INDUCTION OF DOPAMINE SYNTHESIS BY EXPOSURE OF N2a-R CELLS TO TCDD

Akahoshi E, Yoshimura S, Uruno S, Ishihara-Sugano M

Functional Materials Laboratory, Corporate Research & Development Center, Toshiba Corporation, 1, Komukai-Toshiba-cho, Saiwai-ku, Kawasaki, 212-8582, Japan

Abstract

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a potent neurotoxin that causes neurobehavioral abnormalities associated with the cognitive and locomotive systems; however, the underlying mechanism remains unknown. Most TCDD-induced toxicity is mediated by binding to a dioxin receptor, the arylhydrocarbon receptor (AhR). We have previously reported the N2a-R cell line, which is a murine Neuro2a neuroblastoma stably transfected with rat AhR cDNA. In N2a-R cells, mRNA expression of tyrosine hydroxylase (TH), a rate-limiting enzyme in dopamine synthesis, is increased and is correlated with AhR activation. To confirm the affect of TCDD on brain function, in the current study we analyzed TH gene expression and dopamine synthesis in TCDD-treated N2a-R cells. In these cells, the TH mRNA expression level and the amount of dopamine were clearly increased by TCDD treatment. These results suggest that TCDD might affect dopaminergic function due to excess dopamine synthesis.

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is suspected to cause neurological disruption in offspring of humans and experimental animals following perinatal exposure[1-3]. However, the molecular mechanism(s) of TCDD actions in the brain have not been fully investigated. A major participant in the process of dioxin toxicity is the aryl hydrocarbon receptor (AhR)[4, 5]. Since the expression of AhR has been detected in various brain regions, AhR may play a role in the developmental neurotoxicity of dioxins[6, 7]. In a previous report, we constructed murine Neuro2a neuroblastoma cell lines stably transfected with rat AhR cDNA (N2a-R)[8], and showed that the N2a-R cell line is an appropriate experimental model for analyzing the relationship between AhR activation and developmental neurotoxicity. We observed that overexpression of AhR in N2a-R cells caused neural differentiation of the cells, since mRNA expression of tyrosine hydroxylase (TH), a functional marker of catecholaminergic neurons, increased with AhR activation. In the current study we analyzed catecholamine synthesis using N2a-R cells to clarify the mechanism of neurotoxicity of dioxin via AhR.

Materials and Methods

Chemicals and cell cultures

TCDD was purchased from Kanto Chemical (Japan) and maintained as a stock solution (50 μg/ml) in DMSO. N2a-R cell lines were constructed as described previously[8]. N2a-Rβ is a particular N2a-R cell clone with high exogenous AhR expression and N2a-Vc is a control cell line carrying the vector only. The cells were grown in a 1:1 mixture of Dulbecco's modified Eagle medium and medium F12 with 10% (v/v) fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. TCDD treatment was performed by exposing the cells to 10 nM TCDD for 24 hours.

Western blotting

N2a-Rβ and N2a-Vc cells were homogenized in PBS and denatured with SDS. Proteins (30 μg/lane) were fractionated on 10% SDS-PAGE and transferred to a PVDF membrane. Western blotting was performed using anti-AhR antibody (MA1-514, ABR, USA) using a previously described procedure[8].

Reverse transcriptase (RT)-PCR

After TCDD treatment, total RNAs were extracted from N2a-Rβ and N2a-Vc cells with RNeasy (Qiagen, Japan) and reverse transcribed. PCR was performed with gene-specific primers as described previously[8]. PCR products were electrophoresed on an agarose gel and stained with ethidium bromide.

Detection of L-dopa and catecholamine

L-dopa and catecholamines (dopamine, noradrenaline and adrenaline) were extracted from the cells (2×10^7) with 60% $HClO_4$ and determined using fluorescence HPLC. Ethylenediamine or diphenylethylenediamine was used as a fluorescent reagent for the detection of L-dopa and cathechiolamines, respectively.

Immunohistochemistry

Paraffin-embedded sections of rat brain were obtained from Genostaff (Japan). The sections were de-waxed, treated with microwaves for 10 min in citrate buffer (pH 6.0), treated with 3% hydrogen peroxide in methanol for 15 min, with 10% rabbit serum for 30 min, and then with anti-AhR antibody (MA1-513, diluted 1:2500; ABR, USA) or anti-TH antibody (AB152, diluted 1:400; Chemicon, USA) overnight. After washing with TBS, the sections were incubated with biotin-conjugated rabbit anti-mouse IgG (diluted 1:400), washed with TBS, incubated with HRP-conjugated streptavidin for anti-AhR-treated sections or with AP-conjugated streptavidin for anti-TH-treated sections, and then visualized using DAB/H_2O_2 or ALP-RED (Diagnostic Biosystems, USA), respectively. For double-staining, the sections were first stained with anti-AhR antibody followed by staining with anti-TH. The sections were counterstained with Mayer's Hematoxylin and mounted with Malinol (Mutoh, Japan).

Results and Discussion

Induction of TH gene expression in N2a-Rβ cells by TCDD treatment

N2a-Rβ cells were constructed by stably transfecting a murine neuroblastoma Neuro2a cell line with rat AhR cDNA[8]. N2a-Vc cells with the intact vector were constructed as a control cell line. The expression of AhR protein in N2a-Rβ cells is shown in Fig. 1A. To confirm whether the exogenous AhR was activated by TCDD in N2a-Rβ, we analyzed the expression of CYP1A1 mRNA following TCDD treatment, using RT-PCR. AhR is activated by binding to TCDD, and activated AhR transactivates the expression of its target genes by binding to the XRE (xenobiotic response element) consensus sequence. CYP1A1 is a well-known AhR target gene and is used as a functional marker of AhR activation[9]. CYP1A1 mRNA expression was clearly induced by TCDD in N2a-Rβ cells, indicating that the exogenous AhR was activated by TCDD (Fig. 1B). We also analyzed the mRNA expression of TH, a rate-limiting enzyme in dopamine synthesis, and found that TH mRNA was increased by TCDD in N2a-Rβ cells (Fig. 1B). These results show that TCDD induces TH gene expression via AhR activation.

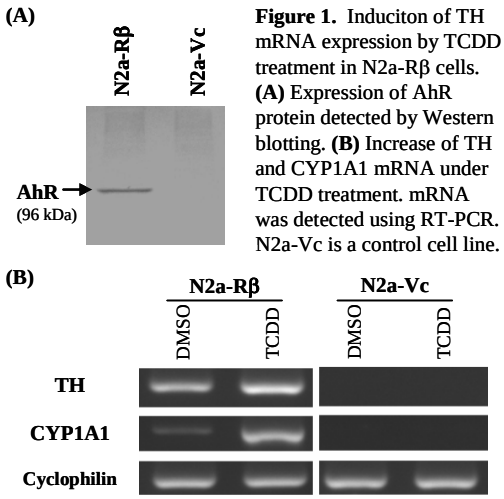

Figure 1. Induciton of TH mRNA expression by TCDD treatment in N2a-Rβ cells. **(A)** Expression of AhR protein detected by Western blotting. **(B)** Increase of TH and CYP1A1 mRNA under TCDD treatment. mRNA was detected using RT-PCR. N2a-Vc is a control cell line.

Induction of dopamine synthesis in N2a-R cells by TCDD treatment

TH catalyzes the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa), the initial step of dopamine synthesis. L-dopa is converted to dopamine, which is a catecholamine neurotransmitter and serves as a precursor of other catecholamine neurotransmitters; that is, noradrenaline and adrenaline. To analyze the relationship between TH gene induction and catecholamine synthesis, we determined the amounts of L-dopa and catecholamines in TCDD-treated and untreated N2a-Rβ cells. L-dopa and dopamine levels increased by more than 1.5-fold with TCDD treatment (Table 1), but there was no significant difference in the level of noradrenaline between TCDD-treated and untreated cells. Adrenaline could not be detected in N2a-Rβ cells.

Table 1. Amounts of L-dopa and catecholamines in TCDD-treated and untreated N2a-Rβ cells

L-dopa and catecholamines	Amount		Fold increase
	DMSO	TCDD (10 nM)	
L-dopa (ng/2x10^7 cells)	118.8 ± 18.9 (n=4)	208.8 ± 18.0 (n=4)	1.75*
Dopamine (ng/2x10^7 cells)	17.7 ± 2.7 (n=4)	28.1 ± 2.9 (n=4)	1.59*
Noradrenaline (pg/2x10^7 cells)	66.8 ± 11.1 (n=4)	71.5 ± 8.6 (n=4)	1.07^{NS}
Adrenaline (pg/2x10^7 cells)	N.D.	N.D.	N.D.

Values are means ± SD; n = no. of experiments; N.D. = not detected. Fold increase represents the ratio of the amounts in TCDD-treated cells versus untreated cells. *P < 0.01. NS = not significant.

These results show that TCDD induces dopamine synthesis by increasing TH gene expression.

Co-localization of TH and AhR in the substantia nigra pars compacta

To examine the possibility that TCDD affects dopaminergic function *in vivo*, we analyzed the localization of AhR and TH proteins in rat brain. AhR was detected mainly in the midbrain, CA1-CA3 region of the hippocampus, and the dentate gyrus. It is of note that AhR was detected in the substantia nigra pars compacta (SNc) in the midbrain, since the SNc contains a major population of dopaminergic neurons, which express TH. Double staining with antibodies against AhR and TH revealed co-localization of these proteins in the SNc (Fig. 2).

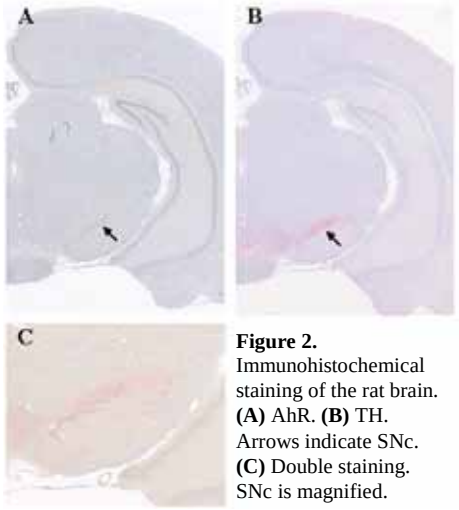

Figure 2. Immunohistochemical staining of the rat brain. **(A)** AhR. **(B)** TH. Arrows indicate SNc. **(C)** Double staining. SNc is magnified.

In conclusion, we have shown that TCDD induces TH mRNA expression and dopamine synthesis via AhR activation in N2a-Rβ cells. We also showed that AhR is localized in TH-positive neurons; that is, in dopaminergic neurons in the SNc. These results suggest that TCDD may affect dopaminergic function *in vivo*. Consequently, excess dopaminergic transmission may play an important role in the neurotoxicity of dioxin.

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