# DIFFERENTIAL RESPONSIVENESS OF THE AH RECEPTOR-DEPENDENT GENE EXPRESSION INDUCED BY HAHS AND PAHS

Bin Zhao, Jessica E. Bohonowych and Michael S. Denison

Department of Environmental Toxicology, University of California, Davis, CA, USA

## Introduction

The Ah receptor (AhR) is a basic helix-loop-helix PAS-containing transcription factor which activates gene expression in a ligand-dependent manner.[1,2] The best-characterized high affinity ligands for the AhR include a variety of halogenated aromatic hydrocarbons (HAHs), such as the polychlorinated dibenzo-p-dioxin, dibenzofurans, and biphenyls and polycyclic aromatic hydrocarbons (PAHs) such as 3-methylcholanthrene, beta-naphthoflavone (BNF) and dibenz(a,h)anthracene.[3] Recently, we and others have demonstrated that the AhR can bind and be activated by a wide variety of chemicals that are structurally distinct from the classical HAH and PAH ligands for the AhR.[3,4] Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin), the prototypical and most potent HAH AhR ligand, produces a wide variety of species- and tissue-specific toxic and biological responses, such as tumor promotion, immuno-, hepato- and dermal toxicity, lethality, wasting, endocrine disruption, birth defects, persistent oxidative stress and induction of numerous enzymes, including that of microsomal cytochrome P4501A1 (CYP1A1). Biochemical, genetic and knockout animal studies have demonstrated that the majority of the above toxic and biological effects of TCDD and related dioxin-like HAHs are mediated by the AhR.[1,2,5,6] Although both HAHs and PAHs can bind to the AhR with relatively high affinity, only HAHs produce TCDD-like AhR-dependent spectrum of toxicity, PAHs rarely produce TCDD-like adverse effects even though they can produce many of the same biological effects, albeit more transiently. The greater toxicological potency of HAHs compared to PAHs has been hypothesized to result from their significantly higher AhR binding affinity, which is expected to result in decreased HAH ligand dissociation from AhR and also to their resistance of HAHs to metabolism. The exact molecular mechanisms responsible for AhR-dependent toxicity and the inability of PAHs to produce these effects are not clear. Toxicity in vivo results from a combination of biological, biochemical, pharmacodynamic and pharmacokinetic events, and given that AhR functions as a transcription factor, it is clear that the ability of chemicals to produce AhR-dependent toxicity appears to be primarily driven by persistent activation of AhR-dependent gene expression in target cells. Therefore, we examined the similarities and differences between the ability of HAHs and PAHs to bind to and transform the AhR and induce AhR-dependent gene expression using a variety of in vitro and in vivo bioassays in order to attempt to examine this difference in toxic/biological potency of AhR ligands.

## Materials and Methods

Chemicals**:** TCDD, 2,3,7,8-tetrachlorodibenzofuran (TCDF) and [$^3$H]TCDD were generously provided by Dr. Steven Safe (Texas A&M University, College Station, TX). BNF and DMSO were from purchased from Aldrich (St. Louis, MO) and [$^3$H]BNF was from Dr. Mark Hahn (Woods Hole Oceanographic Institute). Cell culture reagents and media were purchased from Gibco/BRL (Grand Island, NY) and G418 was from Gemini Bio-Products (Woodland, CA).

Cell culture and enhanced green fluorescent protein (EGFP) bioassay: The stably transfected H4G1.1c2 cells that contained the EGFP gene under the control of DREs were maintained and assayed as previously described.[7,8,9]

AhR ligand binding: The AhR [$^3$H]TCDD or [$^3$H]BNF ligand binding and dissociation experiments were carried out as described previously in detail by using the hepatic cytosol from various species (rat, mouse, guinea pig, and hamster).[10]

**Results and Discussion**

TCDD and BNF dose response experiments in H4G1.1c2 cells for 24 hours revealed that both TCDD and BNF induced AhR dependent GFP expression to a comparable maximum activity in a dose dependent manner, although BNF was at least 1,000-fold lower in inducing potency. This difference in relative inducing potency for BNF and TCDD is significantly greater than their relative ligand binding affinities. The greater AhR binding affinity of TCDD compared to BNF has been suggested to contribute to the relative differences in toxic potency, in that it may allow BNF to more readily dissociate from the AhR, leading to more rapid inactivation of BNF-occupied AhR and hence more transient induction. However, our ligand dissociation experiments clearly demonstrate that binding of either [$^3$H]TCDD or [$^3$H]BNF to cytosolic AhR from various species was essentially irreversible in the window of the tested time (24 hours) indicating that the dissociation hypothesis with these ligands and species is incorrect and that differences in HAHs and PAHs persistence of gene expression results from other factors.

Another possibility is that the toxicity of a given AhR ligand is simply based on its metabolic persistence, rather than its relative affinity for the AhR. To test this hypothesis, the EGFP assay provides us with a unique avenue in which to easily examine ligand-induced AhR-dependent gene expression in a "real-time" manner for an extended period of time in the same cells which can not be accomplished by some other systems.[8] While induction of EGFP reporter gene expression in stably transfected rat hepatoma (H4IIe) cells by a single dose of 1 nM TCDD was maximal at 5 days and remained elevated for up to 10 days, induction by 0.1 μM BNF was maximal by 2 days and it decreased to background by day 10 (Figure 1A). Interestingly, the induction of EGFP expression by 0.1 μM BNF reached the same maximal level as TCDD at 1 day, while less induction level was observed for BNF after 2 days. If the persistence of activation is simply due to metabolism of BNF, then initial treatment of cells with increasing concentrations should result in greater levels of EGFP accumulation and persistence of induction. In fact, BNF at higher concentrations (1 and 10 μM) induced progressively higher and more persistent levels of EGFP expression. It should be noted that because EGFP is extremely stable (T1/2 >23 h), reporter gene signal output at any time point is the combination of newly expressed EGFP, but also existing EGFP that will be very slowly lost. These results demonstrated that decreased PAH induction response compared to that induced by TCDD at later time points is very likely due to metabolism/inactivation of BNF, which progressively results in lower levels of EGFP expression over time. Another avenue in which to test the metabolism theory is to examine the effects of daily treatment of cells to BNF on the induction response compared to TCDD. When cells were repeatedly treated daily with 1 μM BNF, a relatively persistent EGFP expression was observed that was fairly comparable to that obtained with a single treatment with 1 nM TCDD (Figure 1B). These results demonstrate that chronic exposure of cells to BNF results in persistent induction of AhR-dependent gene expression and further support the hypothesis that the transient induction response with BNF was primarily due to its metabolism. Relatively persistent binding of both ligands and the relatively rapid turnover of occupied AhR in treated cells are inconsistent with ligand dissociation being responsible for persistent gene activation. While AhR binding affinity is an important consideration when lower than saturating concentrations of chemicals are present in a response cell, our results are consistent with the hypothesis that the greater persistence of HAH-inducible gene expression is more dependent upon the metabolic stability of the HAH ligand rather than its affinity for and persistence in binding to the AhR. While it is possible that there are differences in the ability of HAH- versus PAH-bound AhR complexes to bind to and activate gene expression, these differences remain to be identified. Overall, we propose that the toxicity of HAHs results from chronic

activation of AhR-responsive genes as a result of continual activation of newly synthesized AhR, rather than persistent activation of the existing pool of AhR in a given cell. In this scenario, PAHs and HAHs activate AhR-dependent gene expression exactly the same, but as CYP1A enzymes are induced by the AhR, they (and other cellular enzymes) subsequently begin to metabolize the PAH reducing its concentration over time; the HAH is unaffected. As new AhR is synthesized it can be bound and activated by the persistent HAH and remaining PAH, but over time there will be less PAH in the cell and progressively less nascent AhR is bound by the PAH and the induction response is attenuated. The results described here would suggest that mechanisms/events that maintain relatively high cellular concentrations of PAHs would result in AhR-dependent toxicity. In fact, such an effect has been observed in fish in vivo. For example, chronic dietary exposure of catfish to relatively high levels of BNF resulted in TCDD-like toxic effects, presumably due to the fact that relatively high body burdens of BNF could be maintained due to the typically low rates of metabolism found in fish.[11] Similarly, work by DiGuilio and coworkers demonstrated that in fish in which PAH metabolism by CYPs was inhibited by chemicals or CYP-morpholinos PAHs could produce TCDD-like toxicity.[12,13] Our results coupled with these data strongly suggest that the lack of AhR-dependent toxicity by PAHs, compared to HAHs, is primarily due to their metabolic lability which results in lower AhR-dependent gene expression over time. Thus, environmental exposures that result in inhibition and/or down-regulation of PAH metabolism has the potential to allow previously nontoxic AhR ligands to produce adverse effects.

**Acknowledgements**

This work was supported by the National Institutes of Environmental Health Science (ES012498, ES04699, ES05707), the American Taxpayers and the California Agricultural Experiment Station.

**References**

1. Hankinson O. *Annu Rev Pharmacol Toxicol* 1995;35:307.
2. Schmidt JV, Bradfield CA. *Annu Rev Pharmacol Toxicol* 1996;12:55.
3. Denison MS, Nagy SR. *Annu Rev Pharmacol Toxicol* 2003;43:309.
4. Denison MS, Seidel SD, Rogers WJ, Ziccardi M, Winter GM, Heath-Pagliuso S. in: Molecular Biology Approaches to Toxicology (Puga A and Wallace KB, eds);1999:393-410, Taylor & Francis.
5. Safe S. *Crit Rev Toxicol* 1990;21:51.
6. Fernandez-Salguero PM, Hilbert DM, Rudikoff S, Ward JM, Gonzalez FJ. *Toxicol Appl Pharmacol* 1996;140:173.
7. Nagy S, Sanborn JR, Hammock BD, Denison MS. *Toxicol Sci* 2002;65:200.
8. Zhao B, Denison MS. *Organohalogen Compounds* 2004;66:3332.
9. Denison MS, Zhao B, Baston D, Clark GC, Murata H, Han D. *Talanta* 2004;63:1123.
10. Bohonowych J, Denison MS. *Organohalogen Compounds* 2004;66:3338.
11. Grady AW, Fabacher DL, Frame G, Steadman BL. *J Aquat Animal Health* 1992;4:7.
12. Wassenberg DM, Nerlinger AL, Battle LP, Di Giulio RT. *Environ Toxicol Chem* 2005;24:2526.
13. Billiard S, Timme-Laragy AR, Wassenberg DM, Cockman C, Di Giulio RT. *Toxicol Sci* 2006; May Epub.

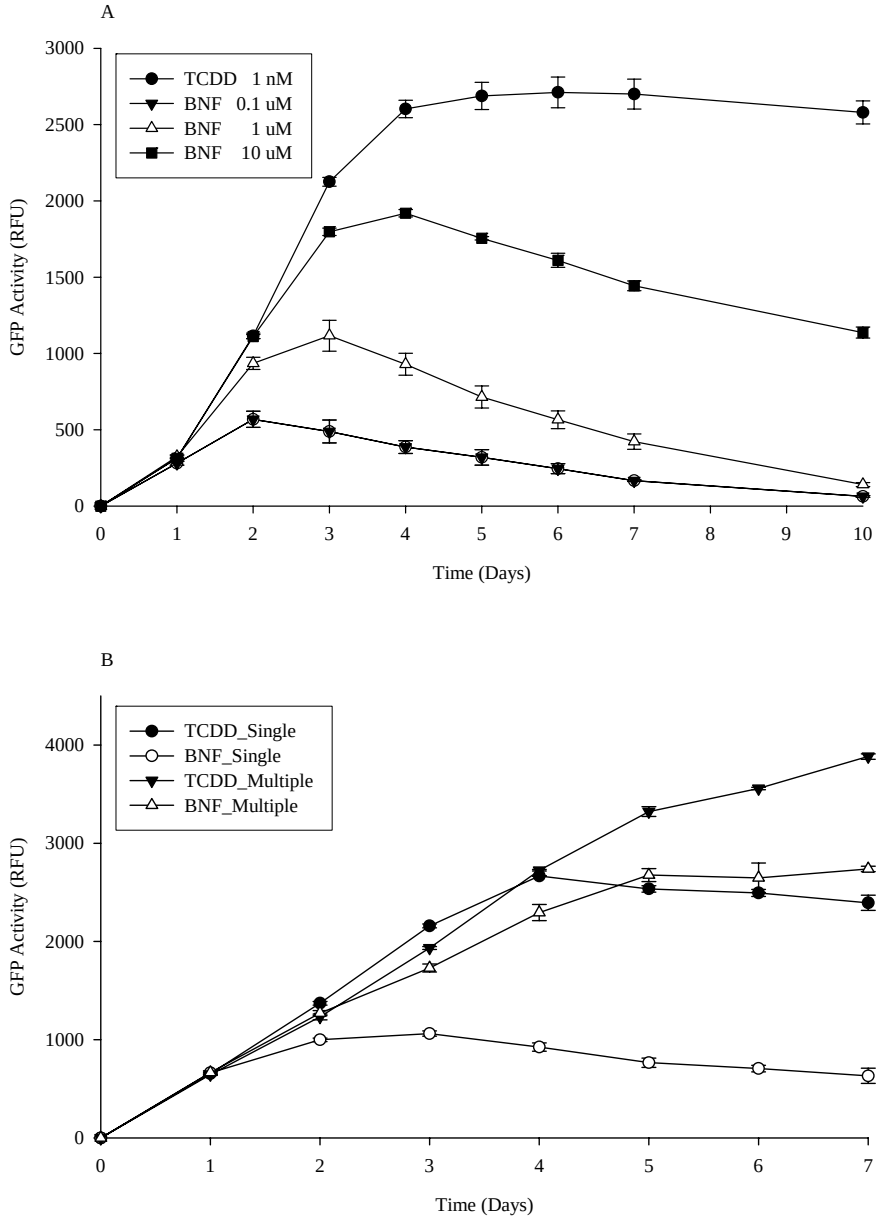


Figure 1. Time-dependent EGFP reporter gene expression in rat H4G1.1c2 cells. Single treatment of the indicated concentrations of TCDD (1 nM) or BNF (0.1, 1 and 10 μM) (A) or single treatment (1 nM TCDD or 1 μM BNF) and multiple treatment that is carried out by changing the medium contained indicated concentration of compound daily (B) was incubated with H4G1.1c2 cells at 33ºC, respectively, and AhR-dependent EGFP induction over time was measured. Values are expressed as relative fluorescence units and represent the mean ± SD of triplicate determinations.