2,3,7,8-Tetrachlorodibenzo-p-dioxin suppress AChE activity in NGF treated PC12 cells

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ABSTRACT

PC12 is a well studied cell model for neuronal differentiation. AChE is also considered as a marker for neuronal differentiation. In this study, we detected the change of AChE activity during the NGF induced differentiation of PC 12 cells, and targeted on the ratio of the activity of AChE on the cell surface, and found that NGF mainly increased the intracellular AChE activity. Dioxin is a kind of persistent organic pollutants which have extreme impact on human health and widely distributed all over the world. Recently, AChE was reported as a target of the toxicity of dioxin. Here we investigated the effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on AChE activity in the PC12 cells, and found that at the later stage of differentiation, TCDD could decrease the AChE activity. This down regulation might not related to transcriptional regulation.

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1. Introduction

Dioxin refers to a group of high persistent organic environmental contaminants with similar chemical structures, including polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) [1]. Hundreds of congeners of dioxins are generated during synthetic processes including waste incineration, metal smelting. Since these environmental contaminants are highly resistant to metabolism in vertebrate species, biomagnification occurs through the food chain, and high tissue concentrations occur in top predator species, including humans [2]. In the family of dioxin, the halogenated aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD, dioxin) is the most intensively studied one, and reported to be exerting various kinds of impacts on human health.

Epidemiology and related studies show that exposure to TCDD results in multiple toxic effects, including immunotoxicity, neurotoxicity, carcinogenesis, endocrine toxicity, productive toxicity etc [3–5].

Acetylcholinesterase (AChE) activity and expression level can be down regulated by TCDD in neuronal cells [3,6]. Thus AChE could be considered as a target of the neuronal toxicity of dioxin. AChE has several types of transcripts by alternative splicing in the 3' region of primary transcripts [7,8]. The “tailed” isoform of AChE (AChE_T) is one of the transcripts that exists in all vertebrates, and can form tetramers associated with a proline-rich membrane anchor (PRiMA) as globular form AChE (G4 AChE) in the brain and muscle [9–11].

The expression level and enzymatic activity of AChE would be increased during neuronal differentiation. Cultured pheochromocytoma PC12 cell line is commonly being used for the detection of neuronal differentiation in responding to various stimuli, e.g. nerve growth factor (NGF) [12,13]. In this study, we used PC12 cells as the study model, investigated the change of AChE activities upon the treatment of NGF and the effect of TCDD on AChE during different stages of NGF induced PC12 differentiation.

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2. Materials and methods

2.1. Materials

2,3,7,8-TCDD was purchased from Wellington Laboratories Inc. (Ontario, Canada), and dissolved in dimethyl sulfoxide (DMSO).

2.2. Cell culture and drug treatment

Pheochromocytoma PC12 cells, a cell line derived from rat adrenal medulla, were obtained from American Type Culture Collection (ATCC, Manassas, VA), and which were maintained in Dulbecco's modified Eagle's medium supplemented with 6% fetal calf serum, 6% horse serum, 100 units/mL penicillin, and 100 µg/mL streptomycin in a humidified CO₂ (7.5%) incubator at 37 °C. Fresh medium was supplied every other day. All culture reagents were purchased from Life Technologies (Carlsbad, CA). During the treatment with NGF and TCDD, cultured PC12 cells were serum starved for 3 h in Dulbecco's modified Eagle's medium supplemented with 1% fetal calf serum, 1% horse serum and penicillin-streptomycin, and then which were treated with the drug for 12, 24, 48 and 72 h.

2.3. RNA extraction, reverse transcription and real-time PCR

To quantify the mRNA expression levels of the synaptic proteins, total RNA from cultured rat cortical neurons were isolated by GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA) according to the manufacture's instruction. 3 µg of total RNA was reverse-transcribed by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacture's instruction, and obtained the cDNA of each sample.

Real-time quantitative PCR was performed on equal amounts of cDNA by using GoTaq Qpcr Master Mix (Promega, Madison, WI) according to the manufacturer's instructions. The SYBR green signal was detected by Roche 480 Real-Time PCR System. PCR products were analyzed by gel electrophoresis, and the specificity of amplification was confirmed by the melting curves. The primers were as follows: Total AChE: 5'-CGCAGCAATACGTGAGCCTGAA-3' (S) and 5'-TGTGGCGCTG AGCAATTGCG-3' (AS) for rat (110bp; BC094521.1). AChET: 5'-CTCCCCAAATTG CTCAGCG-3' (S) and 5'-TCACAGGTCTGAGCAGCG-3' (AS) (147bp; [14]). GAPDH: 5'-AGTTCAACGGCAGACGTAAG-3' (S) and 5'-TACTCAGCACCAGCATCACC-3' (AS) (118bp; NM_017008.4).

2.4. Determination of AChE activity

AChE activities were determined by Ellman assay [9,15].

After treatments, cultured PC12 cells were collected in PBS (3 wells of cells from 6-well-plate were combined in one tube) and washed for 3 times and then resuspended in 1 mL of low salt lysis buffer (pH = 7.5) without Triton X-100 and all of the samples were diluted to equal cell density. Each sample was then equally divided into 3 parts: (1) anchored on the cell surface (cells were suspended in low salt lysis buffer without Triton X-100, AChE activity of the cell suspension was determined), (2) the background reading (cells were centrifuged and AChE activity in the supernatant was determined) (3) the total AChE activity from both intracellular and surface (Triton X-100 was added to the final concentration of 0.5%, AChE activity of total cell lysate was determined). Readings of sample (1) and (3) minus readings of sample (2) obtained the absolute AChE activity on the cell surface and from total cell, respectively.

2.5. Statistical analysis and other assays

Statistical analyses were performed using two way ANOVA. Statistically significant changes were classed as [*] where $p < 0.001$.

3. Results and discussion

3.1. Cell viability

The treatment of NGF and TCDD at the concentration of 1×10^{-11} to 1×10^{-9} did not affect the viability of PC12 cells (Supplementary Fig. 1).

3.2. NGF increases AChE activity

NGF was applied to the cultured PC12 cells at the concentrations of 15 ng/mL and 50 ng/mL. Samples were harvested after the

Fig. 1. AChE activity of NGF induced differentiated PC12 cells. (A): NGF increases total AChE activity in PC12 cells. PC12 cells were harvested at the different times points (12, 24, 48 and 72 h) under the treatment of NGF at the concentration of 15 and 50 ng/mL. Total protein was extracted from each sample to perform the Ellman assay, and results were normalized by the protein concentrations. Values are expressed as the fold of change (X Basal) against the control (the AChE activity of PC12 cells treated by DMSO for 12 h; set as 1), and in Mean ± SEM, $n = 3$, each with triplicate samples. *where $p < 0.01$ compared to the value of NGF 15 ng/mL for 72 h. **(B): Ratio of AChE activity on cell surface in NGF induced differentiated PC 12 cells.** PC12 cells were treated with NGF at the concentration of 15 and 50 ng/mL for 72 h. AChE activity on cell surface and total AChE activity was determined respectively. Values are expressed as the arbitrary unit, and in Mean ± SEM, $n = 3$, each with triplicate samples.

treatment for 12, 24, 48 and 72 h, respectively. Cell differentiation was induced by NGF, and the morphological change can be obviously observed (Supplementary Fig. 2). The enzymatic activity of AChE was measured by Ellman assay. As shown in Fig. 1A, AChE activity could be increased by NGF in time-dependent manners. During the treatment for 48 h, NGF at 15 and 50 ng/mL showed no difference in enhancing the activity of AChE. After being treated for 72 h, NGF at 50 ng/mL showed significantly higher effect compared with NGF at 15 ng/mL. This result implies that AChE activity could be increased during NGF induced neuronal differentiation, and dose dependent manner could be observed at later stage of differentiation in cultured PC12 cells.

3.3. The activity of AChE on the surface and in the cytosome under the treatment of NGF

After treatment of NGF at different concentrations for 72 h, the total AChE activity and activity of AChE on the cell surface were determined. Results show that NGF could increase activity of AChE

both intracellular and on the cell surface (Fig. 1B). Choi et al. reported that both PRiMA-linked G4 AChE and G1 AChE could be up regulated upon the treatment of dibutyrylcAMP (Bt₂-cAMP) and forskolin in cultured PC12 cells. Based on this result, they declared that stimulation of cAMP-dependent signaling pathway triggered the transcription of AChE_T and PRiMA 1 genes, and increase the assembly of PRiMA-linked G4 AChE in PC12 cells [14]. NGF, as an endogenous neurotrophic factor, could activate a lot of signaling pathways mainly including mitogen-activated protein (MAP) kinase pathway, phosphatidylinositol 3-kinase signaling- Akt pathway, cAMP-dependent signaling pathway NF-kappa B dependent pathway, and regulates a variety of cellular activities [16–19]. In PC12 cells, cAMP-dependent signaling pathway is one of the pathways that activated by NGF, but not the dominant one. Thus, in our study, AChE activity on the cell surface did not increase upon the treatment of NGF, even though NGF could up regulate the total AChE activity. That means the assembly of PRiMA-linked G4 AChE in PC12 cells cannot be promisingly enhanced by NGF.

3.4. TCDD affects AChE activity

TCDD at the concentration of 1×10^{-9} M was applied to the cultured PC12 cells to investigate its influence on AChE activity during NGF induced neuronal differentiation. Results show that at the earlier stage of differentiation (NGF treated for 12 h), TCDD cannot affect AChE activity; however, at later stage of differentiation (NGF treated for 72 h), TCDD could slightly decrease AChE activity (Fig. 2A). The ratio of surface AChE activity to total AChE activity was also detected. Result shows that TCDD slightly

Fig. 2. TCDD affects the AChE activity of NGF induced differentiated PC 12 cells. (A): TCDD affects total AChE activity of NGF induced differentiated PC12 cells. PC12 cells were treated with TCDD with NGF, and equal volume of DMSO and NGF for 12 h (left panel) and 72 h (right panel). Total protein was extracted from each sample to perform the Ellman assay, and results were normalized by the protein concentrations. Values are expressed as the fold of change (X Basal) against the control (the AChE activity of PC12 cells treated by DMSO for 12 h; set as 1), and in Mean ± SEM, n = 3, each with triplicate samples. *where $p < 0.01$ compared to the value of NGF 50 ng/mL treatment without TCDD. (B): TCDD affects the ratio of AChE activity on cell surface in NGF induced differentiated PC 12 cells. PC12 cells were treated with TCDD with NGF, and equal volume of DMSO and NGF for 72 h. AChE activity on cell surface and total AChE activity was determined respectively. Values are expressed as the arbitrary unit, and in Mean ± SEM, n = 3, each with triplicate samples.

Fig. 3. TCDD does not change the mRNA level of AChE in NGF induced differentiated PC 12 cells. PC12 cells were treated with TCDD and NGF, and equal volume of DMSO and NGF for 12 h (left panel) and 72 h (right panel). Total RNA was extracted from the cell cultures. The mRNA level of total AChE (A) and AChE_T (B) was analyzed by real-time quantitative PCR. Values are expressed as the fold of change (X Basal) against the control (AChE mRNA level of PC12 cells treated by DMSO for 12 h; set as 1), and in Mean ± SEM, n = 3, each with triplicate samples.

increased the percentage of cell surface AChE activity (Fig. 2B).

The TCDD concentration we used in this experiment is close to environmental levels. Previous work in our laboratory declared that dioxin could decrease AChE activity by about 15% in human-derived neuronal cells SK-N-SH [20]. In the present study, we found that total AChE activity of PC12 cells only could be decreased when the cells were differentiated and showed neuronal properties, while un-differentiated cells did not response to the treatment of TCDD.

3.5. TCDD does not affect AChE mRNA level

Total AChE mRNA level and AChE_T mRNA level were determined by real-time PCR respectively upon the treatment of TCDD and NGF (Fig. 3A and B). Result shows that no significant differences could be observed between the TCDD group and DMSO group. This result implies that the influence of TCDD on AChE activity of differentiated PC12 cells is not a consequence of transcriptional regulation. Meanwhile, even though TCDD slightly increased the percentage of cell surface AChE activity, it did not obtain this effect by changing the mRNA expression level of AChE_T. The mechanism is being determined currently in our laboratory.

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Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.cbi.2016.08.003>.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.cbi.2016.08.003>.

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