known exogenous agonists, implicating a mechanism(s) of activating AhR under physiological conditions in vivo. Activation of AhR by its ligand, such as dioxin, involves a multi-step signal transduction process. 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. The AhR/Arnt dimer binds to a specific nucleotide sequence termed DRE (dioxin responsive element) in the enhancer region of the CYP1A1 gene[16] and mediates the transcription of the gene. TCDD activated AhR is rapidly degraded through the ubiquitin-26S proteasome proteolysis controlled by a labile factor ADPF (AhR degradation promoting factor)[17]. Because of the broad range and the complexity of the biological responses that AhR contributes to, it is conceivable that the signal transduction of AhR involves a complex process during which AhR is regulated through different cellular mechanisms. Furthermore, these regulatory mechanisms may intersect with other signal transduction processes, through which certain non-AhR ligand, environmental/occupational chemicals influence the signal transduction and function of AhR.

Oxidative chemicals represent a broad range of endogenous, dietary, environmental, and occupational chemicals that can cause "oxidative stress" by changing the balance between reactive oxygen species (ROS)/antioxidants in cells, thereby affecting many important cellular processes such as cell division and differentiation, cancer formation, inflammation and immune response[18]. These chemicals include diphenols and quinones, isothiocyanates, peroxides, mercaptans, trivalent arsenicals, heavy metals, flavones, and Michael reaction acceptors. Certain oxidative chemicals exhibit both oxidative and antioxidative properties. Certain biological effects of phenolic chemicals are caused by modulating the expression of target genes through the Nrf2 (NfE2 related factor 2)-mediated, antioxidant response element (ARE)-dependent, gene transcription. For instance, phenolic chemicals induce phase II enzymes such as NADP(H):quinone oxidoreductase (NQOR) and glutathione s-transferase (GstYa)[19,20]; these enzymes are also inducible through the AhR-mediated, DRE-dependent gene transcription[20].

Cumulative evidence indicates that oxidative chemicals can modulate AhR-dependent responses, suggesting that oxidative signal transduction interacts with the AhR signaling process. Because of the broad impact of the oxidative processes, the interaction between AhR and oxidative signal transduction may contribute to the complexity of dioxin toxicity and AhR function. The molecular mechanism of such interaction is not clear at the present. In this study, we examined the interaction of AhR and oxidative signaling from two aspects: (1) the potential interaction between the AhR/DRE and the Nrf2/ARE signaling on the induction of NQOR, and (2) activation of AhR dependent gene regulation by phenolic chemicals. Our results reveal that both AhR/DRE and Nrf2/ARE systems require a labile factor for the induction of NQOR. Phenolic chemicals such as tert- butyl hydroquinone (tBHQ) and hydroquinone (HQ) induce a number of DRE-regulated AhR target genes. The induction is dependent upon the oxidative properties of the chemicals. These findings provide new opportunities for studying the activation of AhR through oxidative pathways and reveal new insights into the mechanism of AhR action upon exposure to mixtures of chemicals.

Methods and Materials

Cell Culture and Treatment. The mouse hepa1c1c7 cells were gifts from Dr. J. P. Whitlock, Jr. (Stanford University). The cells were grown as monolayer in α-minimal essential medium (αMEM), containing 10% fetal bovine serum and 5% CO_2 at 37 °C. The cells were treated with TCDD (1nM), tBHQ (90 uM), HQ (90 uM) or other agents as described in figure legends; DMSO was used as the solvent control.

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 actin, NQOR, TIG1, and CYP1B1 probes, cDNA fragments of the mouse genes were generated by RT-PCR with primers specific for the genes, 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 °C overnight; signals were visualized by chemiluminescence. For all samples analyzed, parallel blots were assayed at the same time for the gene of interest and actin to ensure equal loading. Quantitation of

the blotting results was performed by using the ImageQuaNT program. All data were corrected for loading variations by comparing the amount of actin of each sample analyzed.

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared according to published procedures[16]. EMSA was carried out using nuclear extract from hepa1c1c7 cells, as described, except that 6% polyacrylamide gels were used. The DNA probe contains the DNA recognition sequences for the AhR/Arnt heteromer designated as DRE D, or for the Nrf2 designated as ARE.

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. An affinity-purified Polyclonal antibody against AhR 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).

Results and Discussion

To study the interaction of AhR and oxidative signal transduction, we analyzed the mechanism by which AhR and Nrf2 mediate the induction of NQOR by TCDD and phenolic chemicals, and the mechanism by which phenolic chemicals activate DRE-dependent AhR target genes.

Regulation of AhR/DRE and Nrf2/ARE pathways by a labile factor. NAD(P)H:quinone reductase (NQOR) catalyzes the two electron reduction of quinones and quinoid chemicals. Induction of NQOR constitutes a major mechanism of defense against toxicity of quinones, azo chemicals, and other redox-cycling chemicals. Prototypical inducers of NQOR include TCDD, and tBHQ. Induction of NQOR by TCDD involves binding of TCDD to AhR, AhR/Arnt dimer formation, and binding of the dimer to a dioxin response element (DRE) in the upstream region of NQOR. The antioxidants induce NQOR through a pathway involving activation of the Nrf2 transcription factor, which dimerizes with a Maf protein and binds to an antioxidant response element (ARE). However, the molecular mechanism of the induction by these inducers is not well understood. To gain new insights into the molecular steps of the induction, we examined the effect of cyclohexamide (CHX), a potent inhibitor of protein synthesis, on the transcriptional regulation of NQOR by TCDD and tBHQ. Northern blot analyses reveal that CHX blocks both the basal expression and the induction of the enzyme by both TCDD and tBHQ. The inhibition occurs in a dose- and time- dependent manner, and does not affect the mRNA stability of NQOR. These results demonstrate that CHX inhibits both DRE- and ARE-mediated transcription of NQOR. The blocking involves inhibition of protein synthesis. Time course analyses of the inhibition implicate a labile factor in the induction. Taken together, these results demonstrate that a novel labile factor is required for the transcriptional regulation of NQOR by TCDD and phenolic antioxidants. These findings provide new insights into the interaction and regulation of AhR/DRE and Nrf2/ARE pathways for the induction of phase II enzymes.

Activation of DRE-dependent gene transcription by phenolic chemicals. Phenolic chemicals exhibit both oxidative and antioxidative properties through influencing the balance between ROS and antioxidant species in cells, thereby affecting many cellular functions and processes. In this study, we investigate the effect of phenolic chemicals on AhR and DRE-dependent gene regulation. Our results reveal that phenolic chemicals, such as tBHQ and HQ induce several AhR target genes, including CYP1A1, CYP1B1, and TiPARP (TCDD-inducible poly(ADP-ribose) polymerase), suggesting that phenolic chemicals activate AhR-mediated signal transduction. The induction is

both time and dose dependent, and is mediated through an oxidative pathway(s). These results demonstrate that oxidative chemicals can activate AhR-dependent gene regulation and reveal a link between oxidative chemical exposure and AhR activation through oxidative signal transduction. Current study is aimed at elucidating the molecular mechanism by which AhR interacts with the oxidative signal transduction pathways.

Acknowledgements
We thank Ms. Sheila McGregor and Helen Michael for secretarial assistance.

References

1. Whitlock, J. P., Jr. (1999) *Annu. Rev. Pharmacol. Toxicol.* **39,** 103-125
2. Poland, A., and Kimbrough, R. D. (eds) (1984) *Biological Mechanisms of Dioxin Action* Vol. 18. Banbury Report, Cold Spring Harbor, New York
3. Safe, S. H. (1986) *Annu. Rev. Pharmacol. Toxicol.* **26,** 371-399
4. Hankinson, O. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35,** 307-340
5. Burbach, K. M., Poland, A., and Bradfield, C. A. (1992) *Proc Natl Acad Sci U S A* **89**(17), 8185-9
6. Fernandez-Salguero, P. M., Pineau, T., Hilbert, D. M., McPhail, T., Lee, S. S. T., Kimura, S., Nebert, D. W., Rudikoff, S., Ward, J. M., and Gonzalez, F. J. (1995) *Science* **268,** 722-726
7. Mimura, J., Yamashita, K., Nakamura, K., Morita, M., Takagi, T. N., Nakao, K., Ema, M., Sogawa, K., Yasuda, M., Katsuki, M., and Fujii-Kuriyama, Y. (1997) *Genes Cells* **2**(10), 645-54
8. Lahvis, G. P., Lindell, S. L., Thomas, R. S., McCuskey, R. S., Murphy, C., Glover, E., Bentz, M., Southard, J., and Bradfield, C. A. (2000) *Proc. Natl. Acad. Sci., USA* **2000,** 10442-10447
9. Kolluri, S., Weiss, C., Koff, A., and Gottlicher, M. (1999) *Genes and Dev.* **13**(13), 1742-53
10. Ma, Q., and Whitlock, J. P., Jr. (1996) *Mol. Cell. Biol.* **16**(5), 2144-50
11. Ge, N.-L., and Elferink, C. (1998) *J. Biol. Chem.* **273,** 22708-22713
12. Perdew, G. H. (1988) *J. Biol. Chem.* **263,** 13802-13805
13. Ma, Q., and Whitlock, J. P., Jr. (1997) *J Biol Chem* **272**(14), 8878-84
14. Carver, L., A., and Bradfield, C. A. (1997) *J. Biol. Chem.* **272**(17), 11452-11456
15. Meyer, B. K., Pray-Grant, M. G., Vanden Heuvel, J. P., and Perdew, G. H. (1998) *Mol Cell Biol* **18**(2), 978-88
16. Denison, M. S., Fisher, J. M., and Whitlock, J. P., Jr. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2528-2532
17. Ma, Q., Renzelli, A. J., Baldwin, K. T., and Antonini, J. M. (2000) *J. Biol. Chem.* **275**(17), 12676-12683
18. Dalton, T. P., Shertzer, H. G., and Puga, A. (1999) *Annu. Rev. Pharmacol. Toxicol.* **39,** 67-101
19. Prestera, T., Holtzclaw, W. D., Zhang, Y., and Talalay, P. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2965-2969
20. Favreau, L., and Pickett, C. (1991) *J. Biol. Chem.* **266,** 4556-4561

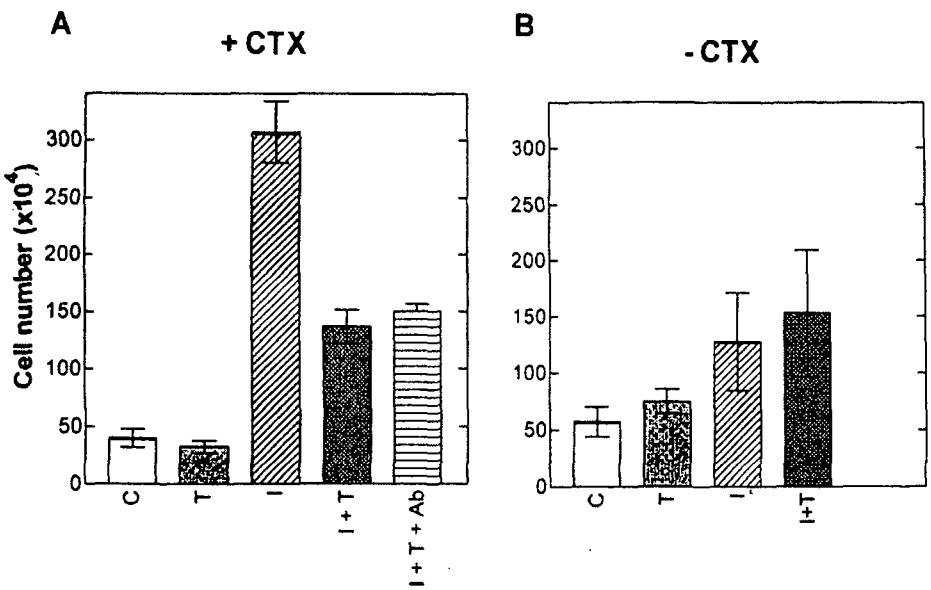

Fig. 1. Proliferation of MCF10A cells by 2,3,7,8-TCDD (30 nM), insulin (800 nM), IGF-1 (0.1 nM) or their combinations in the presence (Figs. 1A) or absence (Fig. 1B) of 100 ng/ml of cholera toxin (CTX). Abbreviations one: C for control, T for TCDD, I for insulin, Ab for a specific antibody against IGF-receptor. Results shown are the means ± SD for representative experiments carried out in triplicate and repeated on at least three occasions. Control is a TCDD solvent (1, 4-Dioxane).

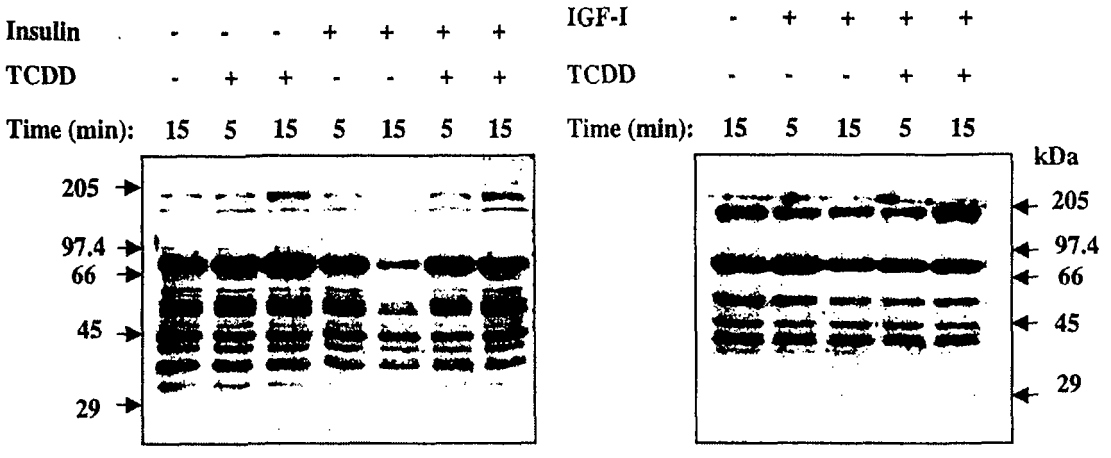

Fig. 2. TCDD activates tyrosine phosphorylation and prevents its down-regulation following stimulation with insulin (left figure) or IGF-I (right figure) in the MCF-10A cells. The cells were grown in the complete growth medium. When the cells reached 70% confluency the medium was changed to serum-free MEBM supplemented with 100 ng/ml cholera toxin. After 24 h of starvation the cells were treated for 5 and 15 min with 30 nM TCDD, insulin (0.8 μM) or TCDD (30 nM) plus insulin (0.8 μM) (Fig. 3 left) and IGF-I (0.1 nM) or IGF-I (0.1 nM) plus TCDD (30 nM) (Fig. 3 right) in fresh serum-free MEBM supplemented with 100 ng/ml cholera toxin. Control cells were incubated with solvent for 15 min. was prepared as described in the Materials and methods section. Tyrosine-phosphorylated proteins were detected in whole cell extracts by immunoblotting with anti-phosphotyrosine antibody (4G10).