TRANSCRIPTIONAL REGULATION OF TYROSINE HYDROXYLASE (TH) EXPRESSION BY AN AHR-NON XRE MEDIATED PATHWAY

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Introduction

Many environmental chemicals, including dioxins and related compounds, affect human health. These chemicals have diverse toxicities in various organs. Thus, development of methods for detecting the toxicities of these chemicals is critical. Dioxins and related compounds are suspected of causing neurological disruption in humans and experimental animal offspring following perinatal exposure[1-3]. The molecular mechanisms of these effects on the brain, however, have not been fully investigated. A major participant in the process of the dioxin toxicity is the aryl hydrocarbon receptor (AhR) [4, 5]. Since the expression of the AhR has been detected in various brain regions, this receptor may play an important role in the neurotoxicity of dioxins[6, 7]. Dioxins induce toxicity through disruption of gene expression via binding to a xenobiotic-responsive element (XRE) mediated by the AhR. Xenobiotic metabolizing enzymes, including cytochrome P450 (CYP) 1A1, CYP1B1, and glutathione S-transferase possess XRE sequences in the promoter regions, and these targets genes are induced by activated AhR. In previous studies, we demonstrated that tyrosine hydroxylase (TH), a functional marker of catecholaminergic neurons, was a target of AhR activated by dioxins [8, 9]. Because the AhR that was activated by 2, 3, 7, 8-tetrachloro dibenzo-*p*-dioxin (TCDD), increased TH mRNA levels in developing neurons, TH may be an appropriate biomarker for detecting the toxic effects of dioxin-like compounds on the nervous system.

In this study we show that the TH gene is regulated by AhR but does not conform to XRE AhR-mediated regulation. Although the AhR ligands, including TCDD, are able to exert toxicities through diverse transcriptional regulation, conventional screening methods of AhR ligands are not sufficient for assessing diverse effects. Therefore, we developed a novel reporter gene assay that is based on transcriptional regulation of the TH gene (TH promoter activation assay or TH assay). In the present paper, we show that the TH assay presents a potential utility for detecting a wide variety of AhR ligands, which are not involved in a xenobiotic response.

Materials and Methods

Chemicals and Cell cultures

TCDD (KANTO CHEMICAL CO., INC. Tokyo, Japan) was maintained as a stock solution (50 μg/ml) in DMSO. Neuro2a, a murine neuroblastoma cell line, was purchased from American Type Culture Collection. The cells were grown in Dulbecco's modified Eagle medium and F12 medium at a 1:1 ratio with 10% fetal calf serum supplementation. Cells were grown at 37 °C in humidified 5% CO_2.

Vector construction

Deletion constructs of the murine TH gene 5'-upstream region (-500 ~ 0bp) were produced by PCR, and the resultant product was cloned into the PGV-B2 luciferase vector (TOYO-B-Net CO., LTD. Tokyo, Japan). PCRs were performed with a common reverse primer and five distinct forward primers for each successive fragment. The primers were designed to contain overhanging restriction enzyme (*Kpn*I and *Nhe*I) sites in order to facilitate directional cloning into the PGV-B2 vector upstream of the firefly luciferase gene. The coding sequence of AhR was obtained by RT-PCR from rat brain RNA. The PCR product was cloned into the mammalian expression vector pcDNA4/V5-His (Invitrogen Japan KK, Tokyo, Japan). The resulting vector was named pcDNA-rAhR. The beta-galactosidase sequence was cloned into the mammalian expression vector pcDNA/V5-His, and the resulting construct (pcDNA4/V5-His/lacZ) was used as an internal standard for transfection efficiency.

Reporter gene assay

Neuro2a cells were seeded at 8 x 10^4 per well in 24-well plates and grown overnight in the appropriate medium. The next day, the cells were transiently transfected with the PGV-B2 luciferase vector containing the deletion construct as well as with the pcDNA-rAhR and the pcDNA4/V5-His/lacZ constructs using Lipofectamine™ 2000 Transfection Reagent (Invitrogen). The cells were exposed to TCDD for approximately 24 hrs before the luciferase assay. The luciferase activities were analyzed in the cell extracts using the PicaGene LT2.0 luminescence kit (TOYO-B-Net CO.), and the substrate was detected in a Mithras LB940 luminometer (Berthold Technologies, Bad Wildbad, Germany). Luciferase activities were normalized by dividing the mean luciferase activity by the mean of beta-galactosidase activity. Values represent the mean ± S.D. for four replicates.

Electrophoretic Mobility Shift Assay

AhR and aryl hydrocarbon receptor nuclear translocator (Arnt) were generated by *in vitro* coupled transcription and translation in rabbit reticulocyte lysates using the TNT system according to the manufacturer's instructions (Promega, Madison, WI). For DNA-protein binding reactions, 5 μL of the *in vitro* translated AhR and Arnt were mixed with 1 μM TCDD and incubated for 2 hrs. The reaction mixture was then mixed with 0.2 μg of poly (dI-dC) in 10 μl of binding buffer supplied by the manufacturer (Roche Diagnostics K.K, Tokyo, Japan). The reaction mixture was incubated for 5 min at room temperature following addition of a 200-fold excess of XRE competitor oligo DNA probe or a 200-fold excess mutated XRE oligo DNA probe. Subsequently, the Alexa Fluor 532-labeled oligo probe containing part of the TH gene 5'-upstream region was added, and the samples were incubated for 15 min at room temperature. AhR/Arnt-oligo DNA probe complexes were resolved on a 6% polyacrylamide gel in 1x Tris borate/EDTA buffer. The gel was scanned by Typhoon 8600 (GE Healthcare Bio-Sciences Corp. NJ, USA).

Results and Discussion:

Promoter deletion analysis of the 5'-upstream region of the murine TH gene

To identify the responsive region of TH gene expression by TCDD, we performed deletion analysis of the 5'-upstream region (-500 ~ 0bp) of the TH gene. The TCDD-responsive element was located within the -291 ~ -173bp fragment (Figure 1). Referring to the database Consite[10], which is the database for transcription factor binding sites, the consensus XRE sequence, which is the known binding sites of AhR, was not found in the sequence between -291~-173bp.

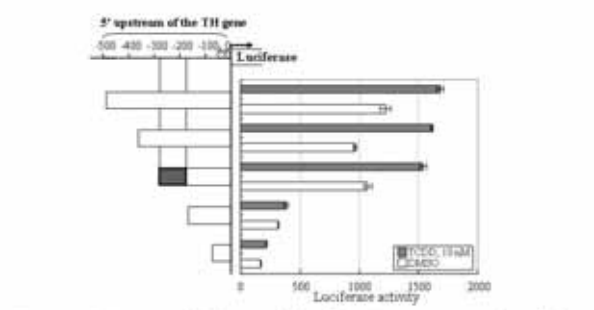

Figure 1. Promoter deletion analysis of the 5'-upstream region of the murine TH gene. Fragments longer than 291 bp increased the luciferase activity, but fragments shorter than 173 bp did not increase this activity. The AhR-responsive element regulating the dioxin response is therefore located in the region between -291 and -173 bp.

Electrophoretic Mobility Shift Assay (EMSA)

In order to verify that the AhR binding site occurred between -291bp and -173bp of the TH gene 5'-upstream region, we divided the region into six partially overlapping fragments and performed EMSA. In the TCDD-treated sample, the AhR/Arnt complex bound to the fragments between -249bp and -224bp of the TH gene 5'-upstream region (a band indicated by the arrow, Figure 2). This binding was inhibited by excess non-labeled XRE oligo probe but not by a non-labeled mutated XRE oligo probe (Figure 2). These results suggested that the 25bp fragment between -249bp and -224bp of the TH gene 5'-upstream region contained a specific AhR binding site; however, this 25-bp fragment does not contain the consensus XRE sequence. Thus, transcription of the TH gene is regulated by AhR recognition of a non-XRE sequence.

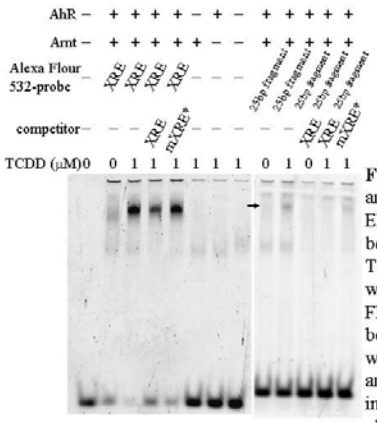

Figure 2. Identification of an AhR binding region by EMSA. The 25-bp fragment between -249 and -224 of TH gene 5'-upstream region was labeled with Alexa Fluor 532. This fragment bound the AhR when treated with TCDD (indicated by an arrow) . An asterisk (*) indicates a mutated XRE oligo.

Development of an in vitro bioassay (TH assay)

We developed a reporter gene assay using the non-XRE AhR transcriptional regulation mechanism as described above. A reporter vector, which contained both the 25-bp fragment of the TH gene 5'-upstream region and the TH gene core-promoter region upstream of a luciferase gene in a PGV vector, was constructed. This reporter

vector was used for the TH assay Figure 3a). Neuro2a cells that had been transfected with the reporter vectors were exposed to TCDD. After 24 hours, luciferase activity was measured in the cells. The response was dose-dependent for concentrations of TCDD between 10 and 1000 pM (Figure 3B). The effects of AhR ligands, such as dioxins, on human health are seen in increased risk of cancer, reproductive dysfunction, and neurological disruption. Although the specific mechanisms of action of the AhR ligands are not fully understood at present, TCDD is a well known AhR ligand and is thought to act through the XRE AhR-mediated pathway. Some reports, however, have suggested that non-XRE transcriptional motifs participated in AhR-regulated gene expression such as human paraoxonase 1 (PON-1) [11]. In addition, the non-XRE AhR-mediated pathway was preferable to the XRE AhR-mediated pathway for the action of 3-methyl cholanthrene, a major AhR ligand, against PON-1. Thus, assessing the diverse properties of the AhR ligands requires an evaluation method based on the non-XRE AhR-mediated mechanism as well as the XRE AhR-mediated mechanism. The TH assay described here is a tool for detecting the action of chemicals specifically through the non-XRE AhR-mediated mechanism.

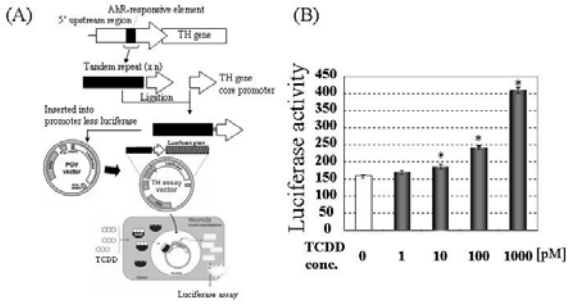

Figure. 3 Scheme of TH assay and assay sensitivity to TCDD.
(A) The vector was transfected into Neuro2a cells, and the luciferase activity was measured. The assay detected the toxic potency of TCDD. (B) The TH assay was performed following incubation of cells with different concentrations of TCDD. A statistically significant increase of luciferase activity was observed at 10 pM TCDD. Mean ± S.D. for four replicates. (*, $p < 0.05$)

Acknowledgements

We are grateful to Dr. T. Ushirogouchi and Dr. S. Itoh for their helpful comments throughout this study.

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